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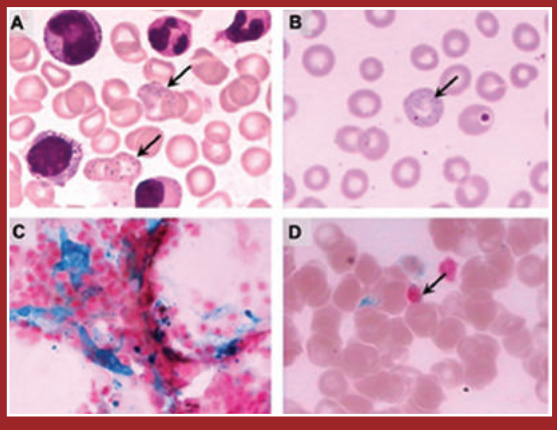
Turkish Journal of Hematology

The Official Journal of the Turkish Society of Hematology

Review

Research Articles



Cover Picture:
Yatian Zhao and Juan Lv
Basophilic Stippling and Chronic Lead Poisoning

4

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25.11.2018

Cover Picture

Yatian Zhao and Juan Lv
Basophilic Stippling and Chronic Lead Poisoning
(A) Bone marrow smears and (B) peripheral blood smears revealing extensive erythrocytes of coarse basophilic stippling (1000x, Wright-Giemsa stain). Bone marrow smears showed 4+ iron stores (C) and ring sideroblasts (D) (1000x, iron stain).

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AIMS AND SCOPE

The Turkish Journal of Hematology is published quarterly (March, June, September, and December) by the Turkish Society of Hematology. It is an independent, non-profit peer-reviewed international English-language periodical encompassing subjects relevant to hematology.

The Editorial Board of The Turkish Journal of Hematology adheres to the principles of the World Association of Medical Editors (WAME), International Council of Medical Journal Editors (ICMJE), Committee on Publication Ethics (COPE), Consolidated Standards of Reporting Trials (CONSORT) and Strengthening the Reporting of Observational Studies in Epidemiology (STROBE).

The aim of The Turkish Journal of Hematology is to publish original hematological research of the highest scientific quality and clinical relevance. Additionally, educational material, reviews on basic developments, editorial short notes, images in hematology, and letters from hematology specialists and clinicians covering their experience and comments on hematology and related medical fields as well as social subjects are published. As of December 2015, The Turkish Journal of Hematology does not accept case reports. Important new findings or data about interesting hematological cases may be submitted as a brief report. General practitioners interested in hematology and internal medicine specialists are among our target audience, and The Turkish Journal of Hematology aims to publish according to their needs. The Turkish Journal of Hematology is indexed, as follows:

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TURKISH JOURNAL OF HEMATOLOGY INSTRUCTIONS FOR AUTHORS

The Turkish Journal of Hematology accepts invited review articles, research articles, brief reports, letters to the editor, and hematological images that are relevant to the scope of hematology, on the condition that they have not been previously published elsewhere. Basic science manuscripts, such as randomized, cohort, cross-sectional, and case-control studies, are given preference. All manuscripts are subject to editorial revision to ensure they conform to the style adopted by the journal. There is a double-blind reviewing system. Review articles are solicited by the Editor-in-Chief. Authors wishing to submit an unsolicited review article should contact the Editor-in-Chief prior to submission in order to screen the proposed topic for relevance and priority.

The Turkish Journal of Hematology does not charge any article submission or processing charges.

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Original Manuscripts

Title Page

Title: The title should provide important information regarding the manuscript's content. The title must specify that the study is a cohort study, cross-sectional study, case-control study, or randomized study (i.e. Cao GY, Li KX, Jin PF, Yue XY, Yang C, Hu X. Comparative bioavailability of ferrous succinate tablet formulations without correction for baseline circadian changes in iron concentration in healthy Chinese male subjects: A single-dose, randomized, 2-period crossover study. Clin Ther 2011;33:2054-2059).

The title page should include the authors' names, degrees, and institutional/professional affiliations and a short title, abbreviations, keywords, financial disclosure statement, and conflict of interest statement. If a manuscript includes authors from more than one institution, each author's name should be followed by a superscript number that corresponds to their institution, which is listed separately. Please provide contact information for the corresponding author, including name, e-mail address, and telephone and fax numbers.

Running Head: The running head should not be more than 40 characters, including spaces, and should be located at the bottom of the title page.

Word Count: A word count for the manuscript, excluding abstract, acknowledgments, figure and table legends, and references, should be provided and should not exceed 2500 words. The word count for the abstract should not exceed 300 words.

Conflict of Interest Statement: To prevent potential conflicts of interest from being overlooked, this statement must be included in each manuscript. In case there are conflicts of interest, every author should complete the ICMJE general declaration form, which can be obtained at http://www.icmje.org/downloads/coi_disclosure.zip

Abstract and Keywords: The second page should include an abstract that does not exceed 300 words. For manuscripts sent by authors in Turkey, a title and abstract in Turkish are also required. As most readers read the abstract first, it is critically important. Moreover, as various electronic databases integrate only abstracts into their index, important findings should be presented in the abstract.

Objective: The abstract should state the objective (the purpose of the study and hypothesis) and summarize the rationale for the study.

Materials and Methods: Important methods should be written respectively.

Results: Important findings and results should be provided here.

Conclusion: The study's new and important findings should be highlighted and interpreted.

Other types of manuscripts, such as reviews, brief reports, and editorials, will be published according to uniform requirements. Provide 3-10 keywords below the abstract to assist indexers. Use terms from the Index Medicus Medical Subject Headings List (for randomized studies a CONSORT abstract should be provided: http://www.consort-statement.org).

Introduction: The introduction should include an overview of the relevant literature presented in summary form (one page), and whatever remains interesting, unique, problematic, relevant, or unknown about the topic must be specified. The introduction should conclude with the rationale for the study, its design, and its objective(s).

Materials and Methods: Clearly describe the selection of observational or experimental participants, such as patients, laboratory animals, and controls, including inclusion and exclusion criteria and a description of the source population. Identify the methods and procedures in sufficient detail to allow other researchers to reproduce your results. Provide references to established methods (including statistical methods), provide references to brief modified methods, and provide the rationale for using them and an evaluation of their limitations. Identify all drugs and chemicals used, including generic names, doses, and routes of administration. The section should include only information that was available at the time the plan or protocol for the study was devised (https://www.strobe-statement.org/fileadmin/Strobe/uploads/checklists/STROBE_checklist_v4_combined.pdf).

Statistics: Describe the statistical methods used in enough detail to enable a knowledgeable reader with access to the original data to verify the reported results. Statistically important data should be given in the

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text, tables, and figures. Provide details about randomization, describe treatment complications, provide the number of observations, and specify all computer programs used.

Results: Present your results in logical sequence in the text, tables, and figures. Do not present all the data provided in the tables and/or figures in the text; emphasize and/or summarize only important findings, results, and observations in the text. For clinical studies provide the number of samples, cases, and controls included in the study. Discrepancies between the planned number and obtained number of participants should be explained. Comparisons and statistically important values (i.e. p-value and confidence interval) should be provided.

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Study Limitations: Limitations of the study should be detailed. In addition, an evaluation of the implications of the obtained findings/results for future research should be outlined.

Conclusion: The conclusion of the study should be highlighted.

References

Cite references in the text, tables, and figures with numbers in square brackets. Number references consecutively according to the order in which they first appear in the text. Journal titles should be abbreviated according to the style used in Index Medicus (consult List of Journals Indexed in Index Medicus). Include among the references any paper accepted, but not yet published, designating the journal followed by "in press".

Examples of References:

1. List all authors

Deeg HJ, O'Donnel M, Tolar J. Optimization of conditioning for marrow transplantation from unrelated donors for patients with aplastic anemia after failure of immunosuppressive therapy. Blood 2006;108:1485-1491.

2. Organization as author

Royal Marsden Hospital Bone Marrow Transplantation Team. Failure of syngeneic bone marrow graft without preconditioning in post-hepatitis marrow aplasia. Lancet 1977;2:742-744.

3. Book

Wintrobe MM. Clinical Hematology, 5th ed. Philadelphia, Lea & Febiger, 1961.

4. Book Chapter

Perutz MF. Molecular anatomy and physiology of hemoglobin. In: Steinberg MH, Forget BG, Higs DR, Nagel RI, (eds). Disorders of Hemoglobin: Genetics, Pathophysiology, Clinical Management. New York, Cambridge University Press, 2000.

5. Abstract

Drachman JG, Griffin JH, Kaushansky K. The c-Mpl ligand (thrombopoietin) stimulates tyrosine phosphorylation. Blood 1994;84:390a (abstract).

6. Letter to the Editor

Rao PN, Hayworth HR, Carroll AJ, Bowden DW, Pettenati MJ. Further definition of 20q deletion in myeloid leukemia using fluorescence in situ hybridization. Blood 1994;84:2821-2823.

7. Supplement

Alter BP. Fanconi's anemia, transplantation, and cancer. Pediatr Transplant 2005;9(Suppl 7):81-86.

Brief Reports

Abstract length: Not to exceed 150 words.

Article length: Not to exceed 1200 words.

Introduction: State the purpose and summarize the rationale for the study.

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Statistics: Describe the statistical methods used in enough detail to enable a knowledgeable reader with access to the original data to verify the reported findings/results. Provide details about randomization, describe treatment complications, provide the number of observations, and specify all computer programs used.

Results: Present the findings/results in a logical sequence in the text, tables, and figures. Do not repeat all the findings/results in the tables and figures in the text; emphasize and/or summarize only those that are most important.

Discussion: Highlight the new and important findings/results of the study and the conclusions they lead to. Link the conclusions with the goals of the study, but avoid unqualified statements and conclusions not completely supported by your data.

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Abstract length: Not to exceed 150 words.

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References: Should not include more than 50 references

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Article length: Not to exceed 200 words.

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CAR-T Cell Therapy: A Door Is Open to Find Innumerable Possibilities of Treatments for Cancer Patients

CAR-T Hücre Tedavisi: Kanser Hastalarına Sayısız Tedavi Olanağı Bulunması için Kapı Aralandı

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Abstract

Seven years ago a chronic lymphocytic leukemia patient was for the first time successfully treated with chimeric antigen receptor (CAR)-modified T cells (CAR-T cells) to target CD19 overexpression in tumor cells. This was the beginning of the development of a new type of immunotherapy treatment in cancer patients. Since then, identification of novel antigens expressed in tumor cells and optimization of both CAR constructs and protocols of administration have opened up new avenues for the successful treatment of other hematological malignancies. However, research still continues to avoid some problems such as toxicities associated with the treatment and to find strategies to avoid tumor cell immune evasion mechanisms. On the other hand, for solid tumors, CAR-T therapy results are still in an early phase. In contrast to hematological malignancies, the complex tumor heterogeneity of solid tumors has led to the research of novel and challenging strategies to improve CAR-T cell activity. Here, we will review the main clinical results obtained with CAR-T cells in hematological malignancies, specifically focusing on CAR-T-19 and CAR-T against B-cell maturation antigen (CAR-T-BCMA). Moreover, we will mention the main problems that decrease CAR-T cell activity in solid tumors and the strategies to overcome them. Finally, we will present some of the first clinical results obtained for solid tumors.

Keywords: CAR-T cell immunotherapy, CD19, BCMA, GD2, HER2, EGFRvIII

Öz

Yedi sene önce kronik lenfositik lösemili bir hasta ilk kez başarılı olarak tümör hücrelerinde aşırı sunulan CD19'u hedefleyen kimerik antijen reseptör (CAR)-ile değiştirilmiş T hücreleri (CAR-T hücreleri) ile tedavi edilmiştir. Bu kanser hastalarında yeni bir tip immünoterapinin gelişiminin başlangıcını oluşturmaktaydı. Bunu takiben, tümör hücrelerinde sunulan yeni antijenlerin tanımlanması ve CAR yapılarını ve uygulama protokolleri diğer hematolojik habis tümörlerin başarılı tedavisi için yeni yollar açmıştır. Ancak, tedavi ile ilişkili toksisite gibi bazı problemlerin önlenmesi ve tümör hücresinin immün kaçış mekanizmalarıyla baş edilmesi ile ilgili çalışmalar halen devam etmektedir. Ayrıca, solid tümörler için, CAR-T tedavi sonuçları halen erken dönemdedir. Hematolojik habis tümörlerin aksine, solid tümörlerin karmaşık tümör heterojenitesi CAR-T hücre aktivitesi arttırmaya yönelik yeni ve zorlayıcı stratejilerinin araştırılmasına yol açmıştır. Burada, CAR-T hücrelerinin hematolojik habis tümörlerdeki, özellikle de CAR-T-19 ve B-hücre matürasyon antijenine karşı CAR-T'nin (CAR-T-BCMA) başlıca klinik sonuçlarını gözden geçireceğiz. Ayrıca, solid tümörlerde CAR-T hücre aktivitesini azaltan problemlerden ve bunların üstesinden gelmeye yarayan stratejilerden bahsedeceğiz. Son olarak, solid tümörlerdeki ilk klinik çalışmaların bazılarını sunacağız.

Anahtar Sözcükler: CAR-T hücre immünoterapisi, CD19, BCMA, GD2, HER2, EGFRvIII

Introduction: Chimeric Antigen Receptor-T Cell Therapy

The last decade has witnessed a huge increase in new immunotherapy modalities to treat cancer patients, such as the infusion of chimeric antigen receptor (CAR) modified-T cells (CAR-T cells), which represents the most important advance made to treat hematological malignancies in patients with relapsed/refractory (r/r) disease. CARs are composed of different synthetic domains combined into a single functional receptor that provides antigen-binding to an antigen present on the tumor cell and T-cell activation after antigen recognition [1]. Once a specific CAR has been designed, CAR-T cell therapy consists on the ex vivo modification of autologous T cells from the patient to express this CAR on their membranes. Afterwards, CAR-T cells are expanded in vitro for 8-10 days and reinfused into the patient, where they will recognize and kill the tumor cells.

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A CAR is composed of three domains: 1) The extracellular region codes for the single-chain variable fragment (scFv) of an antibody against the antigen present in the tumor cell. In this region, there is a spacer/hinge domain derived from CD8 and from immunoglobulin G (IgG) sequences that profoundly affects CAR function and scFv flexibility [2]. 2) The CAR transmembrane domain, derived from T-cell molecules, such as CD3ζ, CD4, CD8a, or CD28, links the extracellular domain with 3) the intracellular domain, which activates the T cells and is composed of CD3ζ T-cell receptor. This is the structure of the first-generation CAR-T cells, which have the benefit of not requiring antigen processing/presentation by the human leukocyte antigen (HLA), allowing them to bypass HLA-I restriction [3,4].

For the first-generation CAR-T cells, it was observed that even when the CAR-T cell mechanism was active, T cells did not proliferate in vivo, and moreover, a robust cytokine response after recognition of a tumor cell was not observed. This finding led to the addition of costimulatory domains in the CAR construct, giving rise to second- and third-generations CAR-T cells. Initially, CD28 was selected as the costimulatory domain by Savoldo et al. [5], who compared two autologous CAR-T types with the same specificity for CD19, one that encoded CD3ζ and CD28, while the other encoded only CD3ζ. The CAR-T cells containing CD28 showed enhanced expansion and persistence, confirming the requirement of costimulatory domains in the CAR construct. At the same time, Porter et al. [6] observed that the inclusion of 4-1BB as a costimulatory domain increased the antitumor activity and the in vivo persistence of CAR-T cells compared to CAR-T cells with the CD3-ζ domain alone. Therefore, costimulatory domains such as CD28, 4-1BB, and OX40 [7,8,9] were included in second-generation CAR-T cells, providing higher in vivo CAR-T cell proliferation than first-generation CAR-T cells. It was observed that whereas CD28 is better to activate T cells, 4-1BB increases CAR-T cell persistence [10]. Therefore, the majority of recent clinical studies on hematological malignancies are infusing CAR-T cells with 4-1BB. Moreover, third- and fourth-generations of CAR constructs have also been added to the CAR-T arsenal. Third-generation CAR-T cells encode more than one costimulatory domain to enhance T-cell activation and proliferation. Fourth-generation CAR-T cells, also known as TRUCKs or "armored CARs", incorporate a constitutive or inducible expression domain for a protein that needs to be induced or constitutively secreted. Therefore, these CARs can deliver a product to the targeted tumor tissue (i.e. a cytokine), but they also could incorporate a peptide to recognize and bind to its ligand (i.e. CD40L) in the target cell, and to interact with other immune cells such as dendritic cells (i.e. 4-1BBL) (Figure 1) [11,12].

Cytokine Release Syndrome Associated with CAR-T Cells

The most common toxicity associated with CAR-T cell therapy is a massive inflammatory response called cytokine release syndrome (CRS), which results from high cytokine levels released after T-cell engagement and proliferation. In most patients, CRS occurs 1-14 days after CAR-T cell infusion. Most patients develop low-grade CRS with fevers and myalgias. However, some patients experience severe CRS with hypotension, pulmonary edema, coagulopathy, vascular leak, and neurotoxicity in some cases, which can result in multiorgan system failure [13]. Interleukin (IL)-6 is a central mediator of CRS and CRS is well managed with tocilizumab, an anti-IL-6 receptor. However, corticosteroids have also been successfully used without compromising CAR-T cell proliferation or efficacy [14,15]. Managing CRS requires performing appropriate grading to define its onset and grading and resolution criteria. Currently, there are three CRS grading scales. The first scale used to define CRS is the one graded by the National Cancer Institute, called the Common Terminology Criteria for Adverse Events; however, this system was not specific for cellular therapeutic approaches. Afterwards, Lee et al. [16] proposed a specific scale for cellular therapeutic modalities, which was slightly modified by the MD Anderson Cancer Center proposing a new grading system [17]. Currently, the most widely used scale is the one proposed by the University of Pennsylvania (UPenn), based on the clinical results of their murine CAR-T-19 (tisagenlecleucel) after treatment of 125 patients with B-cell acute lymphoblastic leukemia (B-ALL) and 42 patients with chronic lymphocytic lymphoma (CLL). This scale is based on easily accessible clinical features and not laboratory values, which makes it possible for it to be applied more widely by

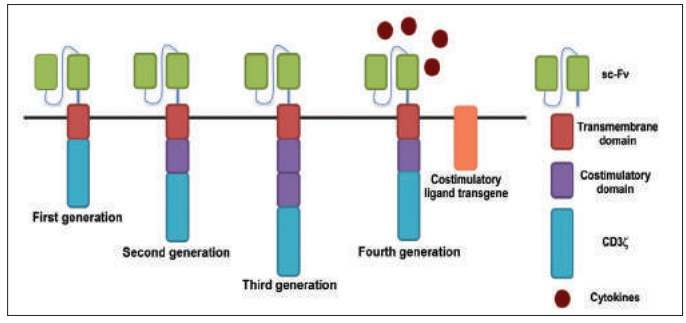

Figure 1. Structure of different chimeric antigen receptor (CAR) generations. First-generation CARs contain the single-chain variable fragment bound to the spacer/hinge domain, a transmembrane domain region with CD8 being the most commonly used, and the T-cell receptor CD3z domain. Second-generation CARs add one costimulatory domain to the construct, and third-generation CARs contain more than one costimulatory domain. Fourth-generation CARs contain an inducible or constitutive domain for another protein such as cytokines or specific ligand receptors.

scFv: Single-chain variable fragment.

many hospitals. It applies to both early-onset and delayed-onset CRS, and it distinguishes between mild, moderate, severe, and life-threatening CRS [18]. This scale was used in two multicenter phase II trials infusing tisagenlecleucel in r/r ALL patients performed in 11 different countries and nine sites in the United States. At all of these centers, using this scale, 81% of the patients experienced some grade of CRS and 45% suffered grade 3 or 4 CRS [18,19]. In addition, this scale has also been adopted for other CAR constructs against B-cell maturation antigen (BCMA) for multiple myeloma (MM) [20] and against mesothelin in epithelial ovarian cancer [21]. Table 1 summarizes the grading and CRS management adopted by our institution.

From the Initial Stages Infusing CAR-T-19 to a High CAR-T Cell Variety to Treat Different Malignancies

More than 20 years have passed from the first studies with first-generation CAR-T cells [22,23] to the design of second-generation CAR-T cells and finally the first successful clinical study in 2011 to treat a CLL patient with CAR-T-19 cells achieving complete remission (CR) [6]. Since then, an increasing number of clinical studies started to be performed, and today almost 200 clinical trials infusing CAR-T cells are being performed around the world. The greatest results have been obtained with CAR-T-19 in B-cell malignancies. Here, we will review some of the most relevant results obtained with CAR-T-19 and CAR-T-BCMA to treat MM. Moreover, we

Table 1. Grading of cytokine release syndrome and management of complications performed at our institution (Hospital Clinic of Barcelona) based on the grading scales of Lee et al. [16] and UPenn Porter et al. [18] and management recommendations.

Grade	Symptoms	Treatment
Grade 1	Not life-threatening symptoms: fever, nausea, fatigue, headache, myalgia, malaise	Conventional treatment to decrease temperature (paracetamol, ibuprofen, naproxen) Maintenance by intravenous fluids for hydration Antibiotics in case of infection Avoid immunosuppressors and steroids
Grade 2	Symptoms require moderate intervention: oxygen requirement <40% or hypotension responsive to fluids or low dose of a vasopressor or Grade 2 organ toxicity	For nonadvanced age and/or without comorbidity: Assess management in intermediate care Manage fever and constitutional symptoms as in Grade 1 Fluid bolus to maintain systolic blood pressure at >90 mmHg Supplementary O_2 to maintain O_2sp at >90% Obtain echocardiogram and initiate methods of hemodynamic monitoring For patients with high risk, consider tocilizumab For advanced age and/or cardiorespiratory comorbidity: Assess management in intermediate care Manage fever and constitutional symptoms as in Grade I Fluid bolus and low noradrenalin doses Supplementary O_2 to maintain O_2sp at >90% Assess tocilizumab administration
Grade 3	Symptoms require severe intervention: oxygen requirement >40% or hypotension requiring high-dose or multiple vasopressors or Grade 3 organ toxicity	Management in an intensive care unit Management as in Grade 2 Supplementary O_2 to maintain O_2sp at >90% Intravenous fluid bolus as needed High doses of vasopressors or multiple vasopressors Tocilizumab ± steroids
Grade 4	Life-threatening symptoms: requirement for ventilator support or Grade 4 organ toxicity	Management in an intensive care unit Management as in Grade 3 Mechanical ventilation Tocilizumab ± steroids
Neurotoxicity	Headache, altered level of consciousness, confusion, delirium, aphasia, dysmetria, ataxia, hallucinations, tremor, seizures	Neurological assessment every day Cranial computed tomography and magnetic resonance, lumbar puncture Dexamethasone administration In cases of seizures, levetiracetam administration Orotracheal intubation Mechanic ventilation

CRS: Cytokine release syndrome.

will mention other CAR constructs employed to treat B-cell malignancies not responding to CAR-T-19.

CAR-T-19 for the Treatment of B-Cell Malignancies

Three different institutions, the National Cancer Institute (NCI), UPenn, and the Memorial Sloan-Kettering Cancer Center (MSKCC), have been the pioneering centers performing clinical studies infusing second-generation CAR-T-19 cells to treat ALL, CLL, and lymphoma patients. Whereas the NCI and MSKCC have employed CAR-T-19 with CD28 as a costimulatory domain, UPenn selected 4-1BB. Their results have contributed to defining critical parameters including the best costimulatory domain, viral vector, gene transfer method, T-cell stimuli used during T-cell production, conditioning chemotherapy, and T-cell dose [24]. For instance, direct comparison by the MSKCC of CAR-T-19 with and without conditioning chemotherapy showed increased T-cell persistence and improved outcome with conditioning chemotherapy [25]. Regarding T-cell dose, whereas for CAR-T-19 this parameter is not so relevant [24], for other CAR constructs, such as BCMA in MM, a minimal CAR-T cell dose is required to achieve response [26]. In more detail, we will describe some clinical results obtained with CAR-T-19 to treat B-cell malignancies.

The first treated CLL patient received $1.46x10^5$ CAR-T-19 cells/kg split into three doses. CAR-T cells persisted for 6 months and remission was ongoing for 10 months. Because of this low CAR-T cell dose, CRS was reported 14 days after the first infusion, coinciding with peak levels of CAR-T-19 in peripheral blood (PB) [6]. Afterwards, two pediatric r/r ALL patients were treated with CAR-T-19. The first patient received $1.2x10^7$ CAR-T-19 cells/kg for three consecutive days without lymphodepletion. Patient 2 received $1.4x10^6$ CAR-T-19 cells/kg in a single dose and etoposide-cyclophosphamide was administered the week before. In both patients, expansion of CAR-T-19 was detected, and CR occurred the first month. However, whereas patient 1 had ongoing CR for 11 months, patient 2 had a CD19-negative relapse 2 months after treatment [14]. This was the first study describing CD19-negative relapses, one of the main problems after CAR-T-19 immunotherapy, which occurs in 78% of relapsed patients [27]. This year updated results on 75 children and young adults receiving CAR-T-19 (tisagenlecleucel) to treat ALL have shown overall response (OR) of 81% within 3 months, including 60% CR. CRS occurred in 77% of patients [27]. These results provided the basis for the approval of the first gene therapy product in the United States in 2017, tisagenlecleucel, commercialized by Novartis to treat B-cell precursor ALL patients up to 25 years old [28].

Recently, the MSKCC published results for CAR-T-19 with CD28 in 53 adult r/r ALL patients. At 29 months 83% CR was obtained, while median disease-free survival (DFS) and overall survival (OS) were 6.1 and 12.9 months, respectively. Severe CRS occurred in 26% of patients. Patients with low disease burden showed higher remission rates with 20.1 and 10.6 months of OS and DFS, respectively, and lower CRS than patients with higher disease burden [29].

Whereas results in ALL have been remarkable, in CLL and lymphoma cases the clinical results have been poorer. Comparison of 14 phase I clinical trials between 1991 and 2014 including 119 patients demonstrated that the OR rate was 73%, with 93% of responses in ALL patients, followed by CLL with 62% and lymphoma patients with 36%. Moreover, lymphodepletion, higher CAR-T cell dose, and no interleukin (IL)-2 administration were associated with better responses [30]. Interestingly, a more recent study of 24 CLL patients showed that CAR-T-19 is highly effective in high-risk CLL relapsed patients after ibrutinib treatment, showing OR of 71% and 83% CRS [31].

The CAR-T-19 from UPenn was used in 28 patients with r/r diffuse large B-cell lymphoma (DLBCL) or follicular lymphoma with CAR-T cell doses from $1x10^8$ to $5x10^8$. CRS occurred in 18% of patients while 90% CR was obtained after 1 month. The CR rate at 3 months was 43% and 71% in DLBCL and follicular lymphoma patients, respectively. At 28.6 months, sustained remissions were maintained in 86% of DLBCL and in 89% of follicular lymphoma patients [32].

On the other hand, the CAR-T-19 from the NCI with CD28 was employed in a phase I study of 7 patients with r/r DLBCL. Patients received $2x10^6$ CAR-T-19 cells/kg. One patient (14%) experienced grade 4 CRS. Grade ≥3 CRS and neurotoxicity were observed in 14% and 57% of patients, respectively. OR and CR were 71% and 57%, respectively. At 12 months, 43% of patients remained in CR [33]. Based on these results, a multicenter phase 2 study was performed to treat 101 r/r patients with DLBCL, primary mediastinal B-cell lymphoma, or transformed follicular lymphoma. Patients received $2x10^6$ CAR-T-19 cells/kg. Grade 3 or higher CRS and neurologic events occurred in 13% and 28% of the patients, respectively. OR was 82% and CR was 54%. At 15.4 months, 42% of the patients continued having a response, with a 40% rate of CR. OS at 18 months was 52%. Of the patients who showed disease progression, 27% of them showed CD19-negative disease [34]. Based on these results, the Food and Drug Administration approved the first CAR-T-19 cell product, called axicabtagene ciloleucel (Yescarta, Kite Pharma), to treat DLBCL, primary mediastinal large B-cell lymphoma, and high-grade B-cell lymphoma [35].

Other CAR Constructs Employed to Treat B-Cell Malignancies Not Responding to CAR-T-19

Other CAR constructs, such as CAR-T cells against CD30 (CAR-T-30), have been used to treat to treat Hodgkin lymphoma (HL) and anaplastic large cell lymphoma (ALCL), which do not

express CD19. Recently, the induction of CR in 9 r/r patients with HL (7 patients) and ALCL (2 patients) even in the absence of a conditioning regimen was reported without CAR-related toxicities. Patients received from $0.2x10^8$ to $2x10^8$ of CAR-T-30 cells/m^2. Seven of 9 patients received two or more infusions of CAR-T-30. Fourteen percent of HL patients entered CR lasting more than 2.5 years after the second infusion, 14% remained in CR for almost 2 years, and 43% had transient stable disease. For ALCL, one patient had CR for 9 months after the fourth infusion of CAR-T cells. Interestingly, although CD30 may be expressed by normal activated T cells, no patients developed impaired virus-specific immunity [36]. Tables 2 and 3 summarize additional studies of other targets.

CAR-T-BCMA for MM and Other B-Cell Malignancies

BCMA has appeared as a promising target to treat MM patients due to specific BCMA expression in plasma cells and its absence in most tissues [37]. Currently, more than 20 clinical trials are infusing CAR-T-BCMA for MM treatment. Due to the restricted BCMA expression pattern, BCMA was defined as the most suitable antigen to treat MM, and the design of novel and effective CAR-T-BCMA with CD28 [38] opened the path for a clinical trial in MM patients in 2016. This study infused CAR-T-BCMA in 12 r/r MM patients. Patients received different CAR-T-BCMA cell doses ($0.33x10^6$, $1x10^6$, $3x10^6$, and $9x10^6$ CAR-T-BCMA cells/kg). The 2 lowest doses achieved limited responses. At the third dose, a partial loss of BCMA expression in MM cells was detected in one patient, and one patient (25%) obtained very good partial response (VGPR). At the highest dose, one patient (50%) achieved CR for 17 weeks before relapse, and the other patient showed VGPR for 28 weeks. Both patients developed CRS [26]. These results were extended to perform a multicenter study to treat 21 patients in a dose-escalation study. CD28 was changed by 4-1BB and the CAR-T-BCMA was now called bb2121. It was found that 71% of patients developed CRS. The lowest dose ($50x10^6$ cells) infused in three patients was not active. The other 18 patients receiving $150x10^6$ (6 patients), $450x10^6$ (9 patients), and $800x10^6$ cells (3 patients) showed 94% OR, 89% VGPR, and 56% CR. Durable responses were ongoing over 1 year, and more importantly, responses continued to improve over time from VGPR to CR [39].

Additional studies with CAR-T-BCMA have been also successful. Cohen et al. [40] treated 21 r/r MM patients with CAR-T-BCMA in split-doses (10% on day 0, 30% on day 1, and 60% on day 2). Patients were assigned to three cohorts: 1-$5x10^8$ CAR-T cells (cohort 1: 9 patients), cyclophosphamide (CXT) 1.5 g/m^2 + 1-$5x10^7$ CAR-T cells (cohort 2: 5 patients), and CTX 1.5 g/m^2 + 1-$5x10^8$ CAR-T cells (cohort 3: 7 patients). Cohort 1 showed the highest CRS at 89% and 1 patient had ongoing CR at 21 months. Cohorts 2 and 3 showed 75% CRS. Cohort 2, with the lowest CAR-T dose, showed the lowest response (40%), which progressed at 4 and 2 months. Cohort 3, with a high CAR-T dose, at 1 month of follow-up showed 83% of any type of response. Interestingly, in 83% of the patients with ≥PR, MM cells showed decreased BCMA intensity [40]. More recently, a human CAR-T-BCMA was developed at the MSKCC, which hopefully will avoid early disappearance of CAR-T cells. Clinical results with this human CAR construct were recently published [41]. At our institution (Hospital Clinic), we have designed a highly effective CAR-T-BCMA. Moreover, we have also humanized the scFv, confirming its high efficacy, and in the next few months it will be used in a multicenter phase I study to treat r/r MM patients.

Moreover, Friedman et al. [42], who designed the CAR-T-BCMA (bb2121) [26], identified BCMA expression in primary lymphoma and CLL cells and confirmed the high efficacy of CAR-T-BCMA against models of MM, Burkitt lymphoma, and mantle cell lymphoma, suggesting that this CAR construct could be also efficient for these malignancies.

One of the problems observed in CAR-T immunotherapy for MM is the proportion of relapsed patients no longer having BCMA expression. Different options to avoid this, such as the use of dual CAR constructs targeting two different antigens, are being tested. Lee et al. [16] confirmed that 100% of primary MM cells expressed BCMA, and 78% of them also expressed TACI. Therefore, they successfully tested a third-generation dual CAR-T-APRIL (a ligand for BCMA and TACI), which eliminated MM cells expressing either BCMA or TACI and demonstrated tumor control in the absence of BCMA [43].

The impressive results in r/r MM patients targeting BCMA [39] suggest that after CAR-T-19, BCMA will be the next area where CAR-T therapy will have a high clinical impact. However, some problems still need to be addressed, such as the high CAR-T cell dose required to achieve responses, which could cause high CRS rates. New clinical protocols will aim to ameliorate severe CRS. Interestingly, for other CAR constructs such as CAR-T-20, Watanabe et al. [44] observed that the threshold of antigen density in the tumor required to induce CAR-T cell lytic activity was around 200 molecules per target cell, and for cytokine production it was 10-fold higher, suggesting a range for antigen density in the tumor cell where cytotoxicity can be performed without development of CRS. Second, the loss of BCMA expression in MM cells after CAR-T-BCMA treatment is a problem. Different strategies, such as dual CAR constructs, are being tested. Third, early disappearance of CAR-T cells in patients may be solved with the use of human and humanized CARs.

Homemade CARs: A Reality?

As previously mentioned, CAR-T-19 cell products have been commercialized by pharmaceutical companies, with prices of

Table 2. Clinical trials ongoing at other institutions other than the National Cancer Institute, University of Pennsylvania, and Memorial Sloan Kettering Cancer Center targeting CD19, CD20, and CD22 for B-cell malignancies, and other targets in other hematological malignancies.

Disease	Type of CAR and/or dose: target	Clinical trial code / location	Phase/n patients [ref]
r/r NHL	Multiple CAR-T cell infusion: CD19, CD20, CD22, CD30	NCT03196830 / First Affiliated Hospital of Soochow University	II/10
DLBCL	Sequential CAR-T cell infusion: CD19 and CD20	NCT02737085 / Southwest Hospital of Third Medical University	I-II/40
B-cell malignancies	CD20	NCT02710149 / Biotherapy Center of Southwest Hospital	I-II/45
r/r BCL	Sequential CAR-T infusion: CD19 and CD20	NCT03207178 / Shanghai Longyao Biotechnology Inc.	I-II/20
r/r BCL	CD20	NCT03576807 / Shanghai Longyao Biotechnology Inc.	I/20
r/r hematological malignancies	Bi-specific CAR-T: CD19 and CD20 or CD22	NCT03398967 / Chinese PLA General Hospital	I-II/80
r/r NHL	CD20	NCT03277729 / Fred Hutchinson Cancer Research Center	I-II/30
r/r B-cell leukemia or lymphoma	Bi-specific CAR-T: CD19/CD20	NCT03097770 / Chinese PLA General Hospital	I/20
r/r CD19+ and CD20+ BCL, ALL, and CLL	Bi-specific CAR-T: CD19/CD20	NCT03271515 / Beijing Doing Biomedical Co.	I/20
CD20+ BCL	CD20	NCT02965157 / Beijing Biohealthcare Biotechnology Co	I-II/15
r/r CD19 or CD20+ B-cell malignancies	Bi-specific CAR-T: CD20/CD19	NCT03019055 / Medical College of Wisconsin	I/24
r/r B-cell leukemia or lymphoma	Bi-specific CAR-T: CD19/CD22	NCT03185494 / Chinese PLA General Hospital	I-II/30
r/r myeloid malignancies	CD33	NCT02958397 / Southwest Hospital	I-II/45
r/r AML	CD33	NCT03126864 / MD Anderson Cancer Center	I/30
r/r AML	Single or double CAR-T cells combining different antigens: Muc1, CD33, CD38, CD56, CD123	NCT03222674 / Shenzhen Geno-Immune Medical Institute	I-II/10
r/r AML	Single or double CAR-T cells combining different antigens: CD38, CD33, CD56, CD123, CD117, CD34, CD133, Muc1	NCT03473457 / Zhujiang Hospital	I-II
r/r myeloid malignancies	CD123	NCT02937103 / Southwest Hospital	I-II/45
r/r BPDCN	Universal CAR-T cells: CD123	NCT03203369 / Cellectis S.A.	I/72
r/r AML	Universal CAR-T cells: CD123	NCT03190278 / Cellectis S.A.	I/156
r/r B cell malignancies	CD19 plus one of CD123, CD20, CD22, CD38, CD70, or I-Cas9	NCT03125577 / Shenzen Geno-Immune Medical Institute	I-II/100
r/r AML	CD123	NCT03556982 / Affiliated Hospital of the Chinese Academy of Military Medical Sciences	I-II/10
AML	Donor CAR-T cells after allo-SCT: CD123-EGFRt	NCT03114670 / Affiliated Hospital of the Academy of Military Medical Sciences	I/20
r/r AML, BPDCN	CD123-EGFRt	NCT02159495 / City of Hope Medical Center	I/60
r/r MM	CD38	NCT03464916 / University of Pennsylvania, Sorrento Therapeutics Inc.	I/72
r/r MM	Single or double CAR-T cells: BCMA/CD138/CD38/CD56	NCT03473496 / Zhujiang Hospital	I-II/50
r/r MM	Multiple CAR-T cells: BCMA/ CD138/CD38/CD56	NCT03271632 / Shenzhen Geno-Immune Medical Institute	I-II/20
r/r HL and NHL	CD30	NCT01316146 / UNC Lineberger Comprehensive Cancer Center	I/10 [36]
r/r AML	CD33	NCT01864902 / Chinese PLA General Hospital	I-II/10 [68]
r/r HL, CD30+ lymphoma	Dose escalation of CAR-T cells: CD30	NCT03049449 / National Cancer Institute	I/76
r/r CD30+ HL and NHL	CD30	NCT02690545 / UNC Lineberger Comprehensive Cancer Center	I-II/34

$475,000 for tisagenlecleucel and $373,000 for Yescarta. If the positive results obtained continue this trend, hopefully CAR-T-BCMA will also be approved for use in MM patients. Unfortunately, these prices are not affordable for many public national health systems. In this sense, at our institution, we have manufactured our CAR-T-19 cell product. This process requires having a good manufacturing practice facility to perform the viral production. Afterwards, the T-cell transfection is performed in the Prodigy device (Miltenyi, Biotec), a sterile isolated system, which performs all the steps required, starting from the apheresis product to the final product of CAR-T cells. This option provides much more affordable prices that can be assumed by a public national health system. With these CAR-T-19 cells (called ARI-0001), 18 patients with r/r B-cell malignancies have already been treated and a phase II clinical trial is about to start.

CAR-T Cells for the Treatment of Solid Tumors

Contrary to hematological malignancies, severe side effects, lack of persistence and effectiveness of CAR-T cells, immunosuppression in the tumor microenvironment, lack of homing, and tumor-off/target-on effects occurring in solid tumors decrease the success of CAR-T therapy for these malignancies [45]. Some strategies employed to improve these problems include the following: 1) Fourth-generation CAR-T cells, by incorporating additional features, such as costimulatory ligands next to the CAR receptor and more than one costimulatory domain, improve the lack of persistence and efficacy of CAR-T cells. In this sense, combining CD28 with OX40 blocks IL-10 production, increasing persistence and conferring higher efficacy to the CAR-T cells [45,46,47]. Combination of CAR-T cells with oncolytic viruses has also been suggested to improve CAR-T efficacy [48]. 2) To overcome the immunosuppressive microenvironment, the preselection of virus-specific CTLs before CAR-T cell transduction achieves a double CAR-T stimulation, either by the TCR or by the CAR, appearing as an option to avoid loss of expression of the tumor antigen [49]. Another option being tested is the combination of CAR-T cells with immunocheckpoint inhibitors, which seems to improve the potency of CAR-T cells [47]. In addition, fourth-generation CAR-T cells can modulate

Table 2. Continued

Disease	Type of CAR and/or dose: target	Clinical trial code / location	Phase/n patients
r/r HL and NHL	CD30	NCT02917083 / Baylor College of Medicine	I/18
AML, MDS, r/r MM	Dose escalation with 4 cohorts, from $1x10^6$ to $3x10^7$ CAR-T cells: CS1	Dana-Farber Cancer Institute, NHLBI	I/12
AML/MM	Lewis Y antigen	NCT01716364 / Peter MacCallum Cancer Centre	I/6

r/r: Relapsed/refractory, NHL: non-Hodgkin lymphoma, BCL: B-cell lymphoma, DLBCL: diffuse large B-cell lymphoma, ALL: acute lymphoblastic leukemia, CLL: chronic lymphocytic leukemia, AML: acute myeloid leukemia, BPDCN: blastic plasmacytoid dendritic cell neoplasm, I-Cas9: Inducible caspase 9, Allo-SCT: allogenic stem cell transplantation, NHLBI: National Heart, Lung, and Blood Institute, MM: multiple myeloma, MDS: myelodysplastic syndrome, PLA: People's Liberation Army Hospital.

Table 3. Clinical studies infusing CAR-T cells published by other institutions than the National Cancer Institute, University of Pennsylvania, and Memorial Sloan Kettering Cancer Center.

Disease	Target / CAR-T cell dose	N patients / clinical outcome	Clinical trial code / location [ref]
r/r HL	CD30 / On day 0: $3.2x10^5$ CAR-T/kg; from day 3 to 5, 5-fold increments	18.39% PR, 33% SD	NCT02259556 / Chinese PLA General Hospital [69]
r/r AML	CD33 / $1.12x10^9$ CAR-T cells; dose escalation over 4 days ($1x10^8$ day 1, $1.2x10^8$ day 2, $4x10^8$ day 3, and $5x10^8$ day 4)	1 / Grade IV toxicity, response at 2 weeks with progression at 9 weeks	NCT01864902 / Chinese PLA General Hospital [70]
r/r CD20+ BCL	CD20 / Escalating doses split into 3-5 doses on consecutive days; total dose from $0.41x10^7$ to $1.46x10^7$ cells/kg	11 / OR 81.8%, 6 CR and 3 PR; no severe toxicity observed; PFS >6 months, 1 patient CR >27 months	NCT01735604 / Chinese PLA General Hospital [71]
r/r AML	Lewis Y / $1.1x10^9$ T cells (ranging from $5x10^8$ to $1.3x10^9$)	5 / III-IV grade toxicity not observed; 1 patient with cytogenetic remission, 1 with reduction in peripheral blood, 1 protracted remission	NCT01716364 / University of Melbourne, Australia, Heidelberg-Australia [72]
r/r B-cell and mantle lymphoma	CD20 / 3 infusions of escalating doses of 10^8, 10^9, and $3.3x10^9$ cells/m^2	4 / 2 patients with PFS of 12 and 24 months; 1 patient in OR and relapsed after 12 months	NCT00621452 / Fred Hutchinson Cancer Research Center, Seattle, WA, USA [73]
r/r HL and ALCL	CD30 / 3 doses from $0.2x10^8$ to $2x10^8$ CAR-T cells/m^2	9 / No toxicities; 7 patients with HL, 1 CR >2.5 years, 1 CR >2 years, 3 transient SD; 2 patients with ALCL, 1 CR >9 months	NCT01316146 / UNC Lineberger Comprehensive Cancer Center [36]

r/r: Relapsed/refractory, HL: Hodgkin lymphoma, AML: acute myeloid leukemia, BCL: B-cell lymphoma, ALCL: anaplastic large cell lymphoma, PR: partial response, SD: stable disease, OR: overall objective response, CR: complete response, PFS: progression-free survival, PLA: People's Liberation Army Hospital.

the tumor environment through the secretion of IL-12 and can also increase tumor cell-CAR-T cell contact by the release of adhesion molecules or enzymes that degrade the extracellular matrix [50,51]. CRISPR/CAS9 technology appears as a further option to generate CAR-T cells resistant to exhaustion and inhibition [52]. 3) Moreover, the high tumor-off/target-on effect occurring in solid tumors can be ameliorated by variations in the administration route for CAR-T cells, cell dose, reduction of scFv affinity, use of "switchable CARs", and the discovery of specific tumor-associated antigens [45,53]. Additional proposals for CAR construct design include the insertion of caspase 9 into the CAR construct, which after administration of a small molecule (AP1903) to the patient will induce apoptosis of 99% of CAR-T cells [54]. Inducible caspase 9 is already being used in clinics, demonstrated to be safe (Table 4). Moreover, the design of transient CAR-T cells by introducing CAR-T mRNA by electroporation has shown antitumor activity in CAR-T-19 for CLL patients [55] and CAR-T-mesothelin for solid tumors [56], and it is being employed in clinical trials (Table 4). Due to all these limitations, positive clinical results with CAR-T cells in solid tumors are scarce, most of them in phase I trials. We will now mention some of the most interesting results obtained with CAR-T cells in solid tumors.

Specific disialoganglioside 2 (GD2) expression in tumor cells and slight expression in normal cells [57] makes GD2 a good candidate for CAR-T therapy, specifically for neuroblastoma. Eight neuroblastoma patients receiving Epstein-Barr virus (EBV)-virus-specific CTLs with CAR-T-GD2 showed evidence of tumor necrosis and one patient remained in CR, suggesting that virus-specific CTLs expressing CAR-T-GD2 show higher persistence in contrast to virus-nonspecific CAR-T cells [49].

Human epidermal growth factor receptor 2 (HER2) is not detected in normal brain tissues, being overexpressed in 25%-30% of breast and ovarian cancers, 60% of osteosarcomas, 80% of glioblastoma multiforme (GBM) cases, and 40% of medulloblastomas [51]. Although HER2 has been successfully targeted with anti-HER2-antibodies (trastuzumab and pertuzumab) in HER2/neu2+ breast cancer, the first breast cancer patient treated with CAR-T-HER2 died because of severe toxicity related to tumor-off/target-on effect [58]. In contrast, gliomas, glioblastomas, GBM, and medulloblastomas showing lower levels of HER2 than breast cancer are not efficiently treated with trastuzumab. Therefore, 17 GBM patients received from 10^6 to 10^8 cells/m^2 of intravenous polyclonal EBV-cytomegalovirus and adenovirus-specific T cells transduced with CAR-T-HER2 (CAR-T-FRP5). Median OS was 11 months, no serious side effects were reported, and CAR-T cells were detected in PB 12 months later [59].

IL-13 receptor alpha-2 (IL-13Rα2) is overexpressed in 75% of glioblastoma patients [60,61,62]. The first study in 3 glioblastoma patients receiving up to 12 local intracranial infusions of virus-specific CTL clones transduced with CAR-IL-13Rα2 (E13Y-zetakine CAR) showed minimal side effects and transient responses in 2 patients [63]. Afterwards, the CAR construct was modified to incorporate 4-1BB and a mutated IgG4-Fc linker to reduce tumor-off/target-on effect. At a dose of $2x10^6$ these CAR-T cells were administrated by intracranial infusion to one glioblastoma patient, followed by five additional infusions of $10x10^6$ CAR-T cells. Severe toxicities did not develop

Table 4. Clinical trials incorporating inducible caspase 9 in CAR-T cells or performing mRNA electroporation to induce the CAR.

Location / NCT code	Antigen (method employed for temporary CAR-T expression)	Malignancy	Phase
MSKCC / NCT02414269	Mesothelin (iCasp9)	Malignant pleural disease, mesothelioma, metastases, LC, BC	I
BCM / NCT01822652	GD2 (iCasp9)	Neuroblastoma	I
MSKCC / NCT02792114	Mesothelin (iCasp9)	BC, metastatic HER2-negative BC	I
Shenzhen Geno-Immune Medical Institute / NCT02992210	GD2 (iCasp9)	Solid tumor	I/II
NCI / NCT02107963	GD2 (iCasp9)	Sarcoma, osteosarcoma, NB, melanoma	I
BCM / NCT01822652	GD2 (iCasp9)	NB	I
Bambino Gesù Hospital / NCT03373097	GD2 (iCasp9)	NB	I/II
BCM / NCT01953900	GD2/VZV vaccine (iCasp9)	Sarcomas	I
Abramson Cancer Center of UPenn / NCT01897415	Mesothelin (mRNA)	PDA	I
UPenn / NCT01837602	cMet (mRNA)	BC, triple negative BC	I
UPenn / NCT03060356	cMet (mRNA)	Melanoma, BC	1

MSKCC: Memorial Sloan Kettering Cancer Center, BCM: Baylor College of Medicine, NCI: National Cancer Institute, UPenn: University of Pennsylvania, PDA: pancreatic ductal adenocarcinoma, BC: breast cancer, VZV: Varicella zoster virus.

and regression of intracranial and spinal tumors during 7.5 months was observed [64].

Most GBM patients overexpress the mutated epidermal growth factor receptor (EGFR) variant III (EGFRvIII), which is associated with tumor progression and poor prognosis [65]. Comparison of humanized second- and third-generation CAR-T cells with 4-1BB and/or CD28/4-1BB against EGFRvIII in vitro and in vivo demonstrated higher efficacy for the third-generation CAR-T cells. Moreover, a lower-affinity scFv was designed to minimize the tumor-off/target-on effects, and finally this CAR-T cell combined with temozolomide was the optimal strategy in a xenograft glioblastoma model [66]. Based on these results, UPenn conducted the first study with 10 newly diagnosed patients with recurrent GBM with residual disease infusing intravenous CAR-T-EGFRvIII cells. No evidence of off-tumor toxicity or CRS was observed. One patient had residual stable disease for over 18 months. All patients demonstrated transient expansion and trafficking of CAR-T cells to regions of active GBM. However, expression of inhibitory molecules and regulatory T-cell infiltration after CAR-T-EGFRvIII infusion was detected in the tumor environment [67]. Many other ongoing clinical studies targeting EGFRvIII, GD2, and HER2 are summarized in Table 5.

Conclusion

In summary, CAR-T immunotherapy has achieved remarkable results in the treatment of hematological malignancies, leading to the commercialization of CAR-T cells as pharmaceutical products. Despite positive results, problems such as loss of expression of the target antigen and CRS could be improved. In solid tumors, additional complications due to intratumoral cell heterogeneity cause low responses and high toxicities. Novel

Table 5. Clinical trials targeting disialoganglioside 2, human epidermal growth factor receptor 2, and epidermal growth factor receptor variant III.

Location / NCT code	Antigen	Malignancy	Phase
Zhujiang Hospital / NCT02765243	GD2	NB	II
NCI / NCT02107963	GD2	NB	I
BCM / NCT03294954	GD2	NB	I
Cancer Research UK / NCT02761915	GD2	NB	R
Bambino Gesù Hospital / NCT03373097	GD2	NB	R
Seattle Children's Hospital / NCT02311621	CD171/EGFRt	NB, GNB	R
Chinese PLA General Hospital, Beijing / NCT01935843	HER2	Advanced HER2-positive ST	I/II
The Methodist Hospital; Center for Cell and Gene Therapy, BCM / NCT02442297	HER2	GB	I
The Methodist Hospital; Center for Cell and Gene Therapy, BCM; Texas Children's Hospital / NCT01109095	HER2	GBM	I
Seattle Children's Hospital / NCT03500991	HER2	Pediatric CNST	I
Fuda Cancer Hospital, Guangzhou / NCT02547961	HER2	BC	I/II
Southwest Hospital, China / NCT02713984	HER2	BC, OC, LC, GC, colorectal, glioma, PC	I/II
Hospital of Harbin Medical University / NCT03267173	HER2	PC	I
Xuanwu Hospital, Beijing / NCT03423992	HER2	Glioma	I
City of Hope Medical Center / NCT03389230	HER2/CD19t	GB, glioma	I
BCM / NCT00902044	HER2	Sarcomas	I
Duke University Medical Center / NCT02664363	EGFRvIII	GB	I
NCI / NCT01454596	EGFRvIII	Glioma, GB, BC, gliosarcoma	I/II
Beijing Sanbo Brain Hospital / NCT02844062	EGFRvIII	GBM	I
NCI, Duke Cancer Institute / NCT03283631	EGFRvIII	GBM	I
Shenzhen Geno-Immune Medical Institute / NCT03170141	EGFRvIII	GBM	I/II
Xuanwu Hospital, Beijing / NCT03423992	EGFRvIII	Glioma	I
NCI / NCT01454596	EGFRvIII	Glioma, GB, BC, gliosarcoma	I/II
Duke University Medical Center / NCT02664363	EGFRvIII	GB	I
UPenn, UCA / NCT02209376	EGFRvIII	GB	I

BCM: Baylor College of Medicine, NCI: National Cancer Institute, UPenn: University of Pennsylvania, UCA: University of California, R: recruitin, GB: glioblastoma, GBM: glioblastoma multiforme, PC: pancreatic cancer, LC: lung cancer, BC: breast cancer, OC: ovarian cancer, GC: gastric cancer, BC: brain cancer, NB: neuroblastoma, GNB: ganglioneuroblastoma, ST: solid tumors, CNST: central nervous system tumor, PLA: People's Liberation Army Hospital, GD2: disialoganglioside 2, HER2: human epidermal growth factor receptor 2, EGFRvIII: epidermal growth factor receptor variant III.

CAR designs, modification of clinical protocols, discovery of novel tumor-specific antigens, and novel molecular strategies will improve clinical results for both hematological and solid tumors.

Ethics

Ethics Committee Approval: Hospital Clinic of Barcelona, approval number: HCB/2017/0438.

Authorship Contributions

Concept: L.P.A., B.M.; Design: L.P.A., B.M.; Literature Search: L.P.A., B.M.; Writing: L.P.A., B.M., B.M.A., M.J., A.U.I.

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References

1. Sadelain M, Brentjens R, Rivière I. The basic principles of chimeric antigen receptor design. Cancer Discov 2013;3:388-398.
2. Dai H, Wang Y, Lu X, Han W. Chimeric antigen receptors modified T-cells for cancer therapy. J Natl Cancer Inst 2016;108:djv439.
3. Chang ZL, Chen YY. CARs: Synthetic immunoreceptors for cancer therapy and beyond. Trends Mol Med 2017;23:430-450.
4. Hartmann J, Schüßler-Lenz M, Bondanza A, Buchholz CJ. Clinical development of CAR T cells-challenges and opportunities in translating innovative treatment concepts. EMBO Mol Med 2017;9:1183-1197.
5. Savoldo B, Ramos CA, Liu E, Mims MP, Keating MJ, Carrum G, Kamble RT, Bollard CM, Gee AP, Mei Z, Liu H, Grilley B, Rooney CM, Heslop HE, Brenner MK, Dotti G. CD28 costimulation improves expansion and persistence of chimeric antigen receptor-modified T cells in lymphoma patients. J Clin Invest 2011;121:1822-1826.
6. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med 2011;365:725-733.
7. Zhong XS, Matsushita M, Plotkin J, Riviere I, Sadelain M. Chimeric antigen receptors combining 4-1BB and CD28 signaling domains augment PI_3kinase/AKT/Bcl-XL activation and $CD8^+$ T cell-mediated tumor eradication. Mol Ther 2010;18:413-420.
8. Carpenito C, Milone MC, Hassan R, Simonet JC, Lakhal M, Suhoski MM, Varela-Rohena A, Haines KM, Heitjan DF, Albelda SM, Carroll RG, Riley JL, Pastan I, June CH. Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. Proc Natl Acad Sci U S A 2009;106:3360-3365.
9. Pule MA, Straathof KC, Dotti G, Heslop HE, Rooney CM, Brenner MK. A chimeric T cell antigen receptor that augments cytokine release and supports clonal expansion of primary human T cells. Mol Ther 2005;12:933-941.
10. Long AH, Haso WM, Shern JF, Wanhainen KM, Murgai M, Ingaramo M, Smith JP, Walker AJ, Kohler ME, Venkateshwara VR, Kaplan RN, Patterson GH, Fry TJ, Orentas RJ, Mackall CL. 4-1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors. Nat Med 2015;21:581-590.
11. Chmielewski M, Abken H. TRUCKs: the fourth generation of CARs. Expert Opin Biol Ther 2015;15:1145-1154.
12. Yeku OO, Brentjens RJ. Armored CAR T-cells: utilizing cytokines and pro-inflammatory ligands to enhance CAR T-cell anti-tumour efficacy. Biochem Soc Trans 2016;44:412-418.
13. Maude SL, Barrett D, Teachey DT, Grupp SA. Managing cytokine release syndrome associated with novel T cell-engaging therapies. Cancer J 2014;20:119-122.
14. Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, Teachey DT, Chew A, Hauck B, Wright JF, Milone MC, Levine BL, June CH. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. N Engl J Med 2013;368:1509-1518.
15. Brentjens RJ, Davila ML, Riviere I, Park J, Wang X, Cowell LG, Bartido S, Stefanski J, Taylor C, Olszewska M, Borquez-Ojeda O, Qu J, Wasielewska T, He Q, Bernal Y, Rijo IV, Hedvat C, Kobos R, Curran K, Steinherz P, Jurcic J, Rosenblat T, Maslak P, Frattini M, Sadelain M. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. Sci Transl Med 2013;5:177-38.
16. Lee DW, Gardner R, Porter DL, Louis CU, Ahmed N, Jensen M, Grupp SA, Mackall CL. Current concepts in the diagnosis and management of cytokine release syndrome. Blood 2014;124:188-195.
17. Neelapu SS, Tummala S, Kebriaei P, Wierda W, Gutierrez C, Locke FL, Komanduri KV, Lin Y, Jain N, Daver N, Westin J, Gulbis AM, Loghin ME, de Groot JF, Adkins S, Davis SE, Rezvani K, Hwu P, Shpall EJ. Chimeric antigen receptor T-cell therapy - assessment and management of toxicities. Nat Rev Clin Oncol 2018;15:47-62.
18. Porter D, Frey N, Wood PA, Weng Y, Grupp SA. Grading of cytokine release syndrome associated with the CAR T cell therapy tisagenlecleucel. J Hematol Oncol 2018;11:35.
19. Maude SL, Grupp SA, Pulsipher MA, Rives R, Myers GD, Verneris MR, Buechner J, Laetsch TW, Bittencourt H, Boyer M, De Moerloose B, Qayed M, Davies S, Martin PL, Bader P, Schlis K, Wood P, Taran T, Zhang Y, Leung M, June CH, Levine J. Analysis of safety data from 2 multicenter trials of CTL019 in pediatric and young adult patients with relapsed/refractory (r/r) B-cell acute lymphoblastic leukemia (B-ALL). European Hematology Association 2017:P517.
20. Cohen AD, Garfall AL, Stadtmauer EA, Lacey SF, Lancaster E, Vogl DT, Dengel K, Ambrose DE, Chen F, Plesa G, Kulikovskaya I, Gonzalez VE, Gupta M, Young RM, Carey T, Ferthio R, Weiss BM, Richardson C, Isaacs RE, Melenhorst JJ, Levine BL, June CH, Milone MC. B-cell maturation antigen (BCMA)-specific chimeric antigen receptor T cells (CART-BCMA) for multiple myeloma (MM): initial safety and efficacy from a phase I study. Blood 2016;128:1147.
21. Tanyi JL, Haas AR, Beatty GL, Stashwick CJ, O'Hara MH, Morgan MA, Porter DL, Melenhorst JJ, Plesa G, Lacey SF, June CH. Anti-mesothelin chimeric antigen receptor T cells in patients with epithelial ovarian cancer. J Clin Oncol 2016;34:5511a (abstract).
22. Brocker T, Karjalainen K. Signals through T cell receptor-zeta chain alone are insufficient to prime resting T lymphocytes. J Exp Med 1995;181:1653-1659.
23. Gong MC, Latouche JB, Krause A, Heston WD, Bander NH, Sadelain M. Cancer patient T cells genetically targeted to prostate-specific membrane antigen specifically lyse prostate cancer cells and release cytokines in response to prostate-specific membrane antigen. Neoplasia 1999;1:123-127.
24. Davila ML, Brentjens R, Wang X, Riviere I, Sadelain M. How do CARs work?: Early insights from recent clinical studies targeting CD19. Oncoimmunology 2012;1:1577-1583.
25. Brentjens RJ, Rivière I, Park JH, Davila ML, Wang X, Stefanski J, Taylor C, Yeh R, Bartido S, Borquez-Ojeda O, Olszewska M, Bernal Y, Pegram H, Przybylowski M, Hollyman D, Usachenko Y, Pirraglia D, Hosey J, Santos E, Halton E, Maslak P, Scheinberg D, Jurcic J, Heaney M, Heller G, Frattini M, Sadelain M. Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. Blood 2011;118:4817-4828.

26. Ali SA, Shi V, Maric I, Wang M, Stroncek DF, Rose JJ, Brudno JN, Stetler-Stevenson M, Feldman SA, Hansen BG, Fellowes VS, Hakim FT, Gress RE, Kochenderfer JN. T cells expressing an anti-B-cell maturation antigen chimeric antigen receptor cause remissions of multiple myeloma. Blood 2016;128:1688-1700.
27. Maude SL, Laetsch TW, Buechner J, Rives S, Boyer M, Bittencourt H, Bader P, Verneris MR, Stefanski HE, Myers GD, Qayed M, De Moerloose B, Hiramatsu H, Schlis K, Davis KL, Martin PL, Nemecek ER, Yanik GA, Peters C, Baruchel A, Boissel N, Mechinaud F, Balduzzi A, Krueger J, June CH, Levine BL, Wood P, Taran T, Leung M, Mueller KT, Zhang Y, Sen K, Lebwohl D, Pulsipher MA, Grupp SA. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. N Engl J Med 2018;378:439-448.
28. U.S. Food & Drug Administration. Release FN. FDA Approval Brings First Gene Therapy to the United States. Washington, FDA, 2017.
29. Park JH, Rivière I, Gonen M, Wang X, Sénéchal B, Curran KJ, Sauter C, Wang Y, Santomasso B, Mead E, Roshal M, Maslak P, Davila M, Brentjens RJ, Sadelain M. Long-term follow-up of CD19 CAR therapy in acute lymphoblastic leukemia. N Engl J Med 2018;378:449-459.
30. Zhang T, Cao L, Xie J, Shi N, Zhang Z, Luo Z, Yue D, Zhang Z, Wang L, Han W, Xu Z, Chen H, Zhang Y. Efficiency of CD19 chimeric antigen receptor-modified T cells for treatment of B cell malignancies in phase I clinical trials: a meta-analysis. Oncotarget 2015;6:33961-33971.
31. Turtle CJ, Hay KA, Hanafi LA, Li D, Cherian S, Chen X, Wood B, Lozanski A, Byrd JC, Heimfeld S, Riddell SR, Maloney DG. Durable molecular remissions in chronic lymphocytic leukemia treated with CD19-specific chimeric antigen receptor-modified T Cells after failure of ibrutinib. J Clin Oncol 2017;35:3010-3020.
32. Schuster SJ, Svoboda J, Chong EA, Nasta SD, Mato AR, Anak Ö, Brogdon JL, Pruteanu-Malinici I, Bhoj V, Landsburg D, Wasik M, Levine BL, Lacey SF, Melenhorst JJ, Porter DL, June CH. Chimeric antigen receptor T cells in refractory B-cell lymphomas. N Engl J Med 2017;377:2545-2554.
33. Locke FL, Neelapu SS, Bartlett NL, Siddiqi T, Chavez JC, Hosing CM, Ghobadi A, Budde LE, Bot A, Rossi JM, Jiang Y, Xue AX, Elias M, Aycock J, Wiezorek J, Go WY. Phase 1 results of ZUMA-1: a multicenter study of KTE-C19 anti-CD19 CAR T cell therapy in refractory aggressive lymphoma. Mol Ther 2017;25:285-295.
34. Neelapu SS, Locke FL, Bartlett NL, Lekakis LJ, Miklos DB, Jacobson CA, Braunschweig I, Oluwole OO, Siddiqi T, Lin Y, Timmerman JM, Stiff PJ, Friedberg JW, Flinn IW, Goy A, Hill BT, Smith MR, Deol A, Farooq U, McSweeney P, Munoz J, Avivi I, Castro JE, Westin JR, Chavez JC, Ghobadi A, Komanduri KV, Levy R, Jacobsen ED, Witzig TE, Reagan P, Bot A, Rossi J, Navale L, Jiang Y, Aycock J, Elias M, Chang D, Wiezorek J, Go WY. Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma. N Engl J Med 2017;377:2531-2544.
35. U.S. Food & Drug Administration. News Release. FDA Approves CAR-T Cell Therapy to Treat Adults with Certain Types of Large B-Cell Lymphoma. Washington, FDA, 2017.
36. Ramos CA, Ballard B, Zhang H, Dakhova O, Gee AP, Mei Z, Bilgi M, Wu MF, Liu H, Grilley B, Bollard CM, Chang BH, Rooney CM, Brenner MK, Heslop HE, Dotti G, Savoldo B. Clinical and immunological responses after CD30-specific chimeric antigen receptor-redirected lymphocytes. J Clin Invest 2017;127:3462-3471.
37. Tai YT, Anderson KC. Targeting B-cell maturation antigen in multiple myeloma. Immunotherapy 2015;7:1187-1199.
38. Carpenter RO, Evbuomwan MO, Pittaluga S, Rose JJ, Raffeld M, Yang S, Gress RE, Hakim FT, Kochenderfer JN. B-cell maturation antigen is a promising target for adoptive T-cell therapy of multiple myeloma. Clin Cancer Res 2013;19:2048-2060.
39. Berdeja JG, Lin Y, Raje N, Munshi N, Siegel D, Liedtke M, Jagannath S, Maus MV, Turka A, Lam LP, Hege K, Morgan RA, Quigley MT, Kochenderfer JN. Durable clinical responses in heavily pretreated patients with relapsed/refractory multiple myeloma: updated results from a multicenter study of bb2121 anti-Bcma CAR T cell therapy. Blood 2017;130:740.
40. Cohen AD, Garfall AL, Stadtmauer EA, Lacey SF, Lancaster E, Vogl DT, Weiss BM, Ambrose DE, Nelson AM, Chen F, Plesa G, Kulikovskaya I, Gonzalez V, Gupta M, Young RM, Dengel K, O'Keefe L, Le S, Richardson C, Isaacs RE, Melenhorst JJ, Levine BL, June CH, Milone MC. Safety and efficacy of B-cell maturation antigen (BCMA)-specific chimeric antigen receptor T cells (CART-BCMA) with cyclophosphamide conditioning for refractory multiple myeloma (MM). Blood 2017;130(Suppl 1):505.
41. Smith EL, Staehr M, Masakayan R, Tatake IJ, Purdon TJ, Wang X, Wang P, Liu H, Xu Y, Garrett-Thomson SC, Almo SC, Riviere I, Liu C, Brentjens RJ. Development and evaluation of an optimal human single-chain variable fragment-derived BCMA-targeted CAR T cell vector. Mol Ther 2018;26:1447-1456.
42. Friedman KM, Garrett TE, Evans JW, Horton HM, Latimer HJ, Seidel SL, Horvath CJ, Morgan RA. Effective targeting of multiple B-cell maturation antigen-expressing hematological malignances by anti-B-cell maturation antigen chimeric antigen receptor T cells. Hum Gene Ther 2018;29:585-601.
43. Lee L, Draper B, Chaplin N, Philip B, Chin M, Galas-Filipowicz D, Onuoha S, Thomas S, Baldan V, Bughda R, Maciocia P, Kokalaki E, Neves MP, Patel D, Rodriguez-Justo M, Francis J, Yong K, Pule M. An APRIL-based chimeric antigen receptor for dual targeting of BCMA and TACI in multiple myeloma. Blood 2018;131:746-758.
44. Watanabe K, Terakura S, Martens AC, van Meerten T, Uchiyama S, Imai M, Sakemura R, Goto T, Hanajiri R, Imahashi N, Shimada K, Tomita A, Kiyoi H, Nishida T, Naoe T, Murata M. Target antigen density governs the efficacy of anti-CD20-CD28-CD3 zeta chimeric antigen receptor-modified effector CD8+ T cells. J Immunol 2015;194:911-920.
45. Lim WA, June CH. The principles of engineering immune cells to treat cancer. Cell 2017;168:724-740.
46. Hombach AA, Heiders J, Foppe M, Chmielewski M, Abken H. OX40 costimulation by a chimeric antigen receptor abrogates CD28 and IL-2 induced IL-10 secretion by redirected CD4+ T cells. Oncoimmunology 2012;1:458-466.
47. Cherkassky L, Morello A, Villena-Vargas J, Feng Y, Dimitrov DS, Jones DR, Sadelain M, Adusumilli PS. Human CAR T cells with cell-intrinsic PD-1 checkpoint blockade resist tumor-mediated inhibition. J Clin Invest 2016;126:3130-3144.
48. Ajina A, Maher J. Prospects for combined use of oncolytic viruses and CAR T-cells. J Immunother Cancer 2017;5:90.
49. Pule MA, Savoldo B, Myers GD, Rossig C, Russell HV, Dotti G, Huls MH, Liu E, Gee AP, Mei Z, Yvon E, Weiss HL, Liu H, Rooney CM, Heslop HE, Brenner MK. Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. Nat Med 2008;14:1264-1270.
50. Chmielewski M, Hombach AA, Abken H. Of CARs and TRUCKs: chimeric antigen receptor (CAR) T cells engineered with an inducible cytokine to modulate the tumor stroma. Immunol Rev 2014;257:83-90.
51. Scarfo I, Maus MV. Current approaches to increase CAR T cell potency in solid tumors: targeting the tumor microenvironment. J Immunother Cancer 2017;5:28.
52. Ren J, Zhao Y. Advancing chimeric antigen receptor T cell therapy with CRISPR/Cas9. Protein Cell 2017;8:634-643.
53. Sadelain M, Brentjens R, Riviere I. The basic principles of chimeric antigen receptor design. Cancer Discov 2013;3:388-398.
54. Straathof KC, Pulè MA, Yotnda P, Dotti G, Vanin EF, Brenner MK, Heslop HE, Spencer DM, Rooney CM. An inducible caspase 9 safety switch for T-cell therapy. Blood 2005;105:4247-4254.
55. Boissel L, Betancur M, Wels WS, Tuncer H, Klingemann H. Transfection with mRNA for CD19 specific chimeric antigen receptor restores NK cell mediated killing of CLL cells. Leuk Res 2009;33:1255-1259.
56. Beatty GL, Haas AR, Maus MV, Torigian DA, Soulen MC, Plesa G, Chew A, Zhao Y, Levine BL, Albelda SM, Kalos M, June CH. Mesothelin-specific

chimeric antigen receptor mRNA-engineered T cells induce anti-tumor activity in solid malignancies. Cancer Immunol Res 2014;2:112-120.

