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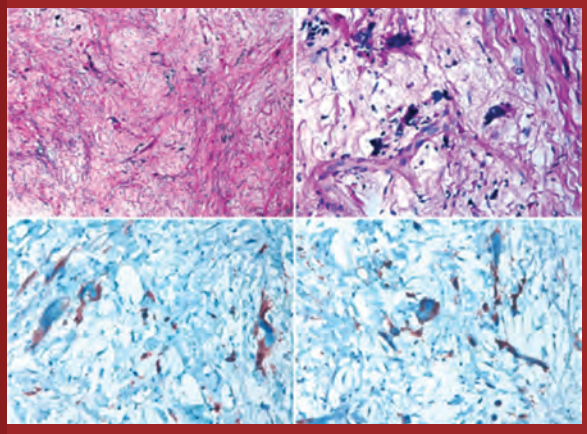
The Official Journal of the Turkish Society of Hematology

Review

Research Articles



Cover Picture:
Kemal Deniz et al.
Sclerosing Extramedullary Hematopoietic Tumor

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Cover Picture

Kemal Deniz et al.,
Sclerosing Extramedullary Hematopoietic Tumor
CD41 and factor 8 positive atypical megakaryocytes in sclerosing extramedullary hematopoietic tumor.

Türk Hematoloji Derneği, 07.10.2008 tarihli ve 6 no'lu kararı ile Turkish Journal of Hematology'nin Türk Hematoloji Derneği İktisadi İşletmesi tarafından yayınlanmasına karar vermiştir.

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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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Manuscripts should be prepared according to ICMJE guidelines (http://www.icmje.org/). Original manuscripts require a structured abstract. Label each section of the structured abstract with the appropriate subheading (Objective, Materials and Methods, Results, and Conclusion). Letters to the editor do not require an abstract. Research or project support should be acknowledged as a footnote on the title page. Technical and other assistance should be provided on the title page.

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.

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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 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.

Discussion: This section should include a discussion of the data. New and important findings/results and the conclusions they lead to should be emphasized. Link the conclusions with the goals of the study, but avoid unqualified statements and conclusions not completely supported by the data. Do not repeat the findings/results in detail; important findings/results should be compared with those of similar studies in the literature, along with a summarization. In other words, similarities or differences in the obtained findings/results with those previously reported should be discussed.

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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.

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Materials and Methods: Clearly describe the selection of the observational or experimental participants. Identify the methods and procedures in sufficient detail. Provide references to established methods (including statistical methods), provide references to brief modified methods, and provide the rationale for their use and an evaluation of their limitations. Identify all drugs and chemicals used, including generic names, doses, and routes of administration.

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.

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Review articles should not include more than 100 references. Reviews should include a conclusion, in which a new hypothesis or study about the subject may be posited. Do not publish methods for literature search or level of evidence. Authors who will prepare review articles should already have published research articles on the relevant subject. The study's new and important findings should be highlighted and interpreted in the Conclusion section. There should be a maximum of two authors for review articles.

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Artificial Intelligence Approaches in Hematopoietic Cell Transplantation: A Review of the Current Status and Future Directions

Hematopoietik Hücre Transplantasyonunda Yapay Zeka Yaklaşımları: Günümüzdeki Durum ve Gelecekteki Yönelimler Hakkında Bir Derleme

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Abstract

The evidence-based literature on healthcare is currently expanding exponentially. The opportunities provided by the advancement in artificial intelligence (AI) tools such as machine learning are appealing in tackling many of the current healthcare challenges. Thus, AI integration is expanding in most fields of healthcare, including the field of hematology. This study aims to review the current applications of AI in the field of hematopoietic cell transplantation (HCT). A literature search was done involving the following databases: Ovid MEDLINE, including In-Process and other non-indexed citations, and Google Scholar. The abstracts of the following professional societies were also screened: American Society of Hematology, American Society for Blood and Marrow Transplantation, and European Society for Blood and Marrow Transplantation. The literature review showed that the integration of AI in the field of HCT has grown remarkably in the last decade and offers promising avenues in diagnosis and prognosis in HCT populations targeting both pre- and post-transplant challenges. Studies of AI integration in HCT have many limitations that include poorly tested algorithms, lack of generalizability, and limited use of different AI tools. Machine learning techniques in HCT are an intense area of research that needs much development and extensive support from hematology and HCT societies and organizations globally as we believe that this will be the future practice paradigm.

Keywords: Artificial intelligence, Machine learning, Hematopoietic cell transplant