57. Doronin II, Vishnyakova PA, Kholodenko IV, Ponomarev ED, Ryazantsev DY, Molotkovskaya IM, Kholodenko RV. Ganglioside GD2 in reception and transduction of cell death signal in tumor cells. BMC Cancer 2014;14:295.
58. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. Mol Ther 2010;18:843-851.
59. Ahmed N, Brawley V, Hegde M, Bielamowicz K, Kalra M, Landi D, Robertson C, Gray TL, Diouf O, Wakefield A, Ghazi A, Gerken C, Yi Z, Ashoori A, Wu MF, Liu H, Rooney C, Dotti G, Gee A, Su J, Kew Y, Baskin D, Zhang YJ, New P, Grilley B, Stojakovic M, Hicks J, Powell SZ, Brenner MK, Heslop HE, Grossman R, Wels WS, Gottschalk S. HER2-Specific chimeric antigen receptor-modified virus-specific T cells for progressive glioblastoma: a phase 1 dose-escalation trial. JAMA Oncol 2017;3:1094-1101.
60. Jain M, Zhang L, He M, Patterson EE, Nilubol N, Fojo AT, Joshi B, Puri R, Kebebew E. Interleukin-13 receptor alpha2 is a novel therapeutic target for human adrenocortical carcinoma. Cancer 2012;118:5698-5708.
61. Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, Kitani A. IL-13 signaling through the IL-13α2 receptor is involved in induction of TGF-β1 production and fibrosis. Nat Med 2006;12:99-106.
62. Bagley SJ, Desai AS, Linette GP, June CH, O'Rourke DM. CAR T cell therapy for glioblastoma: recent clinical advances and future challenges. Neuro Oncol 2018.
63. Brown CE, Badie B, Barish ME, Weng L, Ostberg JR, Chang WC, Naranjo A, Starr R, Wagner J, Wright C, Zhai Y, Bading JR, Ressler JA, Portnow J, D'Apuzzo M, Forman SJ, Jensen MC. Bioactivity and safety of IL13Rα2-redirected chimeric antigen receptor CD8+ T cells in patients with recurrent glioblastoma. Clin Cancer Res 2015;21:4062-4072.
64. Brown CE, Alizadeh D, Starr R, Weng L, Wagner JR, Naranjo A, Ostberg JR, Blanchard MS, Kilpatrick J, Simpson J, Kurien A, Priceman SJ, Wang X, Harshbarger TL, D'Apuzzo M, Ressler JA, Jensen MC, Barish ME, Chen M, Portnow J, Forman SJ, Badie B. Regression of glioblastoma after chimeric antigen receptor T-cell therapy. N Engl J Med 2016;375:2561-2569.
65. Gan HK, Cvrljevic AN, Johns TG. The epidermal growth factor receptor variant III (EGFRvIII): where wild things are altered. FEBS J 2013;280:5350-5370.
66. Johnson LA, Scholler J, Ohkuri T, Kosaka A, Patel PR, McGettigan SE, Nace AK, Dentchev T, Thekkat P, Loew A, Boesteanu AC, Cogdill AP, Chen T, Fraietta JA, Kloss CC, Posey AD Jr, Engels B, Singh R, Ezell T, Idamakanti N, Ramones MH, Li N, Zhou L, Plesa G, Seykora JT, Okada H, June CH, Brogdon JL, Maus MV. Rational development and characterization of humanized anti-EGFR variant III chimeric antigen receptor T cells for glioblastoma. Sci Transl Med 2015;7:275ra22.
67. O'Rourke DM, Nasrallah MP, Desai A, Melenhorst JJ, Mansfield K, Morrissette JJD, Martinez-Lage M, Brem S, Maloney E, Shen A, Isaacs R, Mohan S, Plesa G, Lacey SF, Navenot JM, Zheng Z, Levine BL, Okada H, June CH, Brogdon JL, Maus MV. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. Sci Transl Med 2017;9:0984.
68. Wang QS, Wang Y, Lv HY, Han QW, Fan H, Guo B, Wang LL, Han WD. Treatment of CD33-directed chimeric antigen receptor-modified T cells in one patient with relapsed and refractory acute myeloid leukemia. Mol Ther 2015;23:184-191.
69. Wang CM, Wu ZQ, Wang Y, Guo YL, Dai HR, Wang XH, Li X, Zhang YJ, Zhang WY, Chen MX, Zhang Y, Feng KC, Liu Y, Li SX, Yang QM, Han WD. Autologous T cells expressing CD30 chimeric antigen receptors for relapsed or refractory Hodgkin lymphoma: an open-label phase I trial. Clin Cancer Res 2017;23:1156-1166.
70. Wang QS, Wang Y, Lv HY, Han QW, Fan H, Guo B, Wang LL, Han WD. Treatment of CD33-directed chimeric antigen receptor-modified T cells in one patient with relapsed and refractory acute myeloid leukemia. Mol Ther 2015;23:184-191.
71. Zhang WY, Wang Y, Guo YL, Dai HR, Yang QM, Zhang YJ, Zhang Y, Chen MX, Wang CM, Feng KC, Li SX, Liu Y, Shi FX, Luo C, Han WD. Treatment of CD20-directed chimeric antigen receptor-modified T cells in patients with relapsed or refractory B-cell non-Hodgkin lymphoma: an early phase IIa trial report. Signal Transduct Target Ther 2016;1:16002.
72. Ritchie DS, Neeson PJ, Khot A, Peinert S, Tai T, Tainton K, Chen K, Shin M, Wall DM, Hönemann D, Gambell P, Westerman DA, Haurat J, Westwood JA, Scott AM, Kravets L, Dickinson M, Trapani JA, Smyth MJ, Darcy PK, Kershaw MH, Prince HM. Persistence and efficacy of second generation CAR T cell against the LeY antigen in acute myeloid leukemia. Mol Ther 2013;21:2122-2129.
73. Till BG, Jensen MC, Wang J, Qian X, Gopal AK, Maloney DG, Lindgren CG, Lin Y, Pagel JM, Budde LE, Raubitschek A, Forman SJ, Greenberg PD, Riddell SR, Press OW. CD20-specific adoptive immunotherapy for lymphoma using a chimeric antigen receptor with both CD28 and 4-1BB domains: pilot clinical trial results. Blood 2012;119:3940-3950.

RESEARCH ARTICLE

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Both Granulocytic and Non-Granulocytic Blood Cells Are Affected in Patients with Severe Congenital Neutropenia and Their Non-Neutropenic Family Members: An Evaluation of Morphology, Function, and Cell Death

Ciddi Konjenital Nötropenisi Olan Hastalarda ve Nötropenik Olmayan Ebeveynlerinde Hem Granülositik Hem Granülositik Olmayan Kan Hücreleri Etkilenir: Morfoloji, Fonksiyon ve Hücre Ölümü Yönünden Bir Değerlendirme

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Abstract

Objective: To examine granulocytic and non-granulocytic cells in children with severe congenital neutropenia (SCN) and their non-neutropenic parents.

Materials and Methods: Fifteen patients with SCN and 21 non-neutropenic parents were evaluated for a) CD95, CD95 ligand, annexin V, propidium iodide, cell cycle, and lymphocyte subsets by flow cytometry; b) rapid cell senescence (of leukocytes) by senescence-associated β-galactosidase stain; c) aggregation tests by aggregometer; d) in vitro bleeding time by PFA-100 instrument; e) mepacrine-labeled dense granule number of thrombocytes by fluorescence microscope; and f) hematomorphology by light and electron microscope. *HAX1*, *ELANE*, *G6PC3*, *CSF3R*, and *JAGN1* mutations associated with SCN were studied in patients and several parents.

Öz

Amaç: Ciddi konjenital nötropenisi (CKN) olan hastalar ve nötropenik olmayan ebeveynlerindeki granülositik ve granülositik olmayan kan hücrelerini incelemektir.

Gereç ve Yöntemler: CKN'si olan 15 çocuk ve nötropenik olmayan 21 ebeveynin lenfosit, granülosit ve monositlerinde CD95, CD95 ligand, annexin V, hücre siklusu (periferik lenfositler, granülosiler +/- monositlerde) ve lenfosit alt grupları akım sitometri ile, b) hızlı hücre yaşlanması (lökositlerde) yaşlanma-ilişkili β-galaktozidaz boyası SA-β-galaktosidaz boyası ile, c) agregasyon testleri agregometre ile, d) in vitro kanama zamanı, PFA-100 aleti ile, e) trombositlerde mepakrin işaretli kaba granül sayısı floresan mikroskopu ile, f) hematomorfoloji ışık ve elektron mikroskopu ile değerlendirildi. Hastalarda ve bazı ebeveynlerde CKN ile ilişkili olarak *HAX1*, *ELANE*, *G6PC3*, *CSF3R*, *JAGN1* mutasyonları çalışıldı.

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Results: Significant increase in apoptosis and secondary necrosis in monocytes, lymphocytes, and granulocytes of the patients and parents was detected, irrespective of the mutation type. CD95 and CD95 ligand results implied that apoptosis was non-CD95-mediated. Leukocytes of 25%, 12.5%, and 0% of patients, parents, and controls showed rapid cell senescence. The cell cycle analysis testable in four cases showed G1 arrest and apoptosis in lymphocytes of three. The patients had *HAX1* (n=6), *ELANE* (n=2), *G6PC3* (n=2), and unidentified (n=5) mutations. The CD3, CD4, and NK lymphocytes were below normal levels in 16.6%, 8.3%, and 36.4% of the patients and in 0%, 0%, and 15.4% of the parents (controls: 0%, 0%, 5.6%). The thrombocytes aggregated at low rates, dense granule number/thrombocyte ratio was low, and in vitro bleeding time was prolonged in 37.5%-66.6% of patients and 33.3%-63.2% of parents (vs. 0% in controls). Under electron and/or light microscope, the neutrophils, monocytes, lymphocytes, and thrombocytes in the peripheral blood of both patients and parents were dysplastic and the bone marrow of patients revealed increased phagocytic activity, dysmegakaryopoiesis, and necrotic and apoptotic cells. Ultrastructurally, thrombocyte adhesion, aggregation, and release were inadequate.

Conclusion: In cases of SCN, patients' pluripotent hematopoietic stem cells and their non-neutropenic parents are both affected irrespective of the genetic defect.

Keywords: Severe congenital neutropenia, Monocytes, Lymphocytes, NK cells, Thrombocytes, Phagocytes, Apoptosis, Senescence, Parents, Family

Bulgular: Akım sitometri ile, hasta ve ebeveynlerinin monosit, lenfosit ve granülositlerinde apoptoz ve sekonder nekrozda belirgin artış olduğu ve bunun konjenital nötropeni mutasyonunun cinsi ile ilişkili olmadığı gösterildi. CD95 ve CD95 ligand sonuçları, apoptozun CD95 yolu ile olmadığını gösteriyordu. Hasta, ebeveyn ve kontrol olgularının lökositlerinin %25, %12,5 ve %0'ı SA-β-gal boyası ile boyandı. Dört olguda yapılabilen hücre siklusu analizinde üç olgunun lenfositlerinde G1 arresti ve apoptoz görüldü. Hastalarda *HAX1* (n=6); *ELANE* (n=2); *G6PC3* (n=2) ve belirlenemeyen (n=5) mutasyonlar saptandı. CD3, CD4 ve NK lenfositleri sırasıyla hastaların %16,6; %8,3; %36,4'ünde, ebeveynlerin %0, %0, %15,4'ünde, kontrolün %0, %0, %5,6'sında yaşa göre normal aralığın altında idi. Hasta ve ebeveynlerin trombositleri düşük oranda agrege oluyordu (olguların sırasıyla %66,6 ve %63,2'sinde, kontrolün %0'ında), kaba granül sayısı/trombosit oranı düşük (hasta, ebeveyn ve kontrolün %50, %35 ve %0'ında); in vitro kanama zamanı uzun (farklı kartuşlarla olguların %37,5 ve %33,3'ünde ve ebeveynlerin %18,8 ve %12,5'inde) idi. Işık ve elektron mikroskopta hasta ve ebeveynlerin periferik kanlarındaki nötrofil, monosit, lenfosit ve trombositleri displastik idi; hastaların kemik iliğinde fagosit aktivitesinde artış, dismegakaryopoez, nekrotik ve apoptotik hücreler bulunuyordu. İnce yapısal olarak trombositlerde adezyon, agregasyon, salınım yetersiz idi.

Sonuç: CKN'de, pluripotent hematopoietik kök hücreler ve nötropenik olmayan ebeveynleri genetik bozukluktan bağımsız olarak etkilenirler.

Anahtar Sözcükler: Ciddi kongenital nötropeni, Monositler, Lenfositler, Trombositler, Fagositler, Apoptoz, Yaşlanma, Ebeveyn, Aile

Introduction

Severe congenital neutropenia (SCN) is a heterogeneous bone marrow failure syndrome characterized by recurrent infections, low absolute neutrophil count (<0.5x10^9/L), and maturation arrest at the promyelocyte/myelocyte stage of myelopoiesis in the vast majority of cases and it is due to various genetic defects [1,2,3]. Regular variations [4], giving rise to transient elevations of neutrophil counts to even >1.5x10^9/L with 'intermittent maturation arrest' [5], can be encountered.

Apoptosis in neutrophilic precursors plays a major role in the pathogenesis of SCN [1,2,6]. Reports regarding lymphocyte apoptosis in addition to granulocyte apoptosis have been restricted to a few cases [7,8], and apoptosis in monocytes has not been studied. Reports pertaining to non-granulocytic blood cell lines in SCN and patients' non-neutropenic family members are also too limited [3,7,8,9,10] to make a general characterization of the phenotype of SCN cases with heterogeneous genetic backgrounds.

We have hypothesized that, in SCN, development of all cell lines other than the granulocytic lineage is also impaired and patients' non-neutropenic parents also carry some hematologic abnormalities. Our specific aim in this study is to examine the lymphocytes, monocytes, and granulocytes of patients with SCN and their family members in terms of morphology and cell death parameters [apoptosis and rapid cell senescence (RCS)] and additionally to evaluate thrombocyte morphology and functions and percentage of lymphocyte subsets.

Materials and Methods

Study Participants

Severe congenital neutropenia was defined as persistent neutropenia (neutrophil counts of <0.5x10^9/L) confirmed from two samples a week for 6 weeks and the onset of neutropenia or infections early in life and deficiency in late maturation stages of neutrophils in bone marrow (mature neutrophils being <10%: central neutropenia) [4]. However, subjects whose neutrophils showed some spontaneous variations between <0.5x10^9/L and 1.5x10^9/L were not excluded [4]. Those with syndromic neutropenia were excluded.

Fifteen children with SCN [age: 9.35±4.54 years; range: 1.5-22; 8 female (F), 7 male (M)] and 21 non-neutropenic family members (10 mothers, 11 fathers; age: 35.14±8.92 years; range: 23-55) were included in the study. A 22-year-old female was included since she had been followed in Pediatrics for 8 years.

Patients were prescribed G-CSF (5-25 µg/kg/day), 2-7 times weekly; however, many patients received therapy irregularly for economic and social reasons. Blood was drawn during periods in which patients had stopped therapy and patients and family members had not consumed any other drugs for at least 10 days

Table 1. General characteristics of the patients.

Name, age [year (y)], sex [(female (F), male (M)]	History of symptoms (Infections, bleeding, aphthae)	Bone marrow (myeloid lineage)*	Molecular genetics**	Leukocyte count (WBC; x10^9/L)	Neutrophil count (10^9/L, %)	Monocyte count (x10^9/L, %)	Lymphocyte count (x10^9/L, %)	Thrombocyte count (x10^9/L, %)	GCSF /Latest dose	Time of follow-up
YF[¥], 7 $^{3/12}$ y, M	Skin abscesses, aphthae, gingival enlargement	Maturation arrest	*HAX1* Homozygous [c.130_131insA (p.W44[†])]¶	5.8 (25-50p)[21]	0.5	1.1 (>97.5p)[21]	3.9 (95-97.5p)[21]	372 (50-95p)[21]	5 µg/kg/dx3 days/week Latest dose: 1 month ago	10 years
MNY (Sibling of AY)[¥] 7y, F	ENT*** infections, glossitis	Maturation arrest	*HAX1* Homozygous [c.130_131insA (p.W44[†])]¶	3.1[†] (<25p)[21] 3.53[†] (25-50p)[21]	0[†] 0.77[†]	1.33 (>97.5p)[†21] 0.98 (95-97.5p)[†21]	1.49 (25-50p)[†21] 1.59 (25-50p)[†21]	447 (>97.5p)[†21] 459 (>97.5p)[†21]	5 µg/kg/dx3 days/week Latest dose: 25[†] and 10[‡] days ago	10 years
AY (Sibling of MNY)[¥], 4y, F	ENT infections	Maturation arrest	*HAX1* Homozygous [c.130_131insA (p.W44[†])]¶	4.1[†] (25p)[21] 5.4[†] (25-50)[21]	0.3[†] 0.87[†]	0.7 (50-95p)[†21] 1.48 (>97.5p)[†]	3.0 (50-95p)[†21] 2.4 (25-50p)[†21]	200 (<25p)[†21] 536 (>97.5p)[†21]	5 µg/kg/dx3 days/week[†] Latest dose: 1 month[†] and 10 days[‡] ago	10 years
MK, 9y, F	ENT & pulmonary infections, diarrhea, tuberculosis, aphthae, gingival enlargement, periodontitis, tooth loss	Maturation arrest	*HAX1* Homozygous [c.130_131insA (p.W44[†])]¶	5.2 (25-50p)[21]	0.6	1.3 (>97.5p)[21]	3100 (50-95p)[21]	426 (95-97.5p)[21]	5 µg/kg/dx3 days/week Latest dose unknown	10 years
EÇ (cousin of HY)[¥], 8y, F	ENT infections, pneumonia, aphthae, gingival enlargement , gingival bleeding, nasal bleeding	Slowdown in maturation	*HAX1* Homozygous [c.130_131insA (p.W44[†])]¶	5.2 (25-50p)[21]	0.4	1.4 (>97.5p)[21]	3.1 (50-95p)[21]	276 (25-50p)[21]	5 µg/kg/dx2 days/week Latest dose: 10 days ago	10 years
HY (cousin of EÇ)[¥], 6y, M	ENT infections, pneumonia, decayed teeth	Maturation arrest	*HAX1* Homozygous [c.130_131insA (p.W44[†])]¶	3.0 (<25p)[21]	0.12	1.84 (>97.5p)[21]	0.86 (<25p)[21] (below lowest limit)[23]	563 (>97.5p)[21]	5 µg/kg/dx2 days/week Latest dose: 4 days ago	10 years
OSK, 14y, M	Sometimes aphthae, no infection, hyperlipidemia, Familial Mediterranean Fever (FMF), renal amyloidosis	Maturation arrest	*G6PC3* homozygous, c.194A>C (p.E65A)¶¶¶; No mutation in *HAX1*¶¶/ *ELANE*	3.6 (25-50p)[21]	0.4	0.7 (50-95p)[21]	2.3 (50p)[21]	835 (>97.5p)[21]	No G-CSF	9 years
MeK, 22y, F	ENT infections, epilepsy when toddler, aphthae	Maturation arrest	*G6PC3* homozygous, c.194A>C (p.E65A)¶¶¶; No mutation in *HAX1/ ELANE*	2.4 (<25p)[21#]	0.2	0.2 (<25p)[#21] Below the mean[22##]	1.9 (25-50p)[#21] Within normal range[22##]	696 (>97.5p)[21]	5 µg/kg/dx2 days/week Latest dose: Unknown	9 years
RT[¥], 8y, M	Pneumonia, skin abscesses, aphthae, decayed tooth, easy bruising	Maturation arrest	*ELANE* Heterozygous [c.597+5G>A]; No mutation in *HAX1*¶¶	3.7(25-50p)[21]	0.4	0.6 (50-95p)[21]	2.6 (50-95p)[21]	190 (<25p)[21]	5 µg/kg/dx2 days/week Latest dose: 10 days ago	10 years
NBÖ, 10y, F	ENT infections, aphthae; easy bruising, frequent gingival and prolonged nasal bleeding	Maturation arrest	*ELANE* heterozygous c.416C>T (p.P139L) No mutation in *HAX1/G6PC3*	3.6 (25-50p)[21]	0.06	1.54 (>97.5p)[21]	1.8 (25-50p)[21]	185 (<25p)[21]	5 µg/kg/dx2 days/week Latest dose: 1 month ago	10 years After BMT, hemorrhagic symptoms almost disappeared

Table 1. Continued.

Name, age [year (y)], sex [(female (F), male (M)]	History of symptoms (Infections, bleeding, aphthae)	Bone marrow (myeloid lineage)*	Molecular genetics**	Leukocyte count (WBC; x10^9/L)	Neutrophil count (10^9/L, %)	Monocyte count (x10^9/L, %)	Lymphocyte count (x10^9/L, %)	Thrombocyte count (x10^9/L, %)	GCSF /Latest dose	Time of follow-up
AO, 12y, M	ENT infections	End stage neutrophil+stab <10%	No mutation in *HAX1* ¶¶/ *ELANE/ G6PC3/ CSF3R/JAGN1*	4.4 (25-50p)[21]	0.86	0.82 (95-97.5p)[21]	2.34 (50-95p)[21]	403 (95-97.5p)[21]	5 µg/kg/dx2 days/week Latest dose 10 days ago	10 years
BA, 8y, F	Pneumonia, ENT infections, fronculosis, inflamed urachus cyst , aphthae, gingival enlargement	No maturation arrest	No mutation in *HAX1/ ELANE/ G6PC3/ CSF3R*	3.24 (<25p)[21]	0.76	0.76 (50-95p)[21]	1.54 (25-50p)[21]	275 (25-50p)[21]	No G-CSF	10 years
AG (sibling of ZG)¥, 18/12, M	Pulmonary infections, otitis media; hypoxic labor, developmental delay	Maturation arrest	No mutation in *HAX1/ ELANE/ G6PC3/CSF3R/JAGN1*	9.9 (50-95p)[21]	0.42	2.42 (>97.5p)[21]	6.17 (95-97.5p)[21]	448 (50-95p)[21]	No G-CSF¶¶¶	6 years
ZG (sibling of AG)¥, 3$^{2/12}$, F	ENT infections, pneumonia, easy bruising, frequent nasal bleeding, developmental delay	Maturation arrest	No mutation in *HAX1*	7.34 (50-95pp)[21]	0.44	1.69 (>97.5p)[21]	4.38 (50-95p)[21]	323 (25-50p)[21]	No G-CSF¶¶¶	6 years
KŞ, 12y, M	ENT infections, aphthae, gingivitis	6% neutrophils, 10% neutrophilic (neut) band, 5% neut. metamyelocyte, 5% neut. myelocyte, 2% neut. promyelocyte	Not done	2.8 (<25pp)[21]	0.2	0.3 (25-50p)[21]	2.3 (50p)[21]	425 (95-97.5p)[21]	G-CSF, only during infections Latest dose 7 days ago	Lost to follow-up

*These bone marrow aspirations represent those taken during the follow-up of the patients, after initiation of G-CSF therapy if needed, except AG and ZG whose aspirations represent those taken at diagnosis, ** Peripheral blood cells, *** Ear, nose, throat, ¥ Patients who could be evaluated for cell death parameters, ‡First evaluation for this study, ‡Second evaluation for this study, #evaluation for 15-18 years of age, ##evaluation according to the normal adult data, ¶1591>C homozygous polymorphism in exon 2 of *HAX1* gene, ¶¶ 1591>C heterozygous polymorphism in exon 2 of *HAX1* gene ¶¶¶New mutation, ¶¶¶¶These patients required high dose (10-20 µg/kg/d) G-CSF, but the family refused to administer it due to malignancy risk.

prior to samples being taken to eliminate drug effects on thrombocyte aggregation [11].

For lymphocyte subsets, death parameters (CD95, CD95 ligand, annexin V), dysmorphism, thrombocyte aggregation tests, mepacrine labeling, and in vitro bleeding time, 18, 10, 9, 5, 5, and 9 healthy volunteers were evaluated, respectively. For evaluation of lymphocyte subsets, age-matched normal ranges for healthy Turkish children [12] and our laboratory for adults were used.

Flow Cytometric Evaluation

Peripheral blood was prepared for flow cytometric analysis as reported previously [8,13,14] by direct immunofluorescence (FAC Scan, Becton Dickinson CA and Beckman Coulter, USA). By combining CD45 and CD14 with the forward and right-angle light scatter parameters of blood cells, the lymphocytes, granulocytes, and monocytes were gated (Figure 1A). CD95, CD95 ligand, and annexin V were evaluated in each gate; the CD3, CD4, CD8, and NK cells were evaluated in the lymphocyte gates [13,14]. Kits from Biosciences (USA) (for CD95 and CD95 ligand) and Pharmingen (USA) [for annexin V, propidium iodide (PI), 7-aminoactinomycin D (7-AAD), and CD3, CD4, CD8, and NK cells] were used. Per sample, 10,000 cells were counted. The cell cycles of the lymphocytes and granulocytes were evaluated by the PI florescence histogram method [13].

Rapid Cell Senescence

The leukocytes were stained for senescence-associated β-galactosidase (SA-β-gal kit, Sigma Co., Germany) as per the manufacturer's protocols [15].

Mutation Analysis

Mutation analyses of *HAX1*, *ELANE*, *CSF3R*, *G6PC3*, and *JAGN1* genes were performed by standard techniques (Supplemental Materials and Methods and Supplemental Table 1).

Evaluation of Cellular Morphology

The peripheral blood cells were evaluated by light (Nikon E400) and transmission electron microscopy (TEM) (LEO 906E) for apoptosis and dysplasia [8,10,14,16,17], in a blinded fashion (Supplemental Materials and Methods). The

bone marrow aspiration smears taken at admission were also evaluated under light microscope. Bone marrow aspiration of the parents could not be performed.

Evaluation of the Thrombocytes of the Patients and the Parents

In vitro bleeding time was measured with a PFA-100 instrument (Dade Behring Marburg GmbH, Marburg, Germany) [18] and turbidimetric aggregation tests were measured with a Chrono-Log 560 Ca aggregometer (Chrono-Log Corporation, Havertown, PA, USA) [18].

Dense granules were stained with mepacrine (1 μM, Sigma, St. Louis, MO, USA) [19,20] and thrombocytes were prepared for electron microscopic visualization of aggregation [19], as described previously (Supplemental Materials and Methods).

Statistics Analysis

We used SPSS 15.0 (SPSS Inc., Chicago, IL, USA) to evaluate the data we obtained. A normality test was performed to determine if the data were distributed in a normal fashion (Supplemental Materials and Methods).

Results

History and Physical Examination

In our cohort, there were three pairs of siblings and one pair of cousins, one having coexistent amyloidosis and hypercholesterolemia and the other having hemoglobin C. Their vaccines were administered on time without any complications. Parents of 14 patients were 1st (n=10) or 2nd (n=4) degree relatives. The patients had gingival hypertrophy, aphthous stomatitis, decayed teeth, and tooth loss by 26.6%, 20%, 20%, and 13.3%, respectively. None had any physical malformation. Several patients had monocytosis and thrombocytosis [21,22]. The immunoglobulin (Ig) A, G, and M levels of patient AG and the IgG of patient ZG were higher than normal, while the levels of all the other patients were normal. Four out of 15 SCN patients (26.6%) and 5 of 21 parents (23.8%; 3 mothers, 2 fathers) had frequent nasal bleeding and easy bruising with/without menorrhagia. Investigations of immunoglobulin levels, which could be performed for ten parents, revealed normal results. The other characteristics of the patients and parents are presented in Table 1 and Supplemental Table 2.

Flow Cytometric Evaluation

Percentage of apoptotic cells, necrotic cells, and dead cells (apoptotic + necrotic) in the lymphocyte, granulocyte, and monocyte gates of both the patients and the parents were higher than those of the healthy controls, while they were similar among the patients and parents (Figure 1). CD95 and CD95 ligand results were inconsistent with each other, implying that apoptosis was non-CD95-mediated (Table 2; Figure 1).

The CD3, CD4, and NK cells were below the age-matched normal ranges in 16.6%, 8.3%, and 36.4% of the patients and 0%, 0%, and 15.4% of the parents versus 0%, 0%, and 5.6% of the controls. On the other hand, CD3 and CD8 cells were found to be above the age-matched normal ranges in 16.6% and 27.3% of the patients and in 0% and 7.7% of the parents versus 0% and 16.7% of the controls (Supplemental Table 3).

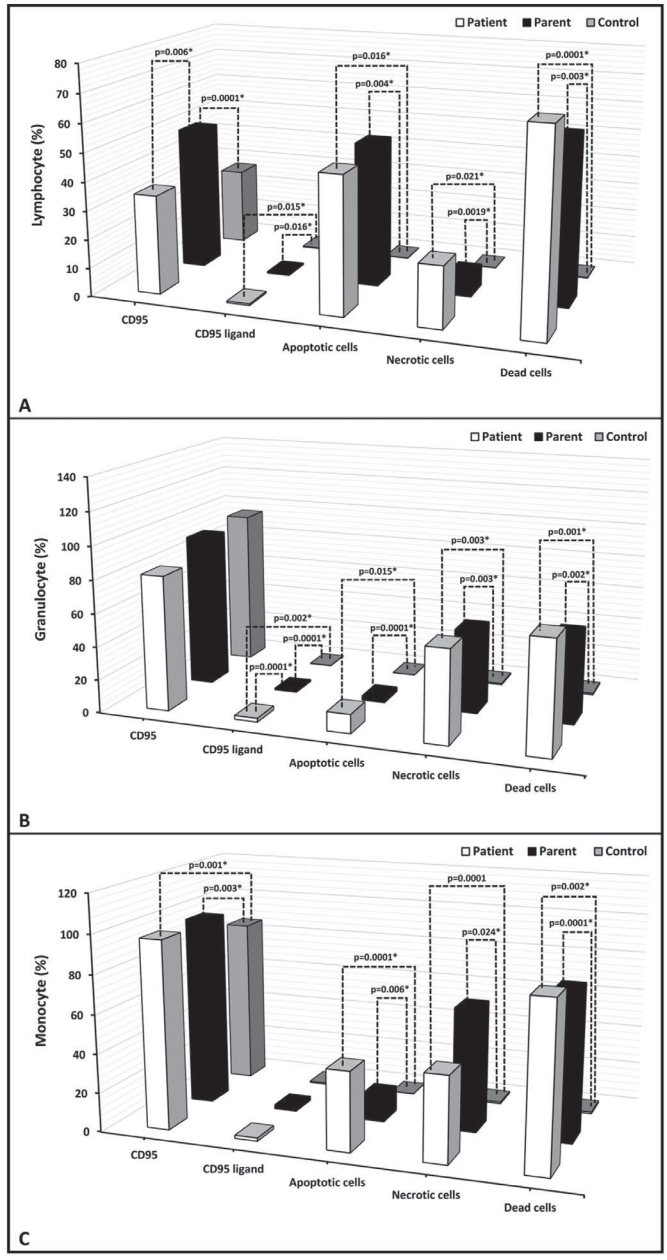

Figure 1. Percentage of CD95, CD95 ligand, annexin (showing apoptotic cells), propidium iodide (PI), or 7-aminoactinomycin D (7-AAD) (showing necrotic cells) and overall dead cells (apoptotic + necrotic cells) in lymphocyte, monocyte, and granulocyte gates.

Table 2. CD95, CD95 ligand, and annexin levels on the lymphocytes, granulocytes, and monocytes of the patients and their parents (%).

		CD95 Lymphocyte	CD95 ligand Lymphocyte	Apoptotic cells* Lymphocyte	Necrotic cells** Lymphocyte	Dead cells*** Lymphocyte	CD95 Monocyte	CD95 ligand Monocyte	Apoptotic cells* Monocyte	Necrotic cells** Monocyte	Dead cells*** Monocyte	CD95 Granulocyte	CD95 ligand Granulocyte	Apoptotic cells* Granulocyte	Necrotic cells** Granulocyte	Dead cells*** Granulocyte
Patient n=11†/8††		34.65±15.66 (12.6-69.0)	0.54±0.31 (0.16-1.3)	47.95±37.81 (0.0-96.9)	21.34±33.82 (0.00-98.00)	69.29±41.22 (1.80-100.00)	96.69±4.84 (86.5-99.8)	1.65±2.15 (0.4-7.9)	40.76±31.52 (1.0-70.70)	43.96±31.19 (0.50-92.40)	84.71±29.31 (20.10-100.00)	81.57±32.28 (7.9-99.6)	2.34±2.06 (0.0-6.6)	11.65±12.64 (0.10-33.50)	56.04±31.60 (2.60-98.40)	67.70±27.11 (24.1-100.0)
Parent n=13†/13††		49.88±8.56 (40.90-71.40)	0.5±0.31 (0.1-1.2)	50.30±40.98 (0.0-99.8)	7.59±12.55 (0.00-36.10)	57.89±41.71 (0.00-100.00)	97.38±2.03 (92.8-99.7)	2.19±2.79 (0.0-9.5)	13.29±12.17 (0.00-37.00)	62.97±33.63 (0.0-97.5)	76.26±37.75 (0.00-100.00)	91.97±22.77 (16.4-99.8)	0.99±0.78 (0.4-2.8)	2.69±3.43 (0.00-12.90)	49.94±46.81 (0.00-100.00)	52.63±45.83 (0.00-100.00)
Control n=12†/11††		27.07±8.42 (11.07-38.75)	0.28±0.31 (0.0-0.99)	0.11±0.10 (0.0-0.20)	0.07±0.08 (0.00-0.20)	0.18±0.18 (0.00-0.40)	85.87±12.59 (63.76-98.3)	0.68±0.61 (0.0-1.46)	0.06±0.21 (0.00-0.70)	0.87±1.65 (0.0-4.70)	0.94±1.82 (0.00-5.40)	94.60±6.17 (79.69-99.65)	0.19±0.14 (0.0-0.48)	0.08±0.10 (0.00-0.30)	0.77±0.81 (0.00-2.30)	0.85±0.88 (0.00-2.60)
P	p^a	0.123	0.015*	0.016*	0.021*	0.0001*	0.001*	0.365	0.0001*	0.0001*	0.002*	0.236	0.002*	0.015*	0.003*	0.001*
	p^b	0.0001*	0.016*	0.004*	0.019*	0.003*	0.003*	0.365	0.006*	0.024*	0.0001*	0.236	0.0001*	0.0001*	0.003*	0.002*
	p^c	0.006*	0.325	0.975	0.789	0.335	0.685	0.365	0.355	0.545	0.874	0.236	0.0001*	0.504	0.651	0.454

*Apoptotic Cells: Those with only annexin positivity; **Necrotic Cells: Those with both annexin and PI positivity; ***Dead Cells: Apoptotic and necrotic cells; p^a: Comparison between the patients and the control group (Kruskal-Wallis test); p^b: Comparison between the parents and the control group (Kruskal-Wallis test); p^c: Comparison between the patients and their parents (Kruskal-Wallis test).

†For CD95 and CD95 ligand.

††For annexin and propidium iodide.

G1 arrest and apoptosis were established in one patient's lymphocytes (ZG) and those of her parents while the cell cycle of lymphocytes in the sibling of ZG (patient AG) was normal. The cell cycles of the parents' granulocytes were normal (Figures 3A-3F).

Rapid Cell Senescence

Eight patients and eight parents were evaluated. The leukocytes of only 2 patients (siblings AG and ZG) and their mother were stained with SA-β-gal by 88%, 76%, and 94%, respectively (Figures 3G, 3H, 3I). These patients were members of a family and were evaluated for cell cycles at the same time (Figures 3A-3F).

Cell Morphology

Neutrophils

The incidences of bizarre nuclei (34.0±17.4% vs. 15.2±4.7%, p=0.015), pseudo Pelger-Huet (PPH) and PPH-like cells (15.7±9.3 vs. 4.1±3.3, p=0.003), striking chromatin clumping (38.1±27.7% vs. 11.0±9.7%, p=0.036), macropolycyte percentage (diameter ≥15 µm) (38.71±27.46% vs. 6.44±6.00%, p=0.0001) of neutrophils, and neutrophil diameter (13.46±1.76 vs. 9.79±1.73, p=0.0001) were significantly higher in the neutrophils of the parents than those of the control group by light microscope.

The few neutrophils of the patients in their peripheral blood and bone marrow also revealed the same abnormalities, but no scoring could be done due to the low number.

Ultrastructural study of the patients and parents revealed that secondary granules of neutrophils were low in number, primary granules were heterogeneous in shape and size, and chromatin clumping and apoptosis were striking (Figures 4D, 4E, 4L, 5D, 5E, and 5H).

Lymphocytes

The peripheral lymphocytes of both patients and parents revealed few lymphocytes with tiny cytoplasmic protrusions. Ultrastructural study of peripheral blood of patients and parents showed that the lymphocytes were abnormal or active (Figures 4G, 5F, and 5G).

Monocytes, Macrophages, Histiocytes, and Other Phagocytes

The peripheral blood of patients and parents revealed monocytes with features of apoptosis, abnormal nucleus, necrosis, or pseudopod formation under light microscope and abnormal mononuclear cells under TEM with or without features of apoptosis (Figures 4F and 5H).

The bone marrow examination of the patients revealed many monocytes, macrophages, histiocytes, neutrophils, bands, eosinophils, and eosinophil myelocytes undergoing phagocytosis. Sea blue-like and Gaucher-like histiocytes in the bone marrow of the patients were striking. The phagocytosed cells were lymphocytes,

Table 3. Laboratory parameters of patients.

Patient/Sex	Mutation*	Lymphocyte subsets (CD3, CD4, CD8, CD4/CD8, NK) as to age matched normal ranges	Dense granule / platelet† Bleeding time (in vitro)‡	Aggregation Defect
YF/7 3/12 y/ Male	*HAX1* Homozygous [c.130_131insA (p.W44†)]¶	Normal	2.95/ Col-epi: 155 s Col-ADP: 109 s	No secondary aggregation with ADP (2 and 6 µM); hypoaggregation with epinephrin (10 µM/mL); normal aggregation with collagen (1 µg/mL) and ristocetin (1.25 mg /mL)
MNY/7y/ Female	*HAX1* Homozygous [c.130_131insA (p.W44†)]¶	NK low, CD8 high, others normal	1.0/ Col-epi: 161 s	Disaggregation with ADP (10 µM); no aggregation with ristocetin (1.25 mg/mL); normal aggregation with collagen (1 µg/mL)
AY/4y/ Female (Sibling of MNY)	*HAX1* Homozygous [c.130_131insA (p.W44†)]¶	Normal	0.37/Col-epi: 147 s	Normal aggregation with collagen (1µg/mL) and ristocetin (1.25 mg/mL) (ADP and epinephrin not available)
MK/9y/Female (sibling of AY)	*HAX1* Homozygous *[c.130_131insA (p.W44†)]*	Not done	Not done/Not done	Hypoaggregation with ADP (10 µM) and collagen (1 µg/mL); normal aggregation with ristocetin (1.25 mg/mL) (epinephrin not available)
EC/8y/Female (cousin of HY)	*HAX1* Homozygous [c.130_131insA (p.W44†)]¶	NK low, others normal	3.36/Not done	No secondary aggregation with ADP (2 µM); hypoaggregation with ADP (10 µM); no aggregation with epinephrin (10 µM/mL) and ristocetin (1.25 mg/mL); normal aggregation with collagen (1 µg/mL)
HY/6y/Male (cousin of EC)	*HAX1* Homozygous [c.130_131insA (p.W44†)]¶	CD8 high, others normal	3.3/Not done	No secondary aggregation with ADP (2 and 6 µM); hypoaggregation with collagen (1µg/mL); no aggregation with epinephrin (10 µM/mL) and ristocetin (1.25 mg/mL)
OSK/14y/ Male (sibling of MeK)	*G6PC3* homozygous, c.194A>C (p.E65A) No mutation in *HAX1*¶¶/ *ELANE*	NK low, CD3 high, others normal	2.65/Not done	Normal aggregation with ADP (2 and 6 µM), epinephrin (10 µM/mL), collagen (1 µg/mL) and, ristocetin (1.25 mg/mL)
MeK/ 22y/ Female (sibling of OSK)	*G6PC3* homozygous, c.194A>C (p.E65A) No mutation in *HAX1/ ELANE*	NK low, CD3 high, others normal	0.83/ Not done	No secondary aggregation with 2µM ADP and disaggregation with 6µM ADP; no aggregation with epinephrin (10 µM/mL); normal aggregation with collagen (1 µg/mL) and ristocetin (1.25 mg/mL)
RT/8y/Male	*ELANE* Heterozygous *[c.597+5G>A]* No mutation in *HAX1*¶¶	Normal	1.4/Col-epi:126 s Col-ADP:100 s	Normal aggregation with ADP (2 and 6 µM), epinephrin (10 µM/mL), collagen (1 µg/mL), ristocetin (1.25 mg/mL)
NBÖ/10y/ Female	*ELANE* heterozygous c.416C>T (p.P139L) No mutation in *HAX1/ G6PC3*	Normal	3.55/Col-epi:120 s	No secondary aggregation with 2µM ADP but normal aggregation with 6 µM ADP; normal aggregation with epinephrin (10 µM/mL), collagen (1 µg/mL), and ristocetin (1.25 mg/mL)
BA / 8 y / Female	No mutation in *HAX1* / *ELANE* / *G6PC3* / *CSF3R*	CD3 low, CD4 low, others normal	0.58 / Col-ADP: 114 s	Disaggregation with 2 µM ADP but normal aggregation with 10 µM ADP; normal aggregation with collagen (2 µg/mL) and ristocetin (1.25 mg/mL) (epinephrine not available)
AG / 18/12 y / Male (sibling of ZG)	No mutation in *HAX1* / *ELANE* / *G6PC3* / *CSF3R* / *JAGN1*	CD3 low, others normal	2.4 / Col-ADP: 155 s	Disaggregation with 2 µM ADP but normal aggregation with 10 µM ADP; normal aggregation with collagen (2 µg/mL) and ristocetin (1.25 mg/mL) (epinephrine not available)
ZG / $3^{2/12}$ y / Female (sibling of AG)	No mutation in *HAX1*	Not done	1.17 / Col-epi: 119 s	Normal aggregation with ADP (2 and 6 µM), epinephrine (10 µM/mL), collagen (1 µg/mL), ristocetin (1.25 µg/mL)
AO / 12 y / Male	No mutation in *HAX1* / *ELANE* / *G6PC3* / *CSF3R* / *JAGN1*¶¶	Not done	1.73 / Col-epi: 213 s Col-ADP: 143 s	Normal aggregation with ADP (2 and 6 µM), epinephrine (10 µM/mL), collagen (1 µg/mL), and ristocetin (1.25 mg/mL)
KŞ / 12 y / Male§	Not done	Not done	Not done / Col-epi: 161 s Col-ADP: 79 s	No secondary aggregation with 2 µM ADP but normal aggregation with 6 µM ADP; hypoaggregation with collagen (1 µg/mL) and ristocetin (1.25 mg/mL)

*Peripheral blood cells; †Control values for dense granule / platelet: 2.78-3.82; ‡Normal values for in vitro bleeding time with collagen-epinephrine (Col-epi) cartridges: 85-157 s; with collagen-ADP (Col-ADP) cartridges: 65-125 s; §Lost to follow-up; ¶159T>C homozygous polymorphism in exon 2 of *HAX1* gene; ¶¶159T>C heterozygous polymorphism in exon 2 of *HAX1* gene; #new mutation (in submission).

Figure 2.A1

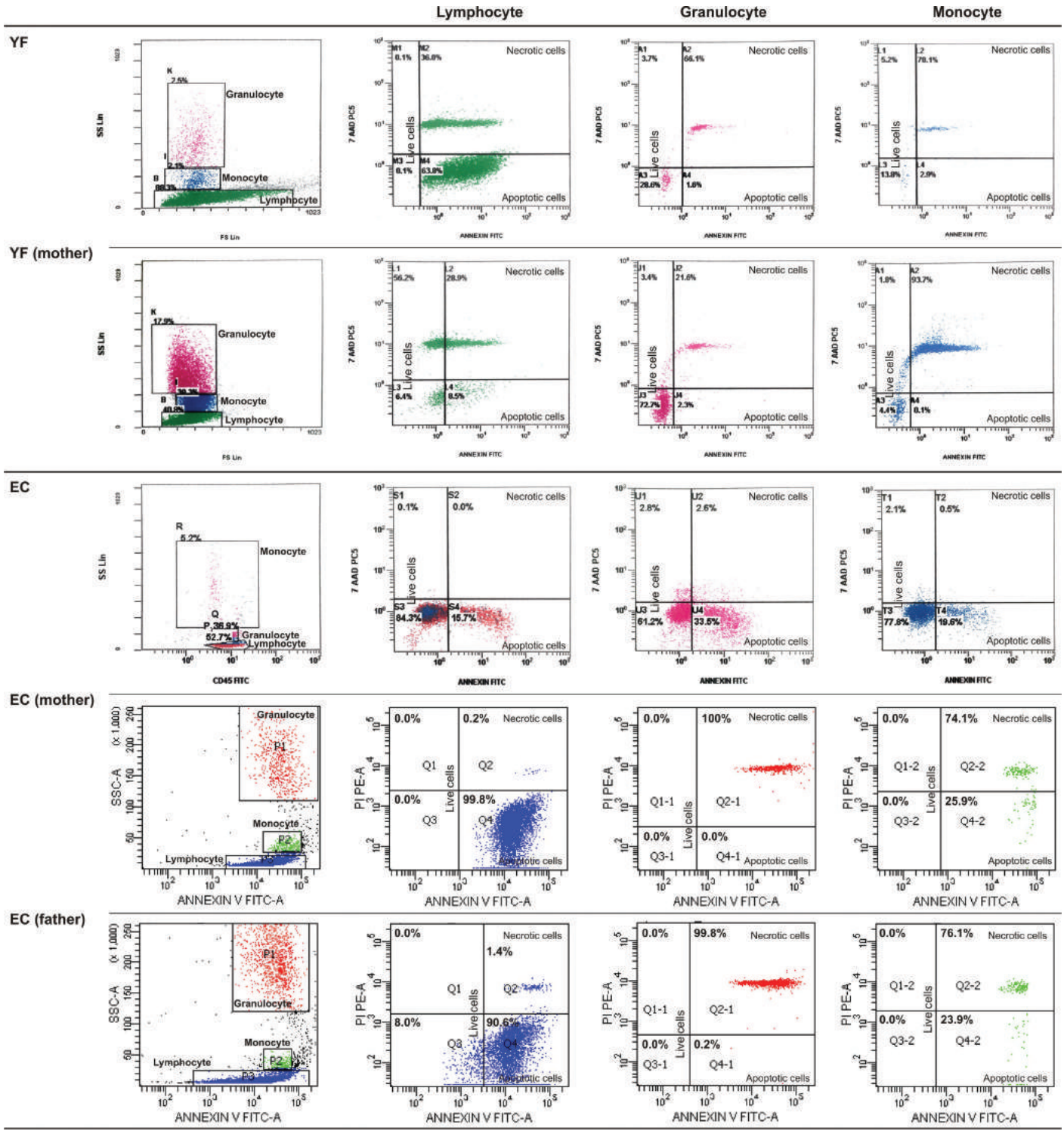

Figure 2. FACS gating and flow cytometric graphics of annexin and PI/7-AAD of lymphocytes, monocytes, and granulocytes of patients with congenital neutropenia and their parents. **2A1 and 2A2:** Those of patients with *HAX1* mutation (YF, EC, AY, MNY, HY) and their parents. The mother and father of AY, MY, and HY and the mother of EÇ were heterozygous for *HAX1*.

Figure 2.A2

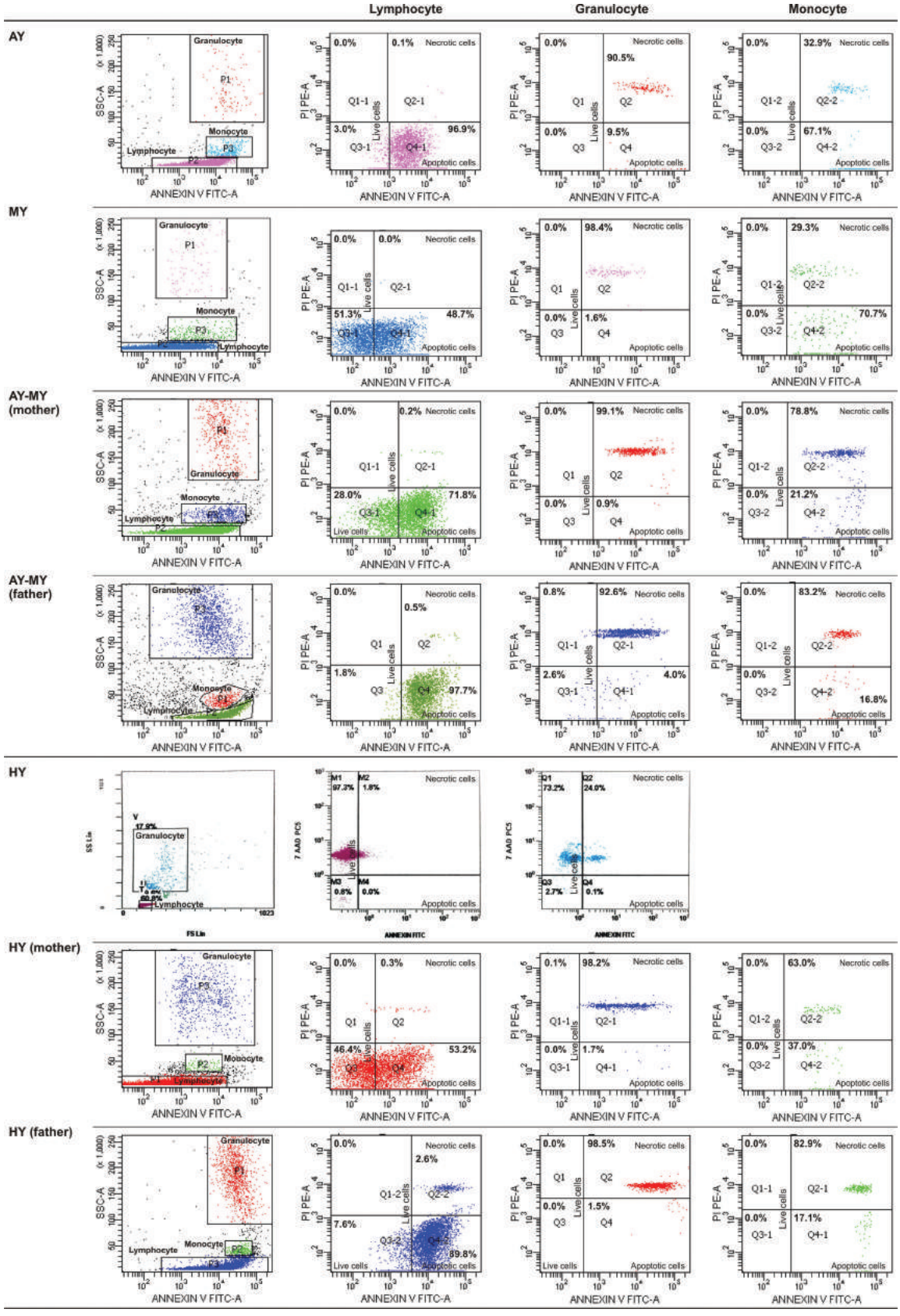

Figure 2. Continued. FACS gating and flow cytometric graphics of annexin and PI/7-AAD of lymphocytes, monocytes, and granulocytes of patients with congenital neutropenia and their parents. **2A1 and 2A2:** Those of patients with *HAX1* mutation (YF, EC, AY, MNY, HY) and their parents. The mother and father of AY, MY, and HY and the mother of EÇ were heterozygous for *HAX1.*

Figure 2.B

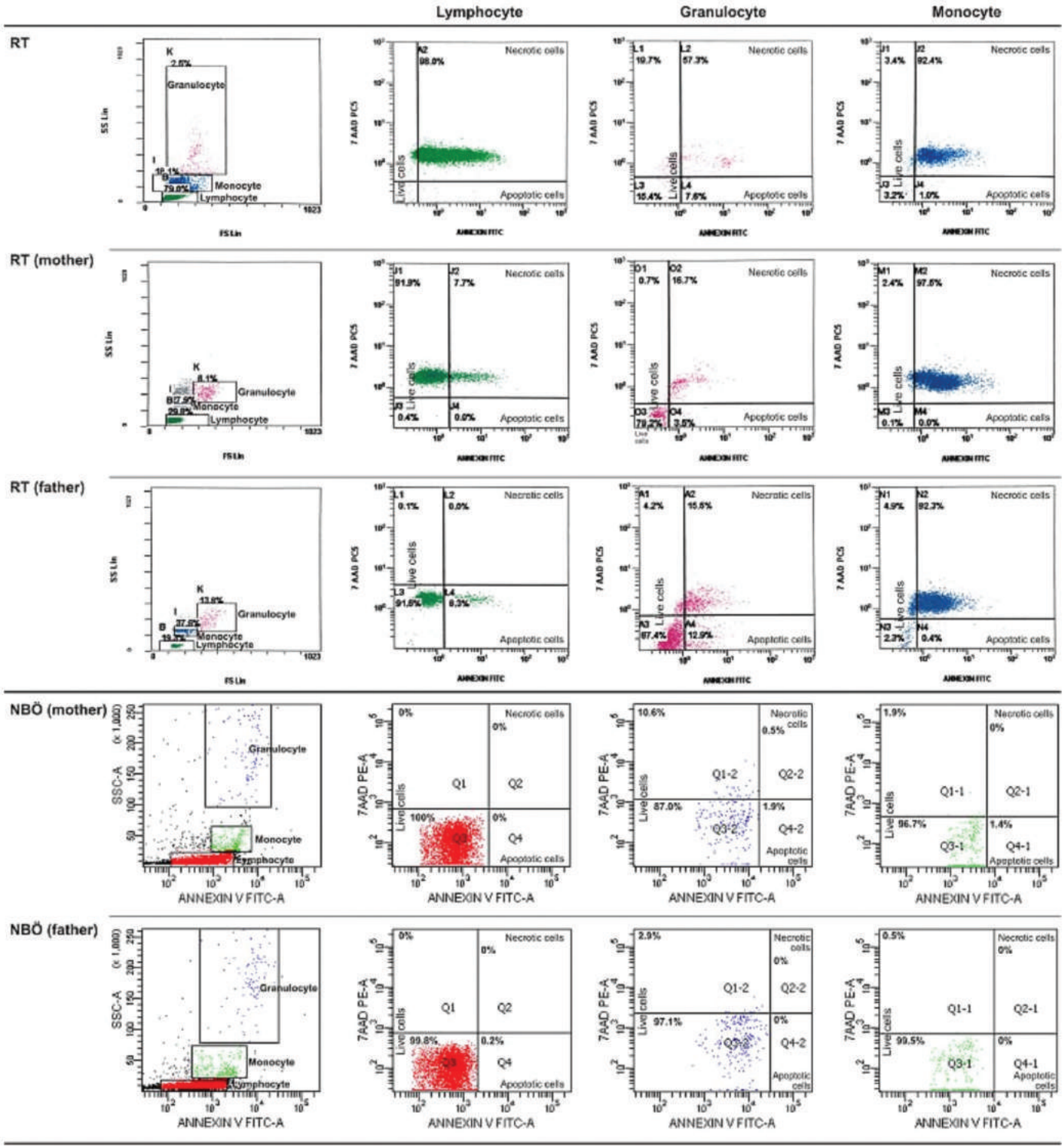

Figure 2. Continued. FACS gating and flow cytometric graphics of annexin and PI/7-AAD of lymphocytes, monocytes, and granulocytes of patients with congenital neutropenia and their parents. **2B:** Those of RT with *ELANE* mutation and his parents and parents of NBÖ with *ELANE* mutation. The cells of patient NBÖ could not be evaluated. Neither of the parents had *ELANE* mutation in peripheral lymphocytes or buccal mucosa.

Figure 2.C

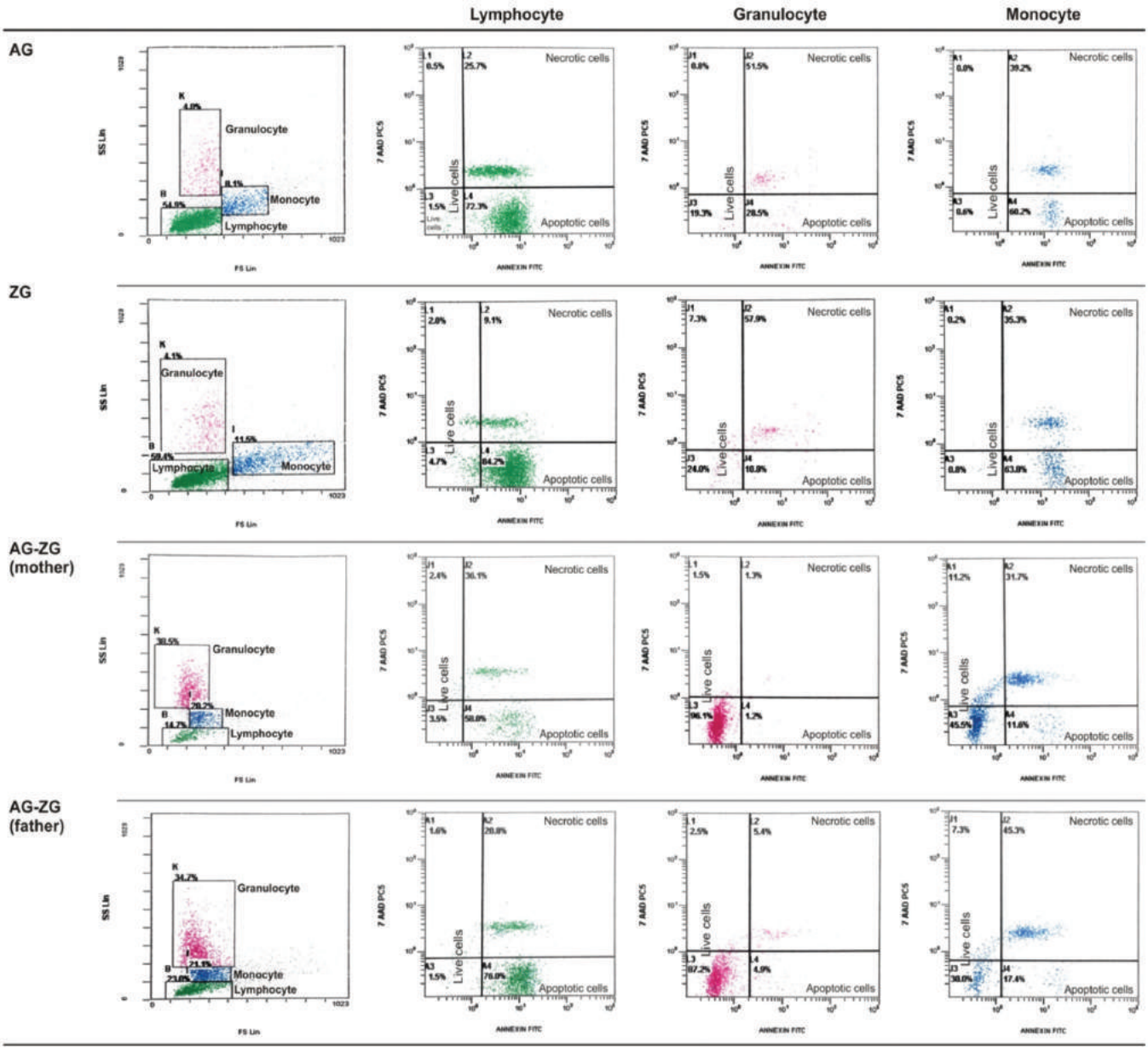

Figure 2.D

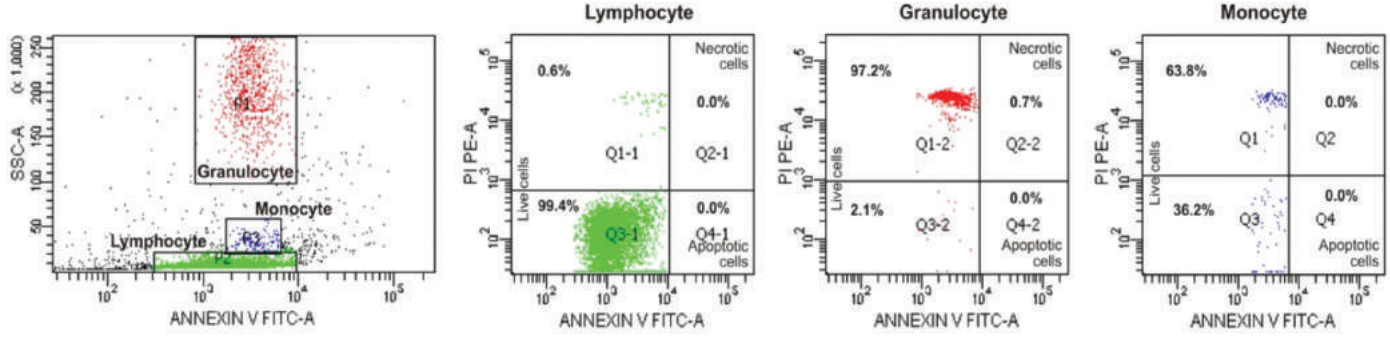

Figure 2. Continued. FACS gating and flow cytometric graphics of annexin and PI/7-AAD of lymphocytes, monocytes, and granulocytes of patients with congenital neutropenia and their parents. **2C:** Those of two siblings with congenital neutropenia with unidentified mutation and their parents (AG, ZG). **2D:** Those of one of the healthy volunteers.

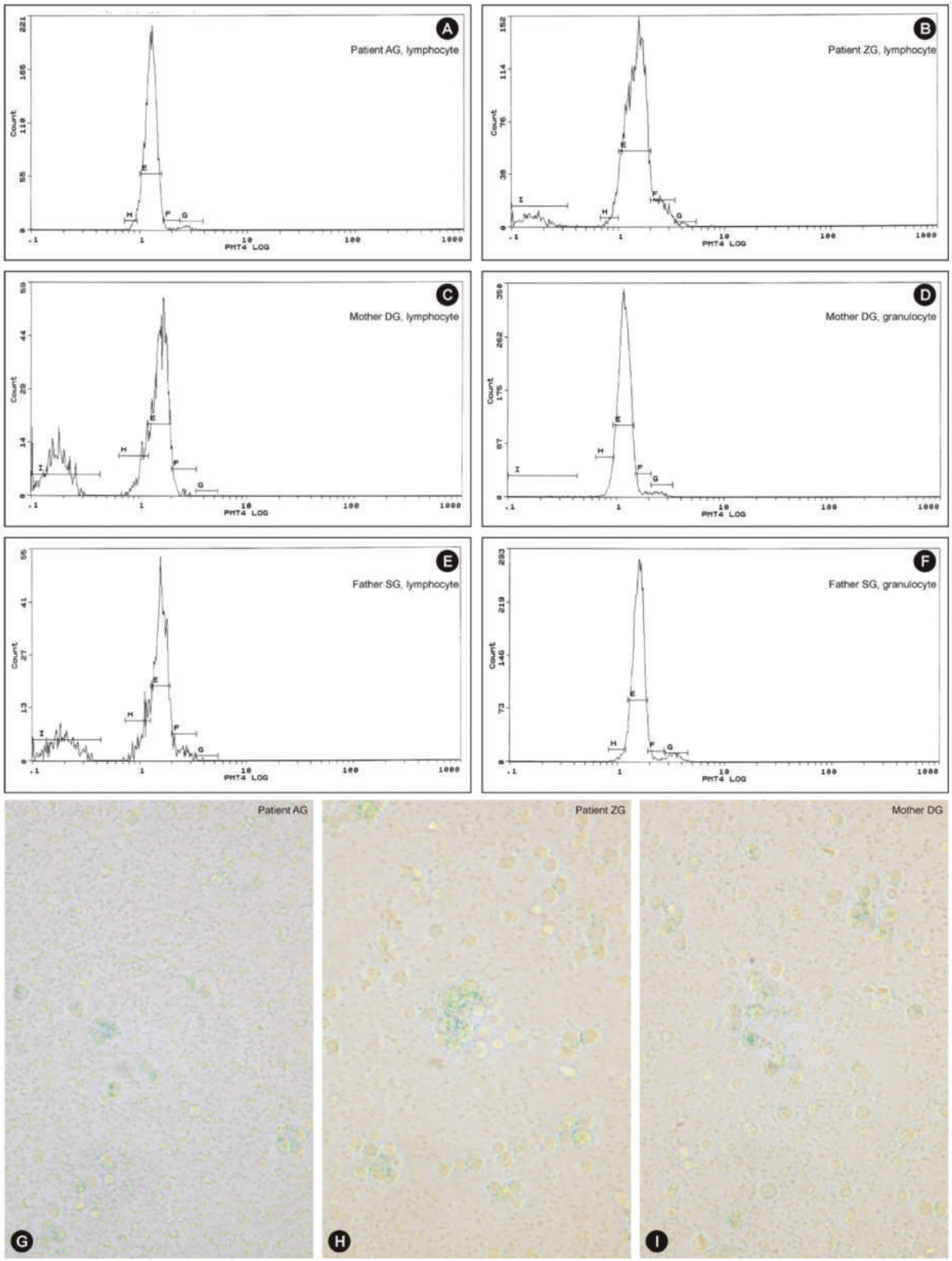

Figure 3. Cell cycle patterns of two sibling patients, their mother, and their father: A) normal cell cycle (patient AG, lymphocyte; sibling of ZG); B) G1 arrest and pre-G1 peak showing apoptosis (patient ZG; sibling of AG); C) G1 arrest and apoptosis (mother DG, lymphocyte); D) normal cell cycle (mother DG, granulocyte); E) G1 arrest and apoptosis (father SG, lymphocyte); F) normal cell cycle (father SG, granulocyte) (the patients' granulocytes could not be evaluated due to granulocytopenia). G-H-I: The leukocytes of two sibling patients and their mother stained by SA-β-gal, as blue granules, from peripheral blood culture (400ˣ). The leukocytes of patient AG (G), patient ZG (H), and their mother DG (I). These patients were members of a family evaluated for cell cycles.

erythroblasts, or apoptotic cells. Necrosis of cells that had phagocytosed other cells was also evident (Figures 6A, 6B, 6C).

Megakaryopoiesis

Megakaryocytes with asynchrony in nucleo-cytoplasmic maturation or those undergoing emperipolesis or transformed/transforming to naked megakaryocyte nuclei were striking. Additionally, naked megakaryocyte cytoplasm just after completing thrombocyte release, many megakaryoblasts, and necrotic, apoptotic, or dysplastic megakaryocytes were also seen in the bone marrow examinations of the patients (Figures 6A, 6B, 6C).

Thrombocytes and Thrombocyte Functions

Thrombocytes with heterogeneous size, abnormal shape, and/or giant forms were observed on the peripheral blood smears of both the parents and patients. Giant and dysplastic thrombocytes were also evident in many patients' bone marrow under light microscope (Figures 6A, 6B, 6C).

The mean dense granule number per thrombocyte was 2.01±1.19 (0.37-3.55) in the patients (n=12), 2.27±1.33 (0.22-4.47) in the parents (n=20), and 3.32±0.40 (2.78-3.82) in the healthy controls (n=5), and these were comparable with each other (p=0.147). However, the percentage of patients, parents, and controls who had fewer than 2 dense granules per thrombocyte was 50%, 35%, and 0% respectively (Supplemental Figure 1).

Ultrastructural examination showed that the thrombocytes had a reduced number of dense granules that were heterogeneous in size, shape, and composition. The open canalicular system (OCS) was enlarged and contained unevacuated components in patients (Figures 4H, 4I, 4J, 4K, and 7A - Case 2) and parents (Figures 5G and 5I).

In vitro bleeding time was prolonged in patients and parents by 37.5% and 18.8% with collagen-epinephrine cartridges and by 33.3% and 12.5% with collagen-ADP cartridges, respectively, vs. 0% in the control group with both cartridges. While in vitro bleeding times in patients and parents were comparable (p=0.293 and 0.233, respectively), only the in vitro bleeding time with collagen-ADP in patients was longer than in the control (p=0.031) (Supplemental Table 4).

Up to 63.6% and 44.4% of the aggregation results performed with various reactive substances in patients and their family members displayed abnormalities (Table 3; Supplemental Tables 5 and 6).

Thrombocyte aggregation at the 2nd, 8th, and 14th minutes under TEM (Figure 7) revealed a lack of adhesion and a lack of or inadequate secretion as also seen in Figures 4H, 4I, 4J, 4K, 5G, and 5I, with delayed or defective centralization, development of pseudopods, and/or secretion, abnormal degranulation, dissociation phenomenon [23,24], and abnormal amoeboid cytoplasmic protrusions (Supplemental Results).

There was inconsistency between the presence of hemorrhagic diathesis and abnormality of laboratory tests (aggregation tests, dense granule number in thrombocytes, in vitro bleeding time, thrombocyte ultrastructure) and vice versa. Not all these abnormalities coexisted all together (Table 3; Supplemental Table 6).

Genetic Mutations

Fourteen of 15 patients and 9 of 21 parents were evaluated for genetic mutations. Patients had homozygous [c.130_131insA (p.W44*)] mutation in the second exon of the *HAX1* gene (n=6), heterozygous *ELANE* mutations [c.597+5G>A and c.416C>T (p.P139L) (n=2)], and homozygous *G6PC3* mutation [c.194A>C (p.E65A), n=2], which is a novel mutation in the literature and is predicted to be disease-causing by SIFT and MutationTaster in silico analysis software [in submission]. Five had unidentified mutations. No tested patient had *CSF3R* mutation. *ELANE* c.597+5G>A splicing mutation was predicted to be disease-causing by NNSPLICE, GeneSplicer, and Human Splicing Finder in silico prediction tools.

Patients with *HAX1* displayed coexistent homozygous c.159T>C polymorphism in the second exon of the *HAX1* gene. Three patients with other mutations were heterozygous for this polymorphism (Tables 1 and 3). Both the mother and father of AY, MNY, and HY and the mother of EÇ were found heterozygous for *HAX1* [c.130_131insA (p.W44*)]. The parents of the two patients with heterozygous *ELANE* mutation revealed no mutation in the *ELANE* gene and their buccal mucosa cells did not reveal mosaicism.

Discussion

In this study, we showed that non-granulocytic blood cells were also affected and that morphologic and functional changes occurred in patients with SCN and in their non-neutropenic family members, and cell death mechanisms other than apoptosis also operated.

Apoptosis and Secondary Necrosis in Granulocytic and Non-Granulocytic Cells

It has been reported that in SCN and other neutropenic states, accelerated apoptosis of bone marrow granulocytic progenitor cells [1,2,6,25,26,27,28,29,30,31] and lymphocyte apoptosis [7,8] took place through different mechanisms. In our study, apoptosis was demonstrated not only in granulocytes and lymphocytes but also in monocytes by elevated annexin V, ultrastructural appearance, and a pre-G1 peak in cell cycle analysis. The absence of a pre-G1 peak is not enough to exclude

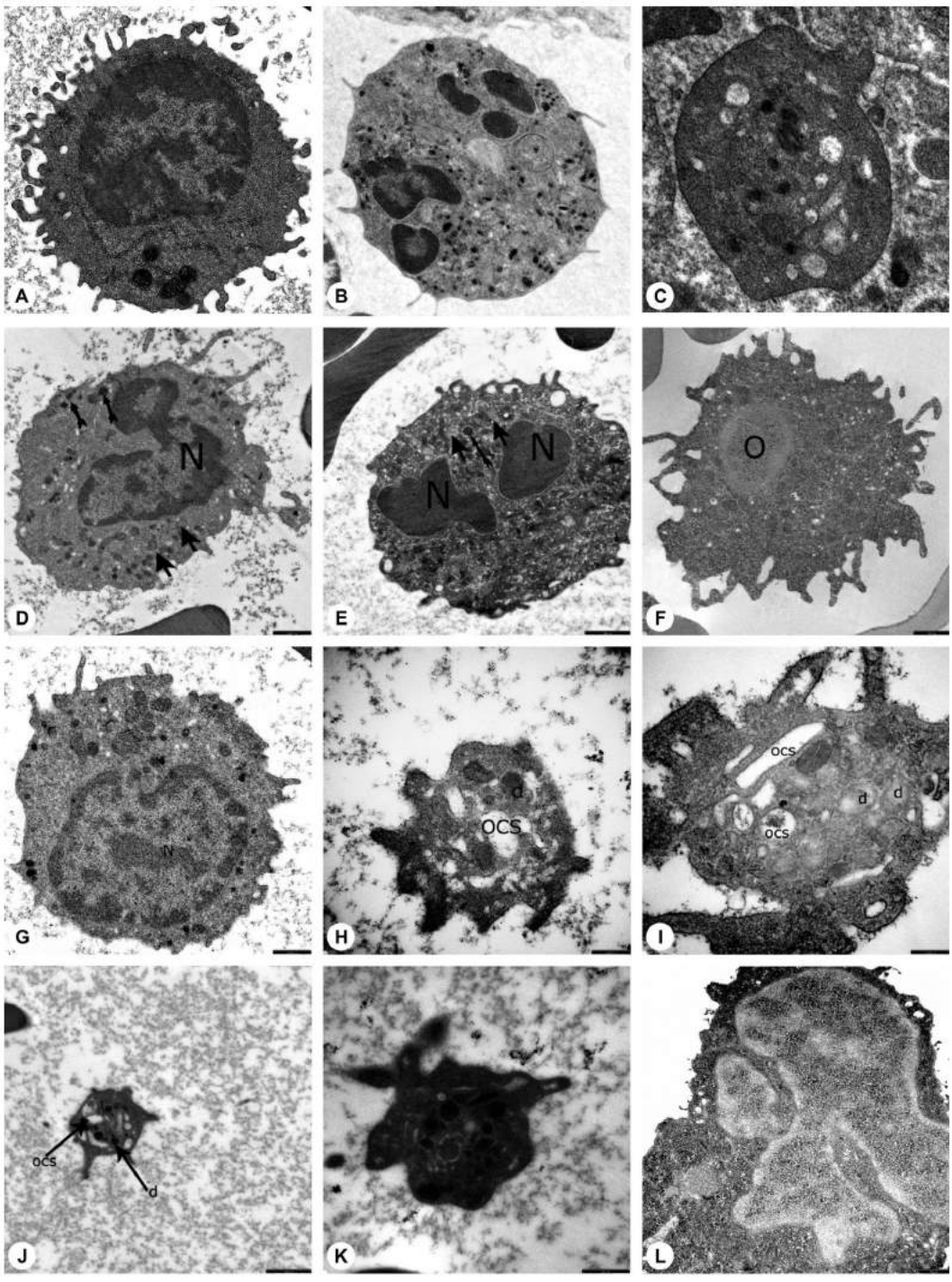

Figure 4. Electron microscopic images of the blood cells of the patients. A) Normal lymphocyte (16700^{x}), B) normal neutrophil (3597^{x}), C) normal thrombocyte (10000^{x}), D) patient EC (with *HAX1* mutation) (12930^{x}), E) patient NBÖ (with *ELANE* mutation) (12930^{x}), F) patient OSK (with *G6PC3* mutation) (10000x), G) patient EC (with *HAX1* mutation) (16700^{x}), H) patient EC (with *HAX1* mutation) (35970^{x}), I) patient MeK (with *G6PC3* mutation) (46460^{x}), J) patient MNY (with *HAX1* mutation) (12930^{x}), K) patient NBÖ (with *ELANE* mutation) (27800^{x}), L) patient MeK (with *G6PC3* mutation) (14000^{x}) (N: nucleus; thick arrow: primary granule; thin arrow: secondary granule; OCS: open canalicular system; d: dense granule; *: segment of non-apoptotic nucleus; arrow head: fusion of granules; O: autophagosome). Decreased number of secondary granules in the neutrophils (Figures 4D, 4E), which were abnormal in shape (4L). Primary granules that were irregular in shape (Figures 4E, 4L) or large (Figure 4D) and had a tendency to combine and condense (Figure 4E). Chromatin clumping in nuclei (Figure 4D) and apoptosis (Figure 4E). An active lymphocyte (Figure 4G). Abnormal giant mononuclear cell with abundant mitochondria and autophagic vacuole (Figure 4F). Dense granules in platelets, which were large and giant (Figure 4H), reduced in number (Figure 4H), in different shapes and dimensions (Figures 4H, 4J, 4K) with varying components (Figure 4I). Enlarged open canalicular system due to unevacuated ingredients (Figures 4H, 4I, 4J, 4K).

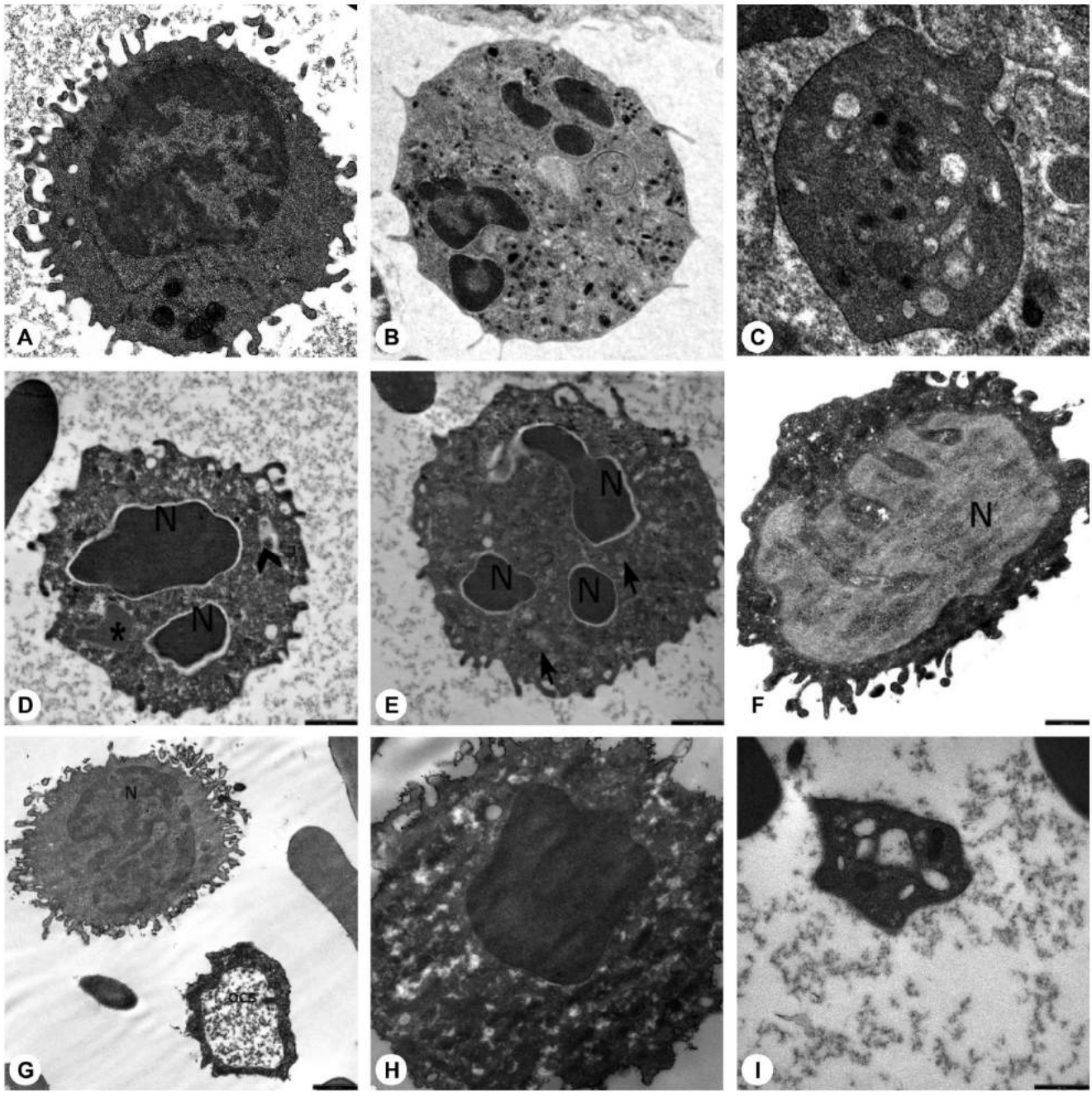

Figure 5. Electron microscopic images of the blood cells of the parents. A) Normal lymphocyte (16700^x), B) normal neutrophil (3597^x), C) normal thrombocyte (10000^x), D) mother of AO (with unidentified mutation) (SO) (12930^x), E) mother of AO (with unidentified mutation) (SO) (12930^x), F) father of MeK and OSK (with *G6PC3* mutation) (MK) (16700^x), G) father of MeK and OSK (with *G6PC3* mutation) (MK) (10000^x), H) father of MeK and OSK (with *G6PC3* mutation) (MK) (21560^x), I) father of MNY and AY (with *HAX1* mutation) (AhY) (27000^x) (N: nucleus; thick arrow: primary granule; thin arrow: secondary granule; OCS: open canalicular system; d: dense granule; *: segment of non-apoptotic nucleus; arrow head: fusion of granules; O: autophagosome). Decreased number of secondary granules in the neutrophils (Figures 5D, 5E). Primary granules in irregular shape (Figure 5D). Chromatin clumping in nuclei (Figure 5D) and apoptosis (Figures 5D, 5E, 5H). Abnormal lymphocytes (Figures 5F, 5G). Dense granules in platelets, which were large and giant (Figure 5I). Open canalicular system enlarged due to unevacuated ingredients (Figures 5G, 5H, 5I).

Figure 6.A

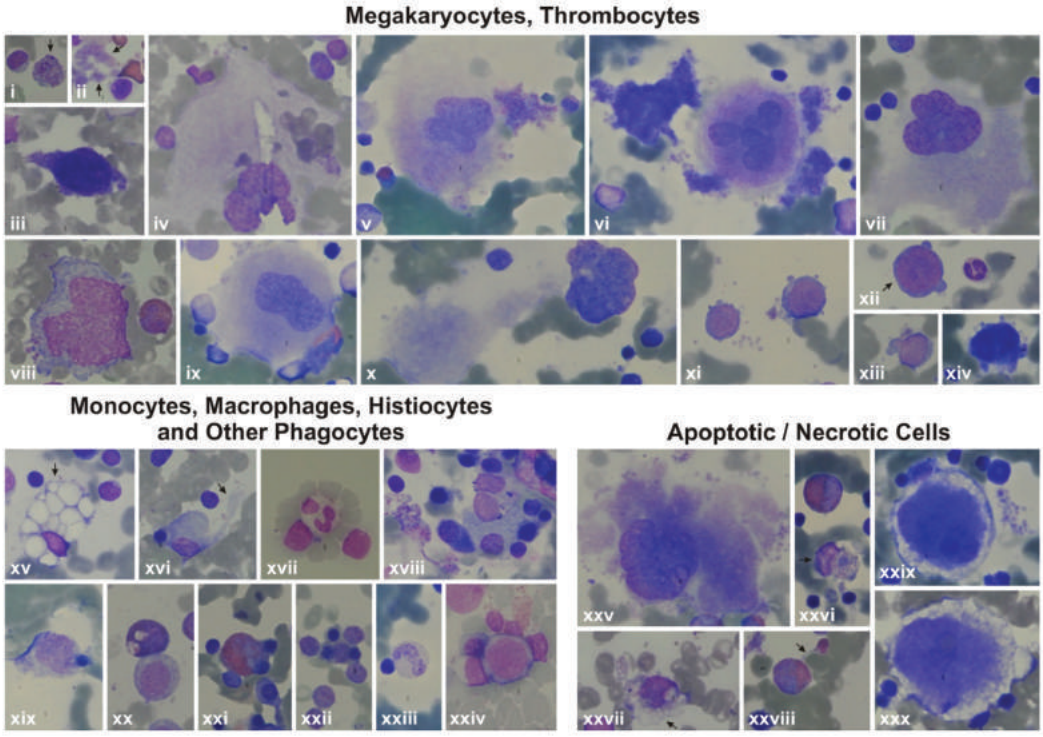

Figure 6.B

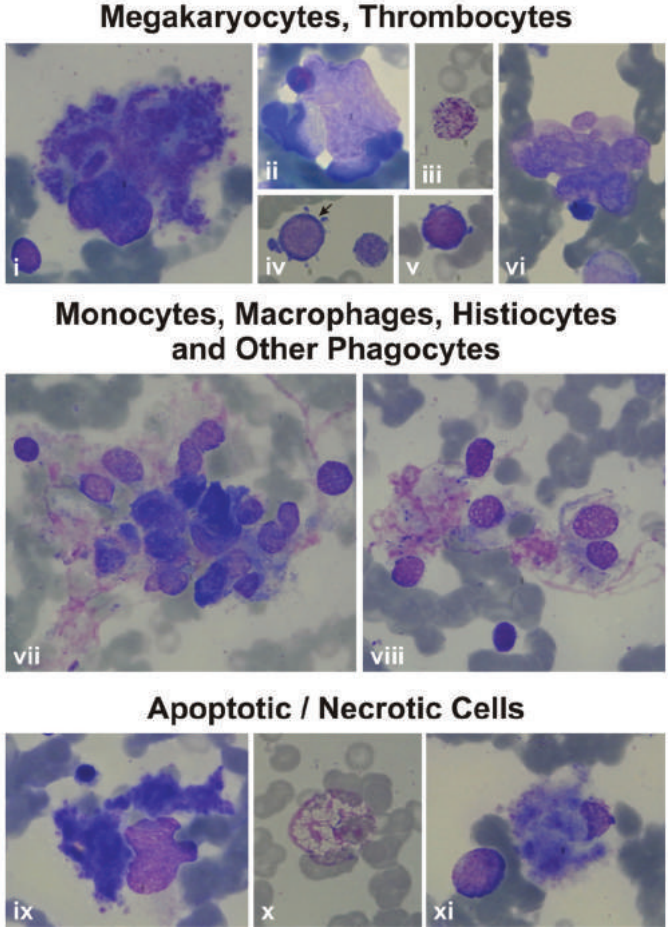

Figure 6.C

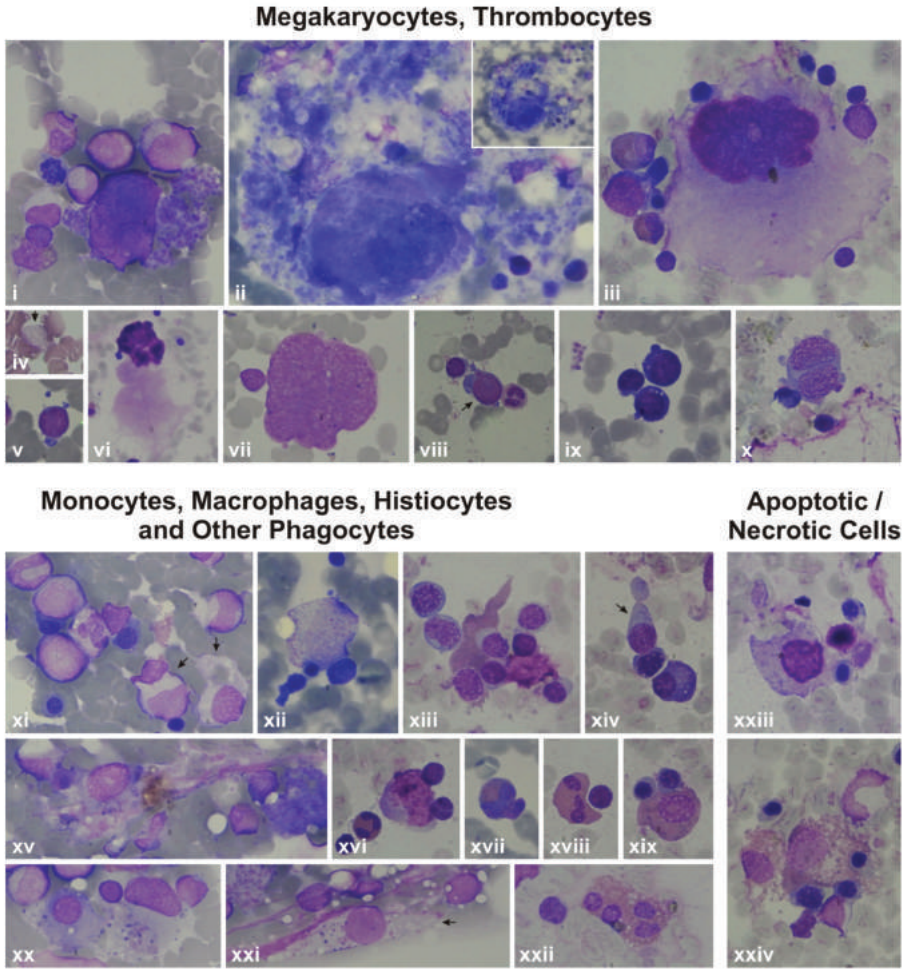

Figure 6. Features of some bone marrow cells (megakaryocytes, thrombocytes, monocytes, macrophages, histiocytes, other phagocytes, and apoptotic/necrotic cells) of the patients by light microscope. **6A:** Features of some bone marrow cells from the patients with *HAX1* mutation. **i, xxvii, xxviii**): Patient AY; ii) Patient EÇ; **iii, iv, viii, xvi, xxx**) Patient MNY; **v, vi, x, xii, xv, xvii, xviii, xxi, xxii, xxiii, xxiv, xxvi**) Patient MK; **vii, xiv, xxix**) Patient YF; **xi, xii, xiii, xix, xx, xxv**) Patient HY (1000^{x}). i) Giant thrombocyte; ii) dysplastic thrombocytes; iii, xi, xii, xiii, xiv) Megakaryoblasts with nucleo-cytoplasmic asynchrony; iv, v, vi, ix) senescent megakaryocytes undergoing the process of being naked megakaryocyte nucleus and naked megakaryocyte cytoplasm, just as producing (v, vi) or after completing production of thrombocytes (iv, vii, ix), with emperipolesis of other bone marrow cells (iv, ix); viii) Abnormal megakaryocyte; x) a megakaryocyte that has just developed into a naked megakaryocyte nucleus and naked megakaryocyte cytoplasm. xv) A macrophage full of fat (Gaucher-like cell); xvi, xix, xxii, xxiv) monocytes that are phagocytosing various mononuclear cells; xx) a monocyte phagocytosing an apoptotic cell; xvii, xxi, xxiii) other phagocytes like a neutrophil (xvii), eosinophilic myelocyte (xxi), and stab (xxiii) that are phagocytosing other bone marrow cells; xxv) A megakaryocyte undergoing necrosis; xxix, xxx) megakaryocytes undergoing apoptosis; xxvi) a monocyte that had performed phagocytosis and is undergoing necrosis; xxvii) a monocyte undergoing necrosis; xxviii) an eosinophilic myelocyte with an apoptotic body attached to the cell. **6B**: Features of some bone marrow cells from the patients with *ELANE* mutation. **i, iii, iv, vii, viii, ix, x, xi**) Patient NBÖ (1000^{x}); **ii, iv, vi**) Patient RT (1000^{x}). i) A degenerating dysplastic megakaryocyte; ii) a mononuclear megakaryocyte undergoing emperipolesis; iii) a giant thrombocyte; iv, v) megakaryoblasts with nucleocytoplasmic asynchrony; vi) naked megakaryocyte nuclei that could not transform to unique nuclei. vii, viii) Histiocytes that phagocytosed many bone marrow cells; ix) A megakaryocyte that is just about to undergo necrosis; x) a ghost-like cell degenerating through secondary necrosis; xi) a necrotic megakaryocyte. **6C:** Features of some bone marrow cells from the patients with unidentified mutations. **i, v, vii, viii, xi, xv, xx, xxi**) Patient AO; **iii, vi, ix, x, xiii, xiv, xvi, xviii, xix, xxii, xxiii, xxiv**) Patient ZG; iv) Patient BA; **ii, xii, xvii**) Patient KŞ (1000^{x}, except Figure vi, which is presented at 400^{x}). i) A megakaryocyte with nucleo-cytoplasmic asynchrony; ii, iii) senescent megakaryocytes undergoing the process of transformation to naked megakaryocyte nucleus and naked megakaryocyte cytoplasm, just as producing thrombocytes (ii) or after finishing production of thrombocytes (iii) or with emperipolesis (iii); iv) a giant thrombocyte; v, viii, ix) megakaryoblasts; vii) a megakaryocyte that is just transforming to naked megakaryocyte nucleus and naked megakaryocyte cytoplasm; vii) a naked megakaryocyte nucleus; x) a dysplastic megakaryocyte with two nuclei and scanty cytoplasm but thrombocyte production; xi, xii, xvi, xix) Monocytes (xi, xiv), macrophages (xii, xvi, xix) that had phagocytosed or are phagocytosing bone marrow cells with eosinophilic cytoplasm (xix) or pseudopods (xiv); xv, xx, xxi) histiocytes that have been phagocytizing bone marrow cells (xv, xx) and/or consist of basophilic debris, which gives the appearance of sea blue-like histiocytes (xv, xx, xxi); xvii, xviii) phagocytosis of other phagocytes like eosinophils (xviii) or eosinophil metamyelocytes (xvii); xxiii, xxiv) Macrophages with normal (xxiii) or eosinophilic (xxiv) cytoplasm that had performed hemophagocytosis and are undergoing necrosis now.

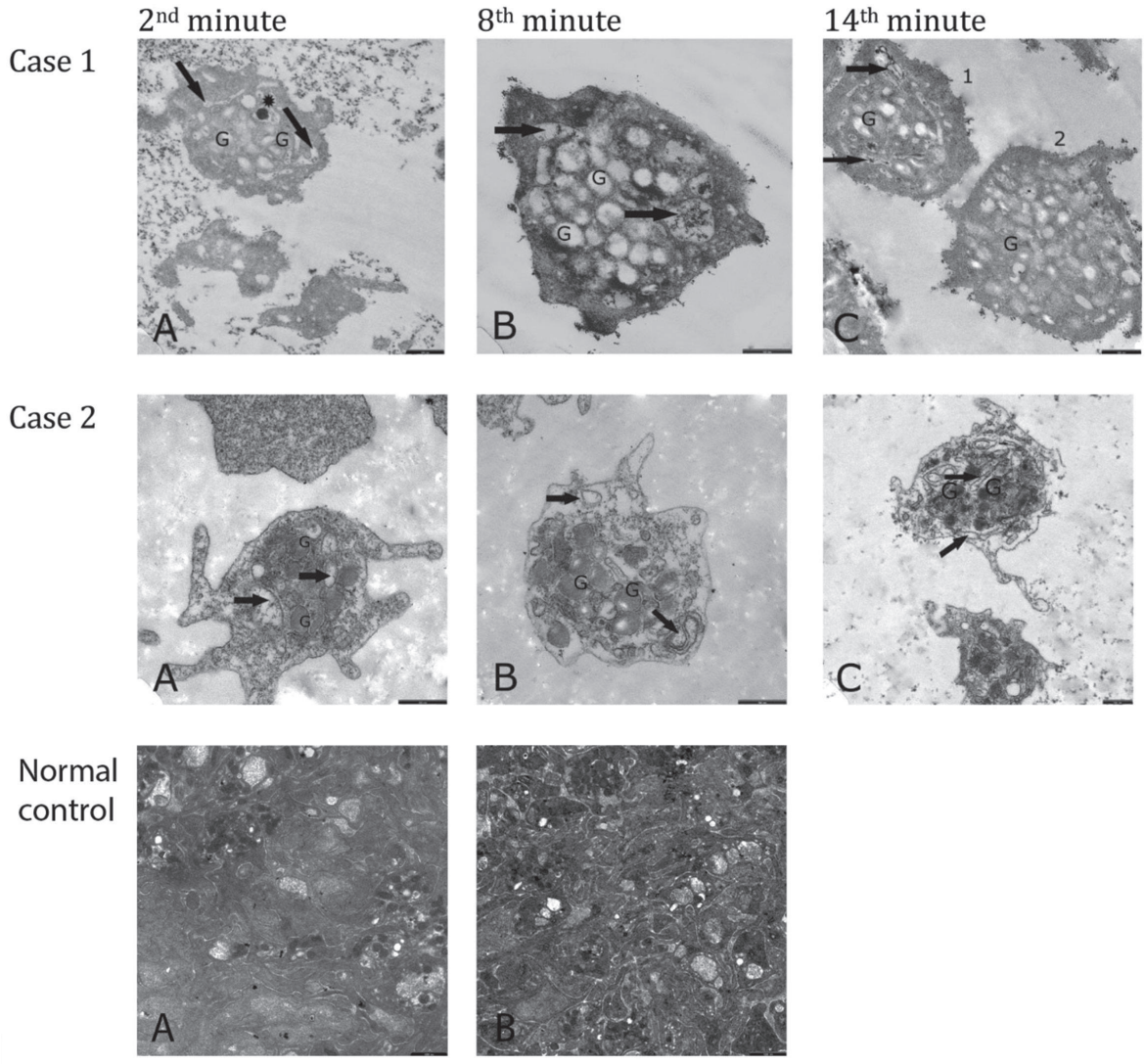

Figure 7. Electron microscopic images of the aggregating thrombocytes after addition of 2 µM ADP. All bars are 500 nm. Case 1 (patient BA, with unidentified mutation): **A)** 2nd minute of aggregation: Thrombocytes are seen to have become close to each other; the granules (G) have centralized; open canalicular system is enlarged (↑); one dense granule is visible in enlarged open canalicular system (*). **B)** 8th minute of aggregation: Platelets are still distant from each other. There are no platelets that fit tightly to each other. However, the granules (G) have centralized and have discharged their ingredients. Open canalicular system is enlarged and consists of residual secretion (↑). There are a few granules that have not evacuated their ingredients yet. **C)** 14th minute of aggregation: The platelets are seen to have become closer but they are still apart from each other. The open canalicular system is enlarged and consists of some secretion (↑). There are and there are not undischarged granules (G) in thrombocytes 1 and 2, respectively. Case 2 (Patient ZG, with unidentified mutation): **A)** 2nd minute of aggregation: Thrombocytes are seen to be apart from each other; the granules (G) have fairly centralized and are intact. **B)** 8th minute of aggregation: The granules (G) are larger than normal and increased in number. They have centralized but have not discharged their contents yet. Pseudopods have developed. The open canalicular system has not enlarged yet (↑). **C)** 14th minute of aggregation: The thrombocytes have not adhered to each other yet. The dense granules are distributed throughout the cytoplasm but the majority have not discharged their contents yet. Upper thrombocyte: The granules (G) are very large and increased in number. None of them have evacuated their contents. Open canalicular system (↑) is apparent. Normal Control: **A)** 2nd minute of aggregation: Thrombocytes are seen to fit tightly together and display abundant pseudopods. They have released almost all of their granules. **B)** 8th minute of aggregation: Thrombocytes fit tightly together. They have degranulated completely, except a few.

apoptosis [32]. Inconsistent elevations in CD95 and CD95 ligand pointed at a non-CD95-mediated apoptosis.

Only in the case of excessive apoptosis, during which the capacity of phagocytes to engulf apoptotic cells is reduced, do the uncleared apoptotic cells and fragments undergo secondary necrosis (delayed apoptotic clearance), which can provoke inflammation [33]. Our flow cytometric and microscopic findings revealed that apoptotic and necrotic cells coexisted in three cell lines in patients irrespective of the type of SCN mutation, and in their parents.

We think that in our patients apoptotic and necrotic cells in the myeloid lineage activated macrophages and other phagocytes extensively (Figures 6A, 6B, 6C), inducing secretion of TNF-alpha, IL-1, IL-6, and IL-12 by activated macrophages, the latter exacerbating macrophage activation through stimulating IFN-gamma production [34,35]. High levels of TNF-alpha [36,37] in SCN patients and their non-neutropenic parents [36] and increased capacity of stimulated monocytes to produce TNF-alpha on stimulation through certain toll-like receptors [38,39] were reported before.

We consider the apoptosis in the non-granulocytic cells to be due to the high TNF-alpha levels, which can give rise to apoptosis in neutrophils [40,41], lymphocytes [42,43], monocytes [44], and thrombocytes [45] in various conditions [46] through TNF alpha-TNFR1 interaction (Supplemental Discussion, Text 1).

Cytopenia in Non-Granulocytic Cells and RCS

Absence of lymphopenia (except 1 case), monocytopenia, and thrombocytopenia (Table 1; Supplemental Table 2) is apparently due to good compensation of the bone marrow of both the patients and the parents.

However, it was striking that both patients and their parents had quantitative abnormalities in T lymphocytes and NK cells, regardless of the type of the SCN mutation carried by the patients.

Abnormalities in B, cytotoxic T, NK, NKT, Th2, and Th7 cells were reported in SCN with *GFI-1* mutation, albinism-neutropenia syndromes, and Wiskott-Aldrich syndrome (WAS) [2,47,48,49,50,51]. None of our cases were clinically compatible with WAS or albinism-neutropenia syndromes. However, 2 SCN patients with *WAS* mutation were reported to have a reduced number of NK and CD4+ cells [7]. Interestingly, SCN patients with *ELANE* and unidentified mutations were reported to have normal numbers of NK cells that were less mature than those of normal controls [52]. NK cell deficiency and dysfunction was reported in some chronic neutropenia patients with morphological abnormalities [53]. In our cases, patients with NK levels lower than the normal range for age had *HAX1* (n=2) and *G6PC3* (n=2) mutations while patients with low CD3 (n=2) and CD4 (n=1) levels had unidentified mutations (Table 3). Mature neutrophils are reportedly necessary for NK cell development [52]. Reduced mature neutrophils may account for low levels of NK cells in the patient group but the reasons why not all SCN patients had low NK cells and why the parents who had low NK levels were not neutropenic require further investigations.

That the patients with low CD3+ and CD4+ lymphocytes (AG, ZG) were those with β-gal positivity of leukocytes suggests that the continual presence of circulating pro-inflammatory factors secreted by activated macrophages kept the immune system in a state of chronic low-level activation, giving rise to immunosenescence through loss of telomeric DNA with each S phase and therefore a decline in the number of T lymphocytes and no change or decline in overall lymphocyte and NK cells [54] during which inflammatory mediators secreted by senescent cells themselves contributed to immunosenescence [55,56] (we could not evaluate B lymphocytes) (Supplemental Discussion, Text 2).

The presence of apoptosis together with RCS was reported in SCN [8] and in cell lines that had been administered cytotoxic drugs [57]. These cases (ZG and parents) in which individuals had not consumed cytotoxic drugs and were exposed to radiation may carry an unknown DNA-disrupting factor.

Cellular senescence is the state of irreversible cell cycle arrest, predominantly in the G1 phase [57,58,59,60,61,62,63,64], being dependent on (replicative senescence) or independent of telomeres (RCS) [60,61]. The latter is due to inappropriately expressed pro-proliferative genes [63], oncogenic mutations [62], DNA-damaging drugs, or gamma irradiation [57,58].

The senescence-like phenotype is characterized by reorganization of heterochromatin [65,66], formation of fragmented nuclei, polyploidy, and enlarged and flattened cell shape, along with expression of SA-β-gal positivity [15,57] and alterations in the cell cycle [65].

Dysplasia of Hematopoietic Cells

In our study, as reported previously [8,10], dysplasia was noted not only in the neutrophil series but also in the monocyte, megakaryocyte, lymphocyte, and eosinophil series in all patients and parents to various degrees. Some dysplasia parameters overlap with the senescence phenotype; however, restriction of RCS to a few cases in our study showed that RCS only partially affected the development of dysplasia. Our previous studies point at the role of inflammatory cytokines to cause dysplasia [36,37] in patients with autoimmune disorders, acute infections, and hemophagocytic histiocytosis [67,56]. The pro-inflammatory cytokines secreted by activated

macrophages can destroy the bone marrow microenvironment and hematopoietic stem cell niches by activating innate immune cells [55] and give rise to hematopoietic stem cell dysfunction, dyshematopoiesis, and thus dysplastic hematopoietic cells.

Morphologic abnormalities due to abnormal differentiation in myeloid cells are also encountered in congenital, cyclic, dysgranulopoietic neutropenia cases [7,10,66,68] with or without the *WAS, GFI-1,* and *G6PC3* mutations [1,7], in myelodysplastic syndrome, and in a number of non-malignant disorders [16,67].

Dysmegakaryopoiesis and Hemorrhagic Diathesis

That the presence of nearly no normal megakaryocytes in our patients and that nearly all megakaryocytes displayed characteristics of naked megakaryocyte nuclei, emperipolesis, or abnormal morphology like peripheral vacuolization (showing non-classical apoptosis: para-apoptosis) and directly destructed megakaryocytes (showing necrosis) and presence of many stage 1 megakaryocytes (megakaryoblasts), some of which were aberrantly releasing thrombocytes, imply defective megakaryocyte maturation, heavy intramedullary premature cell death of megakaryocytes, and increased megakaryopoiesis [14,69,70,71,72,73] (Supplemental Discussion, Text 3).

Defective maturation in megakaryocytes is also expected to be due to the increased levels of pro-inflammatory cytokines, which can destroy the bone marrow microenvironment and hematopoietic stem cell niches [55]. Therefore, thrombocytes derived from megakaryocytes with defective maturation are also expected to be functionally abnormal.

Hence, in our cases, we noted a combination of thrombocyte functional defects and in a few of them a low number of mepacrine-labeled dense granules reminiscent of a delta storage pool defect. The ultrastructural view of aggregating thrombocytes (Figure 7; Supplemental Discussion, Text 3) may reflect defective transmission. From all these aspects, the findings of our patients resemble the thrombocyte disorders encountered in leukemia, refractory anemia, cystinosis, and others [19,23,24,74,75].

Hemorrhagic diathesis is a common finding of albinism-neutropenia syndromes like Chediak-Higashi syndrome, Hermansky-Pudlak syndrome type 2, Griselli's syndrome type 2, Cohen's syndrome, and p14 deficiency [51] but has not been reported in SCN [76] before.

Our results, at the same time, confirmed that not all patients with thrombocyte aggregation defects display laboratory evidence [18,77,78] and the most reliable tool to show thrombocyte aggregation defect is electron microscopic evaluation [78,79,80].

Parents

No parent had cytopenia; however, apoptosis and secondary necrosis to various degrees in granulocytes, monocytes, and lymphocytes with the presence of dysplasia, decreased NK cells, and abnormalities in thrombocyte functions in most of the parents and RCS in one suggest that the parents were also affected by the same genetic abnormality but the cell loss was well compensated by the proliferating compartment. However, only the parents of patients with *ELANE* and those of most patients with *HAX1* could be evaluated genetically.

For the parents of the patients with homozygous *HAX1* mutation (AY, MNY, HY, EÇ) who were heterozygous for the same mutation, we think that cell loss took place through one mutant allele, just like in their children. The apoptotic hematopoietic cells (lymphocytes, neutrophils, monocytes) were the mutant cells that were lost early [81,82], but normal hematopoiesis compensated for the cell loss when the other allele was normal.

As for the parents of patients with *ELANE* mutation (RT, NBÖ), the absence of *ELANE* mutation in the parents' peripheral blood cells led us to consider that the parents were either mosaic for the mutation or actually normal and their children were sporadic cases of *ELANE* mutation. Hence, a number of phenotypically healthy parents were shown to harbor somatic [83], only germline [3], or both somatic and germline [84] mosaicism of *ELANE* mutation. On the other hand, most of the sporadic cases of SCN were reported to have *ELANE* mutations [85].

That we could not detect mosaicism in the buccal mucosa cells of the parents does not rule out mosaicism definitively. A search for mutant alleles in various other cell types like skin or sperm of the father, preferably using more sensitive mutation analysis methods, might have proved mosaicism, like in the reported cases [3,86] in which the mutant allele was negative in DNA from neutrophils, buccal mucosa, and/or lymphocytes and was detected only in spermatozoa. However, the parents felt uneasy about being tested any further.

Additionally, that the parents of patient RT had normal blood cell counts but high cell death parameters in lymphocytes, granulocytes, and monocytes like the parents with heterozygous *HAX1* suggested that they were very probably affected by the same mutation in the same gene, *ELANE*, as their children. However, we cannot exclude the possibility that patient RT was a sporadic case of *ELANE* mutation and did not additionally harbor any other untested/unidentified neutropenia mutation [87] and that his parents were carriers of this mutation. On the other hand, similar death parameters in the hematopoietic cells of the parents of NBÖ to those of the controls suggested that the *ELANE* mutation in NBÖ could be sporadic. As a second possibility, both of the parents of NBÖ might have cyclic hematopoiesis with consecutive normal and abnormal

hematopoiesis [88], just like in the mother of a patient with *JAGN1* mutation who we followed before [8,10, unpublished data]. However, we could not exclude an unidentified mosaicism for the parents of NBÖ definitively due to the same reasons.

As for the parents of patients with *G6PC3* (OSK, MeK) and unidentified mutations (AO, BA, AG, ZG, KŞ), only two siblings with unidentified mutation (AG, ZG) and their parents could be evaluated for cell death parameters and both the parents' blood cells (lymphocytes, granulocytes, monocytes) showed apoptosis and necrosis similar to that of their children. Therefore, we think that the parents of patients with other recessive SCN gene mutations (like *G6PC3* and at least some of the unidentified mutations) might be heterozygous for the same genetic defect, like in the parents who were heterozygous for *HAX1*.

We think that the modifying effects of other genes or factors [3,89] and any other accompanying neutropenia mutations [87] and many other factors that play roles in the transmission of disease, including the 159T>C polymorphism in the same exon of *HAX1* mutation in patients with *HAX1* and other mutations as in other cases [90,91], both in the patients and parents, need to be evaluated in further studies.

The gingival enlargement and oral aphthae of the parents in the present study were thought to be possibly due to dysfunctional neutrophils, which were dysplastic at the same time, like a non-neutropenic mother of a patient with *JAGN1* mutation who we followed before and had apoptosis in addition to morphological and functional abnormalities in neutrophils, lymphocytes, and thrombocytes with low levels of myeloperoxidase and defective chemotaxis [8,10, unpublished data].

Easy bruising and gingival bleeding of the parents were attributed to defective thrombocyte functions stemming from defective megakaryopoiesis.

We attribute apoptosis and secondary necrosis in the myeloid lineage of the non-neutropenic parents to the aforementioned genetic abnormalities relevant to SCN, while those in the non-granulocytic cells to the high TNF-alpha levels [36] that can give rise to apoptosis in blood cells [40,41,42,43,44,45,46], as discussed for children with SCN.

Conclusion

Apoptosis and secondary necrosis in non-granulocytic cell lines, dysplasia of blood cells with/without RCS, and disturbances in lymphocyte subsets and thrombocyte functions were observed in patients with congenital neutropenia and their non-neutropenic parents. Additionally, bone marrow of the patients showed increased phagocytic activity and striking dysmegakaryopoiesis. (Table 4). This study shows that abnormalities in lymphocyte subsets and hemorrhagic diathesis are not restricted to albinism-neutropenia syndromes, as current wisdom holds, but are also encountered in SCN.

Table 4. Summary of the findings.

• In our study, 15 patients with congenital neutropenia (6 had *HAX1*, 2 *G6PC3*, 2 *ELANE*, 5 unknown mutations) and 21 parents (5 had heterozygous *HAX1* mutation) were evaluated.
• Significant increase in apoptosis and secondary necrosis in monocytes, lymphocytes, granulocytes of the patients and their parents was detected by flow cytometry irrespective of the type of congenital neutropenia mutation.
• Rapid cell senescence was additionally shown in 25% and 12.5% of patients and parents respectively.
• Dysplasia was evident in neutrophils, monocytes, lymphocytes, thrombocytes in both patients and parents, by light and electron microscope.
• Bone marrow of the patients showed increased phagocytic activity, striking dysmegakaryopoiesis, necrotic and apoptotic cells.
• CD3 and CD4 lymphocytes and NK cells were decreased in 16.6%, 8.3%, and 36.4% of the patients and 0%, 0%, and 15.4% of parents (controls: 0%, 0%, 5.6%).
• The percentages of patients, parents, and controls with fewer than 2 dense granules/thrombocytes were 50%, 35%, and 0%, respectively.
• In vitro bleeding time was prolonged by 37.5% and 33.3% in patients, and 18.8% and 12.5% in parents with different cartridges.
• Up to 63.6% and 44.4% of the aggregation tests displayed abnormality in patients and parents.
• Electron microscopic evaluation of thrombocyte aggregation revealed abnormality in two evaluated patients.

Moreover, our findings suggest that the pluripotent hematopoietic stem cells in SCN are defective irrespective of the genetic etiology, in contrast to the current thinking that understands the main defect as residing in the progenitor myeloid cells [1,2,6], and myeloid transcriptional factors [92,93].

Study Limitations

The main limitation of our study was that not all sub-studies could be performed for all cases due to daily limitations of our laboratory facilities. Including idiopathic neutropenic patients as a separate control group could have helped evaluate the results more extensively, although these patients were beyond the scope of this study. In spite of this, we believe that our results may lead to further in vivo and in vitro studies involving pluripotent hematopoietic stem cells in SCN so as to better understand the underlying physiopathology. Additionally, the presence of the same abnormalities in non-neutropenic parents shows that the phenotype-genotype relationship is another field that requires further evaluation.

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Ethics

Ethics Committee Approval: Hacettepe University Ethics Committee (Number TBK 05/1-2).

Informed Consent: Informed consent was obtained from all individual participants included in the study.

Authorship Contributions

Surgical and Medical Practices: L.O., Ş.Ü.; Concept: L.O., S.Y.; Design: L.O., S.Y.; Data Collection or Processing: L.O., Ş.Ü., A.M., Y.Y.; Analysis or Interpretation: L.O., H.O., E.E., A.Ö., D.B., H.O., Y.B., A.İ., M.F., G.Ö., S.Y.; Literature Search: L.O., H.O.; Writing: L.O., S.Y.

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Conflict of Interest Statement: The authors of this paper have no conflicts of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

References

1. Özbek N. New insights into the genetics of congenital neutropenia. Turk J Hematol 2009;26:1-8.
2. Klein C. Genetic defects in severe congenital neutropenia: emerging insights into life and death of human neutrophil granulocytes. Ann Rev Immunol 2011;29:399-413.
3. Newburger PE, Pindyck TN, Zhu Z, Bolyard AA, Aprikyan AAG, Dale DC, Smith GD, Boxer LA. Cyclic neutropenia and severe congenital neutropenia in patients with a shared *ELANE* mutation and paternal haplotype: evidence for phenotype determination by modifying genes. Pediatr Blood Cancer 2010;55:314-317.
4. Donadieu J, Fenneteau O, Beaupain B, Mahlaoui N, Chantelot CB. Congenital neutropenia: diagnosis, molecular bases and patient management. Orphanet J Rare Dis 2011;6:26.
5. Boztug K, Järvinen PM, Salzer E, Racek T, Mönch S, Garncarz W, Gertz EM, Schäffer AA, Antonopoulos A, Haslam SM, Schieck L, Puchałka J, Diestelhorst J, Appaswamy G, Lescoeur B, Giambruno R, Bigenzahn JW, Elling U, Pfeifer D, Conde CD, Albert MH, Welte K, Brandes G, Sherkat R, van der Werff Ten Bosch J, Rezaei N, Etzioni A, Bellanné-Chantelot C, Superti-Furga G, Penninger JM, Bennett KL, von Blume J, Dell A, Donadieu J, Klein C. *JAGN1* deficiency causes aberrant myeloid cell homeostasis and congenital neutropenia. Nat Genet 2014;46:1021-1027.
6. Aprikyan AA, Liles WC, Boxer LA, Dale DC. Mutant elastase in pathogenesis of cyclic and severe congenital neutropenia. J Pediatr Hematol Oncol 2002;24:784-786.
7. Ancliff PJ, Blundell MP, Cory GO, Calle Y, Worth A, Kempski H, Burns S, Jones GE, Sinclair J, Kinnon C, Hann IM, Gale RE, Linch DC, Thrasher AJ. Two novel activating mutations in the Wiskott-Aldrich syndrome protein result in congenital neutropenia. Blood 2006;108:2182-2189.
8. Olcay L, Yetgin S, Okur H, Erdemli E. Rapid cell senescence and apoptosis in lymphocytes and granulocytes and absence of GM-CSF receptor in congenital dysgranulopoietic neutropenia. Leuk Res 2008;32:235-242.
9. Ancliff PJ, Gale RE, Watts JM, Liesner R, Hann IM, Strobel S, Linch DC. Paternal mosaicism proves the pathogenic nature of mutations in neutrophil elastase in severe congenital neutropenia. Blood 2002;100:707-709.
10. Olcay L, Yetgin S, Erdemli E, Germeshausen M, Aktaş D, Büyükaşik Y, Okur H. Congenital dysgranulopoietic neutropenia. Pediatr Blood Cancer 2008;50:115-119.
11. Shattil SJ, Abrams CS, Bennett JS. Acquired qualitative platelet disorders due to diseases, drugs and foods. In: Beutler E, Lichtman MA, Coller BS, Kipps TJ, Seligsohn U (eds). Williams Hematology. New York, McGraw-Hill, 2001.
12. İkincioğulları A, Kendirli T, Doğu F, Eğin Y, Reisli İ, Cin Ş, Babacan E. Peripheral blood lymphocyte subsets in healthy Turkish children. Turk J Pediatr 2004;46:125-130.
13. Ormerod MG. Flow Cytometry: A Practical Approach. Oxford, Oxford University Press, 1994.
14. Olcay L, Tuncer AM, Okur H, Erdemli E, Uysal Z, Cetin M, Duru F, Cetinkaya DU. Excessive naked megakaryocyte nuclei in myelodysplastic syndrome mimicking idiopathic thrombocytopenic purpura: a complicated pre- and post-transplantation course. Pediatr Hematol Oncol 2009;26:387-397.
15. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, Peacocke M, Campisi JI. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A 1995;92:9363-9367.
16. Olcay L, Yetgin S, Okur H, Erekul S, Tuncer M. Dysplastic changes in idiopathic thrombocytopenic purpura and the effect of corticosteroids to increase dysplasia and cause hyperdiploid macropolycytes. Am J Hematol 2000;65:99-104.
17. Dykstra MJ. A Manual of Applied Techniques for Biological Electron Microscopy. New York, Plenum Press, 1993.
18. Buyukasik Y, Karakus S, Goker H, Haznedaroglu IC, Ozatli D, Sayinalp N, Ozcebe OI, Dundar SV, Kirazli S. Rational use of the PFA-100 device for screening of platelet function disorders and von Willebrand disease. Blood Coagul Fibrinolysis 2002;13:349-353.
19. Olcay L, Erdemli E, Kesimer M, Büyükasik Y, Okur H, Kalkanoğlu HS, Coskun T, Altay C. High cystine in platelets from patients with nephropathic cystinosis: a chemical, ultrastructural, and functional evaluation. J Clin Pathol 2005;58:939-945.
20. Komarnichi M, Pietrzak I, Zozulinska M. Mepacrine-labeled platelet dense-body number in patients with chronic uremia. Nephron 1988;50:306-307.
21. Brugnara C. Reference values in infancy and childhood. In: Orkin SH, Nathan DG, Ginsburg D, Look AT, Fisher DE, Lux S (eds). Nathan and Oski's Hematology and Oncology of Infancy and Childhood. 8th ed. Philadelphia, Saunders, 2015.
22. Geaghan SM. Normal blood values for neonatal, pediatric and adult populations. In: Hoffman R, Benz EJ, Shattil SJ, Furie B, Cohen HJ, Silberstein LE, McGlave P (eds). Hematology: Basic Principles and Practice. 4th ed. Philadelphia, Churchill Livingstone, 2005.
23. Pintado T, Maldonado JE. Ultrastructure of platelet aggregation in refractory anemia and myelomonocytic leukemia. I. Ultrastructure of aggregation in normal controls and general defects in refractory anemia and myelomonocytic leukemia. Mayo Clin Proc 1976;51:379-392.
24. Pintado T, Maldonado JE. Ultrastructure of platelet aggregation in refractory anemia and myelomonocytic leukemia. II. Individual platelet abnormalities: thrombasthenia-like platelets, surface defects, and dissociation phenomena. Mayo Clin Proc 1976;51:443-451.

25. Courtney PA, Crockard AD, Williamson K, Irvine AE, Kennedy RJ, Bell AL. Increased apoptotic peripheral blood neutrophils in systemic lupus erythematosus: relations with disease. Ann Rheum Dis 1999;58:309-314.
26. Aprikyan AA, Liles WC, Park JR, Jonas M, Chi EY, Dale DC. Myelokathexis, a congenital disorder of severe neutropenia characterized by accelerated apoptosis and defective expression of bcl-x in neutrophil precursors. Blood 2000;95:320-327.
27. Liu JH, Wei S, Lamy T, Epling-Burnette PK, Starkebaum G, Djeu JY, Loughran TP. Chronic neutropenia mediated by Fas ligand. Blood 2000;95:3219-3222.
28. Cohen O, Cicala C, Vaccarezza M, Fauci AS. The immunology of human immunodeficiency virus infection. In: Mandell GL, Bennett JE, Dolin R, (eds). Principles and Practice of Infectious Diseases. New York, Churchill Livingstone, 2000.
29. Uguz A, Coskun M, Yuzbey S, Kizilors A, Karadogan I, Gura A, Yoldas B, Oygur N, Yegin O. Apoptosis of cord blood neutrophils and their response to colony-stimulating factors. Am J Perinatol 2002;19:427-433.
30. Papadaki HA, Eliopoulos AG, Kosteas T, Gemetzi C, Damianaki A, Koutala H, Bux J, Eliopoulos GD. Impaired granulocytopoiesis in patients with chronic idiopathic neutropenia is associated with increased apoptosis of bone marrow myeloid progenitor cells. Blood 2003;101:2591-2600.
31. Kuijpers TW, Maianski NA, Tool AT, Smit GP, Rake JP, Roos D, Visser G. Apoptotic neutrophils in the circulation of patients with glycogen storage disease type 1b (GSD1b). Blood 2003;101:5021-5024.
32. Cohen GM, Sun XM, Snowden RT, Dinsdale D, Skilleter DN. Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. Biochem J 1992;286:331-334.
33. Poon IK, Lucas CD, Rossi AG, Ravichandran KS. Apoptotic cell clearance: basic biology and therapeutic potential. Nat Rev Immunol 2014;14:166-180.
34. Abbas AK, Lichtman AH, Pillai S. Innate immunity. In: Abbas A, Lichtman AH, Pillai S (eds). Cellular and Molecular Immunology. 8th ed. Toronto, Elsevier Saunders, 2015.
35. Abbas AK, Lichtman AH, Pillai S. Differentiation and functions of CD4+ effector T cells. In: Abbas A, Lichtman AH, Pillai S (eds). Cellular and Molecular Immunology. 8th ed. Toronto, Elsevier Saunders, 2015.
36. Olcay L, Ünal Ş, Öztürk A, Erdemli E, Billur D, Metin A, Okur H, İkincioğulları A, Yıldırmak Y, Büyükaşık Y, Yılmaz-Falay M, Özet G, Yetgin S. Granulocytic and non granulocytic lineages in children with congenital neutropenia and their non neutropenic parents: biochemical, functional, morphological and genetic evaluation. Haematologica 1012;97(Suppl 3):O65a (abstract).
37. Shitara T, Yugami S, Ijima H, Sotomatu M, Kuroume T. Cytokine profile during high-dose rhG-CSF therapy in severe congenital neutropenia. Am J Hematol 1994;45:58-62.
38. Koval'chuk LV, Khoreva MV, Nikonova AS, Finogenova NA, Mamedova EA, Polovtseva TV, Fetisova Lla, Gracheva LA, Goldyreva NG. Toll-like receptor-mediated functional activity of mononuclear cells in children with neutropenia. Zh Mikrobiol Epidemiol Immunobiol 2010;2:64-68.
39. Bohn G, Hardtke-Wolenski M, Zeidler C, Maecker B, Sauer M, Sykora KW, Grigull L, Welte K, Klein C. Lethal graft-versus-host disease in congenital neutropenia caused by p14 deficiency after allogeneic bone marrow transplantation from an HLA-identical sibling. Pediatr Blood Cancer 2008;51:436-438.
40. Geering B, Simon HU. A novel signaling pathway in TNFα-induced neutrophil apoptosis. Cell Cycle 2011;10:2821-2822.
41. Ugan Y, Nazıroğlu M, Şahin M, Aykur M. Anti-tumor necrosis factor alpha (Infliximab) attenuates apoptosis, oxidative stress, and calcium ion entry through modulation of cation channels in neutrophils of patients with ankylosing spondylitis. J Membr Biol 2016;249:437-447.
42. Bommhardt U, Chang KC, Swanson PE, Wagner TH, Tinsley KW, Karl IE, Hotchkiss RS. Akt decreases lymphocyte apoptosis and improves survival in sepsis. J Immunol 2004;172:7583-7591.
43. Hotchkiss RS, Tinsley KW, Karl IE. Role of apoptotic cell death in sepsis. Scand J Infect Dis 2003;35:585-592.
44. Kawakami Y, Tsukimoto M, Kuwabara K, Fujita T, Fujino O, Kojima S, Fukunaga Y. Tumor necrosis factor-α-induced mononuclear cell death may contribute to polymorphonuclear cell predominance in the cerebrospinal fluid of patients with bacterial meningitis. J Nippon Med Sch 2011;78:360-366.
45. Cevik O, Adiguzel Z, Baykal AT, Somay G, Sener A. The apoptotic actions of platelets in acute ischemic stroke. Mol Biol Rep 2013;40:6721-6727.
46. Kawada J, Kimura H, Shibata Y, Hara S, Hoshino Y, Kojima S, Nishikawa K, Morishima T. Evaluation of apoptosis in Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis. J Med Virol 2006;78:400-407.
47. Ochs HD, Notarangelo LD. Structure and function of the Wiskott-Aldrich syndrome protein. Curr Opin Hematol 2005;12:284-291.
48. Jung J, Bohn G, Allroth A, Boztug K, Brandes G, Sandrock I, Schäffer AA, Rathinam C, Köllner I, Beger C, Schilke R, Welte K, Grimbacher B, Klein C. Identification of a homozygous deletion in the AP3B1 gene causing Hemansky-Pudlak syndrome, type 2. Blood 2006;108:362-369.
49. Wei ML. Hermansky-Pudlak syndrome: a disease of protein trafficking and organelle function. Pigment Cell Res 2006;19:19-42.
50. Zhu J, Davidson TS, Wei G, Jankovic D, Cui K, Schones DE, Guo L, Zhao K, Shevach EM, Paul WE. Down-regulation of Gfi-1 expression by TGFB is important for differentiation of Th17 and CD103+ inducible regulatory T cells. J Exp Med 2009;206:329-341.
51. Dinauer MC, Newburger PP. The phagocyte system and disorders of granulopoiesis and granulocyte function. In: Orkin SH, Nathan DG, (eds). Nathan and Oski's Hematology of Infancy and Childhood. Philadelphia, Saunders Elsevier, 2009.
52. Jaeger BN, Donadieu J, Cognet C, Bernat C, Ordoñez-Rueda D, Barlogis V, Mahlaoui N, Fenis A, Narni-Mancinelli E, Beaupain B, Bellanné-Chantelot C, Bajénoff M, Malissen B, Malissen M, Vivier E, Ugolini S. Neutrophil depletion impairs natural killer cell maturation, function, and homeostasis. J Exp Med 2012;209:565-580.
53. Komiyama A, Kawai H, Yamada S, Aoyama K, Yamazaki M, Saitoh H, Miyagawa Y, Akabane T, Uehara Y. Impaired natural killer cell recycling in childhood chronic neutropenia with morphological abnormalities and defective chemotaxis of neutrophils. Blood 1985;66:99-105.
54. McElhaney JE, Effros RB. Immunosenescence: what does it mean to health outcomes in older adults? Curr Opin Immunol 2009;21:418-424.
55. Freund A, Orjalo AV, Desprez PY, Campisi J. Inflammatory networks during cellular senescence: causes and consequences. Trends Mol Med 2010;16:238-246.
56. Olcay L, Billur D, Erdemli E, Baskin SE, Balci HF, Yetgin S. Myelodysplastic features and cellular senescence in autoimmune disorders: a pilot study on patients with collagen tissue disorders and immune thrombocytopenic purpura. Turk J Med Sci 2015;45:742-744.
57. Chang BD, Xuan Y, Broude EV, Zhu H, Schott B, Fang J, Roninson IB. Role of p53 and p21waf1/cip1 in senescence-like terminal proliferation arrest induced in human tumor cells by chemotherapeutic drugs. Oncogene 1999;18:4808-4818.
58. O'Connor PM, Jackman J. Assessment of DNA damage cell cycle checkpoints in G1 and G2 phases of mammalian cells. In: Studzinski GP, Robertson GP, Hufford A, Lugo TG, (eds). Cell Growth, Differentiation and Senescence: A Practical Approach. New York, Oxford University Press, 1999.
59. Hubbard K, Ozer HL. Senescence and immortalization of human cells. In: Studzinski GP, Robertson GP, Hufford A, Lugo TG, (eds). Cell Growth, Differentiation and Senescence: A Practical Approach. New York, Oxford University Press, 1999.
60. Ly DH, Lockhart DJ, Lerner RA, Schultz PG. Mitotic misregulation and human aging. Science 2000;287:2486-2492.
61. Gorbunova V, Seluanov A, Pereira-Smith OM. Expression of human telomerase (hTERT) does not prevent stress-induced senescence in normal human fibroblasts but protects the cells from stress-induced apoptosis and necrosis. J Biol Chem 2002;277:38540-38549.