Öz

Sağlık hizmetine yönelik kanıta dayalı literatür günümüzde katlanarak artmaktadır. Makine öğrenimi gibi yapay zeka (YZ) araçlarındaki ilerlemenin sağladığı fırsatlar ile mevcut sağlık hizmetindeki zorlukların pek çoğunun üstesinden gelinecek gibi görünmektedir. Bu yüzden, YZ entegrasyonu sağlık hizmetinin hematoloji alanı dahil çoğu alanında artmaktadır. Bu çalışmada hematopoietik hücre transplantasyonunda (HHT) mevcut YZ uygulamalarının gözden geçirilmesi hedeflenmektedir. İzleyen veri tabanlarını içeren bir literatür taraması yapılmıştır: Ovid MEDLINE, Google Akademik, işlemde olan ve dizine eklenmemiş diğer alıntılar dahil. Aşağıdaki profesyonel derneklerin özetleri de taranmıştır: Amerikan Hematoloji Derneği, Amerikan Kan ve Kemik İliği Transplantasyon Derneği ve Avrupa Kan ve Kemik İliği Transplantasyon Derneği. Bu literatürlerin taranması ile HHT alanına YZ'nin entegrasyonunun son on yılda anlamlı ölçüde arttığı ve transplant öncesi ve sonrası zorlukların hedeflendiği HHT popülasyonun teşhis ve prognozunda umut vaat eden yollar sunduğu görülmüştür. HHT'ye YZ'nin entegrasyonu ile ilgili çalışmalar, yetersiz test edilmiş algoritmalar, genelleştirilebilme eksikliği ve farklı YZ araçlarının sınırlı kullanımı gibi pek çok sınırlamalara sahiptir. Geleceğin uygulama paradigması olacağına inandığımız HHT'de makine öğrenim teknikleri, daha fazla gelişmeye ve hematolojiden, HHT derneklerinden ve organizasyonlarından yoğun desteğe ihtiyaç duyan araştırmanın yoğun olduğu bir alandır.

Anahtar Sözcükler: Yapay zeka, Makine öğrenimi, Hematopoietik hücre transplantasyonu

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Introduction

About sixty years ago, a Dartmouth conference established the basis of artificial intelligence (AI). The name was coined for the use of technology in accomplishing tasks that usually need human intelligence. These tasks include, but are not limited to, interpreting language, making decisions, and applying visual perception [1,2]. Soon after the conference, the AI field started to develop exponentially. One major example was the DENDRAL project of Stanford University that started in the early 1960s. DENDRAL used heuristic programming to provide solutions in the field of science [3].

Integration of AI in medicine started about a decade after the Dartmouth conference [1]. MYCIN was one of the early medical programs developed from DENDRAL to detect bacteria causing infections and to decide on appropriate antimicrobials and their doses. This program achieved a rate of agreement of 60% when compared to decisions based on human expertise. Despite the suboptimal rate of agreement, it was able to cover all treatable pathogens and was showed to decrease the number of antimicrobials used [4]. This was followed by many other AI tools, such as Internist-I, that were developed to help medical practitioners [1,5].

The use of AI in medicine has led to a debate about how beneficial AI is in improving medical practice. Advocates of such integration list advantages such as increasing efficiency and helping medical practitioners to practice medicine in its real meaning. On the contrary, opponents of such integration cite different disadvantages that include concerns about the accuracy of these systems, the risk of having "deskilled" physicians, and fewer future jobs, especially in diagnostic medical fields such as radiology and pathology [6,7].

Despite the possible disadvantages and skepticism, the increasing complexity of medical practice and the opportunities provided by the advancements in AI make integration inevitable. Thus, growing numbers of projects have tried to integrate the tools that AI provides into different fields of medicine including hematology and oncology. Examples of integration are numerous. For instance, Watson for Oncology (WFO) is a project created by IBM Corporation that can cope with expanding evidence and learn from cases [8]. The project's results are promising; for example, a 93% level of concordance was achieved by WFO when compared to physician-led tumor board decisions for breast cancer treatment plans. This level was even higher in stages II and III of breast cancer [9]. The use of AI in the fields of hematology and oncology is not limited to treatment decisions and plans. For example, different studies investigated the use of AI in leukemia diagnosis, management, and prognosis [10,11,12].

The field of hematopoietic cell transplantation (HCT) is expanding, with more than 60,000 procedures being performed annually worldwide [13]. It is also estimated that by 2020 the world will have half a million HCT survivors [14]. The rapid expansion of the field necessitates the augmenting of tools provided by AI to increase efficiency and improve patient care. Thus, this review aims to investigate the status of AI integration in the field of HCT and list some future directions and research agenda.

Methods

The literature review used Boolean logic with terms including "Machine learning", "Deep learning", "Neural networks", and "Artificial intelligence" in combination with terms specific to the field of HCT such as "Bone marrow transplant", "Hematopoietic cell transplant", "Graft-versus-host disease", etc. The search targeted the last 10 years due to the growth of the AI field in hematology, oncology, and HCT. The following databases were used: Ovid MEDLINE, including In-Process and Other Non-Indexed Citations, and Google Scholar. Abstracts presented at the annual meetings of the American Society of Hematology (ASH), American Society for Blood and Marrow Transplantation (ASBMT), and European Society for Blood and Marrow Transplantation (EBMT) were screened as well to avoid file-drawer bias. The terms used to screen the abstracts were "Artificial intelligence" and "Machine learning".

Results

The number of abstracts of studies investigating the use of AI in the field of hematology has increased over the years. Figure 1 shows the number of abstracts presented in the field of AI in the meetings of three major hematological societies (ASH, ASBMT, and EBMT) from 2010 to 2017. It can be noted from the figure that the number of AI abstracts presented in these meetings increased 8 times between 2010 and 2017. This increase indicates the increasing focus on and advancements in potential uses of AI in hematology. On the other hand, the number of such abstracts presented in the field of HCT increased from none in 2010 to 5 in 2017.