62. Wright WE, Shay JW. Historical claims and current interpretations of replicative aging. Nat Biotechnol 2002;20:682-688.

63. Rodier F, Campisi J. Four faces of cellular senescence. J Cell Biol 2011;192:547-556.

64. Ohtani N, Hara E. Roles and mechanisms of cellular senescence in regulation of tissue homeostasis. Cancer Sci 2013;104:525-530.

65. Macieira-Coelho A. Chromatin reorganization during senescence of proliferating cells. Mutat Res 1991;256:81-104.

66. Parmley RT, Crist WM, Ragab AH, Boxer LA, Malluh A, Lui VK, Darby CP. Congenital dysgranulopoietic neutropenia: clinical, serologic, ultrastructural, and in vitro proliferative characteristics. Blood 1980;56:465-475.

67. Olcay L, Yetgin S. Disorders mimicking myelodysplastic syndrome and difficulties in its diagnosis. In: Fuchs O, (ed). Myelodysplastic Syndromes. Rijeka, InTech, 2016.

68. Lightsey AL, Parmley RT, Marsh WL Jr, Garg AK, Thomas WJ, Wolach B, Boxer LA. Severe congenital neutropenia with unique features of dysgranulopoiesis. Am J Hematol 1985;18:59-71.

69. Thiele J, Lorenzen J, Manich B, Kvasnicka HM, Zirbes TK, Fischer R. Apoptosis (programmed cell death) in idiopathic (primary) osteo-/myelofibrosis: naked nuclei in megakaryopoiesis reveal features of para-apoptosis. Acta Haematol 1997;97:137-143.

70. Centurione L, Di Baldassarre A, Zingariello M, Bosco D, Gatta V, Rana RA, Langella V, Di Virgilio A, Vannucchi AM, Migliaccio AR. Increased and pathologic emperipolesis of neutrophils within megakaryocytes associated with marrow fibrosis in GATA-1(low) mice. Blood 2004;104:3573-3580.

71. Aslan D, Yetgin S. Megakaryocyte emperipolesis in a child with chronic neutropenia: an unusual coexistence. Turk J Pediatr 2001;43:255-256.

72. Houwerzijl EJ, Blom NR, van der Want JJ, Esselink MT, Koornstra JJ, Smit JW, Louwes H, Vellenga E, de Wolf JT. Ultrastructural study shows morphologic features of apoptosis and para-apoptosis in megakaryocytes from patients with idiopathic thrombocytopenic purpura. Blood 2004;103:500-506.

73. Erber WN, Jacobs A, Oscier DG, O'Hea AM, Mason DY. Circulating micromegakaryocytes in myelodysplasia. J Clin Pathol 1987;40:1349-1352.

74. Cowan DH, Graham RC, Baunach D. The platelet defect in leukemia. Platelet ultrastructure, adenine nucleotide metabolism, and the release reaction. J Clin Invest 1975;56:188-200.

75. Rao AK. Inherited platelet function disorders: overview and disorders of granules, secretion, and signal transduction. Hematol Oncol Clin North Am 2013;27:585-611.

76. Klein C. Congenital neutropenia. Hematology Am Soc Hematol Educ Program 2009;344-350.

77. Nieuwenhuis HK, Akkerman JW, Sixma JJ. Patients with a prolonged bleeding time and normal aggregation tests may have storage pool deficiency: studies on one hundred and six patients. Blood 1987;70:620-623.

78. Jasty R, Kuerbitz S, Matthew P. Correlation of clinical severity of bleeding with electron microscopy findings in a group of pediatric patients with platelet storage pool deficiencies at a single institution. J Pediatr Hematol Oncol 2001;23:1146a (abstract).

79. Sladky JL, Klima J, Grooms L, Kerlin BA, O'Brien SH. The PFA-100 ® does not predict delta-granule platelet storage pool deficiencies. Hemophilia 2012;18:626-629.

80. White JG. Use of the electron microscope for diagnosis of platelet disorders. Semin Thromb Hemost 1998;24:163-168.

81. Ancliff PJ, Gale RE, Watts JM, Liesner R, Hann IM, Strobel S, Linch DC. Paternal mosaicism proves the pathogenic nature of mutations in neutrophil elastase in severe congenital neutropenia. Blood 2002;100:707-709.

82. Hirata O, Okada S, Tsumura M, Karakawa S, Matsumura I, Kimura Y, Maihara T, Yasunaga S, Takihara Y, Ohara O, Kobayashi M. Mosaicism of an ELANE mutation in an asymptomatic mother in a familial case of cyclic neutropenia. J Clin Immunol 2015;35:512-516.

83. Kim HJ, Song MJ, Lee KO, Kim SH, Kim HJ. Paternal somatic mosaicism of a novel frameshift mutation in ELANE causing severe congenital neutropenia. Pediatr Blood Cancer 2015;62:2229-2231.

84. Malcov M, Reches A, Ben-Yosef D, Cohen T, Amit A, Dgany O, Tamary H, Yaron Y. Resolving a genetic paradox throughout preimplantation genetic diagnosis for autosomal dominant severe congenital neutropenia. Prenat Diagn 2010;30:207-211.

85. Ancliff PJ, Gale RE, Liesner R, Hann IM, Linch DC. Mutations in the *ELA2* gene encoding neutrophil elastase are present in most patients with sporadic severe congenital neutropenia but only in some patients with the familial form of the disease. Blood 2001;98:2645-2650.

86. Sippel KC, Fraioli RE, Smith GD, Schalkoff ME, Sutherland J, Gallie BL, Dryja TP. Frequency of somatic and germ-line mosaicism in retinoblastoma: implications for genetic counseling. Am J Hum Genet 1998;62:610-619.

87. Skokowa J, Dale DC, Touw IP, Zeidler C, Welte K. Severe congenital neutropenias. Nat Rev Dis Primers 2017;3:17032.

88. Haurie C, Dale DC, Mackey MC. Occurrence of periodic oscillations in the differential blood counts of congenital, idiopathic, and cyclical neutropenic patients before and during treatment with G-CSF. Exp Hematol 1999;27:401-409.

89. Jaloma-Cruz AR, Beltran-Miranda CP, Gonzalez-Ramos IA, Lopez-Jimenez JJ, Luna-Zaizar H, Mantilla-Capacho JM, Mundo-Ayala JN, Galvan MJV. Genotype-phenotype interaction analyses in hemophilia. In: Batorova A, (ed). Hemophilia. Rijeka, InTech, 2012.

90. Steinemann D, Praulich I, Otto N, Göhring G, Niemeyer CM, Schlegelberger B. Mutation analysis of the *HAX1* gene in childhood myelodysplastic syndrome. Br J Haematol 2009;145:533-550.

91. Park J, Kim M, Lim J, Kim Y, Cho B, Park JY, Han K. Molecular analysis of two cases of severe congenital neutropenia. Korean J Lab Med 2010;30:111-116.

92. Skokowa J, Welte K. Dysregulation of myeloid-specific transcription factors in congenital neutropenia. Ann N Y Acad Sci 2009;1176:94-100.

93. Skokowa J, Fobiwe JP, Dan L, Thakur BK, Welte K. Neutrophil elastase is severely down-regulated in severe congenital neutropenia independent of ELA2 or HAX1 mutations but dependent on LEF-1. Blood 2009;114:3044-3051.

Appendix: Supplemental Materials and Methods

Mutation Analysis

Mutation analyses of the *HAX1*, *ELANE*, *CSF3R*, and *G6PC3* genes were performed by standard techniques using a DNA sequencing kit (PerkinElmer, Foster City, CA, USA) and the ABI Prism 3100 sequence analyzer (Applied Biosystems, Foster City, CA, USA).

Reference accession number of the genes

HAX1 (NM_006118)

ELANE (NM_001972)

CSF3R (NM_156039)

G6PC3 (NM_138387)

JAGN1 (NM_032492)

Evaluation of Cellular Morphology

The peripheric blood cells were evaluated by light (Nikon Eclipse E400, Nikon Corporation, Japan) and transmission electron microscope (TEM) (LEO 906E, Zeiss, Germany) for apoptosis and dysmorphism [1,2,3,4], in a blinded fashion. One hundred neutrophils of the parents (except YF's mother whose peripheral blood smear enabled us to evaluate only 50 neutrophils) and control group were scored for dysmorphism under light microscope (1000X). Each dysmorphic feature shown by an individual neutrophil was given one score and the total score for a definite dysmorphic feature was presented as '%'. Only for YF's mother the total score for a definite dysmorphic feature was multiplied by two. The patients' neutrophils could not be scored due to neutropenia. The photographs of the representative cells with dysmorphic features under electron microscope were taken and presented.

Evaluation of the Thrombocytes of the Patients and the Parents

In vitro bleeding time: This was measured by a PFA–100 instrument (Dade Behring Marburg GmbH, Marburg, Germany) using collagen-epinephrine and/or collagen-ADP cartridges [5].

Turbidimetric aggregation tests: A Chrono-Log 560 Ca aggregometer was used with minor modifications (Chrono-Log Corporation, Havertown, PA, USA) [5]. Thrombocyte aggregation was studied with collagen (1 and 2 µg/mL), ristocetin (1.25 mg/mL), ADP (2, 6, 10 µM), and epinephrine (10 µM).

Mepacrine labeling of dense granules: Thrombocyte-rich plasma was prepared and stained with mepacrine (1 µM; Sigma, St. Louis, MO, USA) as described previously [6,7]. The greenish-yellow fluorescent dense granules were visualized under a fluorescent microscope (Zeiss Axoscope; Zeiss, Thornwood, NY, USA). The dense granules were counted in 84-106 platelets for each sample. The results were presented as the mean dense granule number per thrombocyte [6,7].

Electron microscopic evaluation of aggregation: After adding 2 µM ADP to thrombocyte-rich plasma obtained from two patients (BA, ZG), samples were removed at the 2nd, 8th, and 14th minutes and were prepared for TEM [6].

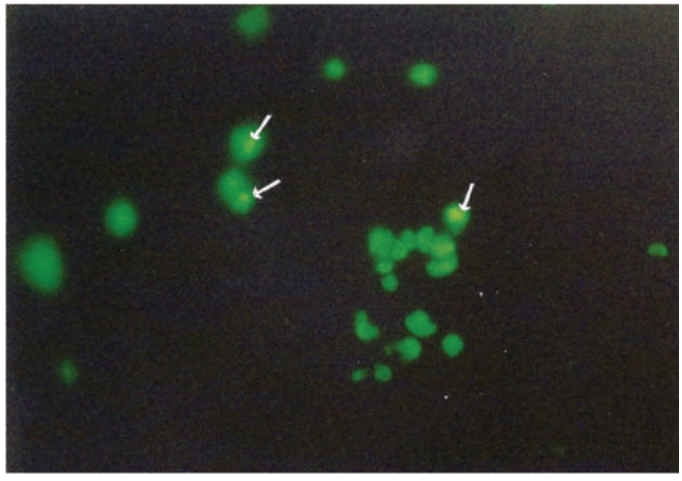
Statistics

We used SPSS 15.0 to evaluate the data we obtained (SPSS Inc., Chicago, IL, USA). A normality test was performed to determine if the data were distributed in a normal fashion. To compare evenly and unevenly distributed data, we used the independent samples t-test and the Mann-Whitney U test (for two groups) and One-way ANOVA and the Kruskal-Wallis H test with Bonferroni correction (for more than two groups). We used the Scheffe test in the one-way ANOVA testing to determine which groups had differences between themselves. One-sample t-tests were used to evaluate whether the means of the variables differed from the means of the reference group. Values of $p<0.05$ and $p<0.017$ were considered significant for inter-group comparison and the Kruskal-Wallis H test, respectively.

Supplemental Results

Ultrastructural View of Aggregating Thrombocytes

Aggregation tests under TEM revealed the following abnormalities: **1) Lack of adhesion:** At the 2nd, 8th, and 14th minutes of the aggregation test, the thrombocytes were still separate from each other. At the second minute, the granules were fairly or completely centralized with no visible pseudopods, whereas normal thrombocytes fit together tightly, displayed abundant blunt pseudopods, and had released almost all of their granules (Case 1, Case 2, and control). **2) Lack of or inadequate secretion:** Organelle centralization took place but the organelles later became dispersed in the cytoplasm, without undergoing secretion and with



Supplemental Figure 1. The platelet dense granules stained by mepacrine (arrow).

Supplemental Table 1. Primers used for the sequencing of the coding regions of *HAX1, ELANE, G6PC3, CSF3R, JAGN1* genes.

HAX1-1F	ACTGGAGGGGTTCAAAGGTT
HAX1-1R	ATCACCCC AGGTTGGAGAA
HAX1-2F	TCC CACTTTGCCACCCATGAGT
HAX1-2R	TTCTCACACTTCCCATCCCC
HAX1-3F	CCTTTCCCATCCCAGCAAACACC
HAX1-3R	CT CACAAGCTCTCACTTCAGGACCA
HAX1-4F	G GGGTTTTGGAGCTCGGGAGTAGTT
HAX1-4R	TTCAGGGAGGGAGAACACACAG A
HAX1-5,6F	CTCCTGCTTCTTCATCTCTC TGCTC
HAX1-5,6R	AGGCAGAAGCAAAGGACAAGGG
HAX1-7F	CTCAAGATTCCTTGGGGAAG
HAX1-7R	CGGAAGTGTTGGAT GGTTCT
ELANE-2,3F	CGGAGGGGCAGAGACCCCGGA
ELANE-2,3R	AGACCGGGACGCGGGGTCCGA
ELANE-4F	CTCGAGCACCTTCGCCCTCAG
ELANE-4R	TCAACGGCCCATGGCGGGTAT
ELANE-5-6F	CCTGCCCTGCAGGATCCCAGA
ELANE-5-6R	GGAGAGTGTGGGTGTGGGCAG
CSF3R-3R	AAGGAAATTCCCAATATCTCTCC
CSF3R-4F	CCAGGGTCTGCTTTTCTCTG
CSF3R-4R	AGGCACCCGCCACTATG
CSF3R-5-6F	AGGAAGCTTTCTGAGTGGTCC
CSF3R-5-6R	TGTGTTTCCCTCTCCATTCC
CSF3R-7-8F	AGAGCCCTCTAGGGTGGG
CSF3R-7-8R	GGGGCCTGGACTGGATAC
CSF3R-9F	GTATCCAGTCCAGGCCCC
CSF3R-9R	CTAAGCCCCGGTTTGTAGG
CSF3R-10F	CCTAGAGGCTCTCCTTGACTC
CSF3R-10R	CCAGGCAGTCTAGCCTTTG
CSF3R-11F	CAGGCTTCTGGCTCAAGG
CSF3R-11R	TCAGATAAGCACTGCCTCCC
CSF3R-12F	GCTCCTGATCATTAGCACAGG
CSF3R-12R	AGAGCCTTGGGAGAGAGAGG
CSF3R-13-14F	AGAAGTCCAACCGGGCTC
CSF3R-13-14R	AAATCAGCATCCTTTGGGTG
CSF3R-15-16F	AGACCCAGCCTTCCCAAC
CSF3R-15-16R	CTTGGCTTCAGAAGGTGTCC
CSF3R-17F	CCAGACAGGGACAGTGGC
CSF3R-17R	ACCCTCCCCTCTTCTCCAG
G6PC3-1F	GTGATAGCCGAGGCGCTACAG
G6PC3-1R	AGGGCTTACACATGACTCAGGG
G6PC3-2F	GAGTACTCTGTGTCCTGCCCG
G6PC3-2R	CTCTGAAATGTTCACCCGAACC
G6PC3-3F	CAGCTGTATTATTGAGGCATCACC
G6PC3-3R	GCCATGCTGCTGTGTCTATTAGG
G6PC3-4F	TTCAACCATGGAGTACCTGGG
G6PC3-4R	GTGGGCTCTACAGAAGCTGGG
G6PC3-5F	ATTCTCTTGCCAAGCTGCACTG
G6PC3-5R	AGGCTAGGTGCAGGCGAGATAG
G6PC3-6aF	GATAGCCTGCCTTGTGCTGG
G6PC3-6aR	CGGAAGCAGATTTAGGGAGGG
G6PC3-6bF	CAGTGGCCTTCTATGTTCCAGC
G6PC3-6bR	GGCCAGAGGGTGTACTTGAGG
JAGN1_1F	GTGTCGTTGCGGTACCAGG
JAGN1_1R	GGGGAGCAAGACCCTGAG
JAGN1_2F	TTGTCTGGCATATAGTTGGTGG
JAGN1_2R	AAGGGATTTTGGAACCGC

unevacuated large granules and no enlargement in the OCS (Case 2) or having made partial secretion with enlarged OCS full of unsecreted material (Cases 1 and 2). **3) Delay in all stages of aggregation:** Each stage of aggregation (centralization, development of pseudopods, and/or absent or impartial secretion) took place with delay (Case 1, Case 2, and control). **4) Abnormal degranulation:** Unlike in normal degranulation, in which the dissolution of the granule's membrane is followed by evacuation of granule contents into the OCS and canalicular dilation, in some samples of the patients the dense granule was evident in the OCS, without membrane dissolution (Case 1). **5) Dissociation phenomenon:** The patients' platelets showed the dissociation phenomenon, a disruption of normal aggregation that normally takes place in a coordinated sequence of pseudopod formation, change in shape, clustering of central granules, degranulation, and canalicular dilation [8,9]. In our cases, all of these changes took place without the adherence of thrombocytes to each other. We observed OCS enlargement and secretion of some material taking place before the degranulation phase ended (Case 1). **6) Abnormal cytoplasmic protrusions:** The cytoplasmic protrusions in normal aggregating thrombocytes were thin, whereas in our patients they were amoeboid (Cases 1 and 2).

Clinical Correlations Between Hemorrhagic Diathesis and Laboratory Parameters

It was striking that not all cases with a history of hemorrhagic diathesis displayed abnormal aggregation test results, low dense granule number in thrombocytes, or prolonged in vitro bleeding time, whereas many cases without any history of hemorrhagic diathesis displayed abnormal aggregation test results or low dense granule number in thrombocytes or prolonged bleeding time or abnormal ultrastructure of thrombocytes. Not all of these abnormalities coexisted all together (Table 2; Supplemental Table 5).

Supplemental Discussion

Text 1

In the steady state apoptotic cells are rarely encountered under physiological conditions and the removal of the apoptotic cells is fast and promotes an anti-inflammatory response [10]. However, in the case of substantial and excessive apoptosis during which the capacity of phagocytes to engulf apoptotic cells is reduced, the uncleared apoptotic cells and fragments undergo secondary necrosis (delayed apoptotic clearance) which can provoke inflammation or autoimmunity. Macrophages that ingest necrotic cells cause increased T cell proliferation [10].

Hence, the flow cytometric graphics show that apoptotic and necrotic cells coexisted in all three cell lines, confirming that necrosis was due to excessive apoptosis. Hence, phagocytosis of both necrotic and apoptotic cells were shown in the bone marrow of the patients.

However, presence of excessive apoptosis and secondary necrosis not only in the patients' neutrophils but in all other cell lines (lymphocytic and monocytic) regardless of the mutation type of congenital neutropenia and their existence even in the non-neutropenic parents of the congenital neutropenia patients seem unreliable at first glance.

We think that macrophages of our patients were activated by increased apoptosis in the myeloid lineage, and the TNF-alpha secreted by activated macrophages gave rise to increased apoptosis (and secondary necrosis) in all cell lineages. Hence, striking phagocytic activity of various phagocytic cells (histiocytes, monocytes, macrophages, neutrophils, bands and even eosinophils and eosinophil myelocytes), mainly being monocytic cells, and our previous findings showing elevated TNF-alpha which is mainly secreted by activated macrophages in a group of congenital neutropenia patients, most of whom also enrolled in this study [11], confirm our explanation.

Supplemental Table 2. Characteristics of the parents.

	Age	Symptoms / findings	Molecular genetics	Leukocyte count (WBC; x10^9/L) (n=4.0-10.0)	Neutrophil count (10^9/L, %) (n=1.9-8.0)	Monocyte count (x10^9/L, %) (n=0.12-1.2)	Lymphocyte count (x10^9/L, %) (n=0.9-5.2)	Thrombocyte count (x10^9/L, %) (n=130-400)
Father of MK	38y	-	NA†	7.0	3.6	0.8	2.5	365
Mother of MNY-AY siblings*	34y	-	*HAX1* heterozygousc.130-131insA (p.W44X)**	8.3	5.2	0.5	2.5	312
Father of MNY-AY siblings*	37y	-	HAX1 heterozygous c.130-131insA (p.W44X)**	8.48	5.77	0.62	1.8	304
Mother of MeK-OSK siblings	55y	-	NA†	9.24	4.38	0.77	3.91	314
Father of MeK-OSK siblings	54y	-	NA†	8.7	5.6	0.8	2.1	322
Mother of AF*	28y	Gingival enlargement	NA†	8.8	5.4	0.8	2.3	247
Father of AF	32y	Frequent aphthae	NA†	8.9	5.6	0.7	2.3	211
Mother of EÇ*	26y	Gingival enlargement gingival bleeding	*HAX1* heterozygous c.130-131insA (p.W44X)**	6.82	4.26	0.61	1.80	241
Father of EÇ*	25y	-	NA*	8.32	5.37	0.57	2.07	238
Mother of HY*	29y	-	*HAX1* heterozygous c.130-131insA (p.W44X)**	7.38	4.75	0.52	1.94	268
Father of HY*	32y	-	HAX1 heterozygous c.130-131insA (p.W44X)**	10.15	5.87	0.70	3.18	287
Mother of RT*	30y	-	No *ELANE****	10.0	7.3	0.3	2.3	261
Father of RT*	34y	-	No *ELANE****	14.1	11.3	0.6	2.1	280
Mother of NBÖ*	46y	Easy bruising	No *ELANE****	4.7	2.8	0.3	1.6	242
Father of NBÖ*	49y	-	No *ELANE****	5.1	2.3	0.5	2.1	251
Mother of AO	29	Easy bruising	NA†	7.9	5.2	0.4	2.2	361
Father of AO	36y	Gingival enlargement	NA†	8.0	4.8	0.48	2.4	328
Mother of BA	32y	-	NA†	6.4	4.0	0.4	1.9	366
Father of BA	38y	Easy bruising	NA†	9.4	6.3	0.6	2.2	248
Mother of AG-ZG siblings*	23y	-	NA†	6.7	3.6	0.56	2.24	205
Father of AG-ZG siblings*	27y	Easy bruising	NA†	6.84	3.88	0.57	2.12	227

*Those who could be evaluated for death parameters by flow cytometry, **Peripheral blood cells, ***Peripheral blood cells and buccal mucosa cells, †NA: Not available.

Supplemental Table 3. The number and percentage of the patients and their parents whose lymphocyte subset levels were below and above age matched normal range.

	CD3		CD4		CD8		CD4/CD8		NK	
	Below normal	Above normal	Below normal	Above normal	Below normal	Above normal	Below normal	Above normal	Below normal	Above normal
Patients (n=11)	2	2	1	0	0	3	0	0	4	0
	16.6%	16.6%	8.3%	0%	0%	27.3%	0%	0%	36.4%	0%
Parents (n=13)	0	0	0	0	0	1	0	0	2	0
	0%	0%	0%	0%	0%	7.7%	0%	0%	15.4%	0%
Control (n=18)	0	0	0	0	0	3	0	0	1	0
	0%	0%	0%	0%	0%	16.7%	0%	0%	5.6%	0%

Supplemental Table 4. In vitro bleeding time of the cases.

	Collagen-epinephrin (n=85-157 sn) / (No of cases)	Collagen-ADP (n=65-125 sn) / (No of cases)
Patients	150.25±28.89 (n=8)	121.0±22.5 (n=6)
Parents	131.9±35.5 (n=16)	110.3±74.1 (n=8)
Control	129.2+28.8 (n=9)	88.3±12.2
P (Patients-parents)	0.293	0.233
P (Patients-control)	0.293	0.031
P (Parents-control)	0.293	1.000

Supplemental Table 5. Platelet aggregation responses to various agonists.

	ADP 2 µM			ADP 6 or 10 µM			Epinephrin 10 µM			Collagen 1 or 2 µg/mL			Ristosetin 1.25 µg/mL		
	Patients (n=11)	Family members (n=10)	Control (n=5)	Patients (n=13)	Family members (n=16)	Control (n=5)	Patients (n=9)	Family members (n=9)	Control (n=5)	Patients (n=13)	Family members (n=17)	Control (n=5)	Patients (n=14)	Family members (n=16)	Control (n=5)
Normal (n)	4	6	5	7	10	5	5	5	5	11	13	5	11	14	5
Abnormal secondary aggregation (n)	7	4	-	4	5	-	-	1	-	-	-	-	-	-	-
No aggregation (n)	-	-	-	-	-	-	3	-	-	-	-	-	3	-	-
Suboptimal / low response (n)	-	-	-	2	1	-	1	3	-	2	4	-	-	2	-
Abnormal/ Total (n, %)	7/11 (63.6%)	4/10 (40%)	0/5 (5%)	6/13 (46.2%)	6/16 (37.5%)	0/5 (5%)	4/9 (44.4%)	4/9 (44.4%)	0/5 (5%)	2/13 (15.4%)	4/17 (23.5%)	0/5 (5%)	3/14 (21.4%)	2/16 (12.5%)	0/5 (5%)

Additionally, studies of other colleagues showing enhanced stimulating effect of monocyte activation through toll-like receptors on TNF-alpha production [12,13], and increased baseline cytokines including TNF-alpha in children with congenital neutropenia [14] also draw attention to the role of TNF-alpha and other inflammatory cytokines in congenital neutropenia.

Monocytes are activated by microbial products as well as molecules expressed or released by dying or stressed cells through toll-like receptors, interferon gamma, Th1 cells or complement fragments [15]; additionally removal mechanisms of necrotic and apoptotic cells may overlap [10]. Once activated, macrophages secrete TNF-alpha, IL-1, IL-6 and IL-12, the latter of which is also secreted by dendritic cells and stimulates IFN-gamma production by NK and T cells thereby exacerbating macrophage activation [15,16]. Hence, elevations of pro-inflammatory cytokines in congenital neutropenia were reported before [11,14].

TNF-alpha which is mainly secreted by macrophages is a well known cytokine to give rise to apoptosis in neutrophils [17,18], lymphocytes [19,20], monocytes [21], and thrombocytes [22] in various conditions like sepsis, HLH [23], stroke, and ankylosing spondilitis and secreted mainly by macrophages and through TNF alpha and TNFR1 interaction.

Therefore, the factor that stimulates macrophage activation in our patients, none of whom had any major infection other than gingivitis or aphthous stomatitis at the time of evaluation, seems to be excessive apoptosis (thereby secondary necrosis) in the granulocytic lineage due to various mutations distrupting normal granulopoiesis. Apoptosis and secondary necrosis of other cell lines like lymphocytes and monocytes as shown by flow cytometry, and in megakaryocytes as shown by light microscopy are thought to be secondary to increased TNF-alpha through excessive macrophage activation.

Text 2

On the other hand, RCS in 25% and 12.5% of the evaluated patients and parents, documented by β-gal positivity of leukocytes may be another reason for quantitative abnormalities in T lymphocytes. That the patients with low CD3+ and CD4+ lymphocytes (sibling patients AG, ZG) were those with β-gal positivity of leukocytes showing RCS and with normal NK levels suggests a relationship between T-lymphopenia and RCS. Presence of immune activation in our patients documented by increased phagocytic activity in their bone marrow suggests that continual presence of circulating pro-inflammatory aforementioned factors secreted by activated macrophages had kept the immune system in a state of chronic low-level activation giving rise to an immunosenescence through loss of telomeric DNA with each S phase, and therefore a decline in the absolute number of T and B lymphocytes, with no change in overall lymphocyte count but a relative increase in NK cells [24] (in our patients we could not evaluate B lymphocytes). A set of mediators including inflammatory mediators secreted by senescent cells themselves contribute to the chronic inflammation [25] and the loss of telomeres. Likewise, a previous study of ours showed RCS (β-gal positivity) of leukocytes in active autoimmune disorders [systemic lupus erythematasus (SLE), juvenile rhematoid arthritis (JRA), and immune thrombocytopenic purpura (ITP)], in all of which pro-inflammatory cytokines are high [26].

Text 3

Dysmegakaryopoiesis and Hemorrhagic Diathesis

In our patients one of the most prominent findings was abnormal megakaryopoiesis evidenced by naked megakaryocyte nuclei (NMN),

Supplemental Table 6. Laboratory parameters of the parents.

Family of the patient that is examined/ mutation of the patient	Parent/molecular genetics	Lymphocyte subsets in comparison to age matched normal ranges	Dense granule per thrombocyte (n=2.78-3.82)/ Bleeding time (in vitro) (Coll-epi* n=85-157 s; Coll-ADP** n= 65-125 s)	Aggregation defect
Family of YF/ *HAX1* homozygous c.130-131insA (p.W44X)	Mother/NA†	NK:25-50p CD3:25-50p CD4:<25p CD8:25-50p CD4/CD8:25-50p	3.2/coll-epi: 120 s	No secondary aggregation with 2 and 6 µM ADP; hypoaggregation with epinephrin (10 µM/mL), normal aggregation with collagen (1µg/mL) and ristocetin (1.25 mg/mL)
	Father/NA†	ND	2.1/coll-epi: 152 s	No secondary aggregation with 2 and 6 µM ADP; normal aggregation with epinephrin (10 µM/mL), collagen (1µg/mL), and ristocetin (1.25 mg/mL)
Family of MNY/AY Both *HAX1*/ Homozygous c.130-131insA (p.W44X)	Mother *HAX1* heterozygous c.130-131insA (p.W44X)***	NK<25p;CD3>75p CD4:25-50p CD8>75p CD4/CD8<25p	4/coll-epi: 154 s	ND
	Father *HAX1* heterozygous c.130-131insA (p.W44X)***	NK<25p CD3:50-75p CD4>75p CD8<25p CD4/CD8>75p	0.23/coll-epi:122 s	Secondary aggregation defect and and disaggregation with ADP (10 µM), hypoaggregation with collagen (2 µg/mL), normal aggregation with ristocetin (1.25 µg/mL) (epinephrin not available)
Family of MK/ *HAX1* homozygous c.130-131insA (p.W44X)	Father/NA†	ND	ND/ND	Hypoaggregation with ADP (10 µM) and collagen (1 µg/mL), normal aggregation with ristocetin (1.25 µg/mL)
Family of EÇ/ *HAX1* homozygous c.130-131insA (p.W44X)	Mother *HAX1* heterozygous c.130-131insA***	NK<25p CD3>75p CD4:50-75p CD8:50-75p CD4/CD8:50p	3.6/coll-epi:134 s; Coll-ADP:103 s	Normal aggregation with ADP (10 µM), collagen (2 µg/mL), ristocetin (1.25 mg/mL)
	Father NA*	NK<25p CD3:25-50p CD4>75p CD8<25p CD4/CD8>75p	3.5/coll-epi:151; coll-ADP:80 s	Normal aggregation with ADP (10 µM), collagen (2 µg/mL), ristocetin (1.25 mg/mL)
Family of HY/ *HAX1* homozygous c.130-131insA (p.W44X)	Mother *HAX1* heterozygous c.130-131insA (p.W44X)***	NK<25p CD3>75p CD4:25-50p CD8:25-50p CD4/CD8:50p	4.5/coll-epi:76 s; Coll-ADP:48 s	Secondary aggregation defect and disaggregation with ADP (10 µM), normal aggregation with collagen (2 µg/mL), ristocetin (1.25 mg/mL)
	Father *HAX1* heterozygous c.130-131insA (p.W44X)***	NK:25-50p CD3:50-75p CD4:25-50p CD8:25-50p CD4/CD8:50p	0.9/coll-epi: 166 s; coll-ADP: 115 s	Secondary aggregation defect and disaggregation with ADP (10 µM), collagen (2 µg/mL), ristocetin (1.25 mg/mL)
Family of OSK/MeK *G6PC3* homozygous, c.194A>C (p.E65A)	Father/NA†	ND	2.3/ND	Normal aggregation with ADP (2 µM), hypoaggregation with epinephrin (10 µM/mL), collagen (1µg/mL) and ristocetin (1.25 mg/mL)
Family of the patient that is examined/ mutation of the patient	Parent/molecular genetics	Lymphocyte subsets in comparison to age matched normal ranges	Dense granule per thrombocyte (n=2.78-3.82)/ Bleeding time (in vitro) (Coll-epi* n=85-157 s; Coll-ADP** n=65-125 s)	Aggregation defect
Family of RT/ *ELANE* Heterozygous IVS4+5G>A	Mother No *ELANE* mutation****	NK<25p CD3:50-75p CD4>75p CD8<25p CD4/CD8>75p	3.95/coll-epi: 142 s; coll-ADP: 96 s	Normal aggregation with ADP (2 and 6 µM), epinephrin (10 µM), collagen (1 µg/mL), ristocetin (1.25 mg/mL)
	Father No *ELANE* mutation****	NK<25p CD3>75p CD4:25-50p CD8:50-75p CD4/CD8:25-50p	3.26/coll-epi: 107 s; coll-ADP:65 s	Normal aggregation with ADP (2 and 6 µM), epinephrin (10 µM), collagen (1 µg/mL), ristocetin (1.25 mg/mL)

Supplemental Table 6. Continued.

Family of the patient that is examined/ mutation of the patient	Parent/molecular genetics	Lymphocyte subsets in comparison to age matched normal ranges	Dense granule per thrombocyte (n=2.78-3.82)/ Bleeding time (in vitro) (Coll-epi* n=85-157 s; Coll-ADP** n=65-125 s)	Aggregation defect
Family of BA Unidentified mutation	Father//NA†	NK<25p CD3:50-75p CD4>75p CD8<25p CD4/CD8>75p	3.5/coll-epi: 97 s	No secondary aggregation with 2µM ADP and epinephrin (10 µM/mL) but normal aggregation with 6 µM ADP, collagen (1µg/mL), and ristocetin (1.25 µg/mL)
Family of AG/ZG Unidentified mutation	Mother//NA†	NK<25p CD3:50-75p CD4>75p CD8<25p CD4/CD8>75p	0.76/coll-epi: 134s	Normal aggregation with ADP (2 and 10 µM), collagen (2 µg/mL), ristocetin (1.25 mg/mL)
	Father//NA†	NK<25p CD3>75p CD4>75p CD8<25p CD4/CD8>75p	0.98/ coll-epi: 193 s; coll-ADP>300 s	Normal aggregation with ADP (10 µM), collagen (2 µg/mL), ristocetin (1.25 mg/mL)
Family of NBÖ/ Heterozygous *ELANE* c.416C>T	Mother No *ELANE* mutation***	ND	1.8/coll-epi: 82 s	Normal aggregation with ADP (2 and 6 µM), epinephrin (10 µM), collagen (1 µg/mL), ristocetin (1.25 mg/mL)
	Father No *ELANE* mutation***	ND	2.0/coll-epi: 91 s	No secondary aggregation with ADP (2 µM) but normal aggregation with ADP (6 µM), epinephrin (10 µM), collagen (1 µg/mL), ristocetin (1.25 mg/mL)
Family of AO Unidentified mutation	Mother/NA†	ND	0.22/coll-epi: 189 s; coll-ADP:103 s	Normal aggregation with ADP (2 and 6 µM), collagen (1 µg/mL), ristocetin (1.25 mg/mL). Prolonged lag phase with epinephrin (10 µM)

*Collagen-epinephrin cartridge, **Collagen-ADP cartridge, ***Peripheral blood cells, ****Peripheral blood cells and buccal mucosa cells, †NA: not available.

megakaryocyte emperipolesis, megakaryocytes with features of apoptosis, paraapoptosis, necrosis, and abnormalities in thrombocyte aggregation.

Naked megakaryocyte nuclei form after progressive folding of lobulated nucleus itself and then contracting (pyknosis), in order to reach the lowest volume that the megakaryocyte can occupy. The surrounding small rim of cytoplasm contains no characteristic MK granules or demarcation membranes. In physiological conditions, NMN are senescent cells which completed thrombocyte shedding and are few in normal bone marrow. However, large numbers of NMN in the bone marrow, like in myeloproliferative diseases and AIDS show defective maturation, a heavy intramedullary premature cell death of megakaryocytes and increased megakaryopoies [27, 28].

Emperipolesis [29,30] is a random passage of one cell through the cytoplasm of another one with no physiological change in neither of them. It is most frequent in megakaryocytes and the most engulfed cell types are neutrophils and erythrocytes, as in our patients.

Naked megakaryocyte nuclei [27] and megakaryocytes with emperipolesis [29] were shown to display the features of paraapoptosis (non classical apoptosis).

While apoptosis is characterized by margination of condensed chromatin, nuclear fragmentation and formation of apoptotic bodies, paraapoptosis of megakaryocytes is characterized by cytoplasmic vacuolization due to mitochondrial swelling and distended demarcation membrane system (DMS), condensed nuclear chromatin with no early chromatin margination and no surface blebbing and apoptotic body formation. All paraapoptotic megakaryocytes were shown to have an intact, thickened peripheral zone which seemed to contain no functional cellular material, like organelles or DMS and their cytoplasm did not contain thrombocyte territories [31], like in our patients.

Additionally presence of many stage 1 megakaryocytes (megakaryoblasts), some of which are aberrantly releasing thrombocytes, unlike what is expected, also show dysmegakaryopoiesis [32].

That presence of nearly no normal megakaryocytes in our patients and that nearly all megakaryocytes displayed characteristics of NMN, emperipolesis or abnormal morphology like peripheral vacuolization (showing non-classical apoptosis: paraapoptosis and directly destructed megakaryocytes (showing necrosis) imply defective megakaryocyte maturation, a heavy intramedullary premature cell death of megakaryocytes and increased megakaryopoiesis.

Defective maturation in megakaryocytes is also expected to be due to the increased levels of aforementioned proinflammatory cytokines secreted by and after macrophage activation which can destroy the bone marrow microenvironment and hematopoietic stem cell niches [25] giving rise to hematopoietic stem cell dysfunction and dyshematopoiesis and thereby generalized

dysplastic findings of bone marrow cells, as it is for the other bone marrow cells.

Supplemental References

1. Olcay L, Yetgin S, Okur H, Erdemli E. Rapid cell senescence and apoptosis in lymphocytes and granulocytes and absence of GM-CSF receptor in congenital dysgranulopoietic neutropenia. Leuk Res 2008;32:235-242.
2. Olcay L, Yetgin S, Erdemli E, Germeshausen M, Aktaş D, Büyükaşik Y, Okur H. Congenital dysgranulopoietic neutropenia. Pediatr Blood Cancer 2008;50:115-119.
3. Dykstra MJ. A Manual of Applied Techniques for Biological Electron Microscopy. New York, Plenum Press, 1993.
4. Olcay L, Yetgin S, Okur H, Erekul S, Tuncer M. Dysplastic changes in idiopathic thrombocytopenic purpura and the effect of corticosteroids to increase dysplasia and cause hyperdiploid macropolycytes. Am J Hematol 2000;65:99-104.
5. Buyukasik Y, Karakus S, Goker H, Haznedaroglu IC, Ozatli D, Sayinalp N, Ozcebe OI, Dundar SV, Kirazli S. Rational use of the PFA-100 device for screening of platelet function disorders and von Willebrand disease. Blood Coagul Fibrinolysis 2002;13:349-353.
6. Olcay L, Erdemli E, Kesimer M, Büyükasik Y, Okur H, Kalkanoğlu HS, Coskun T, Altay C. High cystine in platelets from patients with nephropathic cystinosis: a chemical, ultrastructural, and functional evaluation. J Clin Pathol 2005;58:939-945.
7. Komarnichi M, Pietrzak I, Zozulinska M. Mepacrine-labeled platelet dense-body number in patients with chronic uremia. Nephron 1988;50:306-307.
8. Pintado T, Maldonado JE. Ultrastructure of platelet aggregation in refractory anemia and myelomonocytic leukemia. I. Ultrastructure of aggregation in normal controls and general defects in refractory anemia and myelomonocytic leukemia. Mayo Clin Proc 1976;51:379-392.
9. Pintado T, Maldonado JE. Ultrastructure of platelet aggregation in refractory anemia and myelomonocytic leukemia. II. Individual platelet abnormalities: Thrombasthenia-like platelets, surface defects, and dissociation phenomena. Mayo Clin Proc 1976;51:443-451.
10. Poon IK, Lucas CD, Rossi AG, Ravichandran KS. Apoptotic cell clearance: basic biology and therapeutic potential. Nat Rev Immunol 2014;14:166-180.
11. Olcay L, Ünal Ş, Onay H, Erdemli E, Öztürk A, Billur D, Metin A, Okur H, Yıldırmak Y, Büyükaşık Y, İkincioğulları A, Yılmaz-Falay M, Özet G, Yetgin S. Granulocytic and non granulocytic lineages in children with congenital neutropenia and their non neutropenic parents: Biochemical, functional, morphological and genetic evaluation. Haematologica 1012;97(Suppl 3):O65a (abstract).
12. Koval'chuk LV, Khoreva MV, Nikonova AS, Finogenova NA, Mamedova EA, Polovtseva TV, Fetisova LIa, Gracheva LA, Goldyreva NG. Toll-like receptor-mediated functional activity of mononuclear cells in children with neutropenia. Zh Mikrobiol Epidemiol Immunobiol 2010;2:64-68.
13. Bohn G, Hardtke-Wolenski M, Zeidler C, Maecker B, Sauer M, Sykora KW, Grigull L, Welte K, Klein C. Lethal graft-versus-host disease in congenital neutropenia caused by p14 deficiency after allogeneic bone marrow transplantation from an HLA-identical sibling. Pediatr Blood Cancer 2008;51:436-438.
14. Shitara T, Yugami S, Ijima H, Sotomatu M, Kuroume T. Cytokine profile during high-dose rhG-CSF therapy in severe congenital neutropenia. Am J Hematol 1994;45:58-62.
15. Abbas AK, Lichtman AH, Pillai S. Innate immunity. In: Cellular and Molecular Immunology. 8th ed. Canada, Elsevier Saunders, 2015:51-86.
16. Abbas AK, Lichtman AH, Pillai S. Differentiation and functions of CD4+ effector T cells. In: Cellular and Molecular Immunology. 8th ed. Canada, Elsevier Saunders, 2015:213-230.
17. Geering B, Simon HU. A novel signaling pathway in TNFα-induced neutrophil apoptosis. Cell Cycle 2011;10:2821-2822.
18. Ugan Y, Nazıroğlu M, Şahin M, Aykur M. Anti-tumor necrosis factor alpha (Infliximab) attenuates apoptosis, oxidative stress, and calcium ion entry through modulation of cation channels in neutrophils of patients with ankylosing spondylitis. J Membr Biol 2016;249:437-447.
19. Bommhardt U, Chang KC, Swanson PE, Wagner TH, Tinsley KW, Karl IE, Hotchkiss RS. Akt decreases lymphocyte apoptosis and improves survival in sepsis. J Immunol 2004;172:7583-7591.
20. Hotchkiss RS, Tinsley KW, Karl IE. Role of apoptotic cell death in sepsis. Scand J Infect Dis 2003;35:585-592.
21. Kawakami Y, Tsukimoto M, Kuwabara K, Fujita T, Fujino O, Kojima S, Fukunaga Y. Tumor necrosis factor-α-induced mononuclear cell death may contribute to polymorphonuclear cell predominance in the cerebrospinal fluid of patients with bacterial meningitis. J Nippon Med Sch 2011;78:360-366.
22. Cevik O, Adiguzel Z, Baykal AT, Somay G, Sener A. The apoptotic actions of platelets in acute ischemic stroke. Mol Biol Rep 2013;40:6721-6727.
23. Kawada J, Kimura H, Shibata Y, Hara S, Hoshino Y, Kojima S, Nishikawa K, Morishima T. Evaluation of apoptosis in Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis. J Med Virol 2006;78:400-407.
24. McElhaney JE, Effros RB. Immunosenescence: what does it mean to health outcomes in older adults? Curr Opin Immunol 2009;21:418-424.
25. Freund A, Orjalo AV, Desprez PY, Campisi J. Inflammatory networks during cellular senescence: Causes and consequences. Trends Mol Med 2010;16:238-246.
26. Olcay L, Billur D, Erdemli E, Baskin SE, Balci HF, Yetgin S. Myelodysplastic features and cellular senescence in autoimmune disorders: a pilot study on patients with collagen tissue disorders and immune thrombocytopenic purpura. Turk J Med Sci 2015;45:742-744.
27. Thiele J, Lorenzen J, Manich B, Kvasnicka HM, Zirbes TK, Fischer R. Apoptosis (programmed cell death) in idiopathic (primary) osteo-/myelofibrosis: naked nuclei in megakaryopoiesis reveal features of para-apoptosis. Acta Haematol 1997;97:137-143.
28. Olcay L, Tuncer AM, Okur H, Erdemli E, Uysal Z, Cetin M, Duru F, Cetinkaya DU. Excessive naked megakaryocyte nuclei in myelodysplastic syndrome mimicking idiopathic thrombocytopenic purpura: a complicated pre- and post-transplantation course. Pediatr Hematol Oncol 2009;26:387-397.
29. Centurione L, Di Baldassarre A, Zingariello M, Bosco D, Gatta V, Rana RA, Langella V, Di Virgilio A, Vannucchi AM, Migliaccio AR. Increased and pathologic emperipolesis of neutrophils within megakaryocytes associated with marrow fibrosis in GATA-1(low) mice. Blood 2004;104:3573-3580.
30. Aslan D, Yetgin S. Megakaryocyte emperipolesis in a child with chronic neutropenia: an unusual coexistence. Turk J Pediatr 2001;43:255-256.
31. Houwerzijl EJ, Blom NR, van der Want JJ, Esselink MT, Koornstra JJ, Smit JW, Louwes H, Vellenga E, de Wolf JT. Ultrastructural study shows morphologic features of apoptosis and para-apoptosis in megakaryocytes from patients with idiopathic thrombocytopenic purpura. Blood 2004;103:500-506.
32. Erber WN, Jacobs A, Oscier DG, O'hea AM, Mason DY. Circulating micromegakaryocytes in myelodysplasia. J Clin Pathol 1987;40:1349-1352.

RESEARCH ARTICLE

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Tendency of K562 Chronic Myeloid Leukemia Cells Towards Cell Reprogramming

K562 Kronik Myeloid Lösemi Hücrelerinin Yeniden Hücre Programlanmasına Yatkınlığı

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Abstract

Objective: Cancer cell reprogramming is a potential tool to study cancer progression, disease pathology, and drug sensitivity. Prior to performing cancer reprogramming studies, it is important to evaluate the stemness predisposition of cells that will be reprogrammed. We performed a proof-of-concept study with chronic myeloid leukemia K562 cells in order to evaluate their tendency for cancer cell reprogramming.

Materials and Methods: Expression of reprogramming factors, pluripotency markers, and tumor-suppressor genes was analyzed at gene and protein levels via real-time reverse transcription-polymerase chain reaction and flow cytometry. Human peripheral blood mononuclear cells (PBMCs) were used as a positive control.

Results: K562 cells were shown to express higher levels of most of the reprogramming factors and pluripotency markers. Expression of p53, which is one of the main regulators during the generation of induced pluripotent stem cells, was found to be lower in K562 cells compared to PBMCs, whereas the other tumor-suppressor genes showed higher expression levels.

Conclusion: This study suggested that, similar to healthy human PBMCs, K526 cells could be used in cancer cell reprogramming studies. Generating induced pluripotent stem cells from leukemia cells could help scientists to establish chronic myeloid leukemia models in vitro for a better understanding of therapy resistance and development of novel therapeutic targets.

Keywords: Induced pluripotent stem cells, Chronic myeloid leukemia, K562, Disease modeling, Cell reprogramming

Öz

Amaç: Kanser hücrelerinin yeniden programlanarak hastalık modellerinin oluşturulması, hastalığın ilerleyişini, patolojisini ve ilaç duyarlılığını incelemek için önemli bir teknolojidir. Kanser hücrelerinde yeniden programlama çalışmalarını gerçekleştirmeden önce, hücrelerin programlamaya yatkınlığını değerlendirmek önemlidir. Kronik myeloid lösemi K562 hücrelerinin kanser programlama çalışmalarında kullanılabilirliğini göstermek amacıyla bir kavram kanıtı çalışması gerçekleştirdik.

Gereç ve Yöntemler: Yeniden programlama faktörleri, pluripotensi belirteçleri ve tümör baskılayıcı genlerin ifadeleri, gerçek zamanlı-polimeraz zincir reaksiyonu ve akan hücre sitometrisi ile gen ve protein seviyelerinde analiz edildi. Programlama çalışmalarında en çok kullanılan insan periferik kan mononükleer hücreleri (PBMC) pozitif kontrol olarak kullanıldı.

Bulgular: K562 hücrelerinin, yeniden programlama faktörlerini ve pluripotency belirteçlerini PBMC hücrelerine göre daha yüksek seviyede ifade ettiği gösterilmiştir. Uyarılmış pluripotent kök hücrelerinin oluşumu sırasında ana düzenleyicilerden biri olan p53'ün ifadesi, K562 hücrelerinde PBMC'ye kıyasla daha düşük bulunurken, diğer tümör baskılayıcı genler daha yüksek ifade göstermiştir.

Sonuç: Bu çalışma, sağlıklı insan PBMC'lerine benzer şekilde, K526 hücrelerinin yeniden programlama çalışmalarında kullanılabileceğini göstermiştir. Lösemi kaynaklı uyarılmış pluripotent kök hücreleri kullanılarak in vitro ortamda hastalık modellerinin üretilmesi, bilim insanlarının ilaç dirençlerini daha iyi anlaması ve yeni tedavi hedefleri geliştirilmesi için önemli bir araç olacaktır.

Anahtar Sözcükler: Uyarılmış pluripotent kök hücreler, Kronik myeloid lösemi, K562, Hastalık modelleme, Hücre programlama

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Introduction

Since their discovery, induced pluripotent stem cells (iPSCs) have been extensively used to model diseases and test drugs in vitro [1,2,3,4,5,6]. Hematological disorders including chronic myeloid leukemia (CML) have been modeled by various research groups [4,7,8]. iPSCs capture the genetic alterations present in leukemia cells and their differentiation ability helps scientists to understand disease progression and therapy resistance.

In one of the first such studies, the CML cell line KBM7 was reprogrammed towards pluripotency via retroviral vectors carrying OKSM (Oct3/4, Klf-4, Sox-2, c-Myc) factors [9]. Unlike the untreated cells, the reprogrammed group showed resistance to the chemotherapeutic agent imatinib, which is an inhibitor of the BCR-ABL oncogene. It was hypothesized that the therapeutic agent imatinib targets cells in a specific epigenetic differentiated cell state, which can contribute to its inability to fully eradicate disease in CML patients [10]. Later, Bedel et al. [11] reported that when $CD34^{BCR-ABL+}$ cells from CML patients were reprogrammed, CML-iPSCs lost their BCR-ABL dependency and became resistant to tyrosine kinase inhibitor therapy. The authors suggested that CML-iPSCs can be used to study mechanisms by which leukemic stem cells survive to therapy and are a promising tool for testing and screening new therapeutic targets reducing leukemic stem cell survival [11]. In another CML study, again with the use of retroviral vectors, iPSCs were generated from primary CML patients' cells. Although CML-iPSCs were resistant to the chemotherapeutic agent imatinib, CML-iPSC-derived hematopoietic cells recovered sensitivity to the drug [12]. In another study, whole-genome sequencing of CML-derived iPSCs revealed genocopying of highly mutated primary leukemic cells, which were used to understand the selective growth under tyrosine kinase inhibitor therapies [13]. In 2015, iPSCs were used to identify the leukemia stem cells for primitive CML by Suknuntha et al. [14]. Due to the rarity of leukemia stem cells within the primitive hematopoietic cell compartment, it is difficult to study their contribution. By the generation of CML-iPSCs, the authors discovered olfactomedin 4 as a novel factor that contributes to the survival and growth of somatic lin(-)CD34(+) cells from the bone marrow of patients with CML in the chronic phase, but not primitive hematopoietic cells from normal bone marrow [14]. These contradictory results show that more work is needed to model CML. However, as in the reprogramming of healthy cells, there are various factors affecting reprogramming efficiency, and for this reason, these factors should be first determined for leukemia in order to model such diseases in vitro.

As can be seen from the above studies, reprogramming cancer cells is a potential tool to study cancer progression, disease pathology, and drug sensitivity. Prior to performing cancer reprogramming studies, it is important to evaluate the stemness predisposition of cells that will be reprogrammed [4]. Here, we performed a proof-of-concept study with K562 cells in order to evaluate their tendency for cancer cell reprogramming. We analyzed the endogenous expression of reprogramming and pluripotency factors that are known to be important factors for cell reprogramming. Furthermore, it is well known that the expression of tumor-suppressor genes also determines reprogramming efficiency [15]. Therefore, the levels of important tumor-suppressor genes were identified in K562 cells.

Materials and Methods

Cell Culture

Human CML cell line K562 and human peripheral blood mononuclear cells (PBMCs) were obtained from ATCC and Lonza, respectively. Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 50 U/mL penicillin, 50 µg/mL streptomycin, and 1% L-glutamine at 37 °C in 5% CO_2.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Cells ($1x10^6$ cells) were collected and RNA was extracted with the Macherey-Nagel RNA isolation kit. cDNA synthesis from 1 µg of RNA sample was performed with the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Two microliters of each cDNA sample were used to perform real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) reactions with iQ SYBR Green SuperMix (Bio-Rad). Samples were run on the CFX-96 Connect Real-Time System (Bio-Rad) with the following protocol: 95 °C for 3 min, 1 cycle; 95 °C for 10 s and 60 °C for 30 s, repeated for 40 cycles. GAPDH was used as a reference gene and gene expression levels for OCT3/4, SOX2, KLF4, CMYC, NANOG, REX, CRIPTO, P53, P21, P16, and PRB were normalized to PBMCs.

Flow Cytometry Analysis

Cells ($1x10^6$ cells) were collected by centrifugation, washed with ice-cold methanol for fixation, and then permeabilized with 0.1% Triton-X100 containing 2% bovine serum albumin-phosphate buffered saline (PBS). Following washing with PBS, cells were stained with rabbit anti-Oct3/4, rabbit anti-Nanog, or mouse anti-p53 antibodies. Anti-rabbit-AF543 and anti-mouse-AF488 were used as secondary antibodies. Cells were analyzed with a BD Accuri Plus Flow Cytometer (BD). For 10,000 events, percentages of positive populations were determined by using BD Accuri Plus software (BD).

Statistical Analysis

Triplicates containing required amounts of cells were used during analyses. Delta Ct values were used for the statistical analysis of RT-PCR results. Statistical analysis was performed

by analysis of variance and Tukey's pairwise comparisons using SPSS 16.0 (SPSS Inc.).

Results

In order to test the tendency of K562 cells towards cell reprogramming, we used human PBMCs as a positive control because there are studies that have shown successful reprogramming with PBMCs [16].

As shown in Figure 1, the reprogramming factors (Oct3/4, Klf2, Sox2, cMyc) were all upregulated in K562 cells compared to PBMCs (Figure 1A). Significant differences were observed for the Klf2, Sox2, and cMyc genes. When the cells were analyzed for the expression of pluripotency markers including Nanog, Rex, and Cripto via real-time RT-PCR, we observed higher expression levels compared to PBMCs (Figure 1B). However, we obtained a lower profile when compared to that of the programming factors.

In addition to the programming and pluripotency factors, the expression of tumor-suppressor genes determines the efficiency of reprogramming [15]. For this reason, we analyzed the expression of the P53, P21, P16, and PRB genes and found that P53 was downregulated in K562 cells compared to PBMCs, whereas the others showed higher expression levels (Figure 2).

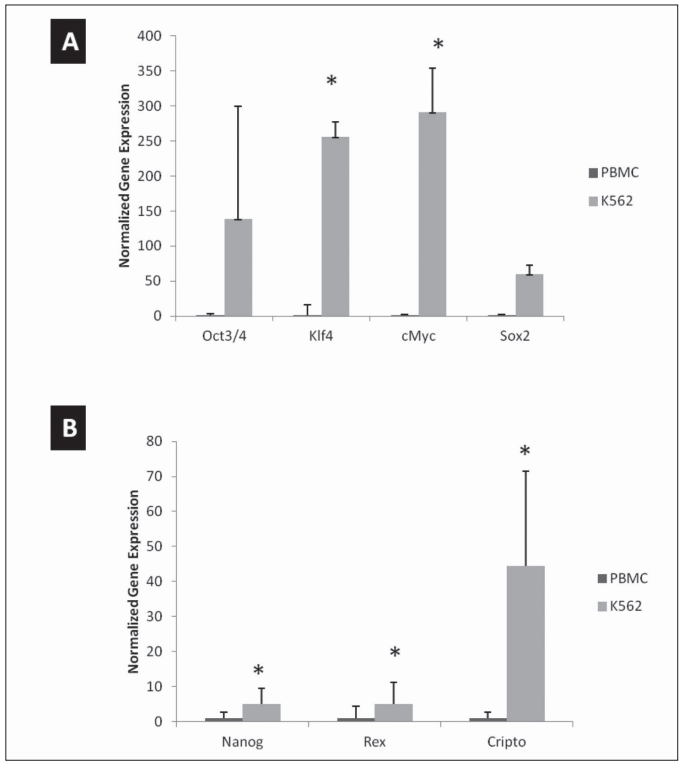

Figure 1. Gene expression of reprogramming factors and pluripotency markers. RNA was isolated from peripheral blood mononuclear cells (PBMCs) and K562 cells and quantitative real-time polymerase chain reaction was performed. Relative gene expression was plotted for A) reprogramming factors and B) pluripotency markers. GAPDH was used as a reference gene and data were normalized to PBMCs.

*p<0.05 compared to PBMCs.

In order to confirm the gene expression data, we performed flow cytometric analysis of programming factor Oct3/4, pluripotency marker Nanog, and tumor suppressor p53 in PBMCs and K562 cells. Flow cytometry analyses confirmed the real-time RT-PCR data. The percentage of cells positive for Oct3/4 and Nanog was increased to 11.7% and 9.5%, respectively (Figure 3). When anti-P53 antibodies were used to stain the cells in flow cytometry, in contrast to real-time RT-PCR data, there was no significant change in the P53-positive cell populations (Figure 4). This may suggest that even though there was a difference at the mRNA level, protein levels do not vary, possibly due to posttranscriptional regulation.

Discussion

It has been previously reported that the expressions of reprogramming and pluripotency factors in the starting cells are limiting factors in cell reprogramming [4,17]. As shown here, higher levels of reprogramming and pluripotent factors at both gene and protein levels increase the tendency towards cellular reprogramming. On the other hand, expression of tumor-suppressor genes needs to be controlled during iPSC generation [15,18,19]. In this study, we observed downregulation of P53 mRNA and similar levels of its protein in K562 cells compared to PBMCs. However, the overall expressions of other tumor-suppressor genes can still be limiting factors. Therefore, the expression of these genes should be carefully monitored during reprogramming. Silencing strategies could be needed to achieve efficient reprogramming. Until now, cancer reprogramming studies for CML have not focused on the expression of the above factors [9,11,13,20,21] and there is no study that has reported

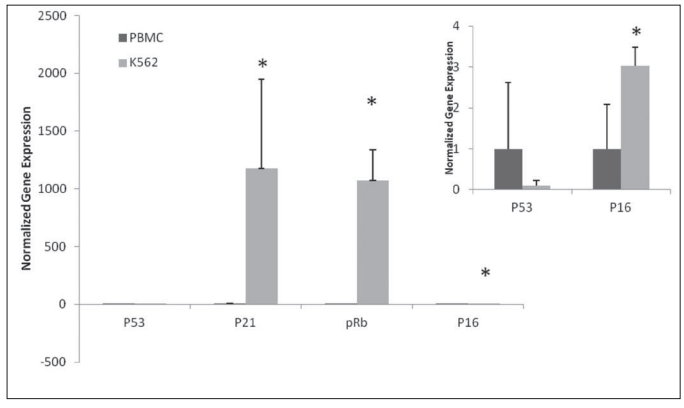

Figure 2. Gene expression of tumor-suppressor genes. RNA was isolated from peripheral blood mononuclear cells (PBMCs) and K562 cells and quantitative real-time polymerase chain reaction was performed. Relative gene expression was plotted for tumor-suppressor genes. GAPDH was used a reference gene and data were normalized to PBMCs.

*p<0.05 compared to PBMCs. Small graph shows the gene expression profile of p53 and p16 in order to better represent the differences.

the link between these factors and the ease of reprogramming. Therefore, this is an important preliminary study that reinforces the importance of these factors.

Screening the levels of reprogramming and pluripotency factors has been one of the ways to assess the efficiency of iPSC generation [4]. On the other hand, expression of these markers has also been linked with multidrug resistance of leukemia cells through modulating the ATP-binding-cassette transporters (ABC-transporters) [9,21]. For example, the expressions of Oct4, Sox2, and Nanog, all of which are studied in the present work, have been shown to be upregulated in K562 cells when they retain multidrug resistance to doxorubicin [20,22]. Therefore, this also suggests that monitoring their expression status is a key step in order to model the disease and study drug resistance.

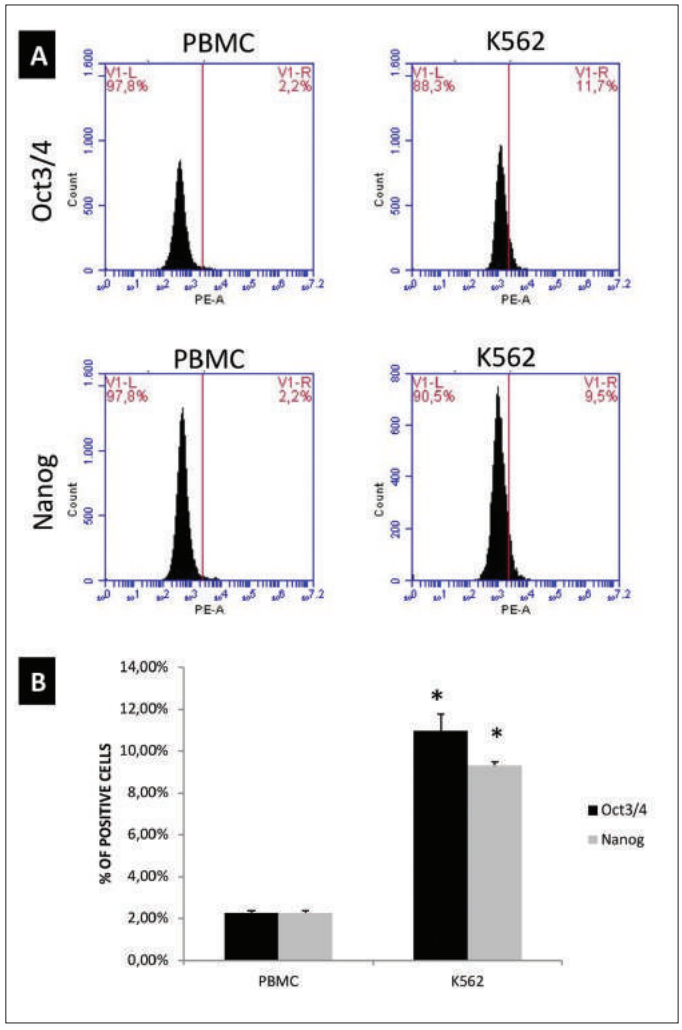

Figure 3. Protein expression of reprogramming factor Oct3/4 and pluripotency marker Nanog. Cells were collected by centrifugation, followed by staining for Oct3/4 and Nanog. Cells were analyzed in a BD Accuri Plus Flow Cytometer (BD).

*p<0.05 compared to peripheral blood mononuclear cells.

PBMC: Peripheral blood mononuclear cell.

In addition to the above factors, there are other factors that limit the efficiency of iPSC generation from cancer cells. These include the proliferation rate of cancer cells, the epigenetic background, long-term culturing conditions during reprogramming, the heterogeneity of tumor cells, and the presence of cancer stem cells [4]. For example, highly proliferating cells will be difficult to reprogram since dividing cancer cells and reprogrammed cancer cells will compete in culture conditions, which would not allow for ground-state pluripotency in the reprogrammed cells [23]. Therefore, future studies should monitor the above parameters in detail during cell reprogramming of CML.

Considering PBMC usage as the cell source in reprogramming protocols, this proof-of-concept study showed that K526 CML cells could also be used in cancer cell reprogramming studies. Generating iPSCs from these cell lines could help scientists to establish CML models in vitro. This can allow us to study disease progression, drug responses, and disease pathology.

Conclusion

This study suggested that, similar to healthy human PBMCs, K526 cells could be used in cancer cell reprogramming studies. Generating iPSCs from leukemia cells could help scientists

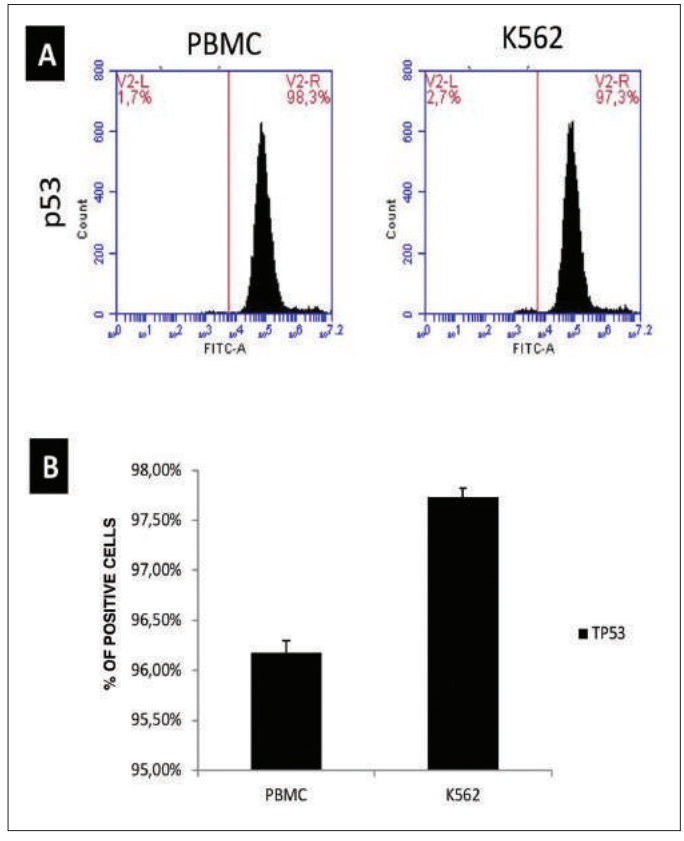

Figure 4. Protein expression of tumor-suppressor gene p53. Cells were collected by centrifugation, followed by staining for p53. Cells were analyzed in a BD Accuri Plus Flow Cytometer (BD).

PBMC: Peripheral blood mononuclear cell.

to establish CML models in vitro, better understand disease progression, and develop novel therapeutic targets.

Ethics

Ethics Committee Approval: N/A.

Conflict of Interest: A.Y.A. acknowledges support by the Scientific and Technological Research Council of Turkey (TÜBİTAK, grant number 113S897). The author confirms that there are no known conflicts of interest associated with this publication.

References

1. Colman A, Dreesen O. Pluripotent stem cells and disease modeling. Cell Stem Cell 2009;5:244-247.
2. Yamanaka S. The winding road to pluripotency (Nobel Lecture). Angew Chem Int Ed Engl 2013;52:13900-13909.
3. de Lázaro I, Yilmazer A, Kostarelos K. Induced pluripotent stem (iPS) cells: a new source for cell-based therapeutics? J Control Release 2014;185:37-44.
4. Yilmazer A, de Lázaro I, Taheri H. Reprogramming cancer cells: a novel approach for cancer therapy or a tool for disease-modeling? Cancer Lett 2015;369:1-8.
5. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126:663-676.
6. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861-872.
7. Hanna J, Wernig M, Markoulaki S, Sun CW, Meissner A, Cassady JP, Beard C, Brambrink T, Wu LC, Townes TM, Jaenisch R. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science 2007;318:1920-1923.
8. Lim KL, Teoh HK, Choong PF, Teh HX, Cheong SK, Kamarul T. Reprogramming cancer cells: overview & current progress. Expert Opin Biol Ther 2016;16:941-951.
9. Carrett-Dias M, Almeida LK, Pereira JL, Almeida DV, Filgueira DM, Marins LF, Votto AP, Trindade GS. Cell differentiation and the multiple drug resistance phenotype in human erythroleukemic cells. Leuk Res 2016;42:13-20.
10. Carette JE, Pruszak J, Varadarajan M, Blomen VA, Gokhale S, Camargo FD, Wernig M, Jaenisch R, Brummelkamp TR. Generation of iPSCs from cultured human malignant cells. Blood 2010;115:4039-4042.
11. Bedel A, Moreau-Gaudry F, Pasquet JM, Taillepierre M, Lippert E, Lagarde V, de Verneuil H, Richard E, Mahon FX. Induced pluripotent stem cells (iPSC) from chronic myeloid leukemia: study of BCR-ABL addiction and effect of tyrosine kinase inhibitors. Blood 2011;118:3754-3754.
12. Kumano K, Arai S, Hosoi M, Taoka K, Takayama N, Otsu M, Nagae G, Ueda K, Nakazaki K, Kamikubo Y, Eto K, Aburatani H, Nakauchi H, Kurokawa M. Generation of induced pluripotent stem cells from primary chronic myelogenous leukemia patient samples. Blood 2012;119:6234-6242.
13. Sloma I, Mitjavila-Garcia MT, Feraud O, Oudrhiri N, Tosca L, El Marsafy S, Gobbo E, Divers D, Proust A, Griscelli F, Tachdjian G, Marra MA, Eaves CJ, Bennaceur-Griscelli A. Whole genome sequencing of chronic myeloid leukemia (CML)-derived induced pluripotent stem cells (iPSC) reveals faithful genocopying of highly mutated primary leukemic cells. Blood 2013;122:514.
14. Suknuntha K, Ishii Y, Tao L, Hu K, McIntosh BE, Yang D, Swanson S, Stewart R, Wang JYJ, Thomson J, Slukvin I. Discovery of survival factor for primitive chronic myeloid leukemia cells using induced pluripotent stem cells. Stem Cell Res 2015;15:678-693.
15. Hong H, Takahashi K, Ichisaka T, Aoi T, Kanagawa O, Nakagawa M, Okita K, Yamanaka S. Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. Nature 2009;460:1132-1135.
16. Soares FAC, Pedersen RA, Vallier L. Generation of human induced pluripotent stem cells from peripheral blood mononuclear cells using Sendai virus. In: Turksen K, Nagy A (eds). Induced Pluripotent Stem (iPS) Cells. Methods in Molecular Biology. New York City, Humana Press, 2015:1357.
17. Miyoshi N, Ishii H, Nagai K, Hoshino H, Mimori K, Tanaka F, Nagano H, Sekimoto M, Doki Y, Mori M. Defined factors induce reprogramming of gastrointestinal cancer cells. Proc Natl Acad Sci U S A 2010;107:40-45.
18. Kawamura T, Suzuki J, Wang YV, Menendez S, Morera LB, Raya A, Wahl GM, Izpisúa Belmonte JC. Linking the p53 tumour suppressor pathway to somatic cell reprogramming. Nature 2009;460:1140-1144.
19. Marión RM, Strati K, Li H, Murga M, Blanco R, Ortega S, Fernandez-Capetillo O, Serrano M, Blasco MA. A p53-mediated DNA damage response limits reprogramming to ensure iPS cell genomic integrity. Nature 2009;460:1149-1153.
20. Xin H, Kong Y, Jiang X, Wang K, Qin X, Miao ZH, Zhu Y, Tan W. Multi-drug-resistant cells enriched from chronic myeloid leukemia cells by doxorubicin possess tumor-initiating-cell properties. J Pharmacol Sci 2013;122:299-304.
21. Kim HB, Lee SH, Um JH, Kim MJ, Hyun SK, Gong EJ, Oh WK, Kang CD, Kim SH. Sensitization of chemo-resistant human chronic myeloid leukemia stem-like cells to Hsp90 inhibitor by SIRT1 inhibition. Int J Biol Sci 2015;11:923-934.
22. Rumjanek VM, Vidal RS, Maia RC. Multidrug resistance in chronic myeloid leukaemia: how much can we learn from MDR-CML cell lines? Biosci Rep 2013;33:e00081.
23. Yılmazer A, Taheri H. Reprogramming human melanocytes and melanoma cells with Yamanaka factors. Hacettepe J Biol Chem 2018;46:35-41.

RESEARCH ARTICLE

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Plasma Ischemia-Modified Albumin Levels and Dynamic Thiol/Disulfide Balance in Sickle Cell Disease: A Case-Control Study

Orak Hücre Hastalığında Plazma İskemi Modifiye Albümin Düzeyleri ve Dinamik Tiyol/Disülfit Dengesi: Bir Olgu Kontrol Çalışması

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Abstract

Objective: Sickle cell disease (SCD), described as a group of inherited blood disorders, affects millions of people throughout the world and is particularly common in the southern part of Turkey. We aimed to determine the relationship between ischemia-modified albumin (IMA) and the dynamic thiol/disulfide balance in SCD.

Materials and Methods: Fifty-four adult SCD patients and 30 healthy controls were included in the study. The 54 adult patients included 30 (56%) males and 24 (44%) females with a mean age of 28.3±8.4 years (minimum-maximum: 18-46 years). Of the 54 patients, 46 had homozygous sickle cell anemia (HbSS) and 8 had sickle/β-thalassemia (HbS/β$^+$-thalassemia). Fasting blood samples were collected. After centrifugation at 1500×g for 10 min, plasma samples were portioned and stored at -80 °C. IMA levels were determined by albumin cobalt binding test, a colorimetric method. Total and native thiols and disulfide were analyzed with a novel spectrophotometric method.

Results: We found significantly lower levels of native thiol (-SH) (284.0±86.3 μmol/L), disulfide levels (14.6±7 μmol/L), and total thiols (-SH + -S-S-) (313.0±89.3 μmol/L) in SCD patients compared to healthy controls (respectively 417.0±54.2, 22.7±11.3, and 462.0±58.7 μmol/L). Plasma albumin levels (34.9±7.9 g/L) were lower and IMA levels (13.6±3.1 g/L) were higher in SCD patients compared to controls (respectively 43.5±3.1 and 8.4±1.6 g/L). Plasma albumin levels were strongly correlated with both plasma native (r=0.853; p=0.0001) and total thiols (r=0.866; p=0.0001).

Conclusion: Decreased plasma native and total thiol levels and increased IMA levels are related to increased oxidative stress and provide an indirect and quick reflection of the oxidative damage in SCD patients.

Keywords: Sickle cell disease, Thiol/disulfide homeostasis, Oxidative stress, Ischemia-modified albumin

Öz

Amaç: Kalıtsal kan hastalıklarının bir grubu olarak tanımlanan orak hücre hastalığı (OHH), tüm dünyada milyonlarca insanı etkilemekte ve özellikle Türkiye'nin güneyinde yaygın olarak görülmektedir. OHH hastalarında, iskemi modifiye albümin (İMA) ve dinamik tiyol/disülfit dengesi arasındaki ilişkiyi belirlemeyi amaçladık.

Gereç ve Yöntemler: Çalışmaya, yaş ortalaması 28,3±8,4 (minimum-maksimum: 18-46) olan 30 (%56) erkek ve 24 (%44) kadın olmak üzere 54 yetişkin OHH hastası ve 30 sağlıklı kontrol dahil edildi. Elli dört yetişkin OHH hastanın 46'sında homozigot orak hücre anemisi (HbSS) ve 8'inde orak/β-talasemi (HbS/β$^+$-talasemi) vardır. Hasta ve kontrol gruplarının açlık kan örnekleri toplandı. Plazma örnekleri 1500×g'de 10 dakika santrifüj edildikten sonra porsiyonlandı ve -80 °C'de saklandı. İMA seviyeleri, kolorimetrik bir test olan albümin kobalt bağlanma testi ile belirlendi. Toplam ve serbest tiyoller ve disülfit düzeyleri yeni bir spektrofotmetrik metot ile analiz edildi.

Bulgular: OHH hastalarında sırasıyla serbest tiyol düzeyleri (-SH) (284,0±86,3 μmol/L), disülfit seviyeleri (14,6±7 μmol/L) ve total tiyol (-SH + -SS-) düzeylerinin sağlıklı kontrollerle karşılaştırıldığında anlamlı derecede düşük olduğu bulundu (313,0±89,3 μmol/L) (417,0±54,2; 22,7±11,3; 462,0±58,7 μmol/L). OHH hastalarında kontrol grubu ile karşılaştırıldığında plazma albümin seviyeleri (34,9±7,9 g/L) daha düşük ve İMA düzeyleri (13,6±3,1 g/L) daha yüksek bulundu (sırasıyla 43,5±3,1; 8,4±1,6 g/L). Ayrıca, plazma albümin düzeylerinin, hem serbest tiyoller (r=0,853; p=0,0001) hem de total tiyoller (r=0,866; p=0,0001) ile güçlü bir korelasyona sahip olduğu bulundu.

Sonuç: Azalmış plazma serbest ve total tiyol seviyeleri ve artmış İMA seviyeleri, artmış oksidatif stres ile ilişkilidir ve OHH hastalarında oksidatif hasarın dolaylı ve hızlı bir yansımasını sağlar.

Anahtar Sözcükler: Orak hücre hastalığı, Tiyol/disülfit dengesi, Oksidatif stres, İskemi modifiye albümin

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Introduction

Sickle cell disease (SCD), described as a group of inherited blood disorders, affects millions of people throughout the world and is particularly common in the southern part of Turkey [1]. It is characterized by chronic anemia and painful events mainly related to tissue and organ damage. The primary cause of this disorder is a single DNA base mutation that results in the substitution of valine for glutamine at position 6 in the β-globin chain of hemoglobin [2]. This single base mutation induces the production of abnormal and insoluble hemoglobin S (Hb S), which is accumulated especially in anoxic conditions, leading to erythrocyte sediments by sickling [3]. Interaction of sickle erythrocytes with capillary endothelium initiates ischemic end-organ injury via a cascade of thrombotic, inflammatory, and oxidative insults that is exacerbated during painful vaso-occlusive crises [4]. These interact by changing the imbalance of reactive oxygen species (ROS) and antioxidants, causing oxidative damage to cell structures such as lipids, membranes, proteins, and nucleic acids [5]. The imbalance between free radicals and antioxidants also affects sulfhydryl groups (-SH) of organic sulfur derivatives (thiols, RSH), which are represented by serum proteins, mainly albumin [6], and play crucial roles in redox homeostasis. The increase in oxidative stress leads to imbalance in the reversible formation of dynamic disulfide bonds between protein thiol groups [7]. Increasing evidence indicates the vital roles of dynamic thiol-disulfide homeostasis in the regulation of intracellular enzymatic activity, antioxidant protection, and apoptosis, which are related to the pathogenesis of a variety of diseases including diabetes mellitus [8], cardiovascular diseases [9], and cancer [10].

Oxidative stress also causes the reduction of the binding affinity of albumin due to free radical damage to the N-terminal of albumin molecules [11]. This new chemically changed albumin is called ischemia-modified albumin (IMA) and is used as a sensitive biochemical marker of ischemia and oxidative stress originating as a consequence of tissue hypoxia [12]. IMA has been shown to increase in patients with ischemic conditions including acute coronary syndrome, stroke, and chronic liver diseases [13,14,15].

However, there is no study in the literature evaluating dynamic thiol/disulfide hemostasis and IMA levels in patient with SCD. In light of those studies that indicated the role of increased oxidative stress in SCD, we hypothesized that thiol/disulfide hemostasis could be related to the pathogenesis of the disease.

In this study, we aimed to evaluate the relationship between IMA and the dynamic thiol/disulfide balance in sickle cell patients.

Materials and Methods

Study and Control Groups

Fifty-four SCD patients, including 30 (56%) males and 24 (44%) females with a mean age of 28.3±8.4 years (minimum-maximum: 18-46 years), in steady-state condition who applied to the hematology department of Mustafa Kemal University Hospital, were enrolled in the study. The control group consisted of 30 age- and sex-matched healthy subjects. Of the 54 patients, 46 had homozygous sickle cell anemia (HbSS) and 8 had sickle/β-thalassemia (HbS/β^+-thalassemia).

Informed written consent was obtained from all patients and healthy controls. Demographic data were collected from the hospital information system. Patients with diabetes mellitus, obesity, kidney or liver failure, coronary heart disease, any hematologic disorder other than SCD, and malignancy were excluded from the study, as were smokers, alcohol drinkers, and patients with a recent history of transfusion in the last 3 months. The institutional Ethics Committee of Mustafa Kemal University approved the study protocol (protocol number: 2016/90).

Specimen Collection

Fasting venous blood samples were collected into vacutainer tubes containing ethylenediaminetetraacetic acid and lithium-heparin from the sickle cell patients and healthy controls and were centrifuged at 1500×g for 10 min within 1 h after sampling. After separation, plasma samples were aliquoted for albumin, total thiol, native thiol, and IMA measurements and stored at -20 °C until the time of assay.

Measurement of Biochemical Parameters

Chemicals and Devices Used in the Assays

All reagents and chemicals were purchased from Sigma-Aldrich. Spectrophotometric analysis was performed with a Shimadzu UV-1800 spectrophotometer with a temperature-controlled cuvette holder and an automated analyzer (Architect Plus C8000, Abbott, USA).

Assay Principle of Thiol/Disulfide Homeostasis Parameters

Native thiol and total thiol levels were assayed with a newly developed spectrophotometric method as described previously by Erel and Neselioglu [7]. Briefly, disulfide bonds were first reduced to form free functional thiol groups using sodium borohydride. The unused sodium borohydride reductant was removed with formaldehyde to prevent reduction of DTNB (5,5'-dithiobis-(2-nitrobenzoic) acid). After the reaction with DTNB, the thiol groups including reduced and native thiol groups were determined spectrophotometrically at 415 nm. Disulfide concentration and disulfide/native thiol percentages were calculated with the following formulas:

Disulfide levels (μmol/L) = (total thiol - native thiol)/2.

Disulfide/native thiol percentage (%) = (disulfide x 100)/native thiol.

Since most of the serum thiols are formed by human serum albumin thiols (HSA-SH, ~80%), we calculated the adjusted total thiol, native thiol, and disulfide levels based on serum albumin concentrations from the following formulas:

Adj. total thiol levels = total thiol (μmol/L)/alb (g/L).

Adj. native thiol levels = native thiol (μmol/L)/alb (g/L).

Adj. disulfide levels = disulfide (μmol/L)/alb (g/L).

Ischemia-Modified Albumin and Plasma Albumin Levels

IMA levels were determined by albumin cobalt binding test, a rapid colorimetric method developed by Bar-Or et al. [16]. The method is based on the binding ability of reduced cobalt ions (Co^{2+}) of HSA due to ischemia. Briefly, a known amount of exogenous Co ($CoCl_2$) was added to serum samples. Albumin, which is altered as a result of ischemic processes, binds to the Co(II) to a far lesser extent and the excess (unbound) amount of Co^{2+} forms a colored complex with dithiothreitol, which is measured spectrophotometrically at 480 nm. Plasma albumin levels were measured in a calibrated and well-controlled autoanalyzer using the bromocresol green method (Architect Plus C8000, Abbott, USA).

Statistical Analysis

Analysis of study data was carried out using MedCalc for Windows, version 15.8. Data normality was examined with the Kolmogorov-Smirnov test. Qualitative data were evaluated using the chi-square test and quantitative data were tested utilizing the Kruskal-Wallis and Mann-Whitney U tests. Correlations were assessed using the Spearman test. Values of $p<0.05$ were regarded as statistically significant.

Results

A total of 54 adult patients, including 30 (56%) males and 24 (44%) females with a mean age of 28.3±8.4 years (minimum-maximum: 18-46 years), were included in this study. Of the 54 patients, 46 had homozygous sickle cell anemia (HbSS) and 8 had sickle/β-thalassemia (HbS/β^+-thalassemia). We found that plasma native thiol (-SH) and total thiol levels (-SH + -S-S-) were significantly lower in SCD patients (respectively 284±86.3 μmol/L and 313±89.3 μmol/L) compared to controls (respectively 417±54.2 μmol/L and 462±58.74 μmol/L). However, plasma disulfide (-S-S-) levels were lower in SCD patients compared to controls (respectively 14.6±7.0 μmol/L and 22.7±11.3 μmol/L). In addition, plasma IMA levels of SCD patients were higher than those of controls (respectively 13.6±3.1 g/L and 8.4±1.6 g/L) (Table 1) and negatively correlated with plasma native thiol (r=-0.730; p=0.0001) and total thiol (r=-0.729; p=0.0001) (Table 2).

Although plasma albumin levels were within the normal reference minimum-maximum (3.4-5.4 g/dL) in the healthy controls and SCD patients, levels were found to be significantly decreased in SCD patients. Albumin levels strongly correlated with both plasma native (r=0.853, p=0.0001) and total thiols (r=0.866, p=0.0001) (Table 2). However, after adjusting disulfide levels according to the albumin concentrations, no significant difference was observed between the patient and control groups.

Disulfide/native thiol ratios were not significantly different between the two groups before or after correction for albumin concentrations. Our study demonstrated that IMA levels significantly increased in SCD patients compared to healthy controls.

Discussion

There are no other reports in the literature investigating the plasma IMA levels and thiol/disulfide balance in patients with SCD. These findings provide a context for understanding the role of the dynamic thiol/disulfide balance and its relation with IMA, an indicator of oxidative stress, in SCD patients.

Our study demonstrated that IMA levels significantly increased and serum native (-SH) and total thiol (-S–S- + -SH) levels decreased in SCD patients. Plasma albumin levels were lower in SCD patients and significantly correlated with plasma native and total thiol levels. The disulfide (-S–S-) levels were lower in SCD patients compared to healthy controls. However, there was no significant difference observed between the study and control groups after adjusting disulfide levels according to albumin concentrations.

It is well known that the repeated polymerization of deoxygenated HbS, under hypoxic conditions, causes hypoxia, increased ROS, and impairment of oxidative balance in patients with SCD [17]. In addition, decreasing antioxidant enzyme activities (i.e. glutathione peroxidase, catalase, and superoxide dismutase) and consumption of non-enzymatic antioxidants (i.e. glutathione) exacerbate the oxidative damage [18,19]. It has been accepted that -SH groups of sulfur-containing proteins are also related to the maintenance of oxidative balance and play a critical role in the prevention of oxidative stress [20]. Oxidation of free cysteine residues (-SH) leads to reversible formation of disulfide bonds (-S-S) between thiols and protein thiol groups and abnormal thiol/disulfide hemostasis [6]. Therefore, dynamic thiol/disulfide homeostasis is being increasingly implicated in many disorders associated with ischemia.

Table 1. Comparison of thiol/disulfide homeostasis parameters and ischemia-modified albumin levels of the study and control groups.

Variables	SCD (n=54)		Controls (n=30)		p
	Mean ± SD	Median (min; max)	Mean ± SD	Median (min; max)	
Total thiol (µmol/L)	313.0±89.3	308.6 (172.3; 620.6)	462.0±58.7	465.4 (325.9; 578.5)	0.0001
Native thiol (µmol/L)	284.0±86.3	275.2 (145.5; 589.2)	417.0±54.2	414.6 (285.7; 498.5)	0.0001
Disulfide (µmol/L)	14.6±7.0	14.5 (2.9; 31.9)	22.7±11.3	25.1 (4.3; 44.5)	0.0006
Adjusted total thiol (µmol/g alb)	9.1±1.4	8.9 (7; 14)	10.6±0.9	10.5 (9; 12)	0.0001
Adjusted native thiol (µmol/g alb)	8.2±1.4	7.9 (6.0; 13.5)	9.6±0.9	9.7 (8; 11)	0.0001
Adjusted disulfide (µmol/g alb)	0.4±0.2	0.4 (0; 1)	0.5±0.3	0.6 (0; 1)	0.0630
Disulfide/native thiol (%)	5.5±2.9	5 (1.7; 10.1)	5.6±2.8	6.2 (1.1; 10.9)	0.8400
Albumin (g/L)	34.9±7.9	36.2 (15; 47)	43.5±3.1	43.8 (33; 48)	0.0001
IMA (g/L)	13.6±3.1	13.1 (10; 20)	8.4±1.6	8.4 (5.4; 13)	0.0001

SD: Standard deviation, IMA: ischemia-modified albumin.

Table 2. Correlations among plasma ischemia-modified albumin, albumin levels, and thiol/disulfide homeostasis parameters in sickle cell disease patients.

	Total thiol (µmol/L)		Native thiol (µmol/L)		Disulfide (µmol/L)	
	r	p	r	p	r	p
IMA (g/dL)	-0.7	**0.0001**	-0.7	**0.0001**	-0.2	0.2400
Albumin (µmol/L)	0.8	**0.0001**	0.8	**0.0001**	0.3	0.0660

IMA: Ischemia-modified albumin.

Intracellular thiols mainly consist of reduced glutathione (GSH), a low-molecular-weight thiol (LMWT) [21]. Intra-erythrocyte GSH depletion has been shown in many studies and is linked to increased oxidative stress in SCD patients [22,23,24,25,26,27]. Even though the exact mechanism remains to be elucidated, erythrocyte GSH loss is attributed to a variety of factors including increased GSH consumption, substrate availability (i.e. NADPH), and dysfunctional GSH recycling [28]. In the present study we evaluated plasma total thiols and dynamic thiol/disulfide balance for the first time by a novel method described by Erel and Neselioglu [7] in SCD patients.

We found significantly lower levels of plasma native and total thiol levels in SCD patients compared to healthy controls (Table 1). Plasma albumin levels were also strongly correlated with both plasma native (r=0.853, p=0.0001) and total thiols (r=0.866, p=0.0001) (Table 2). In a previous study, Ateş et al. [29] evaluated serum thiol levels in patients with chronic kidney disease (CKD) and speculated that low serum native and total thiol levels may have resulted from low serum albumin concentration in the CKD patients. In contrast to the intracellular environment, in plasma, total thiols are at lower concentrations than cells and are mainly formed by HSA-SH (~80%) and slightly formed by LMWTs (i.e. cysteine, cysteinylglycine, gamma glutamylcysteine, glutathione, and homocysteine) [30]. Therefore, we can say that low plasma native and total thiols may be attributed to the low albumin levels in SCD patients. However, this significant difference did not change between the study and control groups after normalization of native and total thiol levels according to albumin concentrations. This suggests that lower thiol levels are related to SCD and cannot be explained simply by depletion of albumin levels. Interestingly, for disulfide levels, the difference between the two groups was nonsignificant after normalization with albumin. This suggests that lower disulfide levels (before adjusting for albumin) may be explained by decreased albumin levels in the SCD group. Two possible processes may contribute to this phenomenon. First, HSA-SH, as the most abundant thiol in plasma, has an antioxidant role due to its cysteine thiols, which react with ROS [30]. In hypoxic conditions, oxidation of thiols with oxidants leads mainly to sulfenic acid (HAS-SOH) formation, which is an oxidized form of albumin. Sulfenic acid can form reversible disulfides or, in the presence of excess oxidants, it can be further oxidized to sulfinic (RSO_2H) and sulfonic acids (RSO_3H), which are irreversible processes. These oxidized forms of albumin have relatively short half-lives and are swept from the circulation by liver cells [30,31]. We suggest that native thiols may change into sulfenic acid forms of albumin, rather than disulfide forms, and be picked up by the liver continuously. Second, glutathione and other LMWTs are necessary for the reduction of oxidized thiol groups. It has been shown that they have relatively low concentrations both in erythrocytes and plasma [30,32]. We can say that the low availability of glutathione and other LMWTs can limit protein mixed-disulfide formation.

IMA (or the albumin cobalt binding test) is used as a novel and sensitive marker of ischemia and oxidative stress mainly for myocardial infarction and various diseases related to ischemia, including peripheral vascular disease, skeletal muscle ischemia,

and diabetes mellitus [33,34,35,36]. SCD causes several harmful conditions including sickling, vaso-occlusion, and ischemia-reperfusion injury, which involve the generation of ROS and impairment of oxidative balance. Many studies have shown relationships between oxidative stress parameters and SCD or other related complications such as acute chest syndrome [37] and pulmonary hypertension [38]. In the present study we showed for the first time that IMA levels were significantly higher in SCD patients. Increased IMA levels can be explained by increased ROS production in SCD patients. These data indicate that increased oxidative stress and ROS production in SCD can play a major role in decreased body thiol reserves and cause increased IMA levels.

Study Limitations

The limitation of our study is that it is a cross-sectional study. Further prospective studies are needed to compare the thiol balance and IMA levels in patients with vaso-occlusive crises and in the steady-state condition.

Conclusion

Our results support the idea that decreased plasma native and total thiol levels are related to increased oxidative stress. Impaired thiol balance plays an important role in the pathogenesis of SCD. Disulfide levels are also important in evaluating thiol hemostasis, but these levels should be adjusted according to albumin concentrations because unadjusted disulfide levels might give misleading results in patients with low albumin levels. In addition, IMA levels are increased by excessive ROS production and measuring IMA as a marker in plasma samples provides an indirect and quick reflection of the oxidative damage in SCD patients.

Ethics

Ethics Committee Approval: The study was approved by the Mustafa Kemal University Local Ethics Committee (protocol number: 2016/90).

Authorship Contributions

Concept: O.Ö., H.E., G.İ., D.D., A.B.G., S.N., Ö.E.; Design: O.Ö., H.E., G.İ., D.D., S.N., Ö.E.; Data Collection: O.Ö., H.E., G.İ., D.D.; Statistical Evaluation: O.Ö., H.E., A.B.G.; Writing: O.Ö., H.E.; Proofreading: O.Ö., H.E., G.İ., D.D., A.B.G., S.N., Ö.E.

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References

1. Yuzbasioglu Ariyurek S, Yildiz SM, Yalin AE, Guzelgul F, Aksoy K. Hemoglobinopathies in the Cukurova Region and neighboring provinces. Hemoglobin 2016;40:168-172.
2. Pauling L, Itano HA, Singer SJ, Wells IC. Sickle cell anemia, a molecular disease. Science 1949;110:543-548.
3. Kaul DK, Finnegan E, Barabino GA. Sickle red cell-endothelium interactions. Microcirculation 2009;16:97-111.
4. Manwani D, Frenette PS. Vaso-occlusion in sickle cell disease: pathophysiology and novel targeted therapies. Blood 2013;122:3892-3898.
5. Chirico EN, Pialoux V. Role of oxidative stress in the pathogenesis of sickle cell disease. IUBMB Life 2012;64:72-80.
6. Ghezzi P, Bonetto V, Fratelli M. Thiol-disulfide balance: from the concept of oxidative stress to that of redox regulation. Antioxid Redox Signal 2005;7:964-972.
7. Erel O, Neselioglu S. A novel and automated assay for thiol/disulphide homeostasis. Clin Biochem 2014;47:326-332.
8. Matteucci E, Giampietro O. Thiol signalling network with an eye to diabetes. Molecules 2010;15:8890-8903.
9. Go YM, Jones DP. Cysteine/cystine redox signaling in cardiovascular disease. Free Radic Biol Med 2011;50:495-509.
10. Prabhu A, Sarcar B, Kahali S, Yuan Z, Johnson JJ, Adam KP, Kensicki E, Chinnaiyan P. Cysteine catabolism: a novel metabolic pathway contributing to glioblastoma growth. Cancer Res 2014;74:787-796.
11. Roy D, Quiles J, Gaze DC, Collinson P, Kaski JC, Baxter GF. Role of reactive oxygen species on the formation of the novel diagnostic marker ischaemia modified albumin. Heart 2006;92:113-114.
12. Ellidag HY, Bulbuller N, Eren E, Abusoglu S, Akgol E, Cetiner M, Yilmaz N. Ischemia-modified albumin: could it be a new oxidative stress biomarker for colorectal carcinoma? Gut Liver 2013;7:675-680.
13. Abboud H, Labreuche J, Meseguer E, Lavallee PC, Simon O, Olivot JM, Mazighi M, Dehoux M, Benessiano J, Steg PG, Amarenco P. Ischemia-modified albumin in acute stroke. Cerebrovasc Dis 2007;23:216-220.
14. Cakir M, Karahan SC, Mentese A, Sag E, Cobanoglu U, Polat TB, Erduran E. Ischemia-modified albumin levels in children with chronic liver disease. Gut Liver 2012;6:92-97.
15. Reddy CB, Cyriac C, Desle HB. Role of "ischemia modified albumin" (IMA) in acute coronary syndromes. Indian Heart J 2014;66:656-662.
16. Bar-Or D, Curtis G, Rao N, Bampos N, Lau E. Characterization of the Co^{2+} and Ni^{2+} binding amino-acid residues of the N-terminus of human albumin. An insight into the mechanism of a new assay for myocardial ischemia. Eur J Biochem 2001;268:42-47.
17. Eaton WA, Hofrichter J. Sickle cell hemoglobin polymerization. Adv Protein Chem 1990;40:63-279.
18. Conran N, Franco-Penteado CF, Costa FF. Newer aspects of the pathophysiology of sickle cell disease vaso-occlusion. Hemoglobin 2009;33:1-16.
19. Tappel A, Zalkin H. Inhibition of lipid peroxidation in microsomes by vitamin E. Nature 1960;185:35.
20. Bektas H, Vural G, Gumusyayla S, Deniz O, Alisik M, Erel O. Dynamic thiol-disulfide homeostasis in acute ischemic stroke patients. Acta Neurol Belg 2016;116:489-494.
21. Staal FJ, Roederer M, Herzenberg LA, Herzenberg LA. Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus. Proc Natl Acad Sci U S A 1990;87:9943-9947.
22. Reid M, Badaloo A, Forrester T, Jahoor F. In vivo rates of erythrocyte glutathione synthesis in adults with sickle cell disease. Am J Physiol Endocrinol Metab 2006;291:73-79.
23. Fujii S, Kaneko T. Selenium, glutathione peroxidase and oxidative hemolysis in sickle cell disease. Acta Haematol 1992;87:105-106.
24. Saad S, Salles SI, Velho PE. Decreased reduced glutathione and glutathione reductase activity in subjects with hemoglobin C. Nouv Rev Fr Hematol 1991;33:11-14.
25. Adelekan DA, Thurnham DI, Adekile AD. Reduced antioxidant capacity in paediatric patients with homozygous sickle cell disease. Eur J Clin Nutr 1989;43:609-614.

26. Varma RN, Mankad VN, Phelps DD, Jenkins LD, Suskind RM. Depressed erythrocyte glutathione reductase activity in sickle cell disease. Am J Clin Nutr 1983;38:884-887.
27. Wetterstroem N, Brewer GJ, Warth JA, Mitchinson A, Near K. Relationship of glutathione levels and Heinz body formation to irreversibly sickled cells in sickle cell anemia. J Lab Clin Med 1984;103:589-596.
28. Griffith OW. Biologic and pharmacologic regulation of mammalian glutathione synthesis. Free Radic Biol Med 1999;27:922-935.
29. Ateş İ, Özkayar N, Yılmaz FM, Bayrakçı N, Neşelioğlu S, Erel Ö, Yenigün EC, Dede F. Oxidative stress level in patients with chronic kidney disease. Ortadogu Medical Journal 2018;10:45-50.
30. Turell L, Radi R, Alvarez B. The thiol pool in human plasma: the central contribution of albumin to redox processes. Free Radic Biol Med 2013;65:244-253.
31. Peters T Jr. All About Albumin: Biochemistry, Genetics, and Medical Applications. San Diego, Academic Press, 1995.
32. Lavoie S, Berger M, Schlögelhofer M, Schäfer MR, Rice S, Kim SW, Hesse J, McGorry PD, Smesny S, Amminger GP. Erythrocyte glutathione levels as long-term predictor of transition to psychosis. Transl Psychiatry 2017;7:e1064.
33. Roy D, Quiles J, Sharma R, Sinha M, Avanzas P, Gaze D, Kaski JC. Ischemia-modified albumin concentrations in patients with peripheral vascular disease and exercise-induced skeletal muscle ischemia. Clin Chem 2004;50:1656-1660.
34. Montagnana M, Lippi G, Volpe A, Salvagno GL, Biasi D, Caramaschi P, Cesare Guidi G. Evaluation of cardiac laboratory markers in patients with systemic sclerosis. Clin Biochem 2006;39:913-917.
35. Piwowar A, Knapik-Kordecka M, Warwas M. Ischemia-modified albumin level in type 2 diabetes mellitus - Preliminary report. Dis Markers 2008;24:311-317.
36. Chang D, Sha Q, Zhang X, Liu P, Rong S, Han T, Liu P, Pan H. The evaluation of the oxidative stress parameters in patients with primary angle-closure glaucoma. PLoS One 2011;6:e27218.
37. Klings ES, Farber HW. Role of free radicals in the pathogenesis of acute chest syndrome in sickle cell disease. Respir Res 2001;2:280-285.
38. Morris CR, Suh JH, Hagar W, Larkin S, Bland DA, Steinberg MH, Vichinsky EP, Shigenaga M, Ames B, Kuypers FA, Klings ES. Erythrocyte glutamine depletion, altered redox environment, and pulmonary hypertension in sickle cell disease. Blood 2008;111:402-410.

RESEARCH ARTICLE

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Does Reinfusion of Stem Cell Products on Multiple Days Affect Engraftment?

Çoklu Gün Kök Hücre Reinfüzyonu Engrafmanı Etkiler mi?

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Abstract

Objective: High-doses of melphalan treatment with autologous stem cell transplantation in multiple myeloma (MM) remains a major treatment modality in suitable patients. A minimal dose of $2x10^6$/kg CD34+ cells is preferred to achieve engraftment. Some patients need multiple leukapheresis procedures to achieve the necessary number of CD34+ cells, but this can cause a high volume of stem cell product that cannot be given in a single day. Whether or not the number of infusion days affects engraftment has not been studied before. We aimed to evaluate the impact of reinfusion of stem cells on multiple days on engraftment results.

Materials and Methods: Demographic features, CD34+ cell doses, neutrophil and platelet engraftment days, hospitalization days, and number of infusion days of 149 autologous transplantations of 143 MM patients were evaluated retrospectively.

Results: The data of 143 MM patients who were transplanted were analyzed retrospectively. Median age was 55±8.5 (range: 26-70) years with a male/female ratio of 91/58. Hospitalization days for all patients were 24±6 (range: 14-50) days. Mean CD34+ cell number was (7.5±5.3) $x10^6$/kg (range: 1.5-31$x10^6$/kg). CD34+ cells were reinfused in 1 day in 80.5% (n=120) of the patients, 2 days in 18.2% of the patients (n=27), and 3 days in 1.3% of the patients (n=2). For 29 patients, reinfusion was applied in more than 1 day because of the high volume of stem cell product. We did not see any dimethyl sulfoxide toxicity, cardiac arrhythmia, or volume overload complications. Hypertensive attacks during infusion were easily controlled by furosemide treatment. In the group with multiple infusions, the infused CD34+ cell numbers had a mean of (4.8±2.8)$x10^6$/kg, and in the single infusion group the mean was (8.1±5.5)$x10^6$/kg. There were no statistical differences between the two groups regarding platelet and neutrophil engraftment days (p=0.850, r=0.820 and p=0.500, r=0.440). There was no statistical difference between the two groups for hospitalization days (p=0.060, r=0.050).

Öz

Amaç: Multipl myelom (MM) hastalarında yüksek doz melfalan ve otolog kök hücre nakli fit hastalarda hala majör tedavi modalitesidir. Yeterli engrafman için en az $2x10^6$/kg CD34+ hücre dozu tercih edilir. Bazı hastalar gerekli sayıda CD34+ hücre sayısına ulaşmak için çoklu lökoferez prosedürüne ihtiyaç duyarlar, fakat bu tek günde verilemeyen yüksek miktarda kök hücre ürününe neden olabilir. İnfüzyon günü sayısının engrafmanı etkileyip etkilemediği daha önce çalışılmamıştır. Çoklu gün kök hücre reinfüzyonunun engrafman sonuçlarına etkisini değerlendirmeyi amaçladık.

Gereç ve Yöntemler: Demografik özellikler, CD34+ hücre dozları, nötrofil ve trombosit engrafman günleri, hastanede yatış günleri ile 143 MM hastasının 149 otolog transplantasyonunun infüzyon gün sayısı retrospektif olarak değerlendirildi.

Bulgular: Transplantasyon yapılan 143 MM hastasının verileri retrospektif olarak incelendi. Ortalama yaş 55±8,5 idi (en düşük: 26 - en fazla: 70) ve erkek/kadın oranı 91/58 idi. Tüm hastalar için hastanede kalış günleri 24±6 gündü (en düşük: 14 - en fazla: 50). Ortalama CD34+ hücre sayısı (7,5±5,3)$x10^6$/kg idi (en düşük: 1,5 $x10^6$/kg - en yüksek: 31$x10^6$/kg). Hastaların %80,5'inde (n=120) tek günde CD34+ hücre reinfüzyon, hastaların %18,2'sinde (n=27) 2 günde CD34+ hücre reinfüzyon ve hastaların %1,3'ünde (n=2) 3 günde CD34+ hücre reinfüzyon yapılmıştı. Yirmi dokuz hastada yüksek hacimde kök hücre ürünü olması nedeniyle 1 günden fazla reinfüzyon uygulandı. Herhangi bir dimetil sülfoksit toksisitesi, kardiyak aritmi veya hacim yükü komplikasyonları görmedik. İnfüzyon sırasında hipertansif ataklar furosemid tedavisi ile kolayca kontrol altına alındı. CD34+ hücre sayıları ortalama çoklu gün reinfüzyonu yapılan grupta (4,8±2,8)$x10^6$/kg ve tek gün reinfüzyon grubunda ise (8,1±5,5)$x10^6$/kg idi. İki grup arasında trombosit ve nötrofil engrafman günleri açısından istatistiksel olarak anlamlı fark yoktu (p=0,850, r=0,820 ve p=0,500, r=0,440). İki grup arasında hastanede yatış günleri açısından istatistiksel fark yoktu (p=0,060, r=0,050).

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Conclusion: In cases with a high volume of stem cell product to acquire adequate stem cells, reinfusion can be safely applied across multiple days without any delay in engraftment.

Keywords: Multiple myeloma, Autologous transplantation, Multiple reinfusion days

Sonuç: Yeterli kök hücre elde etmek için yüksek miktarda kök hücre toplanması durumunda, engrafmanda herhangi bir gecikme olmaksızın reinfüzyon çoklu günde güvenli bir şekilde uygulanabilir.

Anahtar Sözcükler: Multipl myelom, Otolog transplantasyon, Çoklu gün reinfüzyon

Introduction

Multiple myeloma (MM) is a hematologic malignancy with a median age at presentation of 60-65 years [1]. High-dose melphalan therapy together with autologous stem cell transplantation (ASCT) in MM remains a major treatment modality in suitable patients [2]. Advanced age is a poor prognostic factor in studies using conventional chemotherapy even if the biological and clinical features in older MM patients are the same as to those of younger patients [3,4]. This procedure can be applied safely even in selected patients older than the age of 65. The use of peripheral stem cells was found to accelerate the speed of hematopoietic recovery, leading to a significant decrease in mortality and morbidity. ASCT has been a part of myeloma treatment in these patient groups [5,6,7]. Adequate collection and application of enough CD34+ hematopoietic stem cells are needed for successful transplantation. The determination of a suitable minimum CD34+ cell dose for MM patients aged 65 years and older is critical given that the median age of diagnosis for MM is generally between 65 and 70 years of age and many of these patients may be transplant-eligible [8]. In recent practice, a CD34+ dose of at least $2x10^6$/kg is preferred for sufficient neutrophil and platelet engraftment, and $\geq 5x10^6$/kg CD34+ was shown to be related to a shortened time to platelet recovery [9,10].

The aim of our study was to examine the effect of CD34+ reinfusion on multiple days on engraftment results in MM patients. We evaluated the demographic features, infused CD34+ cell doses, neutrophil and platelet engraftment days, hospitalization days, and number of infusion days in 149 ASCTs of 143 MM patients retrospectively.

Materials and Methods

Between February 2009 and September 2017, 143 patients with MM underwent ASCT at the Dokuz Eylül University Hospital Division of Hematology. Data were collected from the electronic and patient files of the medical archives retrospectively. Baseline patient characteristics are shown in Table 1. All patients were suitable for ASCT therapy and had enough stem cell collection. However, second ASCTs were planned for six patients due to late relapse of the disease. All patients were informed about the benefits and risks related to stem cell collection and transplantation. The majority of the patients had one or two lines of prior chemotherapy (range: 1-3) in the pre-ASCT period (vincristine, adriablastin, and dexamethasone [VAD] therapy; bortezomib and dexamethasone therapy; or lenalidomide and dexamethasone therapy).

Transplant details including mobilizing agents, CD34+ cell doses, neutrophil and platelet engraftment days, hospitalization days, and number of CD34+ reinfusion days were analyzed. The data were examined according to the number of CD34+ cell reinfusion days. The neutrophil and platelet engraftment days, hospitalization days, and collected CD34+ cell count of the patients for whom CD34+ cells had been reinfused in one day were compared to those of the patients for whom CD34+ cells been reinfused on multiple days. At the time of transplant, only 11.4% (n=17) of patients had complete response (CR). The majority (n=86, 57.6%) had achieved a very good partial response (PR), while 28% (n=42) of the patients had reached PR and 3% (n=4) had refractory or progressive disease. Second autologous transplantations were planned for six patients because of progressive disease.

Peripheral blood stem cells were collected in 1-4 apheresis procedures (mean: 1.7), following mobilization regimens. We used intravenous cyclophosphamide at 2.4 g/m^2 for one day with mesna and granulocyte colony-stimulating factor

Table 1. Patients' characteristics.

	Number (n)	%
Patients	143	100
Age, years, median (range)	55 (26-70)	
Sex (male/female)	91/58	61/39
Status at transplantation		
CR	17	11.3
VGPR	86	57.7
PR	42	28
Progressive/refractory	4	3
Mobilization regimens		
Cyclophosphamide/G-CSF	133	89.3
Plerixafor/G-CSF	9	6
G-CSF	7	4.7
Conditioning		
Melphalan, 200 mg/m^2	124	83.2
Melphalan, 140 mg/m^2	25	16.8
Death	37	24.8

CR: Complete remission, VGPR: very good partial remission, PR: partial remission, G-CSF: granulocyte colony-stimulating factor.

(G-CSF, 5 µg/kg/day subcutaneously) in 133 patients (89.3%), G-CSF alone in 7 patients (4.7%), and plerixafor plus G-CSF in 9 patients (6%) for mobilization. Apheresis was initiated when the CD34+ cells of peripheral blood samples increased to >10 µL. Each sample was tested by flow cytometric analysis for the proportion of cells expressing CD34. The minimum goal CD34+ stem cell dose as a target for collection was >2x10^6 CD34/kg for all autologous transplantations.

The regimen for conditioning consisted of melphalan for all patients. Melphalan was given at a dose of 200 mg/m^2 for 124 patients (83.2%) and at a reduced dose of 140 mg/m^2 for 25 patients (16.8%) due to reduced creatinine clearance (<50 mL/min). Patients received G-CSF once a day starting on day 1 after the infusion of stem cells until the time of engraftment.

Statistical Analysis

Data were entered and analyzed using SPSS 21.0. Descriptive statistics were used for baseline characteristics, transplant-related factors, and posttransplant results. Differences in the distribution of variables between patient subsets were analyzed using the Pearson chi-square test/correlation test/t-test. All statistical analyses were performed at a critical significance level of 0.05, and p-values were reported.

Results

We analyzed 149 autologous transplantations of 143 MM patients between February 2009 and September 2017 retrospectively. Mean age was 55±8.5 (range: 26-70) years with a 91/58 M/F ratio. There were no significant differences in platelet engraftment days, neutrophil engraftment days, reinfusion days, hospitalization days, or infused CD34+ dose with regards to sex distribution (Table 2).

Patients were separated into two age groups: those aged younger than 60 years and those older. There was no significant difference between the two groups in terms of platelet and neutrophil engraftment days, multiple day reinfusion rate, hospitalization days, or infused CD34+ cell dose (Table 3).

When we analyzed all patients, hospitalization days were 24±6 (range: 14-50). Mean CD34+ cell count was (7.5±5.3)x10^6/kg (range: 1.5-31x10^6/kg). Platelet engraftment days were 13.9±3 (range: 9-30) and neutrophil engraftment days were 11.5±1.5 (range: 8-17).

Higher reinfused CD34+ cell doses were associated with faster platelet and neutrophil engraftment (p=0.034 and p=0.001). Hospitalization days decreased because of a better transplantation outcome with the higher reinfused CD34+ cell doses (p=0.001).

CD34+ cells were reinfused in one day in 80.5% of patients (n=120), 2 days in 18.2% of patients (n=27), and 3 days in 1.3% of patients (n=2). For 29 patients, reinfusion was performed on more than one day, because of the higher volume of stem cell product and according to the tolerability of the patients. However, reinfusion of peripheral blood mononuclear cells cryopreserved with dimethyl sulfoxide (DMSO) can be associated with toxic reactions. We know that infusion of product containing more than 1 g/kg of DMSO per day can lead to increased DMSO toxicity. As a result, the days of reinfusion were determined according to the performance status of our patients, the amount of product they had, and the amount of DMSO contained in the products. There were

Table 2. Results in regards to sex distribution.

	Male	Female	p-value
Patient number, n/%	91/61	58/39	
Platelet engraftment days	14±3.4	14±3.6	0.700
Neutrophil engraftment days	11.7±1.6	11.3±1.3	0.150
Reinfusion days	1.2±0.4	1.1±0.4	0.330
Infused CD34+ dose	(7.5±5.2)x10^6 CD34/kg	(7.4±5.3)x10^6 CD34/kg	0.800
Hospitalization days	24±5	24±6	0.530

Table 3. Results in regards to age (<60 years and ≥60 years).

	<60 years	≥60 years	p-value
Patient number, n/%	91/61	58/39	
Platelet engraftment days	14±3.7	13.7±2.9	0.560
Neutrophil engraftment days	11.5±1.4	11.5±1.6	0.900
Reinfusion days	1.1±0.3	1.2±0.4	0.260
Infused CD34+ dose	(7.7±5.3)x10^6 CD34/kg	(7.1±5.2)x10^6 CD34/kg	0.500
Hospitalization days	24±6.4	23.5±4.7	0.310

also statistical differences between the two groups in terms of mobilization days. Mobilization days were found higher in the multiple day infusion group than in the single day infusion group (2.2 days vs. 1.6 days, p=0.0001). We did not see any DMSO toxicity, cardiac arrhythmia, or volume overload complications. Hypertensive crisis was easily controlled by diuretic treatment at the during infusion. CD34+ cell levels were a mean of (4.8±2.8)x10^6/kg in the multiple day infusion group and (8.2±5.5)x10^6/kg in the single day infusion group. The infused CD34+ cell count was found higher in the single day infusion group than in the multiple day infusion group (p=0.003). There were no statistical differences between the two groups in the case of platelet and neutrophil engraftment days (p=0.850, r=0.820 and p=0.500, r=0.420) or hospitalization days (p=0.060, r=0.050) (Table 4).

Discussion

MM is a disease of the elderly. ASCT is an important treatment modality in symptomatic myeloma patients. Treatment options have expanded in the last decade with novel drugs, but ASCT still maintains its place in the treatment of myeloma. We offer the results of a retrospective analysis of MM patients with autologous transplantation in our center in the last decade. The median age was 55 years and there was male dominance in our study. The median age was relatively young in our study, similar to the study of O'Shea et al. [11].

Recent population-based studies have shown increasing use of ASCT in elderly patients with MM [12]. However, different age cut-off values of 60 years, 65 years, or 70 years were given that estimate survival independently in different studies [13,14].

In our study, ASCT was planned for transplant-eligible patients who had adequate stem cell collection. We mostly applied cyclophosphamide and G-CSF as the mobilization regimen. The target CD34+ stem cell dose for collection was >2x10^6 CD34/kg for each autologous transplantation. The mean CD34+ cell number was (7.5±5.3)x10^6/kg in our study. The mean platelet engraftment days were 13.9 and the mean neutrophil engraftment days were 11.5 in our study. In other studies, the median time to neutrophil engraftment and the median time to platelet engraftment were reported as 9-14 days [15] and 13.5-25 days [16], respectively. The prior studies used the total infused CD34+ cells as a predictor of neutrophil and platelet engraftment. We also demonstrated a significant correlation between the infused CD34+ cell dose and the period to platelet engraftment and neutrophil engraftment [17,18,19].

The collected stem cell product is mostly given as a single day infusion, but in some situations, the product can be reinfused on multiple days due to patient characteristics or concern about complications related to higher volumes.

We chose multi-day reinfusion to overcome possible volume overload in older patients. DMSO toxicity could be another problem if the patient is given the infusion in a single day. Patients requiring multiple days to collect an adequate number of CD34+ cells may be at risk of exposure to serious doses of DMSO. Davis et al. [20] suggested that toxicities related to the infusion of cryopreserved cells are related to the volumes of cryoprotectants, but our study did not demonstrate a difference in toxicity with multiple day infusions, like the study of Abdel-Razeq et al. [21]. We did not see any other toxicity, such as cardiac arrhythmia or volume overload complications. We also wondered about the effect of multi-day infusion on engraftment. The effect of multi-day infusion of stem cells on engraftment was evaluated in the study of Abdel-Razeq et al. [21], as well. They showed there was no effect on engraftment. However, that study consisted of a heterogeneous group of patients with non-Hodgkin lymphoma, Hodgkin lymphoma, and breast cancer, and it did not include myeloma patients. There is no other study evaluating the effect of multi-day stem cell infusion on engraftment in the literature. If we consider all patients in our study, there were no statistical differences between the two groups (multiple reinfusion days and single reinfusion day) regarding platelet and neutrophil engraftment days and hospitalization days.

We also observed that multi-day infusion of stem cells due to higher volumes was mostly done in patients older than 60 years. The ages of these patients were between 26 and 70 years; 39% of them were older than 60 years and 61% were younger than 60. On the other hand, the number of patients older than 65 years old was found to be 14%. In the Italian Group for Bone Marrow Transplantation-Working Group study, age over 65 was described as a poor mobilizing factor, like previous cytotoxic chemotherapy, radiotherapy, bone marrow involvement, and

Table 4. Neutrophil and platelet engraftment days, hospitalization days, and infused CD34+ cell dose according to reinfusion days.

	Multiple infusions group	Single infusion group	p-value
Patient number, n/%	29/19.5	120/80.5	
Platelet engraftment days	14.3±3.2	13.7±3.5	0.850
Neutrophil engraftment days	11.9±1.4	11.5±1.5	0.500
Infused CD34+ dose	(4.8±2.8)x10^6 CD34/kg	(7.1±5.2)x10^6 CD34/kg	**0.003**
Hospitalization days	26±7	23.5±5	0.060

platelet count before mobilization [22]. We did not observe any problem in the mobilization of myeloma patients regarding age.

In our study, we also compared findings between two age groups (<60 years old, ≥60 years old). There were no significant differences in platelet engraftment days, neutrophil engraftment days, reinfusion days, hospitalization days, or infused CD34+ doses in these two groups. However, Sanchez et al. [23] found a significant difference in mean hospitalization days (18.6 days in older versus 16.8 days in younger patients, $p<0.01$) and no significant difference in hospital mortality between older and younger patients.

Conclusion

In cases with high volumes of stem cell product to acquire adequate amounts of stem cells, reinfusion can be safely applied across multiple days without any delay in engraftment.

Ethics

Ethics Committee Approval: Retrospective study.

Informed Consent: N/A.

Authorship Contributions

Surgical and Medical Practices: Ş.S.M., İ.A., G.H.Ö., B.Ü.; Concept: Ş.S.M., İ.A., G.H.Ö., M.A.Ö., Ö.P.; Design: Ş.S.M., İ.A., F.D.; Data Collection or Processing: Ş.S.M., İ.A., D.T.; Analysis or Interpretation: Ş.S.M., İ.A., Ö.G.S.; Literature Search: Ş.S.M., İ.A., C.A.; Writing: Ş.S.M.

Conflict of Interest: The authors of this paper have no conflicts of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

References

1. Grenlee RT, Hill-Harmon MB, Murray T, Thun M. Cancer statistics, 2001. CA Cancer J Clin 2001;51:15-36.
2. Bai L, Xia W, Wong K, Reid C, Ward C, Greenwood M. Factors predicting haematopoietic recovery in patients undergoing autologous transplantation: 11-year experience from a single center. Ann Hematol 2014;93:1655-1664.
3. Riccardi A, Mora O, Brugnatelli S, Tinelli C, Spanedda R, De Paoli A, Barbarano L, Di Stasi M, Bergonzi C, Giordano M, Delfini C, Nicoletti G, Rinaldi E, Piccinini L, Valentini D, Ascari E. Relevance of age on survival of 341 patients with multiple myeloma treated with conventional chemotherapy: updated results of the MM87 prospective randomised protocol. Cooperative Group of Study and Treatment of Multiple Myeloma. Br J Cancer 1998;77:485-491.
4. Quaglino D, Di Leonardo G, Pasqualoni E, Furia N, Di Simone S. Therapeutic management of hematological malignancies in elderly patients. Biological and clinical considerations. Part IV: Multiple myeloma and Waldenström's macroglobulinemia. Aging (Milano) 1998;10:5-12.
5. Badros A, Barlogie B, Siegel E, Morris C, Desikan R, Zangari M, Fassas A, Anaissie E, Munshi N, Tricot G. Autologous stem cell transplantation in elderly multiple myeloma patients over the age of 70 years. Br J Haemetol 2001;114:600-607.
6. Wildes TM, Vij R, Petersdorf SH, Medeiros BC, Hurria A. New treatment approaches for older adults with multiple myeloma. J Geriatr Oncol 2012;3:279-290.
7. Sharma M, Zhang MJ, Zhong X, Abidi MH, Akpek G, Bacher U, Callander NS, Dispenzieri A, Freytes CO, Fung HC, Gale RP, Gasparetto C, Gibson J, Holmberg LA, Kindwall-Keller TL, Klumpp TR, Krishnan AY, Landau HJ, Lazarus HM, Lonial S, Maiolino A, Marks DI, Mehta P, Mikhael Med JR, Nishihori T, Olsson R, Ramanathan M, Roy V, Savani BN, Schouten HC, Scott E, Tay J, To LB, Vesole DH, Vogl DT, Hari P. Older patients with multiple myeloma derive similar benefit from autologous transplantation. Biol Blood Marrow Transplant 2014;20:1796-1803.
8. Kumar SK, Dingli D, Lacy MQ, Dispenzieri A, Hayman SR, Buadi FK, Rajkumar SV, Litzow MR, Gertz MA. Autologous stem cell transplantation in patients of 70 years and older with multiple myeloma: results from a matched pair analysis. Am J Hematol 2008;83:614-617.
9. Stiff PJ, Micallef I, Nademanee AP, Stadtmauer EA, Maziarz RT, Bolwell BJ, Bridger G, Marulkar S, Hsu FJ, DiPersio JF. Transplanted CD34(+) cell dose is associated with long-term platelet count recovery following autologous peripheral blood stem cell transplant in patients with non-Hodgkin lymphoma or multiple myeloma. Biol Blood Marrow Transplant 2011;17:1146-1153.
10. Ungerstedt JS, Watz E, Uttervall K, Johansson BM, Wahlin BE, Näsman P, Ljungman P, Gruber A, Axdorph Nygell U, Nahi H. Autologous hematopoietic stem cell transplantation in multiple myeloma and lymphoma: an analysis of factors influencing stem cell collection and hematological recovery. Med Oncol 2012;29:2191-2199.
11. O'Shea D, Giles C, Terpos E, Perz J, Politou M, Sana V, Naresh K, Lampert I, Samson D, Narat S, Kanfer E, Olavarria E, Apperley JF, Rahemtulla A. Predictive factors for survival in myeloma patients who undergo autologous stem cell transplantation: a single- centre experience in 211 patients. Bone Marrow Transplant 2006;37:731-737.
12. Morris C, Iacobelli S, Brand R, Bjorkstrand B, Drake M, Niederwieser D, Gahrton G; Chronic Leukaemia Working Party Myeloma Subcommittee, European Group for Blood and Marrow Transplantation. Benefit and timing of second transplantations in multiple myeloma: clinical findings and methodological limitations in a European Group for Blood and Marrow Transplantation registry study. J Clin Oncol 2004;22:1674-1681.
13. Siegel DS, Desikan KR, Mehta J, Singhal S, Fassas A, Munshi N, Anaissie E, Naucke S, Ayers D, Spoon D, Vesole D, Tricot G, Barlogie B. Age is not a prognostic variable with autotransplants for multiple myeloma. Blood 1999;93:51-54.
14. Sirohi B, Powles R, Treleaven J, Mainwaring P, Kulkarni S, Pandha H, Bhagwati N, Horton C, Singhal S, Mehta J. The role of autologous transplantation in patients with multiple myeloma aged 65 years and over. Bone Marrow Transplant 2000;25:533-539.
15. Rajkumar SV. Staging and prognostic studies myeloma. UpToDate 2011;19:1-20.
16. Kapoor P, Fonseca R, Rajkumar SV, Sinha S, Gertz MA, Stewart AK, Bergsagel PL, Lacy MQ, Dingli DD, Ketterling RP, Buadi F, Kyle RA, Witzig TE, Greipp PR, Dispenzieri A, Kumar S. Evidence for cytogenetic and fluorescence in situ hybridization risk stratification of newly diagnosed multiple myeloma in the era of novel therapies. Mayo Clin Proc 2010;85:532-537.
17. Martin RMG, Ricci MJ, Foley R, Mian HS. The relationship of CD34 dosage and platelet recovery following high dose chemotherapy and autologous CD34+ reinfusion in multiple myeloma. Transfus Apher Sci 2017;56:552-557.
18. Jillella AP, Ustun C. What is the optimum number of CD34+ peripheral blood stem cells for an autologous transplant? Stem Cells Dev 2004;13:598-606.
19. Siena S, Schiavo R, Pedrazzoli P, Carlo-Stella C. Therapeutic relevance of CD34 cell dose in blood cell transplantation for cancer therapy. J Clin Oncol 2000;18:1360-1377.
20. Davis BJ, Rowley SD, Braine HG, Piantadosi S, Santos GW. Clinical toxicity of cryopreserved bone marrow graft ınfusion. Blood 1990;75:781-786.

21. Abdel-Razeq H, Pohlman B, Andresen S, Kalaycio M, Overmoyer B, Wise K, Dannley R, DeMars D, Goormastic M, Bolwell B. A randomized study of multi-day infusion of autologous peripheral blood progenitor cells. Bone Marrow Transplant 1998;21:221-223.
22. Barosi G, Boccadoro M, Cavo M, Corradini P, Marchetti M, Massaia M, Merlini G, Tosi P, Tura S; Italian Society of Hematology; Italian Society of Experimental Hematology; Italian Group for Bone Marrow Transplantation. Management of multiple myeloma and related-disorders: guidelines from Italian Society of Hematology (SIE), Italian Society of Experimental Hematology (SIES) and Italian Group for Bone Marrow Transplantation (GITMO). Haemotologica 2004;89:717-741.
23. Sanchez L, Sylvester M, Parrondo R, Mariotti V, Eloy JA, Chang VT. In-hospital mortality and post-transplantation complications in elderly multiple myeloma patients undergoing autologous hematopoietic stem cell transplantation: a population-based study. Biol Blood Marrow Transplant 2017;23:1203-1207.

RESEARCH ARTICLE

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The Outcome of Antifungal Prophylaxis with Posaconazole in Patients with Acute Myeloid Leukemia: A Single-Center Study

Akut Myeloid Lösemili Hastalarda Posakonazol ile Antifungal Profilaksi Sonuçları: Tek Merkez Çalışması

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Abstract

Objective: Invasive fungal infections (IFIs) are a significant cause of morbidity and mortality among neutropenic patients undergoing chemotherapy for acute myeloid leukemia (AML) and stem cell transplantation. The aim of this study was to evaluate the real-life impact of posaconazole prophylaxis.

Materials and Methods: Eighty-four adult patients were included with AML under remission induction chemotherapy and posaconazole prophylaxis. The 34 patients in the control group did not receive primary antifungal prophylaxis. The period between June 2006 and January 2009, when antifungal prophylaxis was not administered (control group), was retrospectively compared to the period between December 2010 and May 2012 when primary oral posaconazole prophylaxis was administered in similar conditions (posaconazole group) according to the use of antifungal agents for treatment, breakthrough infections, galactomannan performance, and the necessity for performing bronchoalveolar lavage (BAL) procedures.

Results: The two groups were compared according to the use of antifungal agents; progression to a different antifungal agent was found in 34/34 patients (100%) in the control group and in 9/84 patients (11%) in the posaconazole group (p<0.001). There were four breakthrough IFIs (4/84, 4.8%) in the posaconazole group and 34 IFIs in the control group (p<0.001). In addition, 15/34 patients (44%) in the control group required BAL compared to 11/84 patients (13%) in the posaconazole group (p<0.001). Posaconazole treatment was discontinued within 7-14 days in 7/84 patients (8.3%) due to poor oral compliance related to mucositis after chemotherapy.

Conclusion: Posaconazole appears to be effective and well-tolerated protection against IFIs for AML patients.

Keywords: Acute myeloid leukemia, Invasive fungal infections, Antifungal prophylaxis, Posaconazole

Öz

Amaç: İnvaziv fungal enfeksiyonlar (İFE) akut myeloid lösemili (AML) ve kök hücre nakli yapılan hastalarda önemli bir mortalite ve morbidite nedenidir. Bu çalışmanın amacı posakonazol profilaksisinin gerçek yaşamdaki etkisini değerlendirmektir.

Gereç ve Yöntemler: AML ve remisyon indüksiyon kemoterapisi alan ve posakonazol profilaksisi uygulanan 84 erişkin hasta çalışmaya dahil edildi. Kontrol grubunda primer antifungal profilaksi almayan 34 hasta dahil edildi. Haziran 2006 ile Ocak 2009 tarihleri arası primer oral posakonazol profilaksisi almayan (kontrol grubu) ile Aralık 2010 ile Mayıs 2012 arası primer oral posakonazol profilaksisi (posakonazol grubu) uygulanan hastaları geriye dönük olarak; tedavi için antifungal ajan kullanımı, tedavi altında (breakthrough) enfeksiyonlar, galaktomannan performansı ve bronko-alveolar lavaj (BAL) gerekliliği gibi benzer durumlar için karşılaştırdık.