This literature search revealed many studies that investigated the use of AI tools in improving different aspects of HCT. These studies have targeted both pre- and post-transplant applications and are discussed below.

Pre-transplant Applications

Selection of donor and recipient pairs for HCT is a major challenge that could affect the prognosis of HCT recipients. An HLA-matched sibling can be found only in 30% of cases of HCT in the United States [15]. Lee et al. [16] found that one locus mismatch in donors can decrease 1-year survival to 43% from 52% in fully matched recipient-donor pairs, and this risk increases when more loci mismatches are present. Different

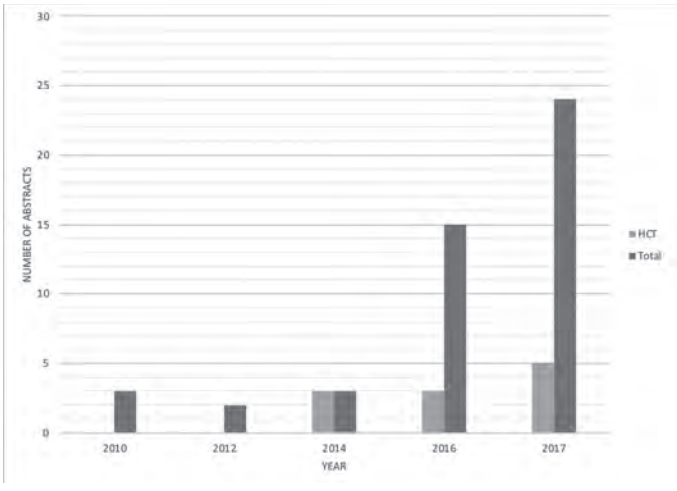

Figure 1. Number of artificial intelligence (AI) abstracts presented at American Society of Hematology, American Society for Blood and Marrow Transplantation, and European Society for Blood and Marrow Transplantation meetings from 2010 to 2017. The number of AI abstracts presented at these meetings increased 8 times during this time period, whereas the number of abstracts presented in the field of hematopoietic cell transplantation increased from none in 2010 to 5 in 2017.

HCT: Hematopoietic cell transplantation.

studies investigated the possible use of AI methods and tools to tackle this challenge. Marino et al. [17] identified 19 amino acid substitutions related to at least one bad outcome following HCT using random forest and logistical regression methods. These included overall survival, treatment-related mortality, incidence of graft-versus-host disease (GVHD), etc. However, none of these substitutions were able to pass the validation test in an independent cohort. This was also the case for a recent study by Buturovic et al. [18], in which different factors that included donor, recipient, and transplantation characteristics were used to create an algorithm using machine learning (ML). This algorithm aimed to increase survival of HCT recipients secondary to acute leukemia (AL) by improving the selection of donors. Despite optimistic preliminary results, the algorithm failed the validation study.

More methods have been proposed to develop algorithms that can help in the selection of donor-recipient pairs. For instance, two abstracts [19,20] proposed the use of different ML tools to aid this process. Sarkar and Srivastava [19] developed an algorithm that used both HLA and killer-cell immunoglobulin-like receptor to improve the selection of donors for recipients with acute myelogenous leukemia (AML). The algorithm was able to increase the accuracy of predictions by 3%-4% compared to the usual analysis. Sivasankaran et al. [20] proposed a black-box model in developing a system that uses secondary non-HLA characteristics in selecting donors, though no data on the validation or improvement of accuracy have been reported to date.

Post-transplant Applications

Despite all the advances in HCT, recipients of HCT are at risk of many complications that might increase their mortality and morbidity, including GVHD [21,22]. Thus, predicting recipients' risk of developing these complications and their prognosis would aid clinicians in making better decisions that would improve patients' quality of life and survival.

One of the major projects in this field is AL-EBMT. In 2015, the EBMT developed the AL-EBMT predictive model to stratify AL patients according to their prognosis following allogeneic HCT [23]. AL-EBMT (http://bioinfo.lnx.biu.ac.il/~bondi/web1.html) [24] was externally validated using an Italian transplantation network cohort [GITMO (Gitmo Onlus Gruppo Italiano Trapianto Midollo Osseo)]. The results showed that AL-EBMT was a valid tool in stratifying the risk of AL patients undergoing HCT. It was able to predict 100-day mortality, leukemia-free survival, 2-year overall survival, and non-relapse-related mortality with values of the area under the receiver operating curves ranging from 0.651 to 0.698 [25]. However, the tool cannot be generalized to other non-European populations.

Studies have also investigated the use of AI tools in predicting the outcomes of HCT. Li et al. [26] proposed using an AI approach in predicting allogeneic HCT outcome in AML and Myelodysplastic syndrome (MDS) by using pre-transplant minimal residual disease (MRD). MRD detection traditionally takes place using flow cytometry with physicians' interpretations, and this leads to considerable variability in interpretations. The ML approach was applied to a training set and then confirmed using a validation set. The approach was found to differentiate between abnormal (MDS or AML) and normal cases by 90.8% in the training set and 84.4% in the validation set. The system was also 100 times faster than experts in getting interpretations of results.