Bulgular: İki grup antifungal ajan kullanımına göre karşılaştırıldığında farklı antifungal ajana geçiş kontrol grubunda 34/34 (%100) idi ve posakonazol grubunda bu oran 9/84 (%11) bulundu (p<0,001). Posakonazol grubunda 4 tedavi altında (breakthrough) IFE (4/84, %4,8) ve kontrol grubunda ise 34 İFE vardı (p<0,001). İlaveten kontrol grubunda BAL gereken hasta 15/34 (%44) iken, posakonazol grubunda BAL gerekliliği 11/84 (%13) bulundu (p<0,001). Posakonazol tedavisi hastaların 7/84'ünde (%8,3) kemoterapi sonrası mukozite bağlı oral alım bozukluğu nedeniyle 7-14 gün içinde kesilmişti.

Sonuç: Posakonazol AML'li hastaların invaziv fungal enfeksiyonlarına karşı korumada etkili ve iyi tolere ediliyor görünmektedir.

Anahtar Sözcükler: Akut myeloid lösemi, İnvaziv fungal enfeksiyonlar, Antifungal profilaksi, Posaconazol

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Introduction

Invasive fungal infections (IFIs) are a significant cause of morbidity and mortality among neutropenic patients undergoing chemotherapy for acute myeloid leukemia (AML) and stem cell transplantation. Antifungal prophylaxis is an important aspect of treatment because these infections are often difficult to diagnose due to their lack of specific clinical features [1,2]. The use of mold-specific prophylaxis has increased in recent years, particularly in AML patients, because of the high mortality rate of IFIs [1,2,3]. Posaconazole has been recommended as the drug of choice for AML patients undergoing induction chemotherapy based on the results of randomized controlled trials [4,5,6,7,8].

The aim of this study was to evaluate the real-life impact of posaconazole prophylaxis. Patients under posaconazole prophylaxis who were followed from 2010 to 2012 were compared with historical control patients without posaconazole prophylaxis who were followed from 2006 to 2009 in similar conditions according to the use of antifungal agents for treatment, breakthrough infections, galactomannan (GM) performance, and the requirement for bronchoalveolar lavage (BAL) procedures.

Materials and Methods

A retrospective single-center study on primary prophylaxis with posaconazole was conducted in the Department of Hematology at the Uludağ University Hospital, a tertiary care hospital with 900 beds accredited by the Joint Commission International. Patients had to meet the following inclusion criteria to be eligible for this study: 18 years or older age, AML diagnosis, under remission induction or salvage chemotherapy, and under treatment at the hospital between December 2010 and May 2012. There were no patients with myelodysplastic syndrome (MDS) in either group. This retrospective study (number 2012-13/1; 19 June 2012) was approved by the local ethics committee for data collection.

Eighty-four adult patients were included with AML under remission induction chemotherapy and posaconazole prophylaxis who were followed from December 2010 to May 2012. In accordance with the indications for high-risk episodes, prophylactic treatment was started 24 h after the last day of chemotherapy and continued until neutrophil levels recovered to $>0.5x10^9$/L. Posaconazole (200 mg, oral suspension) was given orally three times daily. Thirty-four patients undergoing remission induction chemotherapy for AML who were not under posaconazole prophylaxis and who were followed from 2006 to 2009 were included as a control group. The control group did not receive any antifungal prophylaxis. The posaconazole-treated patients were compared with the control group according to the use of antifungal treatment, breakthrough infections, GM performance, and the need for BAL.

In 2008, the Infectious Disease Society of America (IDSA) recommended posaconazole for antifungal prophylaxis in hematopoietic stem cell transplantation recipients with graft-versus-host disease and for neutropenic patients with AML or MDS [9]. The protocol for treating febrile neutropenia was based on the clinical practice guidelines for the use of antimicrobial agents in neutropenic patients with cancer that were introduced by the IDSA in 2002 and updated in 2010.

According to the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) criteria, the clinical decision to replace prophylaxis with intravenous antifungal therapy was based in all cases on an individualized clinical judgment. This decision took into account the patient's general condition, the patient's signs and symptoms, the test results, and the patient's treatment compliance. The incidence and reason for early discontinuation of prophylaxis and the cause of death were recorded in all applicable cases.

Aspergillus galactomannan tests (Platelia *Aspergillus*; Bio-Rad Laboratories, Marnes-la-Coquette, France) were performed for the BAL and bronchial lavage specimens according to the manufacturer's instructions [10,11]. The patients were followed by high-resolution pulmonary computerized tomography (CT), serum GM, BAL, and BAL GM during the course of antifungal treatment in our clinic to refine the overall treatment strategy. The levels of GM in serum were measured twice a week for all of the patients. The serum GM test results were available within 2 days and were considered to be positive if the optical index was >0.7 in one sample or ≥0.5 in two consecutive samples. The BAL results for GM were considered to be positive if the BAL GM was ≥1.5.

A high-resolution pulmonary CT scan was performed between days 5 and 7 of febrile neutropenia or in the case of clinical deterioration.

These data were used to direct the treatment strategy and guide preemptive antifungal therapy at the study center. A multidisciplinary approach was used to make treatment decisions; a hematologist, an infectious disease specialist, a medical microbiologist, and a pulmonologist were consulted. Special attention was given to clinical, radiographic, and microbiological signs of infection; the duration of neutropenia; and the antimicrobial therapy. There was no difference in the daily diagnostic and therapeutic approaches and the physical and environmental conditions during the entire period. There were no HEPA filters or constructional changes in our inpatient clinic in either period. If there was evidence of invasive fungal disease, it was classified according to the 2008 revised EORTC/MSG criteria as 'possible', 'probable', or 'proven' IFI [12,13].

Breakthrough IFI was considered if IFI occurred four or more days after the initiation of primary antifungal prophylaxis with posaconazole [14].

Statistical Analysis

Statistical analyses were performed with SPSS 20.0 for Windows (IBM Corp., Armonk, NY, USA). The data are expressed as means ± standard deviation and were compared as follows: continuous variables were compared using the Mann-Whitney U test, categorical variables were compared using the chi-square test, and $p<0.05$ was considered significant. Data analyses were performed using Fisher's exact test and chi-square analysis.

Results

The patients' characteristics are summarized in Table 1. The average age, distribution of sex, medical history, and underlying disease status were similar in the two groups. In the posaconazole group, there were 84 patients with AML. The median age of the patients was 49.5 years (min-max: 20-71) and 54% of the patients in the posaconazole group were female. Of these 84 patients, 68 had received remission induction chemotherapy for a newly diagnosed disease and 16 had received salvage chemotherapy for relapsed disease. The median duration of primary posaconazole prophylaxis was 28 days (min-max: 7-60) in the posaconazole group and there was no toxicity related to posaconazole treatment. Posaconazole treatment was discontinued within 7-14 days in seven of 84 patients (8.3%) due to poor oral compliance related to mucositis after chemotherapy (Table 2). Two of these patients developed IFIs (1 possible, 1 probable). In addition to that, two patients without mucositis were diagnosed with breakthrough IFIs (1 possible, 1 probable) during posaconazole prophylaxis and their antifungal treatment was changed. There was no breakthrough IFI in 75 patients who completed posaconazole prophylaxis. Totally, there were four breakthrough infections in the posaconazole prophylaxis group (4/84, 4.8%). There were 28 possible and 6 probable IFIs in the control patients.

Antifungal therapy was given to seven of these patients. The antifungal drugs used were conventional amphotericin B, itraconazole, liposomal amphotericin B, voriconazole, and fluconazole (Table 2). Serum GM positivity was detected in 5/84 patients (6%) in the posaconazole group and in 5/34 patients (15%) in the control group (p=0.149). BAL GM positivity was detected in 4/15 patients (27%) in the control group and in 6/11 patients (55%) in the posaconazole group (p=0.227). However, 15/34 patients (44%) required the BAL procedure in the control group and 11/84 patients (13%) required this procedure in the posaconazole group (p<0.001).

There was no mortality within 3 months of the completion of chemotherapy cycles among the AML patients with posaconazole prophylaxis. However, 18/34 patients (53%) in the control group died within 3 months of completion of their chemotherapy cycles. The 3-month mortality rate was significantly higher in control group (p<0.001).

Discussion

Antifungal prophylaxis in hematology patients is important and reduces the use of antifungal therapy for suspected or proven IFIs, total mortality, and fungal infection-related mortality and minimizes the costs of management of either suspected or proven IFIs [15].

This study showed that antifungal prophylaxis with posaconazole significantly reduced IFIs and the need for antifungal treatment. Several recent studies supported the finding that posaconazole

Table 1. Characteristics of the patients and controls.

Patient characteristics	Control group	Posaconazole group	p-value
Period	June 2006-January 2009	December 2010-May 2012	
AML diagnosis	34	84	
New diagnosis / Relapse	31 (91%)/3 (9%)	68 (81%)/16 (19%)	p=0.274
Female / Male	11/23	45/39	p=0.059
Age, median (min-max)	48.5 (24-79)	49.5 (20-71)	p=0.863
Remission induction / Salvage chemotherapy	31 (91%)/3 (9%)	68 (81%)/16 (19%)	p=0.274
Discontinuation of prophylaxis	-	9 (11%)	-
Need for antifungal treatment	34/34	9/84	**p<0.001**
Duration of prophylaxis, days (min-max)	-	28 (7-60)	-
Duration of neutropenia, days (min-max)	17 (13-25)	20 (14-27)	**p=0.299**
Empirical approach / Preemptive approach IFI	17/17 34/34	5/4 4/84	**p<0.001** **p<0.001**
Alive / Exitus	16/18	84/0	**p<0.001**

Remission induction: 3+7 (idarubicin plus cytarabine), Salvage chemotherapy: etoposide-mitoxantrone-cytarabine.
AML: Acute myeloid leukemia, IFI: invasive fungal infection.

Table 2. The clinical characteristics of seven patients with mucositis under posaconazole prophylaxis.

Patient	Age	Sex	Disease status	GM positivity	Clinic status	IPA	Antifungal treatment	IFI	Outcome
1	65	M	New diagnosis AML	Serum: - (GM: -)	Diarrhea, grade III-IV Mucositis	-	Conv. Amp-B (4 days)	Possible	Alive
2	44	M	New diagnosis AML	Serum: + (GM: 1.26) BAL - (GM: 0.92)	Mucositis Diarrhea	+	Conv. Amp-B (11 days)	-	Alive
3	61	M	New diagnosis AML	Serum: - (GM: -)	Fever (continued) Extensive mucositis	-	Conv. Amp-B (8 days)	-	Alive
4	43	F	Relapsed AML	Serum: - (GM: -)	Fever (20 day) Diarrhea Pleural effusion *S. hominis*	-	Fluconazole (4 days)	-	Alive
5	59	F	New diagnosis AML	Serum: + (GM: 1.42) BAL: + (GM: 2)	Diarrhea, grade III	-	Itraconazole (1 day) Liposomal Amp-B (10 days) Voriconazole (10 days)	Probable	Alive
6	41	M	Relapsed AML	Serum: - (GM: -)	Diarrhea, grade IV	-	Fluconazole (16 days)	-	Alive
7	27	M	New diagnosis AML	Serum: - (GM: -)	Mucositis	-	Fluconazole (14 days)	-	Alive

AML: Acute myeloid leukemia, GM: galactomannan, BAL: bronchoalveolar lavage, IPA: invasive pulmonary aspergillosis, IFI: invasive fungal infection.

prophylaxis reduces the incidence of IFIs and invasive aspergillus in patients with AML/MDS or hematopoietic cell transplantation recipients when tested against comparable antifungal agents [16,17,18]. Prophylactic posaconazole was associated with statistically significantly fewer febrile days, shorter duration of hospitalization, and longer fungal-free survival; however, overall and attributable mortality did not differ [19]. In a study of 424 AML or MDS patients by Cho et al. [20], 140 received posaconazole and 284 received fluconazole prophylaxis. Fungal infection-free survival was significantly higher in the posaconazole group (74.7% vs. 87.1%, p=0.028). Investigators in Singapore created a network meta-analysis of randomized controlled trials evaluating posaconazole, concluding that it significantly reduced all-cause deaths compared to a fluconazole and itraconazole solution [21].

In patients receiving mold-active systemic antifungal prophylaxis with posaconazole, breakthrough IFIs occurred in 7.5% of patients [22]. Breakthrough infections are a major problem in patients receiving long-term prophylaxis [23]. Hoenigl et al. [24] proposed that GM testing is a useful diagnostic method for diagnosing breakthrough invasive aspergillosis in patients receiving mold-active prophylaxis and empirical therapy. In the study by Auberger et al. [25], breakthrough IFIs due to non-*Aspergillus* species, especially *Mucorales* spp., were noticed in a considerable proportion of patients at a high risk for IFIs receiving posaconazole prophylaxis. Bose et al. [26] reported that life-threatening *Fusarium* spp. infection may occur in immunocompromised patients despite prophylactic posaconazole.

It is assumed that azole-resistance could become a major problem in the future. Hamprecht et al. reported the first culture-proven case of invasive aspergillosis caused by azole-resistant *Aspergillus fumigatus* in a patient with AML in Germany, and this aspergillosis presented as a breakthrough infection under posaconazole prophylaxis [15]. Data from previous studies indicated that posaconazole is well tolerated, even following long-term administration. Several studies have shown that the most commonly reported adverse events were fever, nausea, diarrhea, vomiting, and headache [1,4,27,28,29]. In our study, posaconazole was discontinued within 7-14 days in 9/84 patients (11%) patients due to mucositis and diarrhea after chemotherapy. In our experience, prophylactic antifungal treatment is infrequently interrupted due to mucositis. Girmenia et al. [30] reported that posaconazole suspension might be used without the stringent need for monitoring plasma posaconazole concentrations in patients without diarrhea.

BAL GM has been recently explored as an additional method to diagnose invasive pulmonary aspergillosis. In those studies, the sensitivity of detection ranged from 57% to 88% and the

specificity ranged from 87% to 95.8% [31]. In this study, there was no difference for serum and BAL GM positivity between the two groups. We found similar GM positivity within the two groups. We think that the low number of patients in the control group could be responsible for this result. On the other hand, it was shown that prophylaxis with posaconazole negatively affected GM test performance. It was shown that the serum GM test was unreliable in asymptomatic patients under anti-mold prophylaxis [32,33,34]. Previous exposure to antifungal agents should be considered when interpreting GM results.

Study Limitations

The present study has some limitations. First, it is a retrospective study. Second, our control group was historical with a small sample size of controls, which was not matched numerically with the posaconazole prophylaxis group even at the minimum required optimal ratio of 1:1 to ensure reliable statistical analysis. Third, we did not measure plasma posaconazole levels. Finally, our study is a single-center study. In spite of these limitations of our study, we think that our results demonstrate the advantage of posaconazole prophylaxis in a real-life setting.

Conclusion

This study showed that antifungal prophylaxis with a second-generation azole (posaconazole) can significantly reduce the need for antifungal treatment without the risk of increasing the rate of adverse events.

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Ethics

Ethics Committe Approval: Uludağ University, approval number: 2012-13/1; 19 June 2012.

Informed consent: Retrospective study.

Authorship Contributions

Medical Practice: V.Ö., F.Ö., S.S., T.E.; Concept: V.Ö., H.A.; Design: V.Ö.; Data Collection or Processing: V.Ö., S.S., F.Ö., B.E., A.U., T.E., E.D., E.K., R.M., H.A.; Statistical Analysis: İ.E.; Literature Search: V.Ö.; Writing: V.Ö., H.A.

Conflict of Interest: The authors of this paper have no conflicts of interest, including spesific financial interests, relationships, and or affiliations relevant to the subject matter or materials included.

References

1. Xu SX, Shen JL, Tang XF, Feng B. Newer antifungal for fungal infection prevention during hematopoietic cell transplantation: a meta-analysis. Transplant Proc 2013;45:407-414.
2. Gomes MZ, Mulanovich VE, Jiang Y, Lewis RE, Kontoyiannis DP. Incidence density of invasive fungal infections during primary antifungal prophylaxis in newly diagnosed acute myeloid leukemia in a tertiary cancer center, 2009-2011. Antimicrob Agents Chemother 2014;58:865-873.
3. Pagano L, Caira M, Cuence-Estrella M. The management of febrile neutropenia in the posaconazole era: a new challenge? Haematologica 2012;97:963-965.
4. Girmenia C, Frustaci AM, Gentile G, Minotti C, Cartoni C, Capria S, Trisolini SM, Matturro A, Loglisci G, Latagliata R, Breccia M, Meloni G, Alimena G, Foa R, Micozzi A. Posaconazole prophylaxis during front-line chemotherapy of acute myeloid leukemia: a single-center, real-life experience. Haematologica 2012;97:560-567.
5. Cornely OA, Maertens J, Winston DJ, Perfect J, Ullmann AJ, Walsh TJ, Helfgott D, Holowiechi J, Stockelberg D, Goh YT, Petrini M, Hardalo C, Suresh R, Angulo-Gonzalez D. Posaconazole vs. fluconazole or itraconazole prophylaxis in patients with neutropenia. N Engl J Med 2007;356:348-359.
6. Ullman AJ, Lipton JH, Vesole DH, Chandrasekar P, Langston A, Tarantolo SR, Greinix H, Morais de Azenodo W, Reddy V, Boparai N, Pedicone L, Patino H, Durrant S. Posaconazole or fluconazole for prophylaxis in severe graft-versus-host disease. N Engl J Med 2007;356:335-347.
7. Tissot F, Agrawal S, Pagano L, Petrikkos G, Groll AH, Skiada A, Lass-Flörl C, Calandra T, Viscoli C, Herbrecht R. ECIL-6 guidelines for the treatment of invasive candidiasis, aspergillosis and mucormycosis in leukemia and hematopoietic stem cell transplant patients. Haematologica 2017;102:433-444.
8. Clark NM, Grim SA, Lynch JP 3rd. Posaconazole: Use in the prophylaxis and treatment of fungal infections. Semin Respir Crit Care Med 2015;36:767-785.
9. Walsh TJ, Anaissie EJ, Denning DW, Herbrecht R, Kontoyiannis DP, Marr KA, Morrison VA, Segal BH, Steinbach WJ, Stevens DA, van Burik JA, Wingard JR, Patterson TF; Infectious Diseases Society of America. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis 2008;46:327-360.
10. Meerseman W, Lagrou K, Maertens J, Wilmer A, Hermans G, Vanderschueren S, Spriet I, Verbeken E, Van Wijngaerden E. Galactomannan in bronchoalveolar lavage fluid: a tool for diagnosing aspergillosis in intensive care unit patients. Am J Respir Crit Care Med 2008;177:27-34.
11. Ağca H, Ener B, Yılmaz E, Ursavaş A, Kazak E, Özkocaman V, Çetinoğlu ED, Dilektaşlı AG, Akalın H, Özkalemkaş F, Ali R. Comparative evaluation of galactomannan optical density indices and culture results in bronchoscopic specimens obtained from neutropenic and non-neutropenic patients. Mycoses 2014;57:169-175.
12. Peterson L, Ostermann J, Rieger H, Ostermann H, Rieger CT. Posaconazole prophylaxis-impact on incidence of invasive fungal disease and antifungal treatment in haematological patients. Mycoses 2013;56:651-658.
13. Maertens JA, Nucci M, Donvely JP. The role of antifungal treatment in hematology. Haematologica 2012;97:325-327.
14. Auberge J, Lass-Flörl C, Aigner M, Clausen J, Gasti G, Nachbaur D. Invasive fungal breakthrough infections, fungal colonization and emergence of resistant strains in high-risk patients receiving antifungal prophylaxis with posaconazole: real-life data from a single centre institutional retrospective observational study. J Antimicrob Chemother 2012;67:2268-2273.
15. Ping B, Zhu Y, Gao Y, Yue C, Wu B. Second-versus first-generation azoles for antifungal prophylaxis in hematology patients: a systematic review and meta-analysis. Ann Hematol 2013;92:831-839.
16. Girmenia C, Perrone S. The diagnostic role of galactomannan during antifungal prophylaxis. Comment on: 'The role of antifungal treatment in hematology'. Haematologica 2012;97:325-327.
17. Xu XH, Zhang L, Cao XX, Li J, Zhang W, Zhu TN, Cai HC, Chen M, Han X, Yang C, Han B, Zhang Y, Zhuang JL, Zhou DB, Duan MH. Evaluation of the

implementation rate of primary antifungal prophylaxis and the prognosis of invasive fungal disease in acute leukemia patients in China. J Infect Chemother 2017;23:360-367.

18. Berking S, Doedens D, Horns H, Fiegl M, Ostermann H, Rieger CT. Antifungal prophylaxis in newly diagnosed AML patients-Adherence to guidelines and feasibility in a real life setting. Mycoses 2017;60:600-606.
19. Vehreschild JJ, Rüping MJ, Wisplinghoff H, Farowski F, Steinbach A, Sims R, Stollorz A, Kreuzer KA, Hallek M, Bangard C, Cornely OA. Clinical effectiveness of posaconazole prophylaxis in patients with acute myelogenous leukemia (AML): a 6 year experience of the Cologne AML cohort. J Antimicrob Chemother 2010;65:1466-1471.
20. Cho SY, Lee DG, Choi SM, Choi JK, Lee HJ, Kim SH, Park SH, Choi JH, Yoo JH, Kim YJ, Kim HJ, Min WS. Posaconazole for primary antifungal prophylaxis in patients with acute myeloid leukemia or myelodysplastic syndrome during remission induction chemotherapy: a single-centre retrospective study in Korea and clinical considerations. Mycoses 2015;58:565-571.
21. Zhao YJ, Khoo AL, Tan G, Teng M, Tee C, Tan BH, Ong B, Lim BP, Chai LY. Network meta-analysis and pharmacoeconomic evaluation of fluconazole, itraconazole, posaconazole, and voriconazole in invasive fungal infection prophylaxis. Antimicrob Agents Chemother 2015;60:376-386.
22. Winston DJ, Barton K, Territo MC, Schiller GJ. Efficacy, safety, and breakthrough infections associated with standard long-term posaconazole antifungal prophylaxis in allogeneic stem cell transplantation recipients. Biol Blood Marrow Transplant 2011;17:507-515.
23. Biehl LM, Vehreschild JJ, Liss B, Franke B, Markiefka B, Persigehl T, Bücker V, Wisplinghoff H, Scheid C, Cornely OA, Vehreschild MJ. A cohort study on breakthrough invasive fungal infections in high-risk patients receiving antifungal prophylaxis. J Antimicrob Chemother 2016;71:2634-2641.
24. Hoenigl M, Seeber K, Koidl C, Buzina W, Wölfler A, Duettmann W, Wagner J, Strenger V, Krause R. Sensitivity of galactomannan enzyme immunoassay for diagnosing breakthrough invasive aspergillosis under antifungal prophylaxis and empirical therapy. Mycoses 2013;56:471-476.
25. Auberger J, Lass-Flörl C, Aigner M, Clausen J, Gastl G, Nachbaur D. Invasive fungal breakthrough infections, fungal colonization and emergence of resistant strains in high-risk patients receiving antifungal prophylaxis with posaconazole: real-life data from a single-centre institutional retrospective observational study. J Antimicrob Chemother 2012;67:2268-2273.
26. Bose P, Parek HD, Holter JL, Greenfield RA. Disseminated fusariosis occurring in two patients despite posaconazole prophylaxis. J Clin Microbiol 2011;49:1674-1675.
27. Gedik H, Yildirmak MT, Simsek F, Aydin D, Demirel N, Yokus O, Arica D. Fungal pathogens and primary antifungal prophylaxis in patients with hematological malignancies: one year experience. Afr Health Sci 2012;12:390-394.
28. Egerer G, Geist MJ. Posaconazole prophylaxis in patients with acute myelogenous leukemia-results from an observational study. Mycoses 2011;54(Suppl 1):7-11.
29. Hahn J, Stifel F, Reichle A, Holler E, Andreesen R. Clinical experience with posaconazole prophylaxis-a retrospective analysis in a haematological unit. Mycoses 2011;54(Suppl 1):12-16.
30. Girmenia C, Annino L, Mariotti B, Fanci R, Minotti C, Spadea A, Carotti A, Piedimonte M, Chierihini A, Cerchiara E, Caselli D, Cupelli L, Arcioni F, Bertaina A, Ribersani M, Proia A, Mengarelli A, Perriello V, Torelli GF, Di Gioia M, Del Principe MI, Cassetta MI, Fallani S, Novelli A. Posaconazole oral suspension primary prophylaxis in acute leukemia and allogeneic stem cell transplant patients: can it be used without measurement of plasma concentration? Med Mycol 2016;54:445-458.
31. Brownback KR, Pitts LR, Simpson SQ. Utility of galactomannan antigen detection in bronchoalveolar lavage fluid in immunocompromised patients. Mycoses 2013;56:552-558.
32. Duarte RF, Sanchez-Ortega I, Cuesta I, Arnan M, Patino B, Fernandz de Sevilla A, Gudiol C, Ayats J, Cuenca-Estrella M. Serum galactomannan-based early detection of invasive aspergillosis in hematology patients receiving effective antimold prophylaxis. Clin Infect Dis 2014;59:1696-1702.
33. McCulloch E, Ramage G, Rajendran R, Lappin DF, Jones B, Warn P, Shrief R, Kirkpatrick WR, Patterson TF, Williams C. Antifungal treatment effects the laboratory diagnosis of invasive aspergillosis. J Clin Pathol 2012;65:83-86.
34. Vena A, Bouza E, Alvarez-Uria A, Gayoso J, Martin-Rabadan P, Cajuste F, Guinea J, Gomez Castella J, Alonso R, Munoz P. The misleading effect of serum galactomannan testing in high-risk hematology patients receiving prophylaxis micafungin. Clin Microbiol Infect 2017;23:1000.

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Diagnostic Problems in Chronic Basophilic Leukemia

Kronik Bazofilik Lösemide Tanısal Sorunlar

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Abstract

Chronic basophilic leukemia (CBL) is an extremely rare type of leukemia. A literature review revealed six cases reported as primary CBL and five patients with secondary CBL. Patients with primary CBL may present with symptoms not related to leukemia. Dysplastic changes in peripheral blood and bone marrow were described and demonstrated in cases of primary and secondary CBL. The literature review also revealed that differential counts made by automated blood cell counters may not characterize cells as basophils in patients with primary and secondary CBL and may mislead physicians in making a differential diagnosis. For these reasons, laboratory studies for the diagnosis of CBL are required, including metachromatic staining by toluidine blue and antigen expressions by flow cytometric analysis, to detect the nature of the neoplastic cells as basophils for a reliable diagnosis of CBL. The literature review failed to reveal specific cytogenetic findings in patients with primary and secondary types of CBL.

Keywords: Myelodysplastic syndrome, Dysplasia, Interleukin-6, Chronic myeloid leukemia, Chronic basophilic leukemia, Mast cell leukemia

Öz

Kronik bazofilik lösemi (KBL) son derece nadir bir lösemi tipidir. Literatürün gözden geçirilmesi ile primer KBL olarak altı olgu ve sekonder KBL olarak beş olgu rapor edildiği görüldü. Primer KBL'li hastalar, lösemi ile ilgili olmayan yakınmalar ile başvurabilirler. Primer ve sekonder KBL hastalarının periferal kan ve kemik iliği yaymalarında anormal değişiklikler tanımlandı ve gösterildi. Literatür taraması, primer ve sekonder KBL hastalarında otomatik kan hücre sayıcıları tarafından yapılan ayırıcı sayımların hücreleri bazofil olarak karakterize edemeyeceğini ve doktorları ayırıcı tanıda yanıltabileceğini ortaya koymuştur. Bu nedenle güvenilir bir KBL tanısı için neoplastik hücre türünün bazofil olarak saptanması, toluidine mavisi ile metakromatik boyanmayı ve membranlarındaki antijen ekspresyonlarını akım sitometrik analizi ile yapan kanıtlayıcı laboratuvar incelemelerini gerektirir. Literatürün gözden geçirilmesi primer ve sekonder KBL hastalarında özel sitogenetik bulgular olmadığını göstermiştir.

Anahtar Sözcükler: Myelodisplastik sendrom, Displazi, Interleukin-6, Kronik myeloid lösemi, Kronik bazofilik lösemi, Mast hücre lösemi

Introduction

Basophils are one of the members of granulocytes in the myeloid lineage and are formed by proliferation and differentiation of committed myeloid progenitors. Basophils in peripheral blood (PB) or tissues range in size from 10 to 15 µm and have nuclei that are purplish or dark blue and cytoplasmic granules of dark blue to purple and even blackish color as seen on Wright-stained PB and bone marrow (BM) smears [1]. A review of the international literature revealed 6 cases reported as primary chronic basophilic leukemia (CBL) [2,3,4] and 5 cases reported as chronic myeloid leukemia (CML) with transformation to CBL, as a secondary CBL [5,6,7,8,9]. The purpose of this review is to remind readers of the importance of diagnostic problems as automated blood cell counters (ABCCs) may not characterize cells as basophils in patients with primary and secondary CBL, but may simply flag them [3,4,9]. The literature review failed to reveal lymphoma or non-hematologic neoplasia with transformation to secondary CBL.

Age and sex distribution, splenic size, presenting symptoms, results of complete blood counts, and cytogenetic and molecular studies in patients with primary and secondary CBL are shown in Table 1 and Table 2, respectively.

Presenting Symptoms

Presenting symptoms may not be related to leukemia, as in Case #6 with primary CBL (Table 1). The patient presented with recurrent occurrence of febrile episodes and abdominal pain at about 8-week intervals, associated with simultaneous cyclic oscillation in neutrophil leukocyte counts and in the levels of C-reactive protein (CRP) when leukocyte counts climbed to the peak level and remained with the consumption of analgesics-antipyretics in

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Table 1. Characteristics of chronic and accelerated phases of primary chronic basophilic leukemia patients [2,3,4,5].

Characteristics	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7 (Accelerated phase of Case 6)
Age/Sex	30/Male	67/Male	52/Male	32/Male	81/Male	68/Female	At the age of 73
Presenting symptoms	Polyurea, Diabetes insipidus, Polydisplasia, Pituitary lesion	Progressive fatigue, Severe motor neuropathy	Progressive fatigue, Epigastric pain, Pruritis urticaria, Weight loss	Progressive fatigue	Decrease in appetite, Weight loss	Febrile episodes occuring with 8-week intervals with abdominal pain, Weight loss	Febrile episodes when basophil count >40x10^9/L with abdominal pain
Splenomegaly	Absent	Marked	Absent	Marked	3-4 cm	Absent	4 cm
Hemoglobin (g/dL)	11.1	12.6	13.2	9.1	10.5	11.2	5.7-7.1
WBC (x10^9/L)	9.9	9.3	52.0	47.5	50.0	59.1	59-315
Platelet (x10^9/L)	200	319	227	240	874	226	134-41
BM blast (%)	7	2	8	5	NR	<1	6
BM basophil (%)	15	26	20	NR	40	75	51
BM eosinophil (%)	3	3	6	6	15	8.5	13
BM dysplasia	Atypical mega-karyocytic hyperplasia	Atypical mega-karyocytic hyperplasia	Atypical mega-karyocytic hyperplasia	Dysmegakaryopoiesis	See text	See text	See text
PB basophil (%)	NR	NR	NR	NR	38	70	54-65
PB dysplasia	NR	NR	NR	NR	See text	See text	See text
Toluidine blue	NP	NP	NP	NP	NP	Positive	Positive
Antigen expression by flow cytometry	NP	NP	NP	NP	CD11a, CD11b, CD13, CD18, CD33, CD45, CD123	CD10 (dim), CD11c (dim), CD13, CD15, CD22 (dim), CD25, CD33, CD38, CD45, CD123, MPO, IgDR	CD10 (dim), CD11c (dim), CD13, CD15, CD22 (dim), CD25, CD33, CD38, CD45, CD123, MPO, IgDR, CD117
Cytogenetic findings	+8, 11q-	-7, -7/-4	46, XY	46, XY	46, XY, t(5;12) (q31;p13)	47, XX, der(6) t(6;?)(q25-27;?) +mar(16)	47, XX, der(6) t(6;?)(q25-27;?) der(17) t(17;?) (p13;?) +mar(16)
Molecular genetic studies	-	-	-	-	Absence of JAK2, (V617F) mutation, BCR/ABL rearrange-ment by PCR	IL-6 gene expression in neoplastic basophils and absence of JAK2 mutation detected by real time PCR	Heterozygote substitution of C1650A>T (K550N) on exon 11, wild type sequence on exons 9, 13, 17. Absence of PIF1L1-PDGFRA rearrangement
IL-6 (pg/mL)	NP	NP	NP	NP	NP	15.8	38.5
Tryptase (µg/L)	NP	NP	NP	NP	NP	NP	42.9-51.2
Treatments	ALLO-SCT	Hydroxyurea prednisone	Imatinib	ALLO-HSCT	Hydroxyurea	Hydroxyurea	Hydroxyurea, Imatinib, Etoposide, Leukapheresis, Prednisone CTX, RBC transfusion
Follow-up (months)	35, alive	48, dead	2, alive	10, dead	9, dead	>50, alive	5, dead

CBL: Chronic basophilic leukemia, WBC: white blood cells, BM: bone marrow, PB: peripheral blood, NR: not recorded, NP: not performed, IL-6: interleukine-6, ALLO-SCT: allogeneic-human stem cell transplantation, RBC: red blood cells, PCR: polymerase chain reaction.

2 h. Physicians were misled in making a differential diagnosis of familial Mediterranean fever because they relied on differential counts made by ABCCs [4]. However, manual differential counts made on PB smears during a febrile episode revealed that the ABCCs wrongly characterized 70% of basophils as neutrophils; this was confirmed by toluidine blue stain and antigen expression by flow cytometric analysis. Marked elevation in plasma interleukin-6 (IL-6) level of 15.8 pg/mL (normal: <5.8 pg/mL) was detected during the febrile episode. Real-time polymerase chain reaction showed IL-6 gene expression in neoplastic basophils, revealing that IL-6 was released from neoplastic basophils infiltrating the BM [4] (Table 1). IL-6 production and release by normal human basophils has not been reported in the literature [10].

Values of PB and BM Basophil Percentages in the Diagnosis of CBL

As shown in Tables 1 and 2, BM basophil percentages of 15%, 26%, and 20% in 3 of 4 patients with primary CBL were reported by Pardanani et al. [2]. These values are almost equal to the BM basophil percentage of ≥20% in 11% of 25 patients in the accelerated phase of CML reported by Kantarjian et al. [11] and also BM basophil percents greater than 1% (range 1 to 27%) in 34 cases and median 19% (range 14 to 27%) in 6 patients (in 4 of whom toluidine blue staining was positive) were reported by Hoyle et al. [12] in their series of 750 patients with acute myeloid leukemia. However, the BM basophil percentages in patients reported by Pardanani et al. [2] were markedly lower compared to the BM basophil percentages (40%,75%) of two patients in the chronic phase [3,4] and the value recorded (51%) in a patient in the accelerated phase of primary CBL [5] (Table 1). BM basophil percentages of 55%, 51.4%, 63%, 72%, and 66% in six patients with secondary CBL were also respectively recorded (Table 2) [6,7,8,9].

Dysplastic Changes in Primary CBL

In Case #5, a patient with primary CBL, dysplasia in the PB smear included hypogranular basophils with small and fine granulations and nuclear hyperlobation. In the BM smear, an increase in cellularity and megakaryocytes and hypogranular, agranular, and hypersegmented forms of basophils and eosinophils with coarse granulations and nuclear hyperlobation in addition to megakaryocytes with many small and hypolobated forms were reported [3].

In Case #6, a patient with primary CBL in the chronic phase, dysplasia appeared as basophils with coarse basophilic granules, occasionally hypersegmented or giant segmented, and band forms of basophils in PB smear [4].

In the BM smear, an increase in cellularity, megakaryocytes, eosinophils, and prominent basophilic hyperplasia with the presence of all stages of maturation that resulted in marked neutrophilic suppression and mild suppression in erythroid lineages were noted. Three-lineage dysplasia manifested as hyposegmented basophils, giant segmented bands, and metamyelocyte forms of basophils and hypogranular basophilic metamyelocytes were noted. Dysplasia seen in eosinophils included binuclear metamyelocyte and myelocyte forms of eosinophils and large eosinophilic myelocytes. Occasional binuclear (Figure 1A) and multinuclear red cell precursors were also noticed. In addition, binuclear agranular immature megakaryocytes, mononuclear giant forms of megakaryocytes, and megakaryocytes with nuclear hyperlobation were observed. Toluidine blue stain showed red (metachromatic) granular staining in about 75% of non-erythroid granular cells in the marrow fields (Figure 1B).

Dysplastic Changes in the Accelerated Phase of Primary CBL

Clinical and hematologic features of the accelerated phase of primary CBL were only observed by Cehreli et al. [5] in their patient in the chronic phase of primary CBL (Case #6) after 53 months of hematologic remission; this is shown as Case #7 in Table 1. She presented with symptoms of anemia and was found to have relapse of her CBL and development of systemic mastocytosis (SM) as a secondary neoplasia. Three months later, the patient showed a rapid downhill clinical course when transformation of primary CBL to the accelerated phase with simultaneous occurrence of mast cell leukemia (MCL) was detected [5]. She experienced febrile episodes with abdominal pain during the accelerated phase of primary CBL with MCL. Although mast cells and eosinophils were shown to produce IL-6 [10], no febrile episodes were observed despite increases in mast cell (MC) counts to $3x10^9$/L and eosinophil counts to $5.3x10^9$/L unless her basophil counts climbed to $>40x10^9$/L when prominent elevation in IL-6 level (38.5 pg/mL) was found. In a Wright-stained BM smear, basophilic hyperplasia with the presence of all stages of maturation that resulted in marked suppression in neutrophilic and erythroid lineages in addition to aggregates of MCs with a new and undefined MC morphology were demonstrated. MCs have round or oval nuclei, one or more nucleoli in immature forms, and mixed orange and dark purplish to black round cytoplasmic granules (Figure 2A). Tryptase immunohistochemical staining of the PB smear showed round, brown, granular cytoplasmic staining in the aggregates of the cells, confirming that these cells demonstrated tryptase activity and represented MCs (Figure 2B), because β-tryptase is a natural serine protease and is the most abundant mediator stored in the granules of MCs [13]. Three-lineage dysplasia manifested as giant hypersegmented basophils, giant binuclear metamyelocytes, binuclear hypogranular basophilic metamyelocytes, binuclear erythroblasts, and Pelger-Hüet anomalies were noted. Additionally, marked pyknosis, manifested as a decrease in both cellular and nuclear sizes, resulted in dense chromatin clumping, inducing a nuclear appearance that resembled a chromatin mass. Pyknotic myelocytes, metamyelocytes, binuclear basophilic metamyelocytes, and drum stick-like nuclear sticks in both pyknotic eosinophils and basophils were observed (Figure 2A). Dysplastic changes in the megakaryocytic lineage were similar to those seen in the chronic phase of the patient [4].

Marked pyknotic changes in the accelerated phase of primary CBL [5] have not been reported in patients with the chronic phase of primary CBL [3,4] as well as in patients with the accelerated [5,7,8] and chronic phase [9] of secondary CBL in the literature.

Table 2. Characteristics of secondary chronic basophilic leukemia patients reported in the literature [6,7,8,9].

Characteristics	Case 1	Case 2	Case 3	Case 4	Case 5
Age/Sex	45/Male	57/Female	61/Male	49/Male	45/Male
Phase of CBL	Accelerated	Accelerated	Accelerated	Accelerated	Chronic
Presenting symptoms	Fatigue, Gas, Fullness	Tiredness, Weight loss, Abdominal pain, Night sweats	Fatigue, Productive cough	Weakness, Myalgia, Intermittent fever	Fatigue, Weight loss
Splenomegaly	Massive	Massive	Massive	NR	3-4 cm
Hemoglobin (g/dL)	7.7	7.7	6.7	9.1	5.1
WBC (x10^9/L)	223	190	175	42	173
Platelet (x10^9/L)	16	162	NR	12	NR
BM blasts (%)	11	5.8	NR	7	9
BM basophils (%)	55.2	51.4	63	72	66
BM eosinophils (%)	2	5	NR	NR	8
BM dysplasia	NR	NR	NR	Large basophilic granules	See text
BM cellularity and findings	Hypercellular, BDSM	Hypercellular, BDSM	Increased, BDSM	90-100%, BDSM	Hypercellular
PB basophils (%)	75	71	NR	90	40
PB dysplasia	NR	NR	NR	NR	NR
Toluidine blue	NP	NP	Metachromatic staining	Metachromatic staining	NP
Antigen expression by flow cytometry	NP	NP	NP	NP	CD11b, CD13, CD33, CD34, CD117, CD123, HLA.DR, CD45, CD7 (aberrant)
Cytogenetic findings	Insufficient analysis	Insufficient analysis	[46, XY, t(8;21), t(9;22)/48, XY, +8, t(8;21), t(9;22) +Ph'/49, XY, +8, t(8;21), t(9;22), 9q+, +Ph']	Diagnosis CML Ph' t(9;22), Diagnosis of CBL, extra chromosomes (8, 10, 17, X)	46, XY, t(9;22)
Interval between CML and CBL (months)	12	31	4.5	48	-
Treatments	Busulfan, Supportive care	Busulfan, Splenic irradiation, Supportive care	Hydroxyurea, +3 cycles CHOP	ALLO-HSCT	Resistant to Gleevec, aggressive treatment
Follow-up (months)	3, dead	1, dead	4, dead	Died after ALLO-HSCT	NR

CBL: Chronic basophilic leukemia, WBC: white blood cells, BM: bone marrow, PB: peripheral blood, NR: not recorded, NP: not performed, ALLO-HSCT: allogeneic-human stem cell transplantation, BDSM: basophils at different stages of maturation, CHOP: cyclophosphamide, doxorubicin, vincristine, and prednisone.

Dysplastic Changes in the Chronic and Accelerated Phases of Secondary CBL

Case #5 in Table 2 was the only patient in the chronic phase of secondary CBL presenting with dysplasia. Diagnosis of CML may possibly be made when transformation of CML to CBL occurs; in this case, the patient had marked basophilia with the presence of 40% and 66% basophils in PB and BM, respectively, and antigen expressions by flow cytometric analysis revealed the nature of cells as basophils. Dysplasia included cytoplasmic hypogranulation or agranulation and nuclear hypersegmentation, eosinophils with abnormal granulation and nuclear hyperlobation, and dyserythropoiesis [9]. No dysplastic findings have been reported in patients in the accelerated phase of secondary CBL [6,7,8] (Table 2).

Confirmatory Laboratory Studies

Both basophils and MCs have electron-dense cytoplasmic granules and produce numerous mediators such as histamine common to both cells. They also both show metachromatic staining with basic dyes, toluidine blue, and Alcian blue [1]. Toluidine blue stain showed a red (metachromatic) granular cytoplasmic staining in both basophils and MCs (Figure 1B) [4,5,14,15]. Peroxidase stain showed black granular cytoplasmic staining in basophils, but MCs do not contain myeloperoxidase and showed negative activity by peroxidase stain (Figure 2C) [16]. Flow cytometric analysis of mononuclear cells (MNCs) of the BM using monoclonal antibodies against the following antigens in Cases #6 and #7 showed that antigen expressions were positive for CD10 (dim), CD11c (dim), CD13, CD15, CD22 (dim), CD25, CD33, CD38, CD45, CD123,

immunoglobulin D (IgD) receptor, and myeloperoxidase and negative for HLA-DR, CD7, CD34, CD71, and CD117 with aberrant expression of CD10 [17,18], thus revealing that the neoplastic cells were basophils.

Expression of the IgD receptor on normal basophils was demonstrated by Chen and Cerutti [19] and was also shown on neoplastic basophils by Cehreli et al. [4,5].

Importance of Confirmatory Laboratory Studies for the Diagnosis of CBL

Tang et al. [3] and Vaidya et al. [9] reported that automated hematology analyzers did not characterize cells as basophils, but simply flagged them. Flow cytometric immunophenotyping became particularly important in confirming the nature of cells as basophils [9]. Cehreli et al. [4,5] reported that ABCCs, even with advanced technology, wrongly characterized basophils as neutrophils, misleading physicians in making differential diagnosis when the physicians relied on differential counts made by ABCCs. These reported observations suggest that manual differential counts should be seen before making a decision for diagnosis and also confirmed by metachromatic staining with toluidine blue stain in PB or BM smears and antigen expressions by flow cytometric analysis in BM MNCs to make an accurate diagnosis of CBL. The authors also proposed that neoplastic basophils with coarse, dark purple basophilic granules as demonstrated in Figure 1A may possibly mimic neutrophils with toxic granules [5]. Neutrophils usually contain light purplish-blue fine granules on Wright-stained PB smears (Figure 1A). Interestingly, in 1932, Kugel and Rosenthal [20] found that during bacterial infections fine neutrophilic granules are replaced by large, dark, irregular basophilic granules, which are called toxic granules, compared to the fine granules of the neutrophils.

Oscillation in Leukocyte Counts

Similar to cyclic oscillations in leukocyte (neutrophil) counts reported in patients with CML [21,22], cyclic oscillations in basophil counts with simultaneous elevations in CRP levels and association with febrile episodes were only demonstrated by Cehreli et al. [5] in patients with primary CBL [4], but not reported in patients with primary [3] and secondary CBL [6,7,8,9].

Literature findings reveal that the diagnosis of CBL is mainly based on basophil morphology and increase in PB and BM basophil percentages. The literature also suggests that higher BM and PB basophil percentages are required to establish a satisfactory morphologic diagnosis of CBL. Based on the reported literature findings [2,3,4,5,6,7,8,9], diagnostic criteria for CBL as shown in Table 3 can be proposed.

Differential Diagnosis

The presence of an increase in megakaryocytes with atypical megakaryocytic hyperplasia and BM basophil percentages of less than 40% in 3 of four patients (Cases #1-3), the absence of diagnostic confirmatory laboratory studies (Table 1), and an abnormal pattern of perivascular atypical MC infiltration detected by tryptase immunohistochemical staining (Cases #2 and #4) suggesting concurrent MC disease were reported as primary CBL by Pardanani et al. [2]. These findings created diagnostic problems in classifying the cases as primary CBL as described by Pardanani et al. [2] because according to the proposed diagnostic criteria for CBL (Table 3) BM basophil percentages were less than ≥40%, increased megakaryocytes with dysplasia have been described in patients with essential thrombocythemia and chronic idiopathic myelofibrosis [23], and presence of abnormal pattern of perivascular atypical MC infiltration detected by tryptase immunohistochemical staining in the BM biopsy is one of the diagnostic criteria for SM [24].

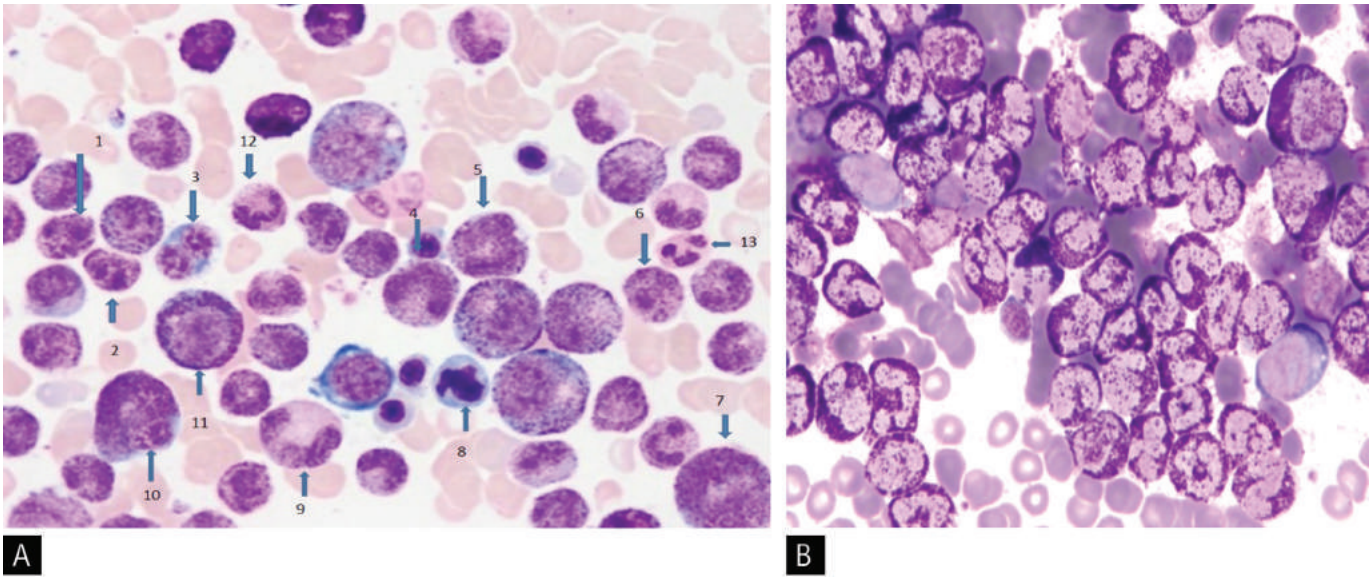

Figure 1. A) Showing hyposegmented basophils (1, 2, 6), binuclear erythroblast (3), giant forms of basophilic bands (4, 5), large eosinophilic myelocyte (7), erythroblast with dysplastic nucleus (8), giant basophilic hypogranular metamyelocyte (9), giant binuclear basophilic metamyelocyte (10), basophilic myelocyte (11), neutrophilic band (12), segmented neutrophil (13) in chronic phase of primary chronic basophilic leukemia (Wright's stain, 100x); B) Demonstrating red color (metachromatic) granular cytoplasmic staining in 70% nucleated cells of the bone marrow (toluidine blue stain, 100x).

Dysplastic changes described in two patients in the chronic phase of primary CBL [3,4] and in a patient in the accelerated phase of primary CBL [5] (Table 1) in addition to a patient in the chronic phase of secondary CBL [9] (Table 2) were not specific for CBL and have been described in patients with myelodysplastic syndrome (MDS) [25,26]. In MDS, dysplastic changes are accompanied by specific cytogenetic abnormalities [25], whereas cytogenetic studies specific for primary and secondary CBL have not been reported in the literature [2,3,4,5,6,7,8,9]. Additionally, laboratory studies with metachromatic staining by toluidine blue stain in both primary and secondary CBL [4,5,7,8] and antigen expressions detected by flow cytometric analysis in both primary and secondary CBL [3,4,5,9] are confirmatory for diagnosis of CBL (Tables 1 and 2), but have no diagnostic value in patients with MDS [25,26]. Patients in the chronic and accelerated phases of CML associated with both PB

Table 3. Proposed diagnostic criteria for chronic basophilic leukemia.

	Diagnostic criteria
CBL	- Hypercellularity of bone marrow, - Increase in megakaryocytes, - Eosinophilia, - Blasts <10%, - BM and PB basophils ≥40%, - Prominent basophilic hyperplasia with presence of all stages of maturation that result in moderate to marked suppression in neutrophilic lineage
Primary CBL	No previous hematologic neoplasia
Secondary CBL	Known previous hematologic neoplasia

CBL: Chronic basophilic leukemia, BM: bone marrow, PB: peripheral blood.

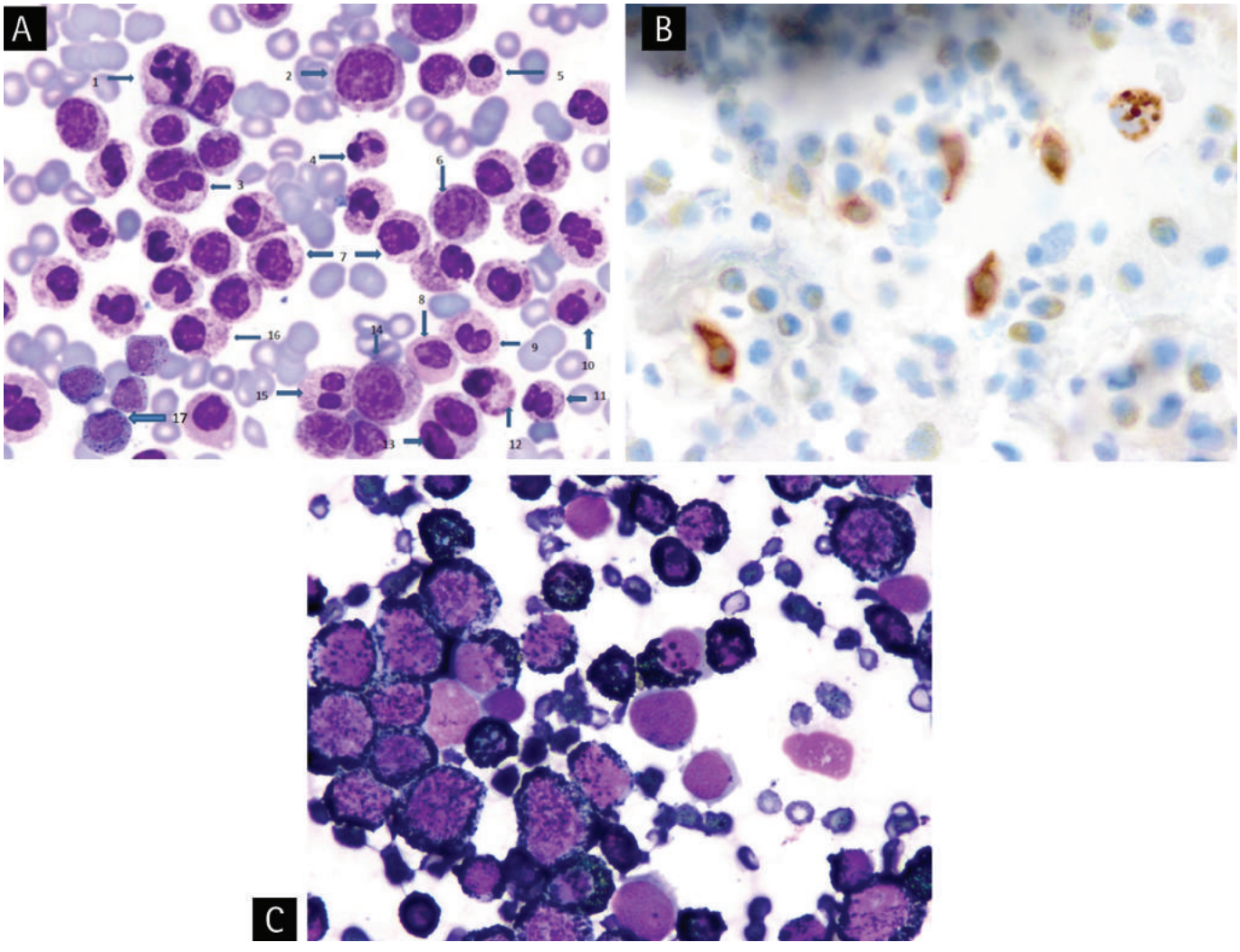

Figure 2. A) Demonstrating hypersegmented basophil (1), basophilic myelocyte (2), giant binuclear basophilic metamyelocyte (3), pyknotic eosinophil and basophil with drum-stick like nuclear sticks (4, 10), normal basophilic metamyelocyte (6), pyknotic myelocyte, metamyelocytes, binuclear basophilic metamyelocyte and basophilic myelocyte (5, 7, 11, 12, 16), agranular and hypogranular metamyelocyte (8, 9), binuclear hypogranular metamyelocyte (13), basophilic myelocyte (14), Pelger-Hüet anomaly (15) and aggregates of mast cells having mixed orange and dark purplish to black color round cytoplasmic granules (17) in accelerated phase of primary chronic basophilic leukemia with mast cell leukemia (Wright's stain, 100x); B) Showing tryptase activity in the round, brown color of cytoplasmic granules of mast cells demonstrated by immunohistochemical staining for tryptase. (tryptase immunohistochemical staining, 100x); C) Demonstrating black granular cytoplasmic staining by peroxidase stain in myeloperoxidase-positive basophils and absence of staining in aggregates of cells representing myeloperoxidase-negative mast cells in the bone marrow (peroxidase stain, 100x).

and BM basophil percentages of ≥40% [6,7,8,9], patients with autoinflammatory diseases manifesting with recurrent attacks of fever and abdominal pain [27] and additionaly, chronic myeloproliferative disorders [28], mastocytosis variants [29], Castleman's disease [30] and the possibility of underlying CBL [4,5] should be considered in the differential diagnosis in the patients presented with progressive leukocytosis associated with eosinophilia and elevations in IL-6 and CRP levels.

Conclusion

ABCCs may not characterize cells as basophils in patients with primary and secondary CBL [3,4,5,9] and may mislead physicians in making a differential diagnosis [4]. The new generation of blood cell counters could be designed to contain toluidine blue stain for the detection of neoplastic basophils, and MCs with atypical or new MC morphology will be beneficial in leading physicians to make a reliable differential diagnosis, like ABCCs containing methylene blue stain for reticulocyte counts. Diagnosis, treatment, and follow-up should be performed with the guidance of manual differential counts in cases of primary and secondary CBL. The frequency and type of dysplastic changes [3,4] observed, especially during the accelerated phase of primary CBL [5] (Figures 1 and 2), are comparable to those seen in patients with MDS and may create problems in the differential diagnosis of MDS [25,26].

Ethics

Conflict of Interest: The author of this paper has no conflicts of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

References

1. Beafus AD, Denburg JA. Basophilic leukocytosis: mast cells and basophils. In: Greer JP (ed). Wintrobe's Clinical Hematology, Twelfth Edition, Volume One. Philadelphia, Wolters Kluwer/Lippincott Williams & Wilkins, 2009.
2. Pardanani AD, Morice WG, Hoyer JD, Tefferi A. Chronic basophilic leukemia: a distinct clinico-pathologic entity. Eur J Haematol 2003;71:18-22.
3. Tang G, Woods LJ, Wang SA, Brettler D, Andersen M, Miron PM, Pechet L, Woda BA, Hao S. Chronic basophilic leukemia: a rare form of chronic myeloproliferative neoplasm. Hum Pathol 2009;40:1194-1199.
4. Cehreli C, Ates H, Cehreli R, Sercan Z, Demirkan F. New paraneoplastic syndrome in chronic basophilic leukemia. Int J Hematol 2013;97:498-504.
5. Cehreli C, Alacacioglu I, Piskin O, Ates A, Cehreli R, Calibasi G, Yüksel E, Ozkal S, Ozsan GH. Mast cell leukemia associated with undefined morphology and chronic basophilic leukemia. BMC Hematol 2014;14:17.
6. Kyle RA, Pease GL. Basophilic leukemia. Arch Intern Med 1966;118:205-210.
7. Xue Y, Guo Y, Lu D, Gu J, Lu D, Gong J, Wang M, Zhu W, Lin B. A case of basophilic leukemia bearing simultaneous translocation t(8;21) and t(9;22). Cancer Genet Cytogenet 1991;51:215-221.
8. Cunningham MT, Ganguly S. Chronic myeloid leukemia in accelerated phase with basophilic transformation of the bone marrow. Am J Hematol 2006;81:380-381.
9. Vaidya S, Madkaikar M, Ghosh K, Vundinti BR. Deletion of ABL/BCR on der (9) associated with severe basophilia. Indian J Hum Genet 2011;17:100-103.
10. Guzmán C, Hallal-Calleros C, López-Griego L, Morales-Montor J. Interleukin-6: a cytokine with a pleiotropic role in the neuroimmunoendocrine network. Open Neuroendocrinology 2010;3:152-160.
11. Kantarjian HM, Dixon D, Keating MJ, Talpaz M, Walters RS, McCredie KB, Freireich EJ. Characteristics of accelerated disease in chronic myelogenous leukemia. Cancer 1988;61:1441-1446.
12. Hoyle CF, Sherrington PD, Fischer P, Hayhoe FG. Basophils in acute leukemia. J Clin Pathol 1989;42:785-792.
13. Payne V, Kamp PC. Mast cell tryptase: a review of its physiology and clinical significance. Anesthesia 2004;59:695-703.
14. Perkins SL. Examination of the blood and bone marrow. In: Greer JP (ed). Wintrobe's Clinical Hematology, Twelfth Edition, Volume One. Philadelphia, Wolters Kumer/Lippincott Williams & Wilkins, 2009.
15. Daniel MT, Flandrin G, Bernard J. Leucémie aiguë a mastocytes: etude cytochimique et ultrastructurale, à propos d'une observation. Nouv Rew Fr Hematol 1975;15:319-332.
16. Pinkus GS, Pinkus JL. Myeloperoxidase: a specific marker for myeloid cells in paraffin section. Mod Pathol 1991;6:733-741.
17. Han X, Jorgensen JL, Brahmandam A, Schlette E, Huh YO, Shi Y, Awagu S, Chen W. Immunophenotypic study of basophils by multiparameter flow cytometry. Arch Pathol Lab Med 2008;132:813-819.
18. Toba K, Koike T, Shiabata A, Hashimoto S, Takahashi M, Masuko M, Azegami T, Takahashi H, Aizawa Y. Novel technique for the direct flow cytoflourometric analysis of human basophils in unseparated blood and bone marrow and the characterization of phenotype and peroxidase of human basophils. Cytometry 1999;35:249-259.
19. Chen K, Cerutti A. The function and regulation of immunoglobulin D. Curr Opin Immunol 2011;23:345-352.
20. Kugel MA , Rosenthal N. Pathologic changes occuring in polymorphonuclear leukocytes during the progress of infections. Am J Med Sci 1932;183:657-667.
21. Gatti RA, Robinson WA, Deinard AS, Nesbit M, McCullough JJ, Ballow M, Good RA. Cyclic leukocytosis in chronic myelogenous leukemia: new perspectives on pathogenesis and therapy. Blood 1973;41:771-782.
22. Mehta BC, Agarwal MB. Cyclic oscillations in leukocyte count in chronic myeloid leukemia. Acta Haematol 1980;63:68-70.
23. George TI. Pathology of myeloproliferative disorders. In: Greer JP (ed). Wintrobe's Clinical Hematology, Twelfth Edition, Volume One. Philadelphia, Wolters Kluwer/Lippincott Williams & Wilkins, 2009.
24. Pardanani A. Systemic mastocytosis in adults: 2012 update on diagnosis, risk stratification, and management. Am J Hematol 2012;87:401-411.
25. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, Sultan C. Proposals for the classification myelodysplastic syndromes. French-American-British (FAB) Cooperative Group. Br J Haematol 1982;51:189-199.
26. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, Harris NL, LeBeau MM, Hellström-Lindberg E, Tefferi A. The 2008 revision of the World Health classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood 2009;114:937-951.
27. Touitou S. Autoinflammatory diseases. Best Pract Res Clin Rheumatol 2008;22:811-829.
28. Panteli KE, Hatzimicael EC, Bouranta PK, Katsaraki A, Seferiadis K, Stebbing J, Bourant KL. Serum interleukin (IL)-1, IL-2, sIL-2Ra, IL-6 and thrombopoietin levels in patients with chronic myeloproliferative diseases. Br J Haematol 2005;130:709-715.
29. Brockow K, Akin C, Huber M, Metcalfe DD. IL-6 levels predict disease variant and extent of organ involvement in patients with mastocytosis. Clin Immunol 2005;115:216-223.
30. El-Osta HE, Kurzrock R. Castleman's disease: from basic mechanisms to molecular therapeutics. Oncologist 2011;16:497-511.

BRIEF REPORT

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Hematologic Adverse Effects of Prolonged Piperacillin-Tazobactam Use in Adults

Erişkinlerde Piperasilin-Tazobaktamın Uzamış Kullanımıyla Gelişen İstenmeyen Hematolojik Etkiler

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Abstract

Objective: We aimed to find the incidence and risk factors of hematologic adverse effects of piperacillin-tazobactam (TZP).

Materials and Methods: Adult patients who used TZP for more than 10 days were included in the study.

Results: The incidence of leukopenia, neutropenia, and eosinophilia in 110 TZP therapy episodes was found to be 16.3%, 10%, and 10%, respectively. Lower Charlson Comorbidity Index score, lower initial leukocyte count, combination of TZP with another antibiotic, and total duration of TZP therapy were found to be independent risk factors for leukopenia, while initial higher eosinophil count (IHEC) and usage of TZP for >20 days were independent risk factors for neutropenia and IHEC and total duration of TZP therapy were independent risk factors for eosinophilia.

Conclusion: Longer duration of therapy, combination with other antibiotics, younger age with fewer comorbidities, and IHEC could result in hematologic adverse effects in patients treated with TZP. Patients with IHEC may be more prone to allergic reactions, so immunological mechanisms may facilitate the development of hematological adverse effects of TZP.

Keywords: Neutropenia, Leukopenia, Eosinophilia, Piperacillin-tazobactam, Adverse effects

Öz

Amaç: Piperasilin-tazobaktamın (TZP) hematolojik istenmeyen etki insidansını ve risk faktörlerini bulmayı amaçladık.

Gereç ve Yöntemler: On günden uzun süre TZP kullanan erişkin hastalar çalışmaya dahil edildi.

Bulgular: Yüz on tedavi epizodunda lökopeni, nötropeni ve eozinofili insidansları sırasıyla %16,3, %10 ve %10 olarak bulundu. Charlson'ın Komorbidite İndeksi'nin düşük olması, başlangıç lökosit sayısının düşük olması, TZP ile başka antibiyotiğin kombine kullanılması ve TZP'nin toplam tedavi süresi; başlangıç eozinofil sayısının yüksek olması (BESYO) ve 20 günden uzun süreli TZP kullanımı; BESYO ve TZP'nin toplam tedavi süresi sırasıyla lökopeni, nötropeni ve eozinofili için bağımsız risk faktörleri olarak bulundu.

Sonuç: TZP ile tedavi edilen hastaların tedavi süresinin uzun olması, kombine antibiyotik tedavisi almaları, daha genç yaşta daha az komorbiditelerinin olması ve BESYO hematolojik istenmeyen etkilerin gelişmesine neden olabilir. BESYO olması hastaların alerjik reaksiyonlara daha yatkın olmasına neden olabilir, bu nedenle immünolojik mekanizmalar TZP kullanımıyla hematolojik istenmeyen etkilerin gelişmesini kolaylaştırabilir.