Graft-Versus-Host-Disease

In addition to the use of AI approaches in diagnosis, Gandelman et al. [27] showed that ML tools offered a chance for classifying chronic GVHD into new phenotypes related to survival. However, this new classification system will need to be validated.

Predicting the development of acute GVHD was investigated by Caocci et al. [28] in 78 thalassemia patients who underwent unrelated allogeneic HCT using artificial neural networks (ANNs). The ANN was compared to results acquired by logistical regression. The authors found that the ANN was significantly more sensitive in predicting acute GVHD in patients who developed it, but no difference was noted in predicting the absence of GVHD. This finding was supported by a recently presented EBMT abstract [29], which showed the superiority of ML models when compared to classical models such as logistical regression in predicting 100-day treatment-related mortalities

after allogeneic HCT. The literature search yielded very few technical studies that explored and compared methods to increase the accuracy of AI approaches and tools.

Furthermore, few studies have indicated the limitations remaining or the methods that will help us to reach optimal use of ML. For instance, Shouval et al. [30] investigated the development of multiple models able to predict 100-day non-relapse mortality after HCT. Their findings suggested the need for broader data input from patients to be able to increase the predictive ability of models developed by AI, including biologic and genetic factors. Elhassan et al. [31] investigated the use of different sampling techniques to improve the accuracy of ML algorithms. They concluded that the use of sampling techniques, including random oversampling, synthetic oversampling, and remote undersampling, improved the accuracy of ML algorithms in predicting 100-day treatment-related mortality in allogeneic HCT.

Discussion

The complexity of the healthcare system and the amount of medical literature and evidence have increased tremendously in the last few decades and it is nearly impossible for a practicing physician to keep up with all published literature, even in a narrow field of practice. This is accompanied by the need for more documentation, especially with the emergence of electronic medical records and electronic health records (EHRs). These electronic records may influence the effectiveness of medical practitioners and can make it difficult for physicians to practice the real meaning of medicine [32,33]. On the other hand, these tools have made it easier to reach patient data, especially in the case of EHRs. In the era of "big data", EHRs act as sources of data that can be used to improve research and healthcare [34,35]. Moreover, data soon might be regional or even international with the help of registries [36]. Thus, EHRs and registries will provide AI systems with sets needed for training and validation. AI systems will also likely revolutionize EHRs to be more automated, thus giving medical practitioners more time to spend with their patients [6,14].

AI approaches using different tools might be an opportunity to use big data to extrapolate and create beneficial algorithms that can be applied for other patients. Despite the many advantages that can be provided by integrating AI in medicine, many disadvantages may also occur. These include endangering some types of medical jobs, possible technical errors, and deskilling [6,37]. Many of these disadvantages might be exaggerated as AI approaches are not alternatives but rather extensions of our currently used statistical tools [38]. These new approaches and tools will play a major role in the future of medicine.

AI integration has been shown to be reliable, accurate, and promising in various instances. For instance, Weng et al. [39] used different ML approaches to create algorithms that can predict the risk of developing cardiac events within 10 years. AI approaches were found to be superior in predicting the risk of developing cardiac events compared to the established American College of Cardiology algorithm. WFO is another example of a project that holds a lot of potential for improving the care delivered to cancer patients [9]. Improvement in diagnosis and efficiency is also expected in diagnostic fields such as radiology [40]. The implementation of AI seems to be inevitable and more applications will soon be in practice.

Moreover, future research is expected to develop more tools that have more ability in detecting patterns in unstructured and unsupervised data. The concurrent development of tools for data collection that is more instant and real-time is important to increase the amount of big data. This is evident in the parallel advancements in the field of the Internet of Things, which will be able to advance our methods in collecting data via connecting the various tools we use in clinical practice (e.g., wearables, thermometers, stethoscopes) directly to our EHRs and databases [41]. This will be an opportunity for us to use more real-time data that will help us to develop more accurate databases that can be later applied by the tools of AI.

In this review, however, we demonstrate that the integration of AI in the field of HCT is still an area that needs much development. The published literature did not tackle many important aspects of HCT including survivorship, risk of infections, or pharmacogenomics. For instance, with the increasing number of HCTs done and improved management, it is expected that there will be half a million long-term HCT survivors by 2020 [14,34]. Thus, AI offers a great opportunity to help provide these patients with optimal longitudinal care.

We have summarized many promising pre- and post-transplant studies of ML in HCT; however, these studies have many limitations. Most of these studies are still in a preliminary phase, a training set applied with a small sample size limits their power, and some of these studies have not confirmed their findings with a validation set. Other limitations include the need for technical studies that investigate the efficiency and accuracy of different AI methods and approaches. One of the concerns about using AI in the field of HCT and other medical fields is the generalizability of the systems. A system such as AL-EBMT [23] needs to be validated on other populations to be eligible for use. However, the horizon includes many opportunities, especially with the increase in the number of registries and data (e.g., CIBMTR, EBMT). Moreover, AI integration should be supported by HCT and hematology societies globally to ensure that AI applications are well validated and can be used. Given the presence of big data in international HCT registries, the HCT community can utilize ML technologies to its benefit to improve both patient outcomes and system efficiency.