Anahtar Sözcükler: Nötropeni, Lökopeni, Eozinofili, Piperasilin-tazobaktam, İstenmeyen etkiler

Introduction

Piperacillin-tazobactam (TZP) is a broad-spectrum semisynthetic antibiotic. It has increased activity against *Pseudomonas aeruginosa* when compared with other penicillins [1]. It is commonly used in nosocomial infections and many other conditions that require broad-spectrum antibiotics, such as febrile neutropenia. Adverse effects of TZP include hypersensitivity reactions and gastrointestinal, renal, and hematologic effects. Although the most frequently reported hematologic adverse effect of TZP is reversible neutropenia, Coombs-positive hemolytic anemia and thrombocytopenia are also reported [1,2]. After the observation of fever and neutropenia in some patients who received prolonged TZP therapy, we aimed to identify the incidence and risk factors for the development of these adverse effects.

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Materials and Methods

Patient Selection

Adult patients (aged >18 years) who were given original TZP for more than 10 days at our faculty from January 2013 to December 2014 were included in the study. Usual adult doses were used and TZP was adjusted to renal function if necessary. Patients with HIV infection and hematologic malignancy, patients with leukopenia and neutropenia, and patients using systemic steroid therapy or chemotherapy within the last 3 months were excluded from the study. If the duration between two episodes of TZP therapy exceeded 1 month, those episodes were evaluated separately.

Data Collection

Patient information was recorded on previously prepared forms by reviewing medical records. The Charlson Comorbidity Index (CCI) was calculated for all patients.

Definitions

Leukopenia was defined as absolute leukocyte count of <4000 cells/mm^3. Anemia was defined as hemoglobin level of <13.5 g/dL in males or <12 g/dL in females, or a decline of 2 g/dL in patients with low hemoglobin levels at the beginning of therapy. Thrombocytopenia was defined as absolute platelet count of <150,000 cells/mm^3, neutropenia was defined as absolute neutrophil count of <2000 cells/mm^3, eosinophilia was defined as absolute eosinophil count of ≥500 cells/mm^3, and hypereosinophilia was defined as absolute eosinophil count of ≥1500 cells/mm^3.

Statistical Analysis

Statistical analyses were performed using SPSS 21 (IBM Corp., Armonk, NY, USA). The univariate analyses were investigated using chi-square tests, Fisher's exact test, Student's t-test, and Mann-Whitney U tests as appropriate. For multivariate analysis, the possible factors identified with univariate analyses were further entered into logistic regression analysis to determine independent risk factors for leukopenia, neutropenia, and eosinophilia. Hosmer-Lemeshow goodness-of-fit statistics were used to assess model fit and $p<0.05$ was considered statistically significant.

Results

One hundred and ten TZP therapy episodes of 102 patients were included in the study. The epidemiological, clinical, and laboratory data of the patients are given in Table 1. Total TZP dose and duration of TZP therapy had no significant effect on the development of anemia or thrombocytopenia. However, they were detected as significant risk factors for the development of leukopenia (16.3%), neutropenia (10%), and eosinophilia (10%).

Drug fever appeared in five of the 11 neutropenic patients and in six of the 18 patients with leukopenia who were afebrile beforehand. All of the patients were alive until the end of TZP therapy. Therapy was continued with another antibiotic in 8 patients with leukopenia and in 5 patients with neutropenia. Body mass index was normal in all patients who developed leukopenia and neutropenia.

Characteristics of patients and statistical analysis with and without leukopenia, neutropenia, and eosinophilia during TZP therapy are given in Table 2. In multivariate analysis, lower CCI score, lower initial blood leukocyte count, combination of TZP with another antibiotic, and total duration of TZP therapy were found to be independent risk factors for leukopenia; initial higher blood eosinophil count (IHEC) and use of TZP for >20 days were found to be independent risk factors for neutropenia; and IHEC and total duration of TZP therapy were found to be independent risk factors for eosinophilia. The characteristics of leukopenia, neutropenia, and eosinophilia episodes are given separately in Tables 3, 4, and 5, respectively.

Table 1. Characteristics of patients within 110 therapy episodes.

Characteristic	Number (%)
Female sex, n (%)	47 (42.7)
Mean age (years), mean ± SD	59.5±16
Charlson Comorbidity Index, mean ± SD	4.07±2.19
Reason for piperacillin-tazobactam usage	
Lower respiratory tract infections, n (%)	60 (54.5)
Bone and joint infections, n (%)	26 (23.6)
Skin and soft tissue infections, n (%)	18 (16.3)
Other infections, n (%)	6 (5.4)
Mean duration of therapy (days), mean ± SD (total)	21±14
Mean dose of therapy (g), mean ± SD (total)	244±149
Combination antibiotic therapy with, n (%)	63 (57.2)
Ciprofloxacin	37 (58.7)
Glycopeptides	17 (26.9)
Others	9 (14.2)
Leukopenia developed during treatment, n (%)	18 (16.3)
Neutropenia developed during treatment, n (%)	11 (10)
Eosinophilia developed during treatment, n (%)	11 (10)
Hypereosinophilia developed during treatment n (%)	1 (0.9)
Anemia developed during treatment, n (%)	21 (19)
Thrombocytopenia developed during treatment, n (%)	7 (6.3)

SD: Standard deviation.

Table 2. Characteristics of patients with and without leukopenia, neutropenia, and eosinophilia.

Characteristics	Patients with leukopenia (n=18)	Patients without leukopenia (n=92)	Univariate analysis p	Multivariate analysis p (OR, 95% CI)	Patients with neutropenia (n=11)	Patients without neutropenia (n=99)	Univariate analysis p	Multivariate analysis p (OR, 95% CI)	Patients with eosinophilia (n=11)	Patients without eosinophilia (n=99)	Univariate analysis p	Multivariate analysis p (OR, 95% CI)
Female sex (n)	6	41	0.378	-	4	43	0.756	-	4	43	0.756	-
Age (mean ± SD)	51±18	60±15	0.071	-	51±18	60±15	0.137	-	53.82±18.59	60±15.78	0.252	-
Age >40 (n)	13	85	0.026	-	9	89	0.343	-	9	89	0.343	-
Charlson Comorbidity Index (mean ± SD)	2.89±2.47	4.30±2.07	0.031	0.014 (0.664, 0.478-0.921)	3.09±2.34	4.18±2.16	0.105	-	3.09±2.07	4.18±2.19	0.124	-
Initial leukocyte count (cells/mm^3)	8801±3143	13,998±6148	<0.001	0.008 (1.00, 1.00-1.00)	11,307±3821	13,352±6249	0.411	-	12,538±5320	13,215±6167	0.846	-
Initial neutrophil count (cells/mm^3)	6101±2601	11,093±5557	<0.001	-	8612±3262	10,461±5678	0.397	-	8950±4575	10,423±5596	0.524	-
Initial eosinophil count (cells/mm^3)	194±140	118±143	0.011	-	242±156	118±119	0.012	0.043 (1.004, 1.000-1.008	272±204	115±128	0.002	0.004 (1.006, 1.002-1.087)
TZP therapy duration (days) (mean ± SD)	26±12	20±14	0.001	0.034 (1.047, 1.004-1.092)	26±13	20±14	0.018	-	30.09±16.61	20.30±13.62	0.002	0.015 (1.047, 1.009-1.087)
TZP total dose (g) (mean ± SD)	320±149	230±145	<0.001	-	321±162	236±146	0.010	-	310±150	237±148	0.047	-
Total hospital stay after the beginning of TZP (days) (mean ± SD)	51±30	35±37	<0.001	-	52±34	36±36	0.015	-	44.09±21.24	37.38±37.97	0.026	-
Combination therapy (n)	16	47	0.004	0.031 (6.58, 1.19-36.31)	10	53	0.023	-	8	55	0.350	-
Ciprofloxacin	11	26	0.007	-	8	29	0.006	-	6	31	0.177	-
Glycopeptides	3	14	0.999	-	1	16	<0.001	-	1	16	0.999	-
TZP therapy duration >14 days (n)	17	69	0.115	-	10	76	0.450	-	11	75	0.177	-
TZP therapy duration >15 days (n)	17	50	0.001	-	10	57	0.057	-	10	57	0.048	-
TZP therapy duration >16 days (n)	16	42	0.001	-	9	49	0.053	-	10	48	0.009	-

Table 2. Continued

Characteristics	Patients with leukopenia (n=18)	Patients without leukopenia (n=92)	Univariate analysis p	Multivariate analysis p (OR, 95% CI)	Patients with neutropenia (n=11)	Patients without neutropenia (n=99)	Univariate analysis p	Multivariate analysis p (OR, 95% CI)	Patients with eosinophilia (n=11)	Patients without eosinophilia (n=99)	Univariate analysis p	Multivariate analysis p (OR, 95% CI)
TZP therapy duration >17 days (n)	16	40	0.001	-	9	47	0.048	-	10	46	0.008	-
TZP therapy duration >18 days (n)	16	36	<0.001	-	9	43	0.023	-	10	42	0.008	-
TZP therapy duration >19 days (n)	15	32	<0.001	-	9	38	0.008	-	9	38	0.007	-
TZP therapy duration >20 days (n)	15	30	<0.001	-	9	36	0.007	0.020 (6.84, 1.36-34.43)	9	36	0.003	-

TZP: Tazobactam, SD: standard deviation, OR: odds ratio, CI: confidence interval.

Discussion

The incidence of leukopenia and neutropenia in patients treated with TZP for more than 10 days were found to be 16.3% and 10% respectively in our study. Incidence of neutropenia was found between 0.04% and 34% in previous studies [3,4,5]. The difference between neutropenia incidences may have resulted from the definitions of neutropenia, duration of TZP therapy, and study design. The total dose and duration of TZP therapy were also found to be the most frequently determined risk factors in the development of these adverse effects in previous studies [4,5,6]. The mechanisms and causes of TZP-induced leukopenia or neutropenia have not been clearly determined. It has been shown that TZP causes reversible proliferation arrest in myeloid cells with cumulative doses [7,8,9].

Duration of TZP therapy was detected as a significant risk factor for the development of leukopenia (21 days), neutropenia (19 days), and eosinophilia (13 days) in our study. Also in a study of 41 patients with bone-related infections, neutropenia developed in patients who used TZP for more than 18 days [4]. In another study that compared risks of neutropenia in patients treated with either TZP or ticarcillin-clavulanate, the risk of neutropenia was higher when children were treated with TZP than with ticarcillin-clavulanate and use of TZP for more than 2 weeks was found to be related to increased risk of neutropenia [5].

In some studies patients who developed neutropenia were found to be younger, as in our study [4,9]. However, these studies could not explain the mechanism behind this. We could find no other study identifying lower CCI as a risk factor for developing leukopenia or neutropenia during TZP therapy in adult patients. This situation could be explained by the role of immunological mechanisms in the hematologic adverse effects of TZP. Hypersensitivity responses against antimicrobial agents may be more effective in younger patients with better immune systems and no comorbid conditions. Additionally, we found IHEC as another independent risk factor for the development of neutropenia with TZP therapy. Patients with higher eosinophil counts were probably allergic to something previously and could be more prone to allergic reactions to antibiotics such as TZP as well; this could also be the reason for neutropenia and leukopenia. In another study, immunoglobulin G antibodies directed against penicillins and neutrophils were described and the authors concluded that an immune-mediated pathogenesis was highly probable in developing neutropenia with penicillin use [10].

Combination antibiotic therapy was found to be a risk factor for the development of leukopenia but not neutropenia in our study, and it was found as a risk factor also in developing neutropenia in another study [4]. Although the hematologic adverse effects of ciprofloxacin, which was the agent most frequently combined with TZP in our study, are mild and rarely seen [11], bone marrow suppression associated with ciprofloxacin use was shown. Combination

Table 3. Characteristics of the 18 episodes of leukopenia.

Sex	Age, year	Time to onset of leukopenia, day	Total dose, g	Initial leukocytes count, $x10^9$/L	Nadir of leukocytes count, $x10^9$/L
F	34	15	180	11.7	2.7
F	76	17	204	5.7	3.7
M	47	10	120	8.9	3.4
F	70	8	96	7.2	3.2
M	46	30	360	13.8	3.2
F	40	15	180	9.8	2.6
M	30	25	300	8.0	3.9
M	69	21	252	7.3	3.2
M	73	21	252	11.8	3.4
M	63	33	396	11.9	3.1
F	60	20	240	6.6	3.4
M	70	29	348	5.2	3.8
M	69	40	480	4.8	3.9
M	22	14	168	6.9	3.1
M	51	20	240	8.0	2.3
M	60	20	240	6.9	3.9
M	32	23	276	6.4	3.8
F	18	21	252	15.9	3.9
Mean ± SD	51.66±18.72	21.22±8.04	254.66±96.48	8.71±3.12	3.36±0.48

SD: Standard deviation, M: male, F: female.

Table 4. Characteristics of the 11 episodes of neutropenia.

Sex	Age, year	Time to onset of neutropenia, day	Total dose, g	Initial neutrophils count, $x10^9$/L	Nadir of neutrophiles count, $x10^9$/L
M	60	25	300	9.9	0.8
M	47	10	120	6.4	0.5
F	70	8	96	6.2	1.4
M	46	30	360	10.5	1.1
M	73	23	276	9.4	1.2
M	63	24	288	8.1	0.8
F	60	20	240	4.3	1.9
M	51	21	252	3.7	1.3
M	60	23	276	4.5	1.9
F	18	21	252	11.0	1.6
F	19	6	72	9.7	1.5
Mean ± SD	51.54±18.39	19.18±7.7	230.18 ±92.45	7.6±2.68	1.27±0.45

SD: Standard deviation, M: male, F: female.

antibiotic therapy with TZP should be limited to patients with severe life-threatening *Pseudomonas aeruginosa* infections and especially those with immunocompromising conditions because of the increased rate of adverse effects, including leukopenia, and the lack of evidence of either improved efficacy or decreased resistance [12].

Study Limitations

Our study is novel in several ways: it includes the largest patient sample among studies on the same subject, we evaluated the hematologic adverse effects of TZP as a whole, and finally we analyzed the independent risk factors for development of leukopenia, neutropenia, and eosinophilia.

Table 5. Characteristics of the 11 episodes of eosinophilia.

Sex	Age, year	Time to onset of eosinophilia, day	Total dose, g	Initial eosinophils count, $x10^9$/L	Nadir of eosinophils count, $x10^9$/L
F	58	1	12	0.3	0.6
M	74	4	24	0.4	0.9
M	65	31	372	0.1	2
F	52	32	384	0.218	1.62
M	30	3	36	0.1	0.7
M	42	7	42	0.748	0.577
M	53	28	336	0.1	1.4
M	74	29	348	0.2	0.5
F	18	3	36	0.126	0.8
M	50	7	84	0.5	0.6
M	76	5	60	0.2	0.5
Mean ± SD	53.81±15.59	13.63±13.12	157.63±161.93	0.272±0.204	0.927±0.512

SD: Standard deviation, M: male, F: female.

Conclusion

It should be kept in mind that if TZP therapy is extended for more than 2-3 weeks, a patient could develop leukopenia, neutropenia, or eosinophilia, especially in cases of combination antibiotic therapy and in younger patients with fewer comorbidities. Although the consequences of TZP-induced hematologic adverse effects were not devastating, duration of hospital stay after the beginning of TZP was longer in patients with leukopenia and neutropenia. Therefore, younger patients with fewer comorbidities and patients with IHEC should particularly be monitored more frequently with complete blood counts. Although combination antibiotic therapy was not found as a risk factor for neutropenia, it was a risk factor for leukopenia and should be avoided unless necessary.

Ethics

Ethics Committee Approval: Retrospective study.

Informed Consent: Informed consent was not required as this was a retrospective study.

Authorship Contributions

Concept: S.Ş.Y., A.B.; Design: S.Ş.Y., A.B.; Data Collection or Processing: A.B., S.Ş.Y.; Analysis or Interpretation: S.Ş.Y., A.B., S.B., A.Ç., H.Ö., H.E.; Literature Search: A.B., S.Ş.Y., S.B., A.Ç., H.Ö., H.E.; Writing: A.B., S.Ş.Y.

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References

1. Doi Y, Chambers H. Penicillins and β-lactamase inhibitors. In: Bennett J, Dolin R, Blaser M, (eds). Mandell, Douglas, and Bennett's Principles and Practice of Infectious Disease. Philadelphia, Elsevier Saunders, 2015.
2. Daley D, Mulgrave L, Munro S, Smith H, Dimech W. An evaluation of the in vitro activity of piperacillin/tazobactam. Pathology 1996;28:167-172.
3. Scheetz MH, McKoy JM, Parada JP, Djulbegovic B, Raisch DW, Yarnold PR, Zagory J, Trifilio S, Jakiche R, Palella F, Kahn A, Chandler K, Bennett CL. Systematic review of piperacillin-induced neutropenia. Drug Saf 2007;30:295-306.
4. Peralta FG, Sanchez MB, Roiz MP, Pena MA, Tejero MA, Arjona R. Incidence of neutropenia during treatment of bone-related infections with piperacillin-tazobactam. Clin Infect Dis 2003;37:1568-1572.
5. Lemieux P, Gregoire JP, Thibeault R, Bergeron L. Higher risk of neutropenia associated with piperacillin-tazobactam compared with ticarcillin-clavulanate in children. Clin Infect Dis 2015;60:203-207.
6. Reichardt P, Handrick W, Linke A, Schille R, Kiess W. Leukocytopenia, thrombocytopenia and fever related to piperacillin/tazobactam treatment - a retrospective analysis in 38 children with cystic fibrosis. Infection 1999;27:355-356.
7. Kumar A, Choudhuri G, Aggarwal R. Piperacillin induced bone marrow suppression: a case report. BMC Clin Pharmacol 2003;3:2.
8. Khan F. Severe neutropenia secondary to piperacillin/tazobactam therapy. Indian J Pharmacol 2005;37:192-193.
9. Ruiz-Irastorza G, Barreiro G, Aguirre C. Reversible bone marrow depression by high-dose piperacillin/tazobactam. Br J Haematol 1996;95:611-612.
10. Neftel KA, Wälti M, Spengler H, von Felten A, Weitzman SA, Bürgi H, de Weck AL. Neutropenia after penicillins: toxic or immune-mediated? Klin Wochenschr 1981;59:877-888.
11. Arcieri GM, Becker N, Esposito B, Griffith E, Heyd A, Neumann C, O'Brien B, Schacht P. Safety of intravenous ciprofloxacin. A review. Am J Med 1989;87:92-97.
12. Vardakas KZ, Tansarli GS, Bliziotis IA, Falagas ME. β-Lactam plus aminoglycoside or fluoroquinolone combination versus β-lactam monotherapy for *Pseudomonas aeruginosa* infections: a meta-analysis. Int J Antimicrob Agents 2013;41:301-310.

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Acanthocytosis and HyperCKemia

Akantositoz ve Kreatin Kinaz Yüksekliği

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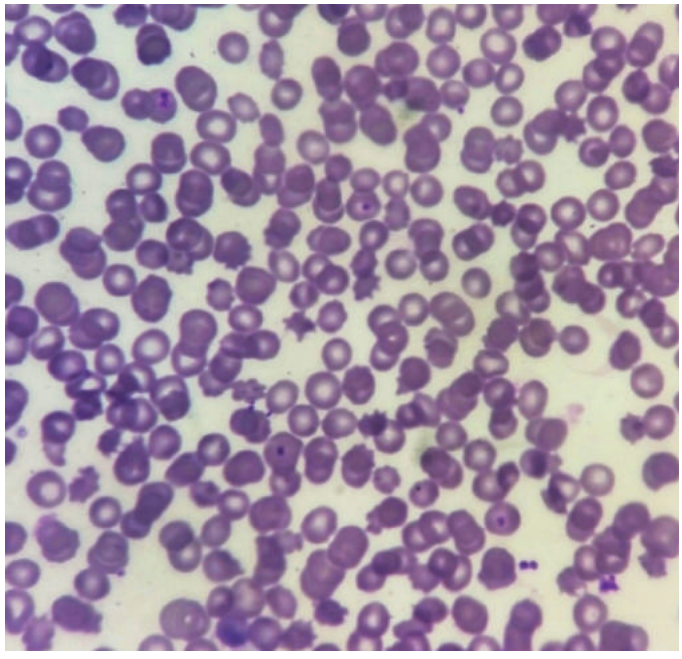

Figure 1. Peripheral blood smear of the patient showing acanthocytosis.

A 14-year-old boy was referred to the neuromuscular clinic for the investigation of hyperCKemia (serum creatinine kinase: 4000 IU/L; normal range: 0-170), detected during laboratory examinations. He was the first child of consanguineous parents. His psychomotor development was normal and he had no past symptoms of a neuromuscular disease. Detailed history taking did not reveal any signs of involuntary movements, bradykinesia, or social problems. Neurologic examination showed normal muscle power in the upper and lower extremities and no signs of muscle atrophy. Deep tendon reflexes could not be elicited. Nerve conduction studies were normal but electromyography revealed combined neurogenic and myogenic potentials in the lower extremity muscles. A muscle biopsy did not show any pathology and was interpreted as normal. Targeted customized Mendeliome panel [1] next-generation sequencing revealed a homozygous splice site mutation in the vacuolar protein sorting-associated protein *(VPS13A)*, NM_015186.3, c.6095+1G>C.

HyperCKemia is a condition characterized by elevated levels of the enzyme creatinine kinase in the blood. Chorea-

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acanthocytosis is an autosomal recessive disease caused by mutations in the *VPS13A* gene, which encodes the protein chorein. The disease is characterized by chorea, dystonias mainly involving the face, parkinsonism, vocal tics, epilepsy, social disinhibition, and distal muscle wasting. The mean age of onset is 35 years [2]. Neuropsychiatric symptoms are also common and may precede movement disorders. Acanthocytes usually constitute 5% to 50% of circulating red blood cells. They may also be absent or may appear late in the course of the disease [3]. Most patients have elevated serum creatinine kinase levels but the cause of this creatinine kinase elevation is unknown. Nerve conduction studies may be normal, but may show sensory axonal neuropathy in some cases. Electromyography may show myogenic or neurogenic potentials. Retrospectively, after the genetic diagnosis, we could confirm the presence of acanthocytes (Figure 1). Our patient had no neurological complaints and no neurological abnormalities. Thus, peripheral blood smears may give important diagnostic clues in cases of idiopathic hyperCKemia. Whole exome sequencing is also a preferable diagnostic modality in cases of idiopathic hyperCKemia, but there are challenges in the counseling of the family in a clinically asymptomatic case in the context of a progressive neurologic disorder.

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Keywords: Acathocytosis, Elevated creatine kinase, Muscle disease

Anahtar Sözcükler: Akantositoz, Kreatin kinaz yüksekliği, Kas hastalığı

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References

1. Haliloglu G, Becker K, Temucin C, Talim B, Küçükşahin N, Pergande M, Motameny S, Nürnberg P, Aydingoz U, Topaloğlu H, Cirak S. Recessive PIEZO2 stop mutation causes arthrogryposis with distal muscle weakness, scoliosis and proprioception defects. J Hum Genet 2017;62:239-242.
2. Jung HH, Danek A, Walker RH. Neuroacanthocytosis syndromes. Orphanet J Rare Dis 2011;6:68-76.
3. Sorrentino G, De Renzo A, Miniello S, Nori O, Bonavita V. Late appearance of acanthocytes during the course of chorea-acanthocytosis. J Neurol Sci 1999;163:175-178.

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Basophilic Stippling and Chronic Lead Poisoning

Bazofilik Noktalanma ve Kronik Kurşun Zehirlenmesi

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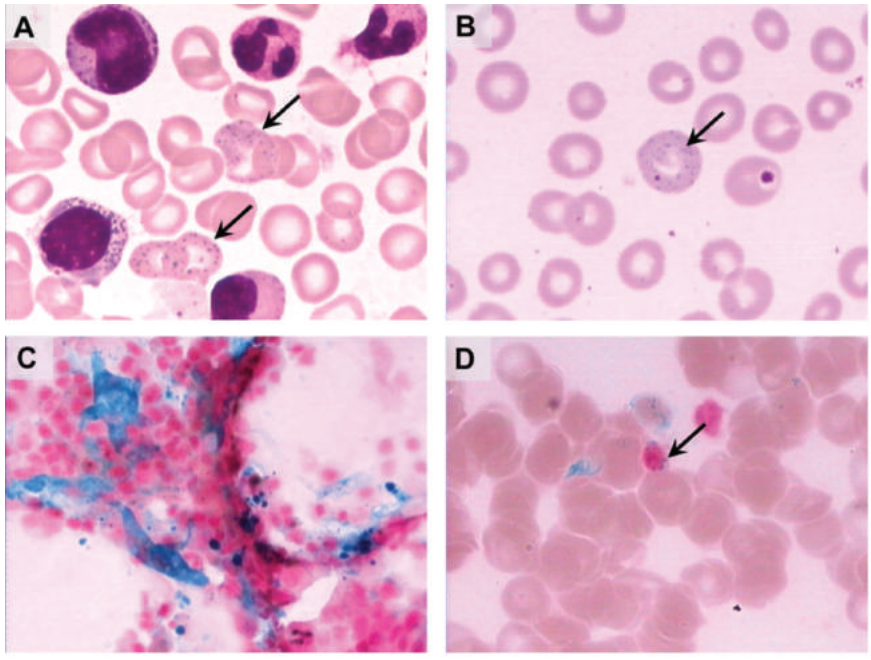

Figure 1. (A) Bone marrow smears and (B) peripheral blood smears revealing extensive erythrocytes of coarse basophilic stippling (1000^{x}, Wright-Giemsa stain). Bone marrow smears showed 4+ iron stores (C) and ring sideroblasts (D) (1000^{x}, iron stain).

A 66-year-old female patient presented with a 9-month history of abdominal colic and fatigue that prompted an abdominal computed tomography scan and gastrointestinal endoscopy with negative assessments. The hemoglobin level was 72-81 g/L with an increase in serum ferroprotein. Bone marrow (Figure 1A) and peripheral blood smears (Figure 1B) revealed extensive erythrocytes with coarse basophilic stippling. It was suggested that there may have been an accumulation of heavy metal in her body. The level of lead in her blood and urine was increased to 1036 µg/L (permissible: <400 µg/L [1]) and 246 µg/L (permissible: <70 µg/L [1]), respectively. Her blood mercury level was below the permissible level (permissible: <15 µg/L [2]). Therefore, she was initially diagnosed with chronic lead poisoning. Further history revealed that she had been taking an adulterated dietary supplement called "Fengwangjiang" from unverified sources for more than the past 1 year. Bone marrow smears showed 4+ iron stores (Figure 1C) and ring sideroblasts (Figure 1D), indicating ineffective heme synthesis. She complied with the doctor's order to stop taking the dietary supplement and received lead-chelation therapy during hospitalization, and her symptoms improved. Basophilic stippling provides a clue to the underlying diagnosis and an understanding of the underlying pathogenesis.

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Keywords: Basophilic stippling, Chronic lead poisoning, Blood

Anahtar Sözcükler: Bazofilik noktalanma, Kronik kurşun zehirlenmesi, Kan

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References

1. China National Occupational Health Standards GBZ 37-2015. Diagnosis of Occupational Chronic Lead Poisoning. Beijing, 2015.
2. China National Occupational Health Standards GBZ 89-2007. Diagnostic Criteria of Occupational Mercury Poisoning. Beijing, 2007.

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The Impact of Small Bowel Endoscopy in Patients with Hereditary Hemorrhagic Telangiectasia

Herediter Hemorajik Telenjiektazi Hastalarında İnce Barsak Endoskopisinin Önemi

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To the Editor,

We have read with interest the article entitled "Thalidomide for the Management of Bleeding Episodes in Patients with Hereditary Hemorrhagic Telangiectasia: Effects on Epistaxis Severity Score and Quality of Life" [1].

This article highlights the use of thalidomide in the management of patients with hereditary hemorrhagic telangiectasia (HHT) who present with epistaxis. The prevalence of HHT is thought to be between 1.5 and 2 cases per 10,000 people [2]. HHT can be associated with other bleeding complications such as bleeding from the gastrointestinal tract and in particular the small bowel (SB). The existence of small bowel angioectasias (SBAs) has been reported to vary between 56% and 91% in the literature [3,4,5,6]. The study by Ingrosso et al. [6] also reported that patients with SBAs were considerably older.

We carried out a study at our tertiary center for the management of patients with HHT where 10 patients (60% males) with genetically confirmed HHT were referred for the management of gastrointestinal-related complications. The impact of small bowel capsule endoscopy (SBCE) and double balloon enteroscopy (DBE) was evaluated. The mean age at first SB endoscopy was 62.6±14.4 years (mean ± standard deviation).

Patients had a total of 39 gastroscopies, 16 colonoscopies, and 6 push enteroscopies. Seven patients underwent SBCE: 6 (85.7%) had proximal, 1 (11.1%) had mid, and 3 (33.3%) had distal SBAs. Two patients had a colon capsule that showed angioectasias.

Several DBEs were carried out for 6 patients (median 4; SD ±6) with a mean of 130.5±133.3 days between DBEs. Fifty-seven SBAs were treated with argon plasma coagulation (APC) on average at each DBE. These procedures take an average of 75 minutes. Mean hemoglobin before and after the procedure was 9.8 and 10.2 g/dL, respectively (p=0.1). Six patients were transfusion-dependent initially but 4 improved following intervention.

Need for transfusion resolved in 1 patient when started on lanreotide (a long-acting somatostatin analog), regular endoscopy, and APC, and in 2 patients upon starting DBEs and APC. One patient passed away from pneumonia. Another patient was switched unsuccessfully from octreotide to lanreotide. She stopped being transfusion-dependent with regular gastroscopies and APC. Another patient was unwilling to undergo further endoscopies due to multiple comorbidities. He improved on lanreotide. In 2 patients, anemia remains persistently problematic. One of them is also on dalteparin for superior mesenteric venous thrombosis. The other patient has recurrent epistaxis, which makes it harder for him to have further endoscopies.

SBCE is a useful screening tool in patients with HHT to assess SBAs. Although classed as invasive endoscopy, DBEs and APC can have a significant impact on mortality and quality of life in patients with HHT. Pharmacotherapy such as somatostatin analogs can additionally help to improve transfusion requirements. They have a good safety profile [7], unlike thalidomide, which can result in teratogenicity [8], peripheral neuropathy (50%) [9], and thromboembolism [10].

Keywords: Hereditary hemorrhagic telangiectasia, Small bowel capsule endoscopy, Argon plasma coagulation

Anahtar Sözcükler: Herediter hemorajik telenjiektazi, İnce barsak kapsül endoskopisi, Argon plazma koagülasyonu

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References

1. Baysal M, Ümit EG, Kırkızlar HO, Özdöver AC, Demir AM. Thalidomide for the management of bleeding episodes in patients with hereditary hemorrhagic telangiectasia: effects on epistaxis severity score and quality of life. Turk J Hematol 2018 (in press).
2. Dakeishi M, Shioya T, Wada Y, Shindo T, Otaka K, Manabe M, Nozaki J, Inoue S, Koizumi A. Genetic epidemiology of hereditary hemorrhagic

telangiectasia in a local community in the northern part of Japan. Hum Mutat 2002;19:140-148.

3. Canzonieri C, Centenara L, Ornati F, Pagella F, Matti E, Alvisi C, Danesino C, Perego M, Olivieri C. Endoscopic evaluation of gastrointestinal tract in patients with hereditary hemorrhagic telangiectasia and correlation with their genotypes. Genet Med 2014;16:3-10.
4. Grève E, Moussata D, Gaudin JL, Lapalus MG, Giraud S, Dupuis-Girod S, Calender A, Plauchu H, Saurin JC. High diagnostic and clinical impact of small-bowel capsule endoscopy in patients with hereditary hemorrhagic telangiectasia with overt digestive bleeding and/or severe anemia. Gastrointest Endosc 2010;71:760-767.
5. Chamberlain SM, Patel J, Carter Balart J, Gossage JR Jr, Sridhar S. Evaluation of patients with hereditary hemorrhagic telangiectasia with video capsule endoscopy: a single-center prospective study. Endoscopy 2007;39:516-520.
6. Ingrosso M, Sabbà C, Pisani A, Principi M, Gallitelli M, Cirulli A, Francavilla A. Evidence of small-bowel involvement in hereditary hemorrhagic telangiectasia: a capsule endoscopic study. Endoscopy 2004;36:1074-1079.
7. Holleran G, Hall B, Breslin N, McNamara D. Long-acting somatostatin-analogues provide significant beneficial effect in patients with refractory small bowel angioectasia: results from a proof of concept open label mono-centre trial. United European Gastroenterol J 2016;4:70-76.
8. Sauer H, Gunther J, Hescheler J, Wartenberg M. Thalidomide inhibits angiogenesis in embryoid bodies by the generation of hydroxyl radicals. Am J Pathol 2000;156:151-158.
9. Izquierdo Navarro Mdel C, Hernando Verdugo M, Cardaba Garcia E, Sanchez Sanchez MT. Therapeutic failure with thalidomide in patients with recurrent intestinal bleeding due to angiodysplasias. Farm Hosp 2016;40:230-232.
10. Palumbo A, Rajkumar SV, Dimopoulos MA, Richardson PG, San Miguel J, Barlogie B, Harousseau J, Zonder JA, Cavo M, Zangari M, Attal M, Belch A, Knop S, Joshua D, Sezer O, Ludwig H, Vesole D, Bladé J, Kyle R, Westin J, Weber D, Bringhen S, Niesvizky R, Waage A, von Lilienfeld-Toal M, Lonial S, Morgan GJ, Orlowski RZ, Shimizu K, Anderson KC, Boccadoro M, Durie BG, Sonneveld P, Hussein MA; International Myeloma Working Group. Prevention of thalidomide- and lenalidomide-associated thrombosis in myeloma. Leukemia 2008;22:414-423.

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Interleukin-2-330T/G and Interleukin-10-1082A/G Genetic Polymorphisms and B-Cell Non-Hodgkin Lymphoma

İnterlökin-2-330T/G ve İnterlökin-10-1082A/G Genetik Polimorfizmi ve B-Hücreli Non-Hodgkin Lenfoma

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To the Editor,

We read the publication "Association of Interleukin-2-330T/G and Interleukin-10-1082A/G Genetic Polymorphisms with B-Cell Non-Hodgkin Lymphoma (B-NHL) in a Cohort of Egyptians" with great interest [1]. Abdel Rahman et al. [1] concluded that "The present study highlights the possible involvement of the [interleukin (IL)] IL-2-330T/G genetic polymorphism in the susceptibility to [B-NHL] B-NHL in Egypt, especially indolent subtypes. Moreover, IL-10-1082A/G is not a molecular susceptibility marker for B-NHL in Egyptians" [1]. In fact, the role of polymorphism of IL is widely mentioned in relationship to NHL susceptibility [2]. We agree with the observation of Abdel Rahman et al. [1]. The differences of the effects of IL-2-330T/G and IL-10-1082A/G can be explained by molecular quantum calculations of molecular weight changes. This is the same phenomenon as seen in other polymorphisms and it can affect the clinical appearance of many medical disorders, such as the effect of CTLA-4 A49G polymorphism on autoimmune blood disease [3]. For IL-2-330T/G and IL-10-1082A/G, the change of molecular weight is equal to -107.07 and +16 per molecule, respectively. This means that a molecule with IL-2-330T/G requires more molecular mass and a molecule with IL-10-1082A/G requires less molecular mass to complete a biological process compared to a naïve molecule.

Keywords: Interleukin, Lymphoma, Polymorphism

Anahtar Sözcükler: İnterlökin, Lenfoma, Polimorfizm

Conflict of Interest: The authors of this paper have no conflicts of interest, including specific financial interests, relationships,

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References

1. Abdel Rahman HA, Khorshied MM1, Reda Khorshid OM2, Mourad HM. Association of interleukin-2-330T/G and interleukin-10-1082A/G genetic polymorphisms with B-cell non-Hodgkin lymphoma in a cohort of Egyptians. Turk J Hematol 2018;35:99-108.
2. Li G, Li D. Relationship between IL-10 gene polymorphisms and the risk of non-Hodgkin lymphoma: a meta-analysis. Hum Immunol 2016;77:418-425.
3. Wiwanitkit V. CTLA-4 A49G polymorphism and autoimmune blood disease: a comment. Turk J Hematol 2011;28:247.

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On Being a "Physician Patient" with His Own Experimental Therapeutic Drug

Deneysel Tedavi Edici İlaç ile "Doktor Hasta" Birlikteliği Üzerine

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Hacettepe University Faculty of Medicine, Department of Hematology, Ankara, Turkey

To the Editor,

We have read with great interest the paper by Patıroğlu et al. [1] on the mucosal healing effects of Ankaferd BloodStopper (ABS), recently published in this journal. They suggested that ABS could be effective for the management of chemotherapy-induced mucositis. We would like to share our own experience with ABS on the burn-induced skin wounds of a patient.

The patient herein is a physician and the senior author of this paper (İ.C.H.). He is also the mentor of the first author (R.Ç.). The experimental therapeutic drug is ABS, which was developed as a medicine with numerous clinical studies (https://www.ncbi.nlm.nih.gov/m/pubmed/?term=ankaferd), mostly authored by İ.C.H. himself. ABS is the first topical hemostatic agent acting on red blood cells and fibrinogen gamma interactions to be tested in clinical trials [2]. ABS is a drug officially approved for the management of clinical hemorrhages in Turkey [3]. However, ABS has never been used in humans for the therapy of burns until İ.C.H. had his left forearm severely burned by a boiling tea kettle. In his physical examination, the burnt areas had acutely developed heavy erythematous lesions, which then complicated into several bullous lesions.

At the time of the burn accident, ABS for burn wound management had only been demonstrated in rats [4,5]. The burns were induced in Wistar albino rats by Kaya et al. [4] they showed that ABS decreases the inflammation and wound diameters and increases the wound contraction and tissue fibrosis in rats with burn injuries. The results of another rat study demonstrated that ABS has a positive effect on second-degree thermal burn healing [6].

The emergency state of the severe burn lesions and the availability of ABS at the time of the accident enabled us to apply it topically to the burn lesions of İ.C.H. The burn lesions were clearly regressed and wound healing occurred with no complications upon the usage of ABS in our physician patient (Figure 1).

In the history of medical science, there are many inventors that applied their own therapeutic tools for the management of their own diseases, such as Dr. Barry J. Marshall. He drank *Helicobacter pylori* bacteria himself and developed stomach ulcers within a few days. He later successfully treated himself with antibiotics and went on to win the Nobel Prize [7].

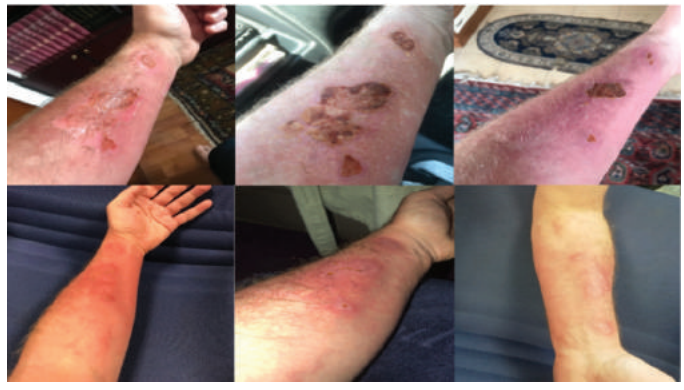

Figure 1. The senior author's burn wounds before and after treatment with Ankaferd BloodStopper.

The conclusions that were drawn from our unique clinical story are as follows:

- Medical inventors and researchers are enthusiastic for the use of their experimental drugs in clinical situations.

- The weakest aspect of evidence-based medicine is the 'lack of evidence' in the related particular clinical problem. This represents a great challenge, particularly for real-life medical emergencies.

- Sometimes medical doctors have to make clinical decisions despite the lack of solid scientific evidence in the presence of urgent medical needs.

- Rat and animal studies may be the only source of evidence for human use in some medical emergencies.

- Nevertheless, the best clinical practice should rely on the best current evidence obtained through randomized controlled clinical trials.

Keywords: Ankaferd, Burn, Physician patient, Mucosal healing

Anahtar Sözcükler: Ankaferd, Yanık, Doktor hasta, Mukozal iyileşme

Informed Consent: Informed consent was obtained from the patient included in the study.

Conflict of Interest: No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References

1. Patıroğlu T, Şahin NE, Ünal E, Kendirci M, Karakükcü M, Özdemir MA. Effectiveness of Ankaferd BloodStopper in prophylaxis and treatment of oral mucositis in childhood cancers evaluated with plasma citrulline levels. Turk J Hematol 2018;35:85-86.
2. Haznedaroglu BZ, Beyazit Y, Walker SL, Haznedaroglu IC. Pleiotropic cellular, hemostatic, and biological actions of Ankaferd hemostat. Crit Rev Oncol Hematol 2012;83:21-34.
3. Gorgulu S, Norgaz T, Sipahi I. Ankaferd blood stopper as a new strategy to avoid early complications after transradial procedures: a randomized clinical trial. J Interv Cardiol 2018;31:511-517.
4. Kaya H, Gokdemir MT, Sogut O, Demir T, Koçarslan S. Effects of folk medicinal plant extract Ankaferd Blood Stopper on burn wound healing. Acta Medica Mediterr 2013;29:497-502.
5. Akalin C, Kuru S, Barlas AM, Kismet K, Kaptanoglu B, Demir A, Astarci HM, Ustun H, Ertas E. Beneficial effects of Ankaferd Blood Stopper on dermal wound healing: an experimental study. Int Wound J 2014;11:64-68.
6. Ozcan E, Senol S, Tokgoz Y, Kuloglu T. The regenerative effects of hemostatic plant extract in second degree burn wound. Ulutas Med J 2017;3:78-84.
7. Marshall BJ. The 1995 Albert Lasker Medical Research Award. *Helicobacter pylori*: the etiologic agent for peptic ulcer. JAMA 1995;274:1064-1066.

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Hematology Laboratory Survey

Hematoloji Laboratuvar Anketi

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To the Editor,

We read the publication "Results of the Hematology Laboratory Survey: What Has Changed in Eight Years?" with great interest. Kozanoğlu et al. [1] noted that "Hematology laboratories have not been defined in the Turkish Medical Laboratories Regulation (2010, 2013), which regulates procedures and principles regarding the planning, licensing, opening, regulating, classifying, monitoring, controlling, and terminating of activities of medical laboratories". We would like to share ideas from our country in Indochina. In Thailand, there is no isolated hematology laboratory. All clinical hematology investigation is performed by a standard clinical laboratory medicine center [2]. Routine laboratory survey and quality surveillance for accreditation is the basic requirement. This might be a good way for quality control of laboratory processes. Indeed, there is a need for clinical pathologists or clinical hematologists for the management of a hematology laboratory. This is necessary for the assurance of the quality of laboratory diagnoses. The concept of isolated hematology and combination with other clinical laboratories in a single laboratory medicine unit is an

interesting topic for further discussion of the advantages and disadvantages.

Keywords: Hematology, Laboratory, Survey

Anahtar Sözcükler: Hematoloji, Laboratuvar, Anket

Conflict of Interest: The authors of this paper have no conflicts of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

References

1. Kozanoğlu İ, Patıroğlu T, Dalva K, Yanıkkaya Demirel G, Soysal T, Demir M. Results of the hematology laboratory survey: what has changed in eight years? Turk J Hematol 2018;35:215-216.
2. Charuruks N, Vanavanitkun Y, Seublinvong T, Werawatanakumpa S, Eiam-Oung S, Jindamaporn A, Chirathaworn C, Panyavorawuch V, Ruangvejvorachai P. Situation of laboratory service and instruments in Thailand: a descriptive study from questionnaires. J Med Assoc Thai 2002;85(Suppl 1):253-261.

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Reply to the Authors

To the Editor,

We read the letter from Mungmunpuntipantip and Wiwanitkit regarding our publication on "Results of the Hematology Laboratory Survey: What Has Changed in Eight Years?"

First of all, we would like to thank the authors, that they have shared their experiences and ideas from their country. We understood that all clinical hematology investigation was performed by a standard clinical laboratory medicine center in Indochina and also, there is no isolated hematology laboratory in their country.

We all agree that the concept of isolated hematology and combination with other clinical laboratories in a single laboratory medicine unit is an interesting topic for further discussion on advantages and disadvantages. Many hematology laboratories have closed and/or were included into a central laboratory system after starting new regulations in Turkey. The authors also agree with us that there is a need for the clinical hematologist for management of the hematology laboratory. However, after the new regulations in our country, the managers of hematology laboratories became biochemistry specialists. But it is very important to evaluate specific tests in the hematology laboratory and to integrate them with the clinical status of patients. Therefore, the removal of clinical hematologists from hematology laboratories was a significant disadvantage. The routine laboratory survey and quality surveillance for accreditation is the basic requirement not only for hematology laboratories but also in all laboratories. However, after the new regulations implemented, only the quality issues will not be sufficient to solve problems related to hematology laboratories in Turkey.

Best Regards,

İlknur Kozanoğlu, Türkan Patıroğlu, Klara Dalva, Gülderen Yanıkkaya Demirel, Teoman Soysal, Muzaffer Demir

Successful Treatment of Recurrent Gastrointestinal Bleeding Due to Small Intestine Angiodysplasia and Multiple Myeloma with Thalidomide: Two Birds with One Stone

İnce Barsak Anjiyodisplazi ve Multipl Miyeloma Bağlı Gelişen Tekrarlayan Gastrointestinal Kanamanın Talidomid ile Başarılı Tedavisi: Bir Taşla İki Kuş

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To the Editor,

Gastrointestinal angiodysplasia (GIA) is the most common digestive tract vascular malformation, often causing recurrent gastrointestinal bleeding. Despite association with certain hereditary diseases [1,2,3], most GIAs are acquired, associated with aortic stenosis, hemodialysis, malignancies, or liver cirrhosis or idiopathic, and they appear among the elderly (>60 years) [4]. Advances in endoscopy brought about management improvements, but due to numerous lesions disseminated over the digestive tract, treatment of GIA remains a clinical challenge. Novel studies suggested that the use of thalidomide might be beneficial in these patients due to its antiangiogenic properties [5,6]. Thalidomide and its modern analogues currently represent a backbone treatment of another disease: multiple myeloma (MM) [7]. Here we would like to present a case of successful MM and GIA treatment with thalidomide.

Our male patient, born in 1947 and suffering from arterial hypertension, benign prostate hyperplasia, and chronic obstructive pulmonary disease, was diagnosed with symptomatic iron deficiency anemia in 2012. He underwent an extensive gastroenterological workup, which revealed multiple small intestine GIAs causing recurrent bleeding. Several attempts at endoscopic argon-plasma coagulation in the following years were not able to control the disease and the patient required regular blood transfusions (every 3-4 weeks) and parenteral iron supplementation. The patient was referred to a hematologist in 2016 for further assessment. Bleeding disorders were excluded (Table 1), but advanced immunoglobulin G kappa MM was found (ISS 1, with 20%-25% clonal plasma cells in the bone marrow and multiple osteolytic lesions), with no signs of bone marrow or gastrointestinal amyloidosis. Treatment with cyclophosphamide (500 mg/week), thalidomide (100 mg/day), and dexamethasone (40 mg/week) together with monthly zoledronate was initiated in March 2016. Cyclophosphamide was discontinued after 3 applications due to development of paroxysmal atrial fibrillation, requiring thromboprophylaxis with enoxaparin. Six months after treatment initiation the

Table 1. Relevant laboratory findings at baseline and during thalidomide treatment.

Key laboratory findings	Baseline (2/2016)	8/2016	2/2017	11/2017
Hemoglobin (g/L)	77	95	117	127
MCV (fL)	71	84.1	85.7	90.9
Fe (µmol/L)	2	5	5	19
Ferritin (µg/L)	<5	25.1	23.8	184.8
PT	1.13	NA	NA	NA
aPTT (s)	22.7	NA	NA	NA
Fibrinogen (g/L)	4.0	NA	NA	NA
VWF (%)	154	NA	NA	NA
FVIII (kIU/L)	2.80	NA	NA	NA
FXIII (kIU/L)	0.85	NA	NA	NA
Total serum protein (g/L)	72	66	67	68
Total serum IgG (g/L)	18.93 (high)	11.8 (normal)	13.09 (normal)	14.37 (normal)
M protein by immunofixation -serum IgG kappa	Present	Present	Present	Present
Serum free light-chains (mg/L), kappa	26.3	13.2	19.6	20.5
Serum free light-chains (mg/L), lambda	21.0	10.2	14.6	17.7
Kappa/lambda ratio serum	1.25 (normal)	1.29 (normal)	1.34 (normal)	1.16 (normal)
M protein-urine	NA	NA	Negative	Negative
Bone marrow plasma cell count (%)	20-25	<5	NA	NA

MCV: Mean cell volume, PT: prothrombin time, aPTT: activated partial thromboplastin time, VWF: Von Willebrand disease, Fe: serum iron, FVIII: factor VIII, FXIII: factor XIII, IgG: immunoglobulin G, NA: not applicable.

patient achieved a very good partial remission (vgPR) of MM. Owing to age, comorbidities, and the patient's preferences, he was considered transplant-ineligible and so thalidomide (100 mg/day) and dexamethasone (20 mg/week) were continued. The patient has had no apparent bleeding since March 2016, he has been transfusion-free since October 2016, and he received the last parenteral iron supplementation in October 2017, so GIA endoscopy was not repeated. MM evaluations revealed continuous vgPR after 22 months of treatment; the patient is asymptomatic, suffers no side effects, and continues with thalidomide maintenance (Table 1).

The efficacy of thalidomide as a first-line treatment in combination regimens and as maintenance therapy for MM is well established [8]. Despite the irrefutable success of some novel therapeutic agents, such as proteasome inhibitors and next-generation immunomodulatory drugs, thalidomide still represents a valid treatment choice, especially in countries with limited healthcare resources. Thalidomide has an emerging role in GIA treatment, with shown efficacy in a small randomized trial [5] and multiple case reports (nicely reviewed by Bauditz [6]). Certain patients, especially those with several susceptible conditions as in the case presented here, seem to achieve utmost clinical benefit and improvement in quality of life. The optimal dosage of thalidomide in GIAs is currently not defined, and the side-effect profile might limit its long-term use for disease control. Nevertheless, its efficacy and side-effect manageability make further research worthwhile.

Keywords: Thalidomide, Angiodysplasia, Recurrent bleeding, Multiple myeloma, Antiangiogenic

Anahtar Sözcükler: Talidomid, Anjiodisplazi, Tekrarlayan kanama, Multipl myelom, Antianjiojenik

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References

1. Makris M, Federici AB, Mannucci PM, Bolton-Maggs PH, Yee TT, Abshire T, Berntorp E. The natural history of occult or angiodysplastic gastrointestinal bleeding in von Willebrand disease. Haemophilia 2015;21:338-342.
2. Duarte BKL, de Souza SM, Costa-Lima C, Medina SS, Ozelo MC. Thalidomide for the treatment of gastrointestinal bleeding due to angiodysplasia in a patient with Glanzmann's thrombasthenia. Hematol Rep 2017;9:6961.
3. Alam MA, Sami S, Babu S. Successful treatment of bleeding gastro-intestinal angiodysplasia in hereditary haemorrhagic telangiectasia with thalidomide. BMJ Case Rep 2011:2011.
4. Becq A, Rahmi G, Perrod G, Cellier C. Hemorrhagic angiodysplasia of the digestive tract: pathogenesis, diagnosis, and management. Gastrointest Endosc 2017;86:792-806.
5. Ge ZZ, Chen HM, Gao YJ, Liu WZ, Xu CH, Tan HH, Chen HY, Wei W, Fang JY, Xiao SD. Efficacy of thalidomide for refractory gastrointestinal bleeding from vascular malformation. Gastroenterology 2011;141:1629-1637.
6. Bauditz J. Effective treatment of gastrointestinal bleeding with thalidomide - chances and limitations. World J Gastroenterol 2016;22:3158-3164.
7. Kumar SK, Vij R, Noga SJ, Berg D, Brent L, Dollar L, Chari A. Treating multiple myeloma patients with oral therapies. Clin Lymphoma Myeloma Leuk 2017;17:243-251.
8. Aguiar PM, de Mendonça Lima T, Colleoni GWB, Storpirtis S. Efficacy and safety of bortezomib, thalidomide, and lenalidomide in multiple myeloma: an overview of systematic reviews with meta-analyses. Crit Rev Oncol Hematol 2017;113:195-212.

This study was previously presented as a poster in the Croatian language at the 7th Croatian Congress of Hematologists in Šibenik, Croatia, in September 2017.

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Treatment of Chronic Back and Chest Pain in a Patient with Sickle Cell Disease Using Spinal Cord Stimulation

Orak Hücre Hastasında Kronik Sırt ve Göğüs Ağrısının Spinal Kord Stimülatörü ile Tedavisi

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To the Editor,

Pain with sickle cell disease can occur in two forms: acute or chronic. Acute pain is often treated with analgesics in emergency services or at home and can intermittently relapse. In the later stages of the disease, chronic pain occurs due to central sensitization. Here we report a patient with sickle cell pain who was treated with a spinal cord stimulator (SCS).

Our patient was a 28-year-old female. She was admitted to the hospital due to painful crises and had a history of operations due to vertebral fracture, femoral head osteonecrosis, and pulmonary hypertension. Her back and chest pain was ranked as 9 on a numeric rating scale when she was referred to the pain clinic. Non-enhancing areas involving vertebral bodies at dorsal and lumbar levels, suggestive of infarcts, were shown by magnetic resonance imaging (Figure 1). Pain control could not be achieved medically; all nonsteroidal anti-inflammatory drugs and opioids had been unsuccessful. After evaluation by the local pain council of the hospital, the patient underwent implantation of an SCS.

In the operating room she was placed in the prone position. Under fluoroscopic guidance a 15-gauge Tuohy needle was inserted into the T6-T7 interlaminar space. Eight-electrode leads were inserted through the needle and advanced until the tip lay at the T1-T4 epidural level (Figure 2). Parameters of stimulation were pulse width of amplitude 2.5 mA and frequency of 10 kHz. Following more than 60% pain relief throughout the trial period, the lead was connected to an implantable pulse generator, which was placed into the left buttock. During 1-year follow-up of the implantation of the high-frequency SCS, excellent pain relief continued with improvement of both the patient's pain and her ability to perform activities of daily living.

SCS has become popular in recent years. It is a neuromodulator and manages cases of certain chronic pain for which other procedures have failed, including failed back syndrome [1], ischemic limb pain [2], angina pectoris [3], and painful peripheral

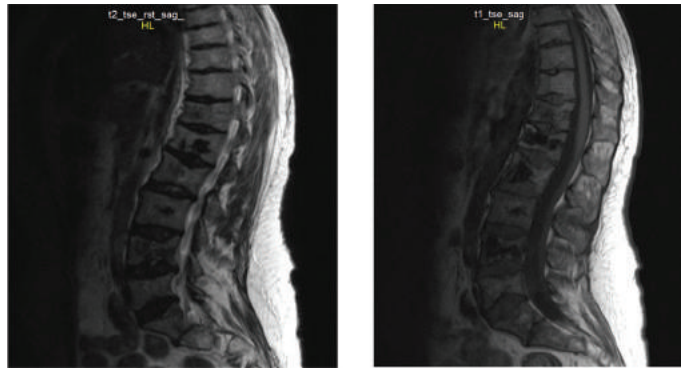

Figure 1. Magnetic resonance imaging shows multiple infarcts involving vertebral column T2 and T1 sagittal images. Non-enhancing areas involving vertebral bodies at dorsal and lumbar levels suggest infarcts.

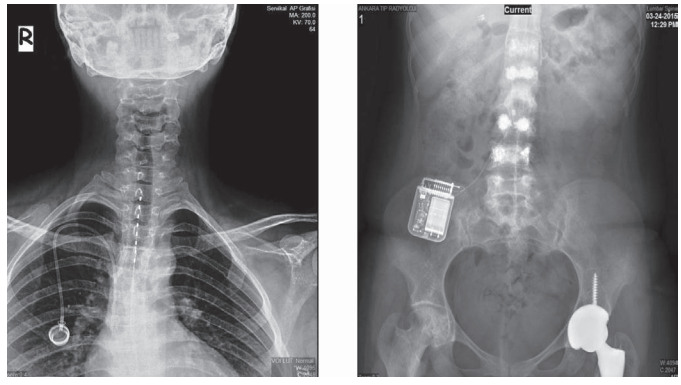

Figure 2. Plain X-ray demonstrating placement of spinal cord stimulator electrodes at the level of T1-T4.

neuropathies [4]. The mechanism of pain relief by the SCS is still not clear [5]. According to the gate control theory, in the peripheral nerve system the afferent activity of large fibers or small fibers is controlled by the dorsal column cells, associated with central transmission of pain. When a surplus of large-fiber activity occurs, this gate closes [6]. The pathophysiology of sickle cell disease could also be related to autonomic nervous system efficiency [7]. Constriction or obstruction of a blood vessel usually causes a reduction in blood flow and oxygen delivery to the tissues and thus insufficient perfusion [8]. Small

fibers, which carry nociceptive information in the sympathetic pain pathway, can be blocked by a high-frequency SCS.

The present report describes the first patient with intractable pain due to sickle cell disease who was treated with a high-frequency SCS successfully. For patients in whom all available treatments have failed or who have an increased risk for more invasive surgical interventions, the SCS might be a therapeutic alternative.

Keywords: Chronic pain, Sickle cell disease, Spinal cord stimulation

Anahtar Sözcükler: Kronik ağrı, Orak hücre hastalığı, Spinal kord stimülatörü

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References

1. Kumar K, Hunter G, Demeria D. Spinal cord stimulation in treatment of chronic benign pain: challenges in treatment planning and present status, a 22-year experience. Neurosurgery 2006;58:481-496.
2. Ghajar AW, Miles JB. The differential effect of the level of spinal cord stimulation on patients with advanced peripheral vascular disease in the lower limbs. Br J Neurosurg 1996;12:402-408.
3. Hautvast RW, DeJongste MJ, Staal MJ, van Gilst WH, Lie KI. Spinal cord stimulation in chronic intractable angina pectoris: a randomized controlled efficacy study. Am Heart J 1998;136:1114-1120.
4. Kumar K, Toth C, Nath RK. Spinal cord stimulation for chronic pain in peripheral neuropathy. Surg Neurol 1996;46:363-369.
5. Melzack RA, Wall PD. Pain mechanisms: a new theory. Science 1965;150:971-979.
6. Stojanovic M, Abdi S. Spinal cord stimulation. Pain Physician 2002;5:156-166.
7. Nebor D, Bowers A, Hardy-Dessources MD, Knight-Madden J, Romana M, Reid H, Barthélémy JC, Cumming V, Hue O, Elion J, Reid M, Connes P; CAREST Study Group. Frequency of pain crises in sickle cell anemia and its relationship with the sympatho-vagal balance, blood viscosity and inflammation. Haematologica 2011;96:1589-1594.
8. Simpson EL, Duenas A, Holmes MW, Papaioannou D, Chilocott J. Spinal cord stimulation for chronic pain of neuropathic or ischaemic origin: systematic review and economic evaluation. Health Technol Assess 2009;13:1-154.

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Simultaneous Presence of Follicular Lymphoma, Diffuse Large B-cell Lymphoma, and Hodgkin-like Lymphoma

Eş Zamanlı Folliküler Lenfoma, Diffüz Büyük B Hücreli Lenfoma ve Hodgkin-Benzeri Lenfoma Varlığı

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To the Editor,

Follicular lymphoma (FL) is a relatively indolent B-cell lymphoma that may transform to a higher-grade lymphoma, most commonly diffuse large B-cell lymphoma (DLBCL) [1]. On the other hand, the occurrence of Hodgkin lymphoma (HL) subsequent to FL as well as composite lymphomas that are composed of HL and FL have rarely been recorded [1,2,3,4,5]. To the best of our knowledge, this is the first reported case of the simultaneous presence of FL, DLBCL, and Hodgkin-like lymphoma in a lymph node. A 65-year-old man developed a palpable mass in his left axilla, which grew larger in a period of 2 months. The patient reported no other symptoms and had no notable medical history. Biopsy of the left axillary lymph node revealed grade 3A-FL with areas of DLBCL. DLBCL was also observed in the biopsy of a mass of the thoracic wall, which was near the enlarged axillary lymph node. Moreover, in the lymph node, Hodgkin and Reed-Sternberg (HRS) cells were identified in extrafollicular areas and some neoplastic follicles (Figure 1). Although some scattered eosinophils, plasma

cells, and histiocytes were observed in the cellular background of the extrafollicular HRS cells, the extent of this cellular infiltrate was less than what would be expected for typical HL (Figure 1). Immunohistochemistry revealed that the follicular neoplastic cells were CD20+, CD10+, BCL6+, BCL2+, PAX-5+, CD30-, CD15-, and MUM1- (Figure 1). The HRS cells were CD30+, CD15+ (20%), CD20-, CD10-, BCL6-, MUM1+, CD3-, CD4-, CD8-, and weakly PAX-5+ (Figure 1). A few reactive follicles with CD10+, BCL6+, and BCL2- germinal center cells were also observed. EBER-in situ hybridization demonstrated Epstein-Barr virus (EBV) positivity in some cells in a few neoplastic follicles (Figure 1), but not in the DLBCL component or in the HRS cells. The above findings were consistent with the simultaneous presence of FL, DLBCL, and Hodgkin-like lymphoma. Computed tomography (CT) and positron-emission tomography (PET)/CT and bone marrow (BM) biopsy were performed. The lymphoma was assigned stage IV because of BM infiltration. The BM lymphoid infiltration was diffuse (15%-20% of the total BM nucleated cells) and composed of medium-sized lymphoid cells with immunophenotype of CD20+, CD10+, BCL6+, PAX-5+, MUM1-, and CD30-. In addition, some cells with the morphology of Hodgkin cells and immunophenotype of CD30+, CD15+, CD45+, CD20-, CD10-, BCL6-, and PAX-5- were also identified in the lymphoid infiltration. The patient subsequently underwent six cycles of rituximab-CHOP chemotherapy without adverse effects. After treatment, the CT scans and PET/CT results were consistent with complete response and BM biopsy showed no lymphoma. He is currently in regular follow-up. In our case, the DLBCL component may correspond to transformation of the FL component, and the EBV-negative Hodgkin-like component may arise from the EBV-negative intrafollicular HRS cells that we detected in the lymph node. The occurrence of HL subsequent to FL as well as composite lymphomas consisting of HL (with classical immunophenotype) and FL without EBV association were rarely reported [2,3,4]. In contrast, Menon et al. [5] described transformation of FL to EBV-positive Hodgkin-like lymphoma. Interestingly, in keeping with the findings of Menon et al. [5], we also observed EBV-positive cells in a few neoplastic follicles. This suggests that EBV infected the cells secondarily in the neoplastic follicles. In conclusion, this is the first reported case of the simultaneous presence of FL, DLBCL, and EBV-negative Hodgkin-like lymphoma.

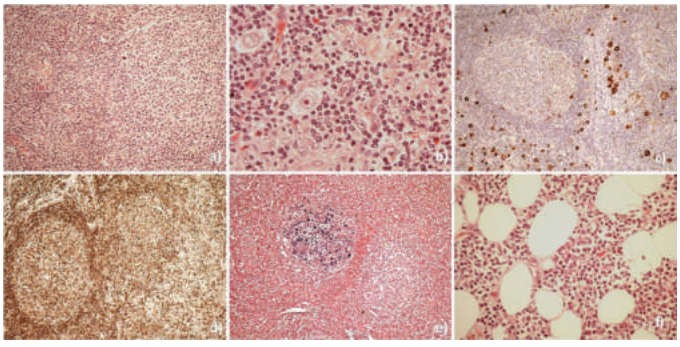

Figure 1. a) Synchronous presence of follicular lymphoma (FL) with Hodgkin-like lymphoma (hematoxylin and eosin staining, 200^x); b) Hodgkin cells admixed with scattered eosinophils, plasma cells, and histiocytes (hematoxylin and eosin staining, 600^x); c) CD30+ Hodgkin and Reed-Sternberg cells in the extrafollicular areas surrounding a neoplastic follicle (immunohistochemical staining, 100^x); d) synchronous presence of FL with Hodgkin-like lymphoma, where the neoplastic follicles express the BCL2 protein (immunohistochemical staining, 100^x); e) Epstein-Barr virus (EBER)-positive cells in the neoplastic follicles (in situ hybridization, 100^x); f) diffuse large B-cell lymphoma (hematoxylin and eosin staining, 400^x).

Keywords: Transformation, Follicular lymphoma, Hodgkin-like lymphoma

Anahtar Sözcükler: Transformasyon, Folliküler lenfoma, Hodgkin-benzeri lenfoma

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References

1. Fischer T, Zing NPC, Chiattone CS, Federico M, Luminari S. Transformed follicular lymphoma. Ann Hematol 2018;97:17-29.
2. Brauninger A, Hansmann ML, Strickler JG, Dummer R, Burg G, Rajewsky K, Küppers R. Identification of common germinal-center B-cell precursors in two patients with both Hodgkin's disease and non-Hodgkin's lymphoma. N Engl J Med 1999;340:1239-1247.
3. Marafioti T, Hummel M, Anagnostopoulos I, Foss HD, Huhn D, Stein H. Classical Hodgkin's disease and follicular lymphoma originating from the same germinal center B cell. J Clin Oncol 1999;17:3804-3809.
4. Nakamura N, Ohshima K, Abe M, Osamura Y. Demonstration of chimeric DNA of bcl-2 and immunoglobulin heavy chain in follicular lymphoma and subsequent Hodgkin lymphoma from the same patient. J Clin Exp Hematop 2007;47:9-13.
5. Menon MP, Hutchinson L, Garver J, Jaffe ES, Woda BA. Transformation of follicular lymphoma to Epstein-Barr virus-related Hodgkin-like lymphoma. J Clin Oncol 2013;31:53-56.

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Skeletal Muscle Diffuse Large B-Cell Lymphoma in the Gluteal Region

Gluteal Bölgede İskelet Kası Diffüz Büyük B Hücreli Lenfoma

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To the Editor,

Diffuse large B-cell lymphoma (DLBCL) is the most common form of non-Hodgkin lymphoma (NHL) [1]. Approximately 30% of NHL cases arise from an extranodal site, including the skin, testes, lungs, bones, gastrointestinal tract, and central nervous system [1,2]. Primary skeletal muscle lymphomas are rare and account for 0.5% of NHL cases [3].