AI integration in HCT is expanding and its role in daily activities of clinical practice is inevitable. It is time for our research and clinical community to step forward and incorporate ML usage with the existing models. Though this would be a cutting-edge advancement, AI's integration should be cautious and must target improvements in patient care rather than a focus on technological improvements. It should be incorporated in practice, but it should not take us away from the sine qua non of medicine as an oral science and our roles as healers.

Conclusion and Future Directions

Implementation of AI in HCT is still suboptimal. Future studies should try to involve more data for both training and validation sets. This necessitates more funding and support from different HCT and hematology societies globally as well as from government agencies. This support will allow AI tools to be of better quality and be generalizable.

Integration of AI in medicine is inevitable. However, this integration should be cautious and well validated to improve patient care. Some concerns regarding AI use are valid and should be considered when using AI tools. The aim of AI should be to improve medical practice and healthcare.

Authorship Contributions

Concept: I.N.M., T.E., S.K.H.; Design: I.N.M., T.E., S.K.H.; Data Collection or Processing: I.N.M., T.E., S.K.H.; Analysis or Interpretation: I.N.M., T.E., S.K.H.; Literature Search: I.N.M., T.E., S.K.H.; Writing: I.N.M., T.E., S.K.H.

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RESEARCH ARTICLE

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FLT3-ITD Compared with DNMT3A R882 Mutation Is a More Powerful Independent Inferior Prognostic Factor in Adult Acute Myeloid Leukemia Patients After Allogeneic Hematopoietic Stem Cell Transplantation: A Retrospective Cohort Study

Allojenik Hematopoetik Kök Hücre Nakli Sonrası Yetişkin Akut Myeloid Lösemi Hastalarında, FLT3-ITD, DNMT3A R882 Mutasyonu ile Karşılaştırıldığında Daha Güçlü Bir Bağımsız Kötü Prognostik Faktördür: Retrospektif Kohort Çalışması

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Abstract

Objective: This study aimed to evaluate DNMT3A exon 23 mutations and their prognostic impacts in the presence of NPM1 and FLT3 mutations in acute myeloid leukemia (AML) patients who underwent allogeneic hematopoietic stem cell transplantation (HSCT).

Materials and Methods: This study comprised 128 adult AML patients referred to the Hematology-Oncology and Stem Cell Research Center of Shariati Hospital. NPM1 and FLT3-ITD mutations were detected by fragment analysis. For DNMT3A exon 23 mutation analysis, we used Sanger sequencing. Overall survival (OS) and relapse-free survival (RFS) curves were estimated by the Kaplan-Meier method and the log-rank test was used to calculate differences between groups.

Results: The prevalence of DNMT3A exon 23 mutations was 15.6% and hotspot region R882 mutations were prominent. RFS and OS were compared in patients with and without DNMT3A exon 23 mutations using univariate analysis and there was no significant difference between these groups of patients. On the contrary, the FLT3-ITD mutation significantly reduced the OS (p=0.009) and RFS (p=0.006) in AML patients after allogeneic HSCT. In the next step, patients with AML were divided into four groups regarding FLT3-ITD and DNMT3A mutations. Patients with DNMT3A R882mut/FLT3-ITDpos had the worst OS and RFS. These results indicate that DNMT3A mutations alone do not affect the clinical outcomes of AML patients undergoing allogeneic HSCT, but when accompanied by FLT3-ITD mutations, the OS was significantly reduced (5-year OS 0% for DNMT3A R882mut/

Öz

Amaç: Bu çalışmada, allogeneik hematopoetik kök hücre nakli (HKHN) geçiren akut myeloid lösemi (AML) hastalarında NPM1 ve FLT3 mutasyonlarının varlığında, DNMT3A ekzon 23 mutasyonlarının prognostik etkilerinin değerlendirmesi amaçlanmaktadır.

Gereç ve Yöntemler: Bu çalışma Shariati Hastanesi Hematoloji-Onkoloji ve Kök Hücre Araştırma Merkezi'ne başvuran 128 erişkin AML hastasını kapsamaktadır. NPM1 ve FLT3-ITD mutasyonları, fragman analizi ile tespit edilmiştir. DNMT3A ekzon 23 mutasyon analizi için Sanger dizi analizi kullanılmıştır. Genel sağkalım (OS) ve relapsız sağkalım (RFS) eğrileri için Kaplan-Meier yöntemi ve gruplar arası farklılıkları hesaplamak için log-rank testi kullanılmıştır.

Bulgular: DNMT3A ekzon 23 mutasyonlarının prevalansı %15,6 olarak bulunmuştur ve bunların içinde sıcak bölge R882 mutasyonları öne çıkmaktadır. Tek değişkenli analiz kullanılarak DNMT3A ekzon 23 mutasyonları olan ve olmayan hastalarda RFS ve OS karşılaştırılmış ve bu hasta grupları arasında anlamlı fark bulunmamıştır. Aksine, FLT3-ITD mutasyonu taşıyan ve allogeneik HKHN geçiren AML hastalarında OS (p=0,009) ve RFS (p=0,006) değerleri anlamlı derecede düşmüş olarak gözlenmiştir. Bir sonraki adımda, AML hastaları FLT3-ITD ve DNMT3A mutasyonları açısından dört gruba ayrılmıştır ve DNMT3A R882mut/FLT3-ITD mutasyonu taşıyan hastalar en kötü OS ve RFS'ye sahip olarak bulunmuştur. Bu sonuçlar, DNMT3A mutasyonlarının tek başına allogenik HKHN yapılan AML hastalarının klinik sonuçlarını etkilemediğini işaret etmektedir, ancak FLT3-ITD mutasyonları eşlik ettiği zaman, OS'nin önemli ölçüde azaldığı (DNMT3A R882mut/FLT3-

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FLT3-ITDpos patients vs. 62% DNMT3A R882wt/FLT3-ITDneg, p=0.025) and the relapse rate increased.

Conclusion: It can be deduced that DNMT3A R882mut/FLT3-ITDpos is an unfavorable prognostic factor in AML patients even after allogeneic HSCT.

Keywords: Allogeneic hematopoietic stem cell transplantation, Acute myeloid leukemia, DNMT3A R882, FLT3-ITD

ITD pozitif hastalarda 5 yıllık OS %0 ve DNMT3A R882wt/FLT3-ITD negatif hastalarda %62 oranında, p=0,025) ve nüksetme oranının arttığı görülmüştür.

Sonuç: AML hastalarında allogeneik HKHN'den sonra bile, DNMT3A R882mut/FLT3-ITD pozitifliğinin kötü prognostik faktör olduğu sonucuna varılabilir.

Anahtar Sözcükler: Allojenik hematopoetik kök hücre transplantasyonu, Akut myeloid lösemi, DNMT3A R882, FLT3-ITD

Introduction

Acute myeloid leukemia (AML) is considered a clonal disorder of the hematopoietic stem cells marked by proliferation of immature myeloid cells in the bone marrow (BM) or peripheral blood. Gene fusion, cell signaling abnormalities, and epigenetic modification affect the destination of hematopoietic stem cells and could lead to leukemogenesis [1,2].

Standard induction chemotherapy, which is a combination of cytarabine and anthracyclines, induces a high rate of complete remission (CR) in patients with AML; however, the rate of relapse is also high. This is more pronounced in elderly patients. Despite this fact, it is hoped that the outcome of patients is better when identifying and evaluating prognostic factors such as cytogenetics and molecular abnormalities [3]. A number of single-gene mutations have served for further risk stratification of AML patients. Risk stratification is one of the most important applications of molecular abnormalities, particularly in determining risk stratification after CR is achieved by induction therapy, and it is important because it prevents the referral of patients to hematopoietic stem cell transplantation (HSCT) centers [4]. As noted above, epigenetic modifications contribute to the formation of tumor cells. Epigenetic regulation refers to the modification of gene transcription and expression in such a way that the genetic code does not change [5].

DNA methylation is one of the most broadly studied mechanisms of epigenetic regulation. Methyltransferases are the key enzymes in the methylation process. DNMT3A belongs to the DNMTS family, which plays a significant role in adding methyl groups to cytosine residues in CpG islands. Actively transcribed genes exhibit a nonmethylated CpG profile. Cancer genomes are usually seen to have overall decrease in 5-methylcytosine, although DNA hypermethylation can be seen in some areas such as the promoter of tumor suppressor genes [5,6,7]. The exact mechanism of the function of DNMT3A mutations in the emergence of leukemia is unclear. Possible mechanisms include a change in enzyme catalytic properties and impaired binding to its ligand. DNMT3A has 23 exons and various mutations have been described to date. More than 60% of mutations are localized in the R882 hotspot region in the methyltransferase domain. DNMT3A mutations are predominantly heterozygous and strongly related to FLT3-ITD, IDH-1, and NPM-1 mutations. Differences in the incidence of DNMT3A mutations in AML patients were observed to range between 4.1% and 25% [8,9,10,11].

BM allogeneic HSCT is the only curative treatment for AML patients with intermediate or poor prognosis outcomes. DNMT3A and FLT3-ITD mutations have been found to be associated with adverse prognosis in patients with AML; however, few studies focused on the prognostic impact of these mutations in AML patients treated with allogeneic HSCT. The present study assessed the prognostic value of DNMT3A R882 and FLT3-ITD mutations in adult AML patients after allogeneic HSCT.

Materials and Methods

Patients

From August 2010 to September 2016, a total of 490 AML patients were referred to our center. DNA samples of 220 AML patients were available. Of those AML patients, 128 treated with allogeneic HSCT were enrolled in our study. Of these, 44 were female and 84 were male with a median age of 34 years (range: 15-67 years). Approval was obtained in writing from all patients in compliance with the Declaration of Helsinki and the ethical guidelines of Iran University of Medical Sciences.

The patients were diagnosed based on cytomorphology using the French-American-British (FAB) classification and immunophenotyping, and patients with AML-M3 with molecularly confirmed PML/RARA fusion gene were excluded from the study. All patients underwent allogeneic HSCT.

The source of hematopoietic stem cells for transplantation was peripheral blood, except for one patient who received BM, and donor types consisted of matched sibling donors (n=100), matched related donors (n=6), matched unrelated donors (n=7), and others (n=15).