A 60-year-old male presented with a 5-month history of a rapidly growing mass in his left buttock accompanied by intense pain and impaired mobilization. He denied weight loss, fever, or night sweats. Physical examination revealed a firm, tender left buttock mass, measuring 19x13 cm (Figure 1a). No palpable lymph nodes were detected. Laboratory tests were unremarkable. Abdominal and pelvic contrast-enhanced CT scan showed a soft tissue tumor in the left gluteal region, affecting the psoas, gluteus maximus, and minor muscles with left retroperitoneal and inguinal lymphadenopathy. Two deep punch biopsies were performed. Histopathological examination revealed diffuse atypical lymphocyte infiltration involving the dermis, subcutaneous tissue, and muscle. Immunohistochemical staining was positive for CD20, with focal positivity of 20% for MUM1, and negative for CD10 and BCL6. The Ki-67 proliferation index was 80%. The final diagnosis was DLBCL, activated B-cell subtype. Six cycles of chemotherapy with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) were started. He obtained complete clinical remission (Figure 1b) with no recurrence.

Extranodal lymphomas (ENLs) are defined as those with no/minimal nodal involvement associated with a dominant extranodal component [4]. However, the definition of primary lymphoma remains a controversial issue, especially in patients where both nodal and extranodal sites are involved. The Lugano classification designates extranodal disease as single extranodal lesions without nodal involvement or patients with state I or II nodal disease with a clinically dominant extranodal component [5,6,7]. ENLs may arise from any site devoid of lymphocytes and almost half represent DLBCL [6].

Involvement of the skeletal muscles in NHL is unusual and has been reported to occur in 1.1% of patients. The most

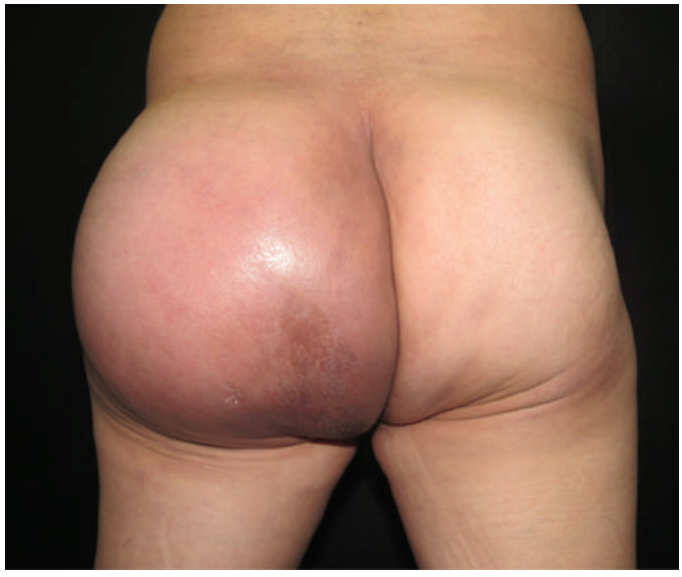

Figure 1a. Diffuse large B-cell lymphoma in the gluteal region before treatment.

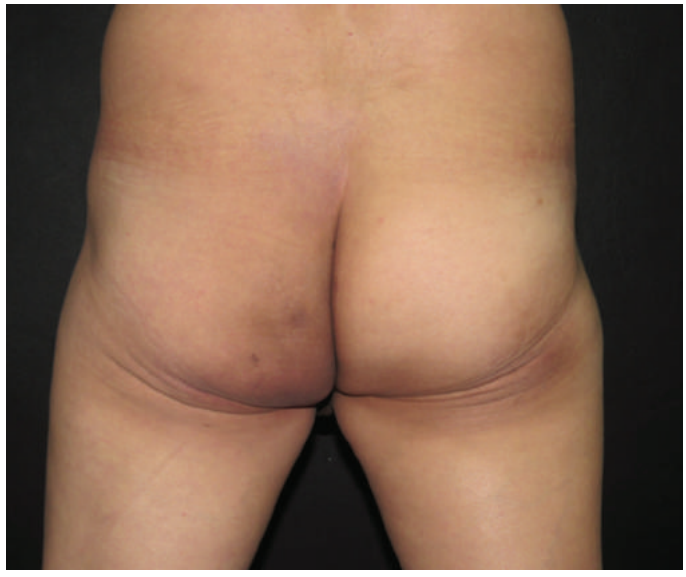

Figure 1b. After 6 cycles of R-CHOP chemotherapy.

common route of muscle involvement is hematogenous, lymphatic, or by contiguous spread, or, very rarely, as a primary extranodal disease [4]. The most commonly affected muscles are those of the extremities, pelvis, and gluteal regions [6]. In a retrospective study from the Mayo Clinic of over 7000 cases of lymphoma, primary muscle lymphoma accounted for only 0.1%, as diagnosed over a 10-year period [8].

The main symptoms include the presence of a mass with progressive enlargement, pain, and swelling [9]. Imaging studies show diffuse enlargement of the muscle involving multiple compartments, distinguishing it from soft tissue sarcomas that usually involve one compartment [9]. Magnetic resonance imaging may aid in diagnosis and enables evaluation of tumor extension and adjacent structure involvement. However, histological analysis and immunohistochemistry is necessary to confirm the diagnosis [10].

Differential diagnosis includes soft tissue sarcoma, metastatic carcinoma, and neurogenic tumors such as malignant peripheral nerve sheath tumors [6]. No specific guidelines for the treatment of skeletal muscle ENLs are available. R-CHOP chemotherapy is usually the preferred regimen [7]. Due to the scarce number of reports, information on the precise prognosis of primary skeletal ENLs is not available.

Acknowledgments

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Keywords: Diffuse large B-cell lymphoma, Extranodal lymphoma, Gluteal lymphoma, Muscle lymphoma

Anahtar Sözcükler: Diffüz büyük B hücreli lenfoma, Ekstranodal lenfoma, Gluteal lenfoma, Kas lenfoması

Informed Consent: Received.

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References

1. Zhang L, Lin Q, Dong L, Li Y. Primary skeletal muscle diffuse large B cell lymphoma: a case report and review of the literature. Oncol Lett 2015;10:2156-2160.
2. Møller MB, Pedersen NT, Christensen BE. Diffuse large B-cell lymphoma: clinical implications of extranodal versus nodal presentation--a population-based study of 1575 cases. Br J Haematol 2004;124:151-159.
3. Katsura M, Nishina H, Shigemori Y, Nakanishi T. Extranodal lymphoma originating in the gluteal muscle with adjacent bone involvement and mimicking a soft tissue sarcoma. Int J Surg Case Rep 2015;7:161-164.
4. Alamdari A, Naderi N, Peiman S, Shahi F. Non-Hodgkin lymphoma with primary involvement of skeletal muscle. Int J Hematol Oncol Stem Cell Res 2014;8:55-57.
5. Cheson BD, Fisher RI, Barrington SF, Cavalli F, Schwartz LH, Zucca E, Lister TA. Recommendations for initial evaluation, staging, and response assessment of Hodgkin and non-Hodgkin lymphoma: the Lugano classification. J Clin Oncol 2014;32:3059-3068.
6. Elkourashy SA, Nashwan AJ, Alam SI, Ammar AA, El Sayed AM, Omri HE, Yassin MA. Aggressive lymphoma "sarcoma mimicker" originating in the gluteus and adductor muscles: a case report and literature review. Clin Med Insights Case Rep 2016;9:47-53.
7. Krol AD, le Cessie S, Snijder S, Kluin-Nelemans JC, Kluin PM, Noordijk EM. Primary extranodal non-Hodgkin's lymphoma (NHL): the impact of alternative definitions tested in the Comprehensive Cancer Centre West population-based NHL registry. Ann Oncol 2003;14:131-139.
8. Travis WD, Banks PM, Reiman HM. Primary extranodal soft tissue lymphoma of the extremities. Am J Surg Pathol 1987;11:359-366.
9. Burton E, Schafernak K, Morgan E, Samet J. Skeletal muscle involvement in B-cell lymphoma: two cases illustrating the contribution of imaging to a clinically unsuspected diagnosis. Case Rep Radiol 2017;2017:2068957.
10. Hongsakul K, Laohawiriyakamol T, Kayasut K. A rare case of primary muscular non-Hodgkin's lymphoma and a review of how imaging can assist in its diagnosis. Singapore Med J 2013;54:179-182.

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Early Direct Antiglobulin Test Negativity after Bendamustine and Rituximab Treatment in Chronic Lymphocytic Leukemia: Two Cases

Bendamustin ve Rituksimab Tedavisi ile Erken Direkt Antiglobülin Testi Negatifliği Elde Edilen İki Kronik Lenfositik Lösemi Olgusu

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To the Editor,

Autoimmune hemolytic anemia (AIHA) can emerge at any stage of chronic lymphocytic leukemia (CLL); furthermore, patients can present with AIHA before diagnosis [1]. Although direct antiglobulin test (DAT) positivity is one of the hallmarks of AIHA, it was also demonstrated to be associated with advanced disease [2] and poor prognosis [3] independent of hemolytic anemia in CLL patients [3]. Here we present two CLL patients with AIHA whose DAT results became negative shortly after receiving bendamustine-rituximab (BR) chemotherapy.

Case 1

A 69-year-old male patient who was being followed without treatment for CLL in Rai stage 2 for 6 months presented with abdominal pain and jaundice. Laboratory tests were as follows: leukocytes: 55,140/µL, lymphocytes: 51,240/µL, hemoglobin: 5.3 g/dL, platelets: 46,000/µL, indirect bilirubin: 2.89 mg/dL, haptoglobin: 2 mg/dL, lactate dehydrogenase (LDH): 1585 U/L, and DAT positive for Immunoglobulin G (IgG) (no titer provided). Imaging studies showed compressing conglomerate lymph node masses in the abdomen. The patient was started on steroid and BR treatments. The hemoglobin value rose to normal levels and DAT became negative after 3 cycles of BR. The patient received 6 cycles of BR chemotherapy and steroids were interrupted at the 5th month of treatment. The patient has been followed in remission for 1 year.

Case 2

A 75-year-old female patient who was being followed without treatment with the diagnosis of CLL in Rai 0 stage for 8 years was admitted due to weakness and fatigue. Laboratory tests were as follows: leukocytes: 78,840/µL, lymphocytes: 67,020/µL, hemoglobin: 6.3 g/dL, platelets: 255,000/µL, indirect bilirubin: 2.58 mg/dL, LDH: 504 U/L, haptoglobin: 1 mg/dL, corrected reticulocyte count: 5.2%, and DAT positive for IgG (4+). The patient was started on steroid treatment and subsequently BR therapy was added due to increased lymphocyte doubling time. After the first cycle, the DAT titer dropped to 3+. Hemoglobin value rose to normal levels and DAT became negative after 3 cycles of BR. Steroids were ceased at the 7th month of treatment; The patient completed 6 cycles of BR and has been followed in remission for 1 year.

While the standard approach in CLL patients with AIHA is steroids, systemic chemotherapy is recommended in refractory cases and in patients requiring treatment for CLL [1]. Although first-line therapy in CLL patients is the fludarabine-cyclophosphamide-rituximab regimen, the wide use of BR chemotherapy, especially in advanced-age patients, has brought up the application of this combination in patients with AIHA [4,5]. In a recent study including 26 CLL patients who had AIHA and received BR, the response rate was 81% for AIHA and 77% for CLL [4]. Similarly, our patients also responded well in terms of CLL and AIHA. The most striking point was that DAT became negative in a short period of time (after 3 cycles of BR).

In conclusion, in addition to being a plausible option in advanced-age CLL patients, BR seems to be an important treatment of choice in terms of eliminating the poor prognostic factor of DAT positivity and assuring safe cessation of steroid treatment due to rapid achievement of DAT negativity.

Keywords: Chronic lymphocytic leukemia, Autoimmune hemolytic anemia, Bendamustine, Rituximab

Anahtar Sözcükler: Kronik lenfositik lösemi, Otoimmün hemolitik anemi, Bendamustin, Ritüksimab

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References

1. Zent CS, Kay NE. Autoimmune complications in chronic lymphocytic leukaemia (CLL). Best Pract Res Clin Haematol 2010;23:47-59.

2. Abbas SA, Zeeshan R, Sultan S, Irfan SM. Direct Coombs test positivity in B-chronic lymphoid leukemia: a marker of advanced clinical disease. Asian Pac J Cancer Prev 2015;16:6007-6010.
3. Quinquenel A, Al Nawakil C, Baran-Marszak F, Eclache V, Letestu R, Khalloufi M, Boubaya M, Le Roy C, Varin-Blank N, Delmer A, Levy V, Ajchenbaum-Cymbalista F. Old DAT and new data: positive direct antiglobulin test identifies a subgroup with poor outcome among chronic lymphocytic leukemia stage A patients. Am J Hematol 2015;90:5-8.
4. Quinquenel A, Willekens C, Dupuis J, Royer B, Ysebaert L, De Guibert S, Michallet AS, Feugier P, Guieze R, Levy V, Delmer A. Bendamustine and rituximab combination in the management of chronic lymphocytic leukemia-associated autoimmune hemolytic anemia: a multicentric retrospective study of the French CLL intergroup (GCFLLC/MW and GOELAMS). Am J Hematol 2015;90:204-207.
5. Hallek M. Chronic lymphocytic leukemia: 2017 update on diagnosis, risk stratification, and treatment. Am J Hematol 2017;92:946-965.

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Demodicidosis Accompanying Acute Cutaneous Graft-Versus-Host Disease after Allogeneic Stem Cell Transplantation

Allojeneik Kök Hücre Nakli Sonrası Akut Graft Versus Host Hastalığına Eşlik Eden Demodisidoz

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To the Editor,

A 39-year-old female with acute myeloid leukemia was admitted to our transplantation clinic with face eruption without any pruritus. The eruption had occurred 28 days after she underwent an allogeneic hematopoietic stem cell transplantation (SCT). She was allografted with $6.12x10^6$ non-manipulated CD34+ cells from a fully matched sibling donor after a conditioning regimen including busulfan (12.8 mg/m^2), fludarabine (150 mg/m^2), anti-thymocyte globulin (30 mg/kg), and total body irradiation (400 Gy/day). Graft-versus-host disease (GVHD) prophylaxis comprised methotrexate at 12 mg/day for 3 days and cyclosporine A at 75 mg twice daily. No recent changes had been made to the medication. Neutrophil and thrombocyte engraftment both occurred on day 11. The toxicity related to the regimen was mild, being assigned the first grade for oral mucosa according to the Bearman scale [1]. The findings of the physical examination were patchy and confluent erythema of the face, suspicious for cutaneous acute GVHD. There were no other skin changes except that of the palms and soles. Neither intestinal nor hepatic acute GVHD occurred. Laboratory evaluation revealed a white blood cell count of 12,000/µL, a hemoglobin level of 11.5 g/dL, a platelet count of 158,000/µL, and an absolute neutrophil count of 8400/µL. A 4-mm skin punch biopsy was performed [2]. There were lymphocytes and polymorphic neutrophils that attacked hair follicles and two Civatte bodies. Histochemically *Demodex folliculorum* was diagnosed with PAS staining within the hair follicles (Figures 1A and 1B). Even with lymphocytes attacking hair follicles and Civatte bodies suggesting GVHD, *Demodex* folliculitis can mimic acute GVHD (Figures 1C and 1D). Demodicidosis was treated successfully with local 1% metronidazole and 5% permethrin. Methylprednisolone was also administered from the beginning of the symptoms and the dosing was reduced by 8 mg every week. The skin eruptions on the face and the neck resolved on day +52.

Demodex folliculitis after allogeneic SCT is seen rarely and, as far as we know, our case is the sixth reported case [3,4,5,6]. The most important differential diagnosis of *Demodex* folliculitis within the first 100 days after allogeneic SCT is acute GVHD. The infestation by *Demodex* sp. can be associated with immune suppression. The differential diagnosis of facial erythema after bone marrow transplantation includes acute GVHD, drug eruptions, systemic lupus erythematosus, viral exanthema, toxic erythema of chemotherapy, drug-induced photosensitivity, and photodermatitis [3]. In our case there

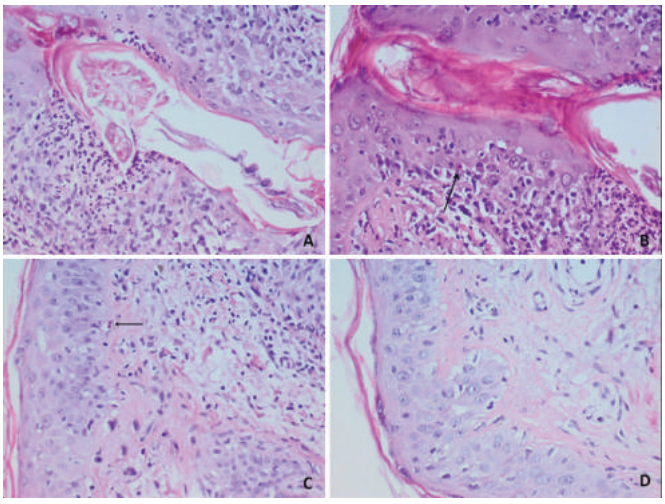

Figure 1. A) *Demodex* mite; B) Civatte body in the follicular epithelium containing *Demodexand* lymphocyte exocytosis; C) Civatte body in the epithelium far from the follicle; D) diffuse basal vacuolization in epidermis (periodic acid-schiff staining, magnification 40ˣ) (159x119 mm; 72x72 DPI).

were eruptions on the cheek, forehead, and jaw regions, which can be distinguished in both acute GVHD and *Demodex* folliculitis. However, the development of palmar erythema of the upper extremities is not a feature of demodicidosis. As confirmed by pathological examination, there were findings of both acute GVHD (presence of Civatte bodies, lymphocyte exocytosis, diffuse basal vacuolization in the epidermis) and demodicidosis (presence of *Demodex* folliculorum).

It should not be forgotten that GVHD may be associated with demodicidosis and *Demodex* infestation should be remembered in the differential diagnosis of eruptions in patients with hematological malignancies receiving chemotherapy and after SCT. For this reason, when the diagnosis of acute GVHD is ambiguous, an early skin biopsy has to be done after allogeneic SCT because early therapy for a possible *Demodex* infestation would prevent the progression of GVHD.

Keywords: *Demodex* folliculitis, Acute graft-versus-host disease, Post-transplantation

Anahtar Sözcükler: *Demodeks* follikülit, Akut graft versus host hastalığı, Nakil sonrası

Informed Consent: Informed consent was received from the patient.

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References

1. Bearman SI, Applebaum FR, Buckner CD, Petersen FB, Fisher LD, Clift RA,Thomas ED. Regimen-related toxicity in patients undergoing bone marrow transplantation. J Clin Oncol 1988;6:1562-1568.
2. Hillen U, Häusermann P, Massi D, Janin A, Wolff D, Lawitschka A, Greinix H, Meyer R, Ziemer M. Consensus on performing skin biopsies, laboratory workup, evaluation of tissue samples and reporting of the results in patients with suspected cutaneous graft-versus-host disease. J Eur Acad Dermatol Venereol 2015;29:948-954.
3. Cotliar J, Frankfurt O. *Demodex* folliculitis mimicking acute graft-vs-host disease. JAMA Dermatol 2013;149:1407-1409
4. Lotze C, Krüger WH, Kiefer T, Swensson O, Herbst EW, Schüler F, Busemann C, Dölken G. Facial rash mimicking cutaneous acute graft-versus-host disease after allogeneic stem cell transplantation for osteomyelofibrosis: was *Demodex* the culprit? Bone Marrow Transplant 2006;37:711-712.
5. Aisa Y, Mori T, Tanikawa A, Takae Y, Kato J, Ikeda Y, Okamoto S. Demodicidosis as a cause of facial eruption developing early after allogeneic hematopoietic stem cell transplantation. Transplant Int 2008;21:1192-1193.
6. Román-Curto C, Meseguer-Yebra C, Cañueto J, Fraile-Alonso C, Santos-Briz A, Vázquez L, Fernández-López E. Demodicidosis stimulating acute graft-versus-host disease after allogeneic stem cell transplantation in one patient with acute lymphoblastic leukemia. Transpl Infect Dis 2012;14:387-390.

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IMBRUVICA™ Kısa Ürün Bilgisi IMBRUVICA™ 140 mg sert kapsül. FARMAKOTERAPÖTİK ÖZELLİKLER: Antineoplastik ajanlar, protein kinaz inhibitörleri.
Etki mekanizması: İbrutinib güçlü bir kovalent BTK (Bruton tirozin kinazı) inhibitörüdür. **ENDİKASYONLARI:** IMBRUVICA en az 3 kür rituksimab ve alkilleyici ajan kombinasyonu sonrası nüks eden veya dirençli olan veya otolog kök hücre nakli sonrası nüks eden mantle hücreli lenfomada (MHL) endikedir. IMBRUVICA, 70 yaş ve üzeri veya Cumulative Illness Rating Scale(CIRS) >6 olması nedeniyle kemoterapiye uygun olmayan ve ilk basamak tedaviye yanıtsız/nüks kronik lenfositik lösemi (KLL) hastalarında ikinci basamak tedavide, 70 yaş üzeri olup CIRS>6 olması nedeniyle kemo-immünterapiye uygun olmayan del17p/TP53 mutasyonu bulunan KLL olgularının ilk basamak tedavisinde, CIRS≤ 6 olup kemo-immünterapiye uygun olan ve del 17p/TP53 mutasyonu olan KLL olgularında ilk basamak tedavi sonrası yanıtsızlık/nüks durumunda ikinci basamak tedavide endikedir. CIRS<6 olup kemo-immünterapiye uygun olan KLL hastalarında İki seri tedaviye rağmen yanıtsızlık/nüks durumunda üçüncü basamak tedavide endikedir. Del17p/TP53 mutasyon pozitiflik sınırı >%20'dir. **POZOLOJİ VE UYGULAMA ŞEKLİ:** Bu ilaç; ile tedavi, anti-kanser ilaçların kullanımında deneyimli bir hekim tarafından başlatılmalı ve gözlem altında tutulmalıdır. Mantle Hücreli Lenfoma (MHL) için önerilen IMBRUVICA dozu günde bir kez ağızdan alınan 560 mg'dır (140 mg'lık dört kapsül). Kronik Lenfositik Lösemi (KLL) için önerilen IMBRUVICA dozu günde bir kez ağızdan alınan 420 mg'dır (140 mg'lık üç kapsül). Tedavi hastalık progresyonuna ya da hasta tarafından tolere edilebildiği sürece devam edilmelidir. **KONTRENDİKASYONLARI:** Etkin madde İbrutinibe ya da yardımcı maddelerden herhangi birine karşı aşırı duyarlılık durumunda ve St. John's Wort (Sarı kantaron) içeren ürünler ile birlikte IMBRUVICA kullanımı kontrendikedir. **UYARILAR/ÖNLEMLER:** Kanama ilişkili olaylar IMBRUVICA ile tedavi edilen hastalarda, trombositopeninin eşlik ettiği ve etmediği hemorajik olay bildirimleri olmuştur. Bunlar arasında kontüzyon, burun kanaması ve peteşi gibi önemsiz kanama olayları ve gastrointestinal kanama, intrakraniyal kanama ve hematüri gibi bazıları ölümcül olan, önemli kanama olayları yer alır. Varfarin ya da diğer K vitamini antagonistleri IMBRUVICA ile eş zamanlı olarak kullanılmamalıdır. Balık yağı, E vitamini ve keten tohumu gibi gıda takviyeleri de alınmamalıdır. Trombosit fonksiyonunu inhibe eden ilaçlara ya da diğer antikoagülan ilaçlara gereksinimi olan hastalarda IMBRUVICA kanama riskini artırabilir. Antikoagülan terapi kullanılması durumunda özel bakım uygulanmalıdır. Konjenital kanama diyatezi olan hastalar incelenmemiştir. IMBRUVICA tedavisi cerrahinin tipine ve kanama riskine bağlı olarak, cerrahi öncesinde ve sonrasında en az 3 ila 7 gün kesilmelidir. IMBRUVICA ile tedavi edilen hastalarda iskostaz vakaları bildirilmiştir. IMBRUVICA tedavisine geçici olarak ara verilmesi düşünülmelidir. Hastalar yakından izlenmelidir. Belirtildiği şekilde hidrasyon ve/veya sitoredüksiyon dahil, destekleyici bakım uygulanmalıdır. IMBRUVICA ile tedavi edilen hastalarda enfeksiyonlar gözlenmiştir (sepsis, nötropenik sepsis, bakteri, virüs ya da mantar enfeksiyonları). Bu enfeksiyonların bazıları hastaneye yatma ve ölümle sonuçlanmıştır. Hastalar ateş, nötropeni ve enfeksiyonlar açısından izlenmeli ve endike olduğunda uygun anti-enfektif tedavi başlatılmalıdır. IMBRUVICA ile tedavi sırasında ortaya çıkan derece 3 ya da 4 sitopenifer (nötropeni, trombositopeni ve anemi) bildirilmiştir. Ayda bir tam kan sayımları alınmalıdır. IMBRUVICA ile tedavi edilen hastalarda İnterstisyel Akciğer Hastalığı (İAH) vakaları bildirilmiştir. Hastalar, İAH'na işaret eden akciğer semptomları bakımından izlenmeli ve semptomların ortaya çıkması durumunda, IMBRUVICA'ya ara verilmeli ve İAH uygun şekilde tedavi edilmelidir. Atriyal fibrilasyon/atriyal çarpıntı. Özellikle kardiyak risk faktörleri, hipertansiyon, akut enfeksiyonlar ve geçmiş bir atriyal fibrilasyon öyküsü olan IMBRUVICA ile tedavi edilen hastalarda atriyal fibrilasyon ve atriyal çarpıntı bildirilmiştir. Hastalar atriyal fibrilasyon açısından periyodik olarak klinik gözlem altında tutulmalıdır.Tümör lizis sendromu IMBRUVICA tedavisinde bildirilmiştir. Tümör lizis sendromu riski taşıyan hastalar, tedavi öncesi tümör yükü yüksek olan hastalardır. Hastalar yakından takip edilmeli ve uygun önlemler alınmalıdır. **GEBELİK VE LAKTASYON:** Gebelik kategorisi: D Çocuk doğurma potansiyeli bulunan kadınlar/Doğum kontrolü (Kontrasepsiyon): İbrutinibin gebe kadınlarda kullanımına ilişkin yeterli veri mevcut değildir. Hayvanlar üzerinde yapılan araştırmalar üreme toksisitesinin bulunduğunu göstermiştir. Gebelik ve Laktasyon dönemi: IMBRUVICA gebelik veya laktasyon sırasında kullanılmamalıdır. Fertilite: İbrutinibin insan fertilitesi üzerine verisi bulunmamaktadır. Özel popülasyonlara ilişkin ek bilgiler: Böbrek yetmezliği: Böbrek yetmezliği olan hastalarda özel klinik çalışmalar yapılmamıştır. Karaciğer yetmezliği: İbrutinib karaciğerde metabolize edilir. Bir karaciğer yetmezliği çalışmasında, veriler ibrutinib maruziyetinde artış ortaya koymuştur Şiddetli kalp hastalığı: Şiddetli kalp hastalığı olan hastalar IMBRUVICA'nın klinik çalışmalarından dışlanmıştır. **Pediyatrik popülasyon:** IMBRUVICA'nın 18 yaş altı çocuklardaki güvenilirliği ve etkililiği ortaya konmamıştır. Geriyatrik popülasyon: Yaşlı hastalarda (65 yaş ve üstü) doz ayarlaması gerekli değildir. Araç ve makine kullanımı üzerindeki etkiler: IMBRUVICA kullanan bazı hastalarda yorgunluk, baş dönmesi ve asteni bildirilmiş olup, bir hastanın taşıt ya da makine kullanma kapasitesini değerlendirirken bu dikkate alınmalıdır. Tedavi sırasında bu olaylardan herhangi birini yaşayan hastalar araç veya makine kullanımı konusunda dikkatli olmalıdır. İstenmeyen etkiler: Güvenlilik profili, IMBRUVICA ile tedavi edilen nüks, dirençli veya yeni tanılı KLL, MHL, WM gibi farklı hastalıklar ile yürütülen üç faz 2, randomize dört faz 3 klinik çalışmada tedavi edilen 981 hastanın verilerinden ve pazarlama sonrası deneyimden elde edilmiştir. En yaygın görülen advers ilaç reaksiyonları (≥ %20) diyare, nötropeni, hemoraji (örneğin morarma), kas-iskelet ağrısı, bulantı, döküntü ve pireksi olmuştur. En yaygın Derece 3 veya 4 advers ilaç reaksiyonları (≥ %5) arasında nötropeni, pnömoni, trombositopeni ve febril nötropeni yer almıştır. Rapor edilen advers ilaç reaksiyonları aşağıdaki sıklık gruplarına özellenmiştir: Çok yaygın (≥1/10); yaygın (≥1/100 ila <1/10); yaygın olmayan (≥1/1,000 ila <1/100); seyrek (≥1/10,000 ila <1/1,000), çok seyrek (<1/10,000). Enfeksiyonlar ve enfestasyonlar: Çok yaygın; Pnömoni,Üst solunum yolu enfeksiyonu, Sinüzit, Deri enfeksiyonu. Yaygın; Sepsis, Üriner sistem enfeksiyonu. İyi huylu ve kötü huylu neoplazmalar (kist ve polipler de dahil olmak üzere): Yaygın; Non-melanom cilt kanseri, bazal hücreli karsinom, skuamöz hücreli karsinom. Kan ve lenf sistemi hastalıkları: Çok yaygın; Nötropeni, Trombositopeni Yaygın, Febril nötropeni, Lökositoz, Lenfositoz. Bağışıklık sistemi hastalıkları: Yaygın; İnterstisyel akciğer hastalığı. Metabolizma ve beslenme hastalıkları: Yaygın; Tümör lizis sendromu, hiperürisemi. Sinir sistemi hastalıkları: Çok yaygın; Baş ağrısı.Yaygın: Baş dönmesi. **Görme bozuklukları:** Görme bulanıklığı. Kardiyak hastalıklar: Yaygın; Atriyal fibrilasyon. Vasküler hastalıklar: Çok yaygın; Kanama, Çürük (morarma). Yaygın; Subdural hematom, Burun kanaması, Peteşi, Hipertansiyon. Gastrointestinal hastalıklar: Çok yaygın; Diyare, Kusma, Stomatit, Bulantı. Kabızlık.Deri ve deri altı doku hastalıkları: Çok yaygın; Döküntü. Yaygın; Ürtiker, Eritem, tırnak lezyonları. Yaygın olmayan: anjiyoödem. Kas-iskelet bozuklukları, bağ doku ve kemik hastalıkları: Çok yaygın; Artralji, Kas spazmları, Kas-iskelet ağrısı. Genel hastalıklar ve uygulama bölgesine ilişkin hastalıklar: Çok yaygın; Pireksi, Periferik ödem. B-hücreli maligniteler için IMBRUVICA ile tedavi olan 981 hastadan %5'i temelde advers ilaç reaksiyonlarına (örn. pnömoni, atriyal fibrilasyon ve kanama) bağlı olarak tedaviyi kesmiştir. IMBRUVICA ile tedavi gören 981 hastanın %62'si ≥65 yaş ve derece ≥3 pnömoni yaşlı hastalarda daha sık gözlenmiştir (≥65 yaş hastaların %13'üne karşılık <65 yaş hastaların %7'si). Diğer tıbbi ürünler ile etkileşimler ve diğer etkileşim: İbrutinib primer olarak CYP3A4 enzimi ile metabolize edilir. IMBRUVICA'nın güçlü ya da orta dereceli CYP3A4 inhibitörleri ile eş zamanlı kullanımı ibrutinib maruziyetini artırabilir ve dolayısıyla toksisite için yüksek risk oluşturabilir. Öte yandan, CYP3A4 enzimini indükleyen ilaçların eş zamanlı kullanımı IMBRUVICA maruziyetini azaltabilir ve dolayısıyla etkililikte azalma riski ortaya çıkar. Bu sebeple, IMBRUVICA'nın güçlü ya da orta dereceli CYP3A4 inhibitörleri/indükleyiciler ile eş zamanlı kullanımından mümkün olduğunca kaçınılmalı ve eş zamanlı kullanım sadece potansiyel yararlar zarardan üstün olduğunda düşünülmelidir. Doz aşımı ve tedavisi: IMBRUVICA doz aşımının etkilerine ilişkin veriler sınırlıdır. Hastaların2.5 mg/kg/gün'e kadar dozlar kullandığı (1,400 mg/gün) faz 1 çalışmasında maksimum tolere edilen bir doza ulaşılmamıştır. IMBRUVICA'nın spesifik bir antidotu yoktur. Önerilen dozdan fazlasını alan hastalar yakından izlenmeli ve uygun destekleyici tedavi almalıdır **SAKLAMA KOŞULLARI:** Orijinal ambalajında ve 30˚C' nin altındaki oda sıcaklığında saklayınız. **RAF ÖMRÜ:** 24 ay. **Ambalajın niteliği ve içeriği:** 90 ya da 120 sert kapsül içeren, çocuk emniyetli kapaklı HDPE şişe. **RUHSAT SAHİBİ:** Johnson and Johnson Sıhhi Malz. San. ve Tic. Ltd. Şti. Ertürk Sok. Keçeli Plaza No: 13 Kavacık/Beykoz/İSTANBUL 34810 Tel: 0216 538 20 00 Faks: 0216 538 23 69 KDV dahil PF5 (26.02.2018): 90 kapsül/kutu: 17.840,83 TL; 120 kapsül/kutu: 23.870,20 TL. **"DAHA GENİŞ BİLGİ İÇİN FİRMAMIZA BAŞVURUNUZ" ÜRETİM YERİ:** Catalent CTS, LLC, Kansas City, MO 64137, ABD. **İLK RUHSAT TARİHİ:** 09.09.2016 **RUHSAT NO:** 2016/642. **KÜB'ÜN YENİLENME TARİHİ:** 07.04.2017.

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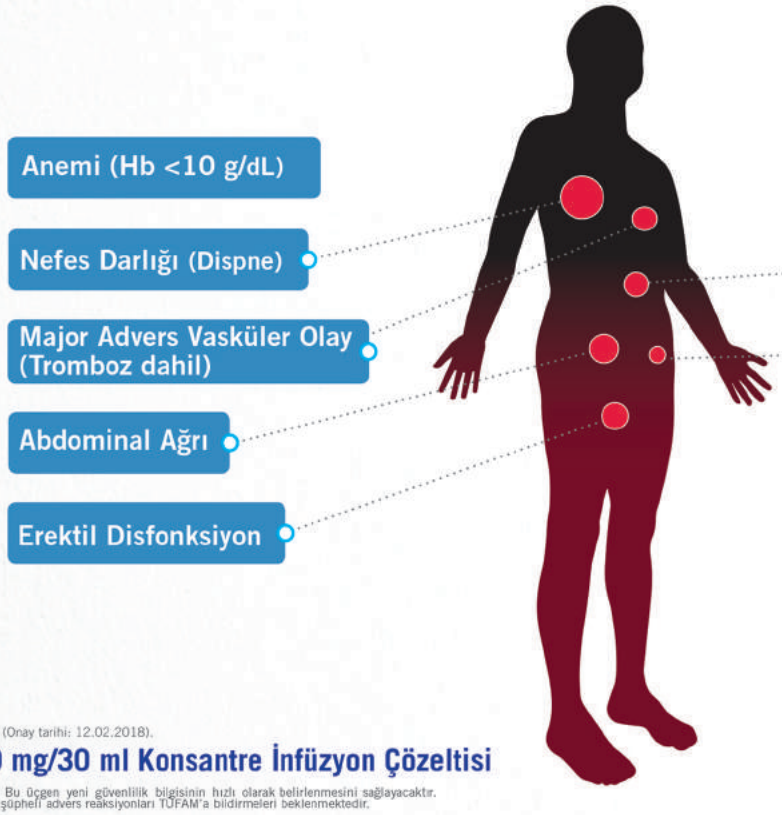

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▼SOLIRIS® 300 mg/30 ml Konsantre İnfüzyon Çözeltisi

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PNH hastaları, serum laktat dehidrogen (LDH) düzeyleri de dahil olmak üzere intravasküler hemoliz bulgu ve belirtileri açısından takip edilmelidir. aHÜS hastaları, trombotik mikroanjiyopatinin (TMA) bulgu ve belirtileri açısından takip edilmelidir. **Kontrendikasyonlar:** Ekulizumab, mürin proteinleri ya da diğer yardımcı maddelere karşı aşırı duyarlılığı olduğu bilinen hastalarda kontrendikedir. İyileşmemiş Neisseria meningitidis enfeksiyonu olan hastalarda, aşılama sonrasında 2 haftaya kadar uygun antibiyotiklerle profilaktik tedavi almamış ve hali hazırda Neisseria meningitidis'e karşı aşılanmamış hastalarda SOLIRIS® tedavisi başlatılmamalıdır. **Özel kullanım uyarıları ve önlemleri:** Meningokokkal enfeksiyon: SOLIRIS® etki mekanizması gereği ciddi veya ölümcül meningokokkal enfeksiyon riskinde artışa neden olmaktadır. Bu nedenle hastalara SOLIRIS® kullanımından en az iki hafta önce meningokokkal enfeksiyonlara karşı koruyucu aşı uygulaması mutlak suretle yapılmalıdır. Ayrıca, aşılama sonrasında iki haftadan daha kısa süre içinde SOLIRIS® tedavisinin başlanması söz konusu olduğu durumlarda ise aşı uygulamasına ilave olarak mutlaka meningokok enfeksiyonuna karşı, ilgili resmi kılavuzlar da göz önüne alınarak antimikrobiyal kemoprofilaksi uygulanmalıdır. Mevcut durumda dört bileşenli (A, C, Y ve W135) konjuge meningokok aşısı mutlaka yapılmalıdır; ayrıca konjuge meningokok B aşısının yapılması da tercih edilmelidir. Bütün bu önlemlere rağmen meningokokkal enfeksiyon için aşılama yeterli olmayabilir. Bu nedenle SOLIRIS® kullanan tüm hastalar meningokokkal enfeksiyonun erken bulguları açısından izlenmeli, enfeksiyondan şüphelenildiği durumlarda acilen değerlendirilmeli ve gerekirse uygun antibiyotiklerle tedavi edilmelidir. Ayrıca SOLIRIS® kullanan hastalar bu bulgu ve belirtiler ile acilen tıbbi bakım alınması için atılması gereken adımlar konusunda bilgilendirilmelidir. Hekimler SOLIRIS® tedavisinin yararlarını ve risklerini hastalarla tartışmalı ve hastalara bilgi broşürü ve hasta güvenilirlik bilgisi kartı vermelidir. Aşılama, komplemanda ek aktivasyona neden olabilir. Bunun sonucunda, PNH ve aHÜS dahil kompleman-aracılı hastalıkları olan hastaların, altta yatan hastalıklarının hemoliz (PNH) ya da TMA (aHÜS) gibi bulgu ve belirtilerinde artış yaşanabilir. Bu nedenle, önerilen aşılama sonrasında hastalık belirtileri açısından hastalar yakından takip edilmelidir. Aşının kontrendike olduğu veya yaptırmadığı durumlarda ve meningokok aşısı uygulanması takip eden ilk iki hafta içerisinde SOLIRIS® kullanan hastalara veya gerekli durumlarda tedavi periyodu boyunca antibiyotik profilaksisi uygulanmalıdır. Ayrıca SOLIRIS®'in etki mekanizması gereği kapsüllü mikroorganizmalara karşı verilen cevapla azalma olması nedeniyle bugüne için kanıtlanmamış olmakla birlikte pnömokokkal hastalık riskinin de artması beklenir ve bu sebeple pnömokok aşısı da önerilmektedir. Mevcut koşullarda çocukluk çağında rutin konjuge pnömokok aşı uygulaması yapıldığı için bunun sorgulanması, daha önce pnömokok aşısı ile aşılanmamış kişilerin bir doz konjuge pnömokok aşısı ile aşılanması ve tercihen en az sekiz hafta sonra da 23 bileşenli polisakkarit pnömokok aşısı ile aşılanması önerilmektedir. Altı yaş altında rutin aşı takvimi sorgulanmalı, Hemofilus influenza tip B ve pnömokok aşısı eksik ise yaşa uygun şekilde yapılmalıdır. Ek olarak Genişletilmiş Bağışıklama Programına uygun olarak aşılama ve takibi yapılmalı ve ayrıca SOLIRIS® kullananların düzenli mevsimsel influenza (grip) aşıları olmaları da önerilmelidir. Diğer sistemik enfeksiyonlar: Etki mekanizmasından dolayı SOLIRIS® tedavisi, aktif sistemik enfeksiyonların olduğu hastalara dikkatli bir şekilde uygulanmalıdır. Hastaların enfeksiyonlara, özellikle kapsüllü bakterilerin nedeni olduğu enfeksiyonlara duyarlılığı artmış olabilir. İnfüzyon reaksiyonları: SOLIRIS®'in uygulanması infüzyon reaksiyonlarına ya da alerjik veya aşırı duyarlılık reaksiyonlarına (anafilaksi de dahil) yol açabilecek immünojenisiteye neden olabilir. Şiddetli infüzyon reaksiyonları görülen tüm hastalarda SOLIRIS® uygulaması durdurulmalı ve uygun tıbbi tedavi uygulanmalıdır. Antikoagülan tedavi: SOLIRIS® tedavisi antikoagülan tedavisini değiştirmemelidir. Laboratuvar takibi: PNH hastaları, serum LDH düzeyleri de dahil olmak üzere intravasküler hemoliz bulgu ve belirtileri açısından takip edilmelidir. SOLIRIS® tedavisi gören PNH hastaları da benzer şekilde, LDH düzeyleri ölçülerek intravasküler hemoliz için takip edilmelidir ve bu hastalarda, idame fazı boyunca önerilen 14±2 gün doz uygulaması takvimi içinde doz ayarlaması gerekli olabilir (her 12. güne kadar). SOLIRIS® tedavisi gören aHÜS hastaları, trombosit sayımları, serum LDH ve serum kreatinin ölçümleri ile TMA için takip edilmelidir ve bu hastalarda, idame fazı boyunca önerilen 14±2 gün doz uygulaması takvimi içinde doz ayarlaması gerekli olabilir (her 12. güne kadar). Tedavinin bırakılması: SOLIRIS® tedavisini bırakan PNH hastaları, ciddi intravasküler hemolizin bulgu ve belirtileri açısından en az 8 hafta boyunca izlenmelidir. SOLIRIS® bırakıldıktan sonra ciddi hemolizin meydana gelmesi durumunda, aşağıdaki işlemleri/tedavileri uygulaması düşününüz: kan transfüzyonu (paketlenmiş eritrositler) ya da akış sitometrisine göre PNH eritrositleri toplam eritrositlerin %50'sinden fazla ise değiş-tokuş transfüzyonu; antikoagülasyon; kortikosteroidler, ya da SOLIRIS® tedavisinin yeniden başlatılması. SOLIRIS® tedavisini bırakan aHÜS hastaları, şiddetli TMA komplikasyonlarının bulgu ve belirtileri açısından yakından izlenmelidir. İzleme, SOLIRIS®'in bırakılmasından sonra aHÜS hastalarında şiddetli TMA komplikasyonlarını önlemek veya öngörmek için yetersiz kalabilir. SOLIRIS®'in bırakılmasından sonra ciddi TMA komplikasyonları ortaya çıkarsa, SOLIRIS® tedavisinin yeniden başlatılması, PD/PI ile destekleyici bakım ya da diyaliz ile böbrek desteği, mekanik ventilasyon ile solunum desteği veya antikoagülasyon içeren uygun, organa spesifik destekleyici önlemler düşünülmelidir. aHÜS klinik çalışmalarında SOLIRIS® tedavisinin bırakılmasını takiben 4 hafta gibi erken bir dönemde ve 127 haftaya kadar TMA komplikasyonları gözlenmiştir. Yardımcı maddeler: SOLIRIS® her bir flakonda 5 mmol sodyum ihtiva eder. Bu durum, kontrollü sodyum diyetinde olan hastalar içinde göz önünde bulundurulmalıdır. **Diğer tıbbi ürünler ile etkileşimler ve diğer etkileşim şekilleri:** SOLIRIS® ile herhangi bir etkileşim çalışması yapılmamıştır. **Çocuk doğurma potansiyeli bulunan kadınlar/Doğum kontrolü (Kontrasepsiyon):** Çocuk doğurma potansiyeli bulunan kadınların, tedavi sırasında ve tedavi bitiminden 5 ay sonrasına kadar etkili doğum kontrol yöntemi kullanması gerekmektedir. **Gebelik dönemi:** SOLIRIS® gebelik döneminde sadece kesin olarak gerekli olduğu durumlarda kullanılmalıdır. **Laktasyon dönemi:** Ekulizumabın insan sütüyle atılıp atılmadığı bilinmemektedir. Birçok tıbbi ürün ve immünoglobulin insan sütüne geçtiğinden ve emzirilen bebeklerde ciddi advers reaksiyon olasılığı bulunduğundan, tedavi sırasında ve tedavi bitiminden 5 ay sonrasına kadar emzirmeye ara verilmelidir. **Araç ve makine kullanımı üzerindeki etkiler:** Etkisi bulunmamaktadır ya da göz ardı edilebilir etkisi bulunmaktadır. **İstenmeyen etkiler:** En yaygın bildirilen advers reaksiyon; baş ağrısı ve tüm meningokokkal enfeksiyonlardan en sık bildirilen ciddi advers reaksiyon meningokokkal sepsistir. Çok yaygın yan etkiler (≥1/10): Baş ağrısı. Yaygın yan etkiler (≥1/100 ila <1/10): Pnömoni, Üst solunum yolu enfeksiyonu, Nazofarenjit, İdrar yolu enfeksiyonu, Ağızda uçuk, Lökopeni, Anemi, İnsomnia, Sersemlik hali, Tat alma duyusunda bozukluk, Tremor, Hipertansiyon, Öksürük, Orofaringeal ağrı, Diyare, Kusma, Bulantı, Abdominal ağrı, Döküntü, Kaşıntı, Alopesi, Artralji, Miyalji, Ekstremitelerde ağrı, Ateş, Üreme, Yorgunluk, Grip benzeri hastalık. Advers reaksiyonların tam listesi için KÜB'e bakınız. PNH ve aHÜS klinik çalışmaları dahil tüm klinik çalışmalarda, en ciddi advers reaksiyon meningokokkal septisemidir. PNH'li hastaların %2'sinde ELISA yöntemi kullanılarak ve aHÜS'lü hastaların %3'ünde EQL köprü formatı testi kullanılarak SOLIRIS®'e karşı antikorlar belirlenmiştir. PNH klinik çalışmalarında SOLIRIS® dozunun unutulduğu ya da geciktirildiği durumlarda hemoliz olguları bildirilmiştir. aHÜS klinik çalışmalarında SOLIRIS® dozunun unutulduğu ya da geciktirildiği durumlarda TMA olguları bildirilmiştir. Pediyatrik hastalarda bildirilen en yaygın advers reaksiyon baş ağrısı olmuştur. Farklı pediyatrik yaş alt gruplarında güvenlilik profillerinin benzer olduğu görülmüştür. **Doz aşımı:** Doz aşımı vakası bildirilmemiştir. **Raf ömrü:** 30 ay. Seyreltildikten sonra, tıbbi ürün hemen kullanılmalıdır. Bununla birlikte kimyasal ve fiziksel olarak 2°C - 8°C'de 24 saat stabil kaldığı gösterilmiştir. **Saklamaya yönelik özel tedbirler:** 2°C - 8°C arasında buzdolabında saklayınız. Dondurmayınız. Işıktan korumak için orijinal ambalajında saklayınız. Orijinal ambalajındaki flakonlar yalnızca bir kez en fazla 3 günlük bir süre için buzdolabından çıkarılabilir. Bu sürenin sonunda ürün tekrar buzdolabına konulabilir. **Ruhsat sahibi:** Alexion İlaç Ticaret Ltd. Şti. Büyükdere Cad. Maya Akar Center 100-102 B Blok 2. Zemin Kat No: 5-6-7-8 34394 Esentepe-Şişli/İstanbul Tel:+90(0)212 355 54 00 Faks:+90(0)212 355 54 29 **Ruhsat Tarihi ve Numarası:** 02.10.2015 - 2015/806 **Güncel KÜB onay tarihi:** 12.02.2018 **KÜB özeti onay tarihi:**01.10.2018 / **versiyonu:** 2.0. Çocukların ulaşamayacakları yerlerde ve ambalajında saklanmalıdır. SOLIRIS® Risk Yönetim programı ve Kontrollü dağıtım kapsamında kısıtlanmış reçete ile satılır. 05.06.2018 tarihi itibariyle Perakende Satış Fiyatı (KDV dahil): SOLIRIS® (Ekulizumab) 300 mg/30 ml konsantre infüzyon çözeltisi 22,719.96 TL. Daha geniş bilgi için firmamıza başvurunuz. www.alexionpharma.com.tr

TR/SOL-P/180023

* Şüpheli advers reaksiyonların raporlanması
Ruhsatlandırma sonrası şüpheli ilaç advers reaksiyonlarının raporlanması büyük önem taşımaktadır. Raporlama yapılması, ilacın yarar/risk dengesinin sürekli olarak izlenmesine olanak sağlar. Sağlık mesleği mensuplarının herhangi bir şüpheli advers reaksiyonu Türkiye Farmakovijilans Merkezine (TÜFAM) bildirmeleri gerekmektedir (www.titck.gov.tr; e-posta: tufam@titck.gov.tr; tel: 0 800 314 00 08; faks: 0 312 218 35 99).

ALEXION

SOLIRIS® (ekulizumab)

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SOLIRIS®
(ekulizumab)

Erişkinlerde ve çocuklarda **paroksismal noktürnal hemoglobinüri (PNH) olan hastaların tedavisinde endikedir.**

Klinik semptom ve hemoliz gösteren yüksek hastalık aktivitesi olan hastalarda klinik fayda kanıtı, transfüzyon öyküsünden bağımsız olarak gösterilmiştir.

Erişkinlerde ve çocuklarda **atipik hemolitik üremik sendrom (aHÜS) olan hastaların tedavisinde endikedir.**

ALEXION

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PNH pediyatrik hast alar (<1 8 ya ş) ≥40 k g idame fazı: Beşinci haftada 900 mg, ardından her iki haftada bir 900 mg. aHÜS pediyatrik hastalar (<18 yaş) ≥40 kg başl angıç fazı: Haftalık 900 mg x 4 hafta. aHÜS pediyatrik hastalar (<18 yaş) ≥40 kg idame fazı: Beşinci haftada 1200 mg, ardından her iki haftada bir 1200 mg. aHÜS ve PNH pediy atrik hastalar 30 il a <40 kg başl angıç fazı: Haftalık 600 mg x 2 hafta. aHÜS ve PNH pediyatrik hastalar 30 ila <40 kg idame fazı: Üçüncü haftada 900 mg, ardından her iki haftada bir 900 mg. aHÜS ve PNH pediyatrik hastalar 20 ila <30 kg başlangıç fazı: Haftalık 600 mg x 2 hafta. aHÜS ve PNH pediyatrik hastalar 20 ila <30 kg idame fazı: Üçüncü haftada 600 mg, ardından her iki haftada bir 600 mg. aHÜS ve PNH pediyatrik hastalar 10 ila <20 kg başlangıç fazı: Haftalık 600 mg x 1 hafta. aHÜS ve PNH pediyatrik hastalar 10 ila <20 kg idame fazı : İkinci haftada 300 mg, ardından her iki haftada bir 300 mg. aHÜS ve PNH pediyatri k hastalar 5 il a <10 kg başlangıç fazı : Haftalık 300 mg x 1 hafta. aHÜS ve PNH pediyatrik hastalar 5 ila <10 kg idame fazı: İkinci haftada 300 mg, ardından her üç haftada bir 300 mg. Eşzamanlı plazma tedavisinde ek SOLİRİS® dozu gereklidir, ayrıntılı bilgi için KÜB'e bakınız. Uygulama şekli: 5 mg/ml konsantrasyona seyreltilir. Seyreltilmiş SOLIRIS® çözeltisi, bir yerçekimi-beslemeli, enjektör tipi pompa ya da bir infüzyon pompası aracılığıyla, erişkinlerde 25-45 dakikalık ve pediyatrik hastalarda 1-4 saatlik intravenöz infüzyon yoluyla uygulanmalıdır. Hastalar infüzyonu takiben bir saat boyunca izlenmelidir. Geriyatrik popülasyon: Sınırlı deneyim olmasına karşın özel bir önlemin gerekli olduğuna işaret eden herhangi bir kanıt mevcut değildir. Karaciğer yetmezliği: Güvenliliği ve etkililiği incelenmemiştir. Böbrek yetmezliği: Doz ayarlaması gerekli değildir. Tedavi takibi: SOLİRİS®'in bırakılması klinik olarak endike olmadıkça, SOLIRIS® tedavisinin hastanın yaşamı boyunca devam ettirilmesi önerilmektedir. PNH hastaları, serum laktat dehidrogenaz (LDH) düzeyleri de dahil olmak üzere intravasküler hemoliz bulgu ve belirtileri açısından takip edilmelidir. aHÜS hastaları, trombotik mikroanjiyopatinin (TMA) bulgu ve belirtileri açısından takip edilmelidir. **Kontrendikasyonlar:** Ekulizumab, mürin proteinleri ya da diğer yardımcı maddelere karşı aşırı duyarlılığı olduğu bilinen hastalarda kontrendikedir. İyileşmemiş Neisseria meningitidis enfeksiyonu olan hastalarda, aşılama sonrasında 2 haftaya kadar uygun antibiyotiklerle profilaktik tedavi almamış ve halihazırda Neisseria meningitidis'e karşı aşılanmamış hastalarda SOLIRIS® tedavisi başlatılmamalıdır. **Özel kullanım uyarıları ve önlemleri:** Meningokokkal enfeksiyon: SOLİRİS® etki mekanizması gereği ciddi veya ölümcül meningokokkal enfeksiyon riskinde artışa neden olmaktadır. Bu nedenle hastalara SOLIRIS® kullanımından en az iki hafta önce meningokokkal enfeksiyonlara karşı koruyucu aşı uygulaması mutlak suretle yapılmalıdır. Ayrıca, aşılama sonrasında iki haftadan daha kısa süre içinde SOLİRİS® tedavisinin başlanması söz konusu olduğu durumlarda ise aşı uygulamasına ilave olarak mutlaka meningokok enfeksiyonuna karşı, ilgili resmi kılavuzlar da göz önüne alınarak antimikrobiyal kemoprofilaksi uygulanmalıdır. Mevcut durumda dört bileşenli (A, C, Y ve W135) konjuge meningokok aşısı mutlaka yapılmalıdır; ayrıca konjuge meningokok B aşısının yapılması da tercih edilmelidir. Bütün bu önlemlere rağmen meningokokkal enfeksiyon için aşılama yeterli olmayabilir. Bu nedenle SOLİRİS® kullanan tüm hastalar meningokokkal enfeksiyonun erken bulguları açısından izlenmeli, enfeksiyondan şüphelenildiği durumlarda acilen değerlendirilmeli ve gerekirse uygun antibiyotiklerle tedavi edilmelidir. Ayrıca SOLIRIS® kullanan hastalar bu bulgu ve belirtiler ile acilen tıbbi bakım alınması için atılması gereken adımlar konusunda bilgilendirilmelidir. Hekimler SOLIRIS® tedavisinin yararlarını ve risklerini hastalarla tartışmalı ve hastalara bilgi broşürü ve hasta güvenlilik bilgisi kartı vermelidir. Aşılama, komplemanda ek aktivasyona neden olabilir. Bunun sonucunda, PNH ve aHÜS dahil kompleman-aracılı hastalıkları olan hastaların, altta yatan hastalıklarının hemoliz (PNH) ya da TMA (aHÜS) gibi bulgu ve belirtilerinde artış yaşanabilir. Bu nedenle, önerilen aşılama sonrasında hastalık belirtileri açısından hastalar yakından takip edilmelidir. Aşının kontrendike olduğu veya yapılamadığı durumlarda ve meningokok aşısı uygulamasını takip eden ilk iki hafta içerisinde SOLIRIS® kullanan hastalara veya gerekli durumlarda tedavi periyodu boyunca antibiyotik profilaksisi uygulanmalıdır. Ayrıca SOLİRİS®'in etki mekanizması gereği kapsüllü mikroorganizmalara karşı verilen cevapta azalma olması nedeniyle bugün için kanıtlanmamış olmakla birlikte pnömokokkal hastalık riskinin de artması beklenir ve bu sebeple pnömokok aşısı da önerilmektedir. Mevcut koşullarda çocukluk çağında rutin konjuge pnömokok aşı uygulaması yapıldığı için bunun sorgulanması, daha önce pnömokok aşısı ile aşılanmamış kişilerin bir doz konjuge pnömokok aşısı ile aşılanması ve tercihen en az sekiz hafta sonra da 23 bileşenli polisakkarit pnömokok aşısı ile aşılanması önerilmelidir. Altı yaş altında rutin aşı takvimi sorgulanmalı, Hemofilus influenza tip B ve pnömokok aşısı eksik ise yaşa uygun şekilde yapılmalıdır. Ek olarak Genişletilmiş Bağışıklama Programına uygun olarak aşılama ve takibi yapılmalı ve ayrıca SOLIRIS® kullananların düzenli mevsimsel influenza (grip) aşıları olmalı da önerilmelidir. Diğer sistemik enfeksiyonlar: Etki mekanizmasından dolayı SOLIRIS® tedavisi, aktif sistemik enfeksiyonların olduğu hastalara dikkatli bir şekilde uygulanmalıdır. Hastaların enfeksiyonlara, özellikle kapsüllü bakterilerin neden olduğu enfeksiyonlara duyarlılığı artmış olabilir. İnfüzyon reaksiyonları: SOLİRİS®'in uygulanması infüzyon reaksiyonlarına ya da alerjik veya aşırı duyarlılık reaksiyonlarına (anafilaksi de dahil) yol açabilecek immünojenisiteye neden olabilir. Şiddetli infüzyon reaksiyonları görülen tüm hastalarda SOLIRIS® uygulaması durdurulmalı ve uygun tıbbi tedavi uygulanmalıdır. Antikoagülan tedavi: SOLİRİS® tedavisi antikoagülan tedavisini değiştirmemelidir. Laboratuvar takibi: PNH hastaları, serum LDH düzeyleri de dahil olmak üzere intravasküler hemoliz bulgu ve belirtileri açısından takip edilmelidir. SOLIRIS® tedavisi gören PNH hastaları da benzer şekilde, LDH düzeyleri ölçülerek intravasküler hemoliz için takip edilmelidir ve bu hastalarda, idame fazı boyunca önerilen 14 ± 2 gün doz uygulaması takvimi içinde doz ayarlaması gerekli olabilir (her 12. güne kadar). SOLIRIS® tedavisi gören aHÜS hastaları, trombosit sayımları, serum LDH ve serum kreatinin ölçümleri ile TMA için takip edilmelidir ve bu hastalarda, idame fazı boyunca önerilen 14 ± 2 gün doz uygulaması takvimi içinde doz ayarlaması gerekli olabilir (her 12. güne kadar). Tedavinin bırakılması: SOLİRİS® tedavisini bırakan PNH hastaları, ciddi intravasküler hemolizin bulgu ve belirtileri açısından en az 8 hafta boyunca izlenmelidir. SOLIRIS® bırakıldıktan sonra ciddi hemolizin meydana gelmesi durumunda, aşağıdaki işlemleri/tedavileri uygulamayı düşününüz: kan transfüzyonu (paketlenmiş eritrositler) ya da akış sitometrisine göre PNH eritrositleri toplam eritrositlerin %50'sinden fazla ise değiş-tokuş transfüzyonu; antikoagülasyon; kortikosteroidler; ya da SOLİRİS® tedavisinin yeniden başlatılması. SOLIRIS® tedavisini bırakan aHÜS hastaları, şiddetli TMA komplikasyonlarının bulgu ve belirtileri açısından yakından izlenmelidir. İzleme, SOLİRİS®'in bırakılmasından sonra aHÜS hastalarında şiddetli TMA komplikasyonlarını önlemek veya öngörmek için yetersiz kalabilir. SOLİRİS®'in bırakılmasından sonra ciddi TMA komplikasyonları ortaya çıkarsa, SOLİRİS® tedavisinin yeniden başlatılması, PD/PI ile destekleyici bakım ya da diyaliz ile böbrek desteği, mekanik ventilasyon ile solunum desteği veya antikoagülasyon içeren uygun, organa spesifik destekleyici önlemler düşünülmelidir. aHÜS klinik çalışmalarında SOLIRIS® tedavisinin bırakılmasını takiben 4 hafta gibi erken bir dönemde ve 127 haftaya kadar TMA komplikasyonları gözlenmiştir. Yardımcı maddeler: SOLİRİS® her bir flakonda 5 mmol sodyum ihtiva eder. Bu durum, kontrollü sodyum diyetinde olan hastalar içinde göz önünde bulundurulmalıdır. **Diğer tıbbi ürünler ile etkileşimler ve diğer etkileşim şekilleri:** SOLİRİS® ile herhangi bir etkileşim çalışması yapılmamıştır. **Çocuk doğurma potansiyeli bulunan kadınlar/Doğum kontrolü (Kontrasepsiyon):** Çocuk doğurma potansiyeli bulunan kadınların, tedavi sırasında ve tedavi bitiminden 5 ay sonrasına kadar etkili doğum kontrol yöntemi kullanması gerekmektedir. **Gebelik dönemi:** SOLİRİS®gebelik döneminde sadece kesin olarak gerekli olduğu durumlarda kullanılmalıdır. **Laktasyon dönemi:** Ekulizumabın insan sütüyle atılıp atılmadığı bilinmemektedir. Birçok tıbbi ürün ve immünoglobulin insan sütüne geçtiğinden ve emzirilen bebeklerde ciddi advers reaksiyon olasılığı bulunduğundan, tedavi sırasında ve tedavi bitiminden 5 ay sonrasına kadar emzirmeye ara verilmelidir. **Araç ve makine kullanımı üzerindeki etkiler:** Etkisi bulunmamaktadır ya da göz ardı edilebilir etkisi bulunmaktadır. **İstenmeyen etkiler:** En yaygın bildirilen advers reaksiyon; baş ağrısı ve tüm meningokokkal enfeksiyonlardan en sık bildirilen ciddi advers reaksiyon meningokokkal sepsistir. Çok yaygın yan etkiler (≥1/10): Baş ağrısı. Yaygın yan etkiler (≥1/100 ila <1/10): Pnömoni, Üst solunum yolu enfeksiyonu, Nazofarenjit, İdrar yolu enfeksiyonu, Ağızda uçuk, Lökopeni, Anemi, İnsomnia, Sersemlik hali, Tat alma duyusunda bozukluk, Tremor, Hipertansiyon, Öksürük, Orofaringeal ağrı, Diyare, Kusma, Bulantı, Abdominal ağrı, Döküntü, Kaşıntı, Alopesi, Artralji, Miyalji, Ekstremitelerde ağrı, Ateş, Ürperme, Yorgunluk, Grip benzeri hastalık. Advers reaksiyonların tam listesi için KÜB'e bakınız. PNH ve aHÜS klinik çalışmaları dahil tüm klinik çalışmalarda, en ciddi advers reaksiyon meningokokkal septisemidir. PNH'li hastaların %2'sinde ELISA yöntemi kullanılarak ve aHÜS'lü hastaların %3'ünde ECL köprü formatı testi kullanılarak SOLIRIS®'e karşı antikorlar belirlenmiştir. PNH klinik çalışmalarında SOLIRIS® dozunun unutulduğu ya da geciktirildiği durumlarda hemoliz olguları bildirilmiştir. aHÜS klinik çalışmalarında SOLIRIS® dozunun unutulduğu ya da geciktirildiği durumlarda TMA olguları bildirilmiştir. Pediyatrik hastalarda bildirilen en yaygın advers reaksiyon baş ağrısı olmuştur. Farklı pediyatrik yaş alt gruplarında güvenlilik profillerinin benzer olduğu görülmüştür. **Doz aşımı:** Doz aşımı vakası bildirilmemiştir. **Raf ömrü:** 30 ay. Seyreltildikten sonra, tıbbi ürün hemen kullanılmalıdır. Bununla birlikte kimyasal ve fiziksel olarak 2°C - 8°C'de 24 saat stabil kaldığı gösterilmiştir. **Saklamaya yönelik özel tedbirler:** 2°C - 8°C arasında buzdolabında saklayınız. Dondurmayınız. Işıktan korumak için orijinal ambalajında saklayınız. Orijinal ambalajındaki flakonlar yalnızca bir kez en fazla 3 günlük bir süre için buzdolabından çıkarılabilir. Bu sürenin sonunda ürün tekrar buzdolabına konulabilir. **Ruhsat sahibi:** Alexion İlaç Ticaret Ltd. Şti. Büyükdere Cad. Maya Akar Center 100 – 102 B Blok 2. Zemin Kat No: 5-6-7-8 34394 Esentepe – Şişli / İstanbul Tel: +90(0)212 355 54 00 Faks: +90(0)212 355 54 29 **Ruhsat Tarihi ve Numarası:** 02.10.2015 - 2015/806 **Güncel KÜB onay tarihi:** 12.02.2018 **KÜB özeti onay tarihi:** 01.10.2018/ **versiyonu:** 2.0. Çocukların ulaşamayacakları yerlerde ve ambalajında saklanmalıdır. 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* Şüpheli advers reaksiyonların raporlanması
Ruhsatlandırma sonrası şüpheli ilaç advers reaksiyonlarının raporlanması büyük önem taşımaktadır. Raporlama yapılması, ilacın yarar/risk dengesinin sürekli olarak izlenmesine olanak sağlar. Sağlık mesleği mensuplarının herhangi bir şüpheli advers reaksiyonu Türkiye Farmakovijilans Merkezine (TÜFAM) bildirmeleri gerekmektedir (www.titck.gov.tr; e-posta: tufam@titck.gov.tr; tel: 0 800 314 00 08; faks: 0 312 218 35 99).

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