Treatment Regimens

The conditioning regimen was non-total body irradiation consisting of oral busulfan at 4 mg/kg (days -6 to day -3) and cyclophosphamide at 60 mg/kg (days -2 and day -1). Antithymocyte globulin (ATG) (r-ATG, 2.5 mg/kg, Thymoglobulin®, Genzyme) was used immediately before transplantation for 3 (in cases of matched related and haploidentical cases) or 2 (in other related cases) days. Graft-versus-host disease (GVHD) prophylaxis consisted of cyclosporine and methotrexate.

Mutation Screenings

DNA was extracted from BM/peripheral blood mononuclear cells according to the standard salting-out extraction method.

DNMT3A exon 23 was amplified by polymerase chain reaction (PCR) using forward (5′-GTGTGGTTAGACGGCTTCC-3′) and reverse (5′-CTCTCCCACCTTTCCTCTG-3′) primers. Polymerase chain reaction cycling conditions were the following: one cycle at 95 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, and then 72 °C for 7 min. Analysis of the PCR products was performed by electrophoresis on a 2% agarose gel using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). The products of PCR were directly sequenced and an automated DNA sequencer (Applied Biosystems 3130 Genetic Analyzer) was employed. Comparing bidirectional sequence data to a normal reference sequence, positive mutations were recognized. Assessment of the NPM1 and FLT3-ITD mutations was performed by fragment analysis as previously described [12].

Definition of Outcomes

The primary endpoints for survival analysis were overall survival (OS), calculated as the duration between the date of transplantation and death or last contact; relapse-free survival (RFS), calculated as time from the date of transplantation to first relapse; non-relapse mortality (NRM), calculated as time from transplantation to death from non-relapse causes; and cumulative incidence of relapse, defined as the time from allogeneic HSCT to the date of hematological relapse and considering death in remission as a competitive event. The grading of acute and chronic GVHD was based on criteria published previously [13].

Statistical Analysis

Comparison of clinical characteristics of patients for continuous variables and categorical variables was done using the Mann-Whitney U test and Pearson's chi-square test, respectively. The Kaplan-Meier method was applied to estimate OS and RFS and the log-rank test was used to compare groups. Multivariate Cox proportional hazards models were employed to assess OS and RFS. The cumulative incidence of NRM and relapse incidence (RI) were calculated considering competing risks. Death due to causes other than recurrence of disease and relapse were considered as competing events for relapse and NRM, respectively. A Fine-Gray proportional hazards regression model was used to evaluate the effects of covariates on RI and NRM. All variables with a p-value at or below 0.1 in the univariate analysis were entered in the multivariate analysis. Data analysis was performed using SPSS 19 and EZR software [14].

Results

Mutation Screening

In 20 patients (15.6%), DNMT3A exon 23 mutations were recognized. The R882 hotspot region harbors 19 mutations, including c.2645G>A, p.(R882H) (n=11); c.2644C>T, p.(R882C) (n=2); and c.2645G>C, p.(R882P) (n=6). One patient was found to be heterozygous for a Q905R missense mutation (Table 1). All mutations were heterozygous. In addition, the G884C synonymous variant was found in 3 patients.

Regarding presenting clinical features, no correlation was found between DNMT3A status and median age, sex, hemoglobin, or platelet and white blood cell (WBC) counts. Analysis of DNMT3A mutations was done with the FAB subtypes of AML. Nine of 20 DNMT3A exon 23 mutations belonged to M4/M5 of the FAB classification. The clinical characteristics and other molecular abnormalities of AML patients with mutated or unmutated DNMT3A are presented in Table 2. The prevalence of FLT3-ITD and NPM1 mutation was 28.1% and 18.8%, respectively. Among molecular aberrations, NPM1 mutations correlated with DNMT3A exon 23 mutations (p=0.014). A statistically significant correlation was observed between FLT3-ITD and NPM1 mutations (p=0.001).

Clinical Outcomes Regarding Molecular Aberration Status after Allogeneic HSCT

Analyses of OS and RFS were carried out with a 5-year follow-up period. Comparing AML patients with and without DNMT3A exon 23 mutations, no statistically significant difference was observed in OS (p=0.3) or RFS (p=0.29). The 5-year OS estimates were 41% in mutated DNMT3A patients versus 56% in AML patients without DNMT3A mutations and the 5-year RFS estimates were 41% versus 57%, respectively (Figure 1).

Analyses of OS and RFS were carried out regarding NPM1 and FLT3-ITD mutations. NPM1 had no impact on OS (p=0.83) or RFS (P=0.71). Regarding RI, a significant difference was observed between AML patients with and without NPM1 mutations (p=0.04). AML patients with FLT3-ITD mutations had lower OS (p=0.015) and RFS (p=0.012) compared to those without FLT3-ITD mutations (Figure 2).

The 5-year RI in the overall population was 30% with significant increase in RI regarding FLT3-ITD mutation (p=0.00003). Among other risk factors, the CR status at the time of hematopoietic cell transplantation was associated with higher RI (p=0.001). The FLT3-ITD mutation by 3.5-fold and the CR status at allogeneic HSCT by 2.5-fold triggered an increase in the risk of relapse.

The cumulative incidence of NRM within 5 years was 19.3%. Neither DNMT3A exon 23 mutations nor the majority of risk factors were found to be in association with NRM. Only in multivariate analysis did chronic GVHD increase the risk of NRM by 5.2-fold (p=0.007, HR=5.23; 95% CI: 1.66-16.5).

In the next step, AML patients based on DNMT3A and FLT3-ITD mutations were divided into four groups: group A (DNMT3A R882wt

Table 1. Frequency of DNMT3A exon 23 mutations in acute myeloid leukemia patients.

Percent	Number of patients	Type	Mutation
8.6%	11	Missense	R882H
4.7%	6	Missense	R882P
1.5%	2	Missense	R882C
0.8%	1	Missense	Q905R
2.3%	3	Synonymous	G884C

/FLT3-ITDneg), group B (DNMT3A R882mut /FLT3-ITDpos), group C (DNMT3A R882wt/FLT3-ITDpos), and group D (DNMT3A R882mut/FLT3-ITDneg). Detailed information about these groups is summarized in Table 3. WBC counts (p=0.003) and CR status at allogeneic HSCT (p=0.022) were statistically significant among groups. Patients with DNMT3Awt/FLT3-ITDpos (group C) had the highest WBC counts compared with other groups. Considering the CR status (p=0.01) and WBC count (p=0.004), the differences were statistically significant in the DNMT3Awt/FLT3-ITDpos group (group C) compared with DNMT3Awt/FLT3-ITDneg (group A). The cumulative incidence of relapse, OS, and RFS rates were compared according to these groups. In univariate analysis, the differences in OS and RFS between AML patients with coexistence of DNMT3Amut/FLT3-ITDpos (group B) and those with DNMT3Awt/FLT3-ITDneg (group A) were statistically significant. The DNMT3Amut/FLT3-ITDpos patients (group B) had the worst OS (p=0.025) and RFS (p=0.011) compared with other groups, revealing a higher RI rate (p=0.0002) (Table 4, Figure 3).

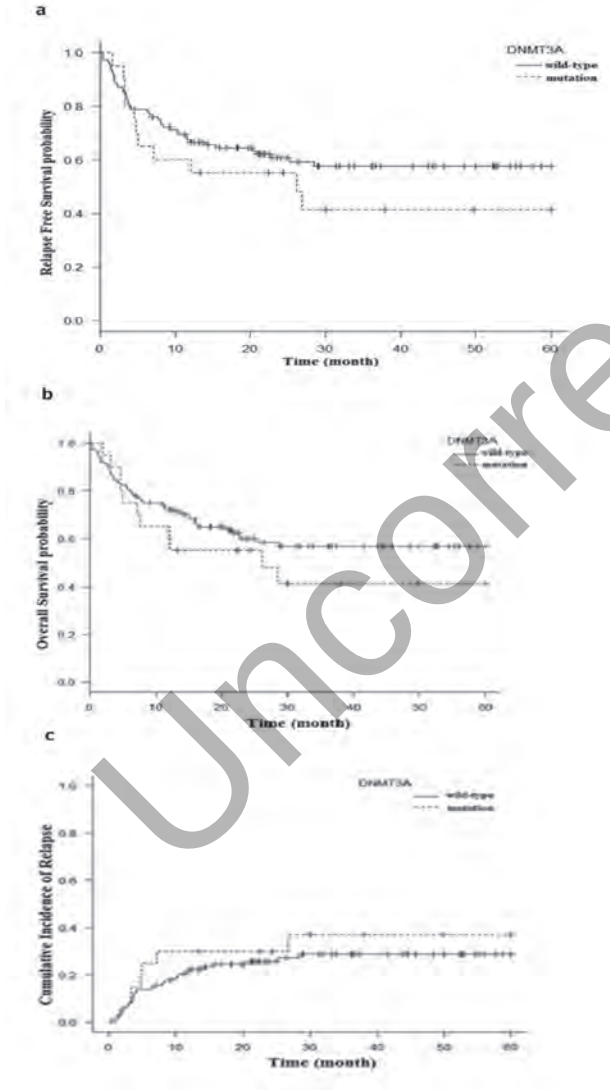

Figure 1. Survival curves of acute myeloid leukemia patients according to mutational status of DNMT3A: a) relapse-free survival, b) overall survival, c) cumulative incidence of relapse.

Multivariate Analyses for OS, RFS, and RI

Multivariate analyses for RFS, OS, and RI were carried out regarding the CR condition (CR1 or CR≥2), the interval from CR1 to transplantation, FLT3-ITD mutation, DNMT3Amut/FLT3-ITDpos, and DNMT3Awt/FLT3-ITDpos. The FLT3-ITD mutation (p=0.03, HR=1.84; 95% CI: 1.05-3.24) and CR status (p=0.04, HR=1.78; 95% CI: 1.02-3.13) were independent factors of inferior survival after allogeneic HSCT. Regarding RI, CR status to transplantation (p=0.0049, HR=2.52; 95% CI: 1.32-4.8) and FLT3-ITD mutation (p=0.00015, HR=3.49; 95% CI: 1.83-6.68) were significant independent prognostic factors for relapse (Table 5).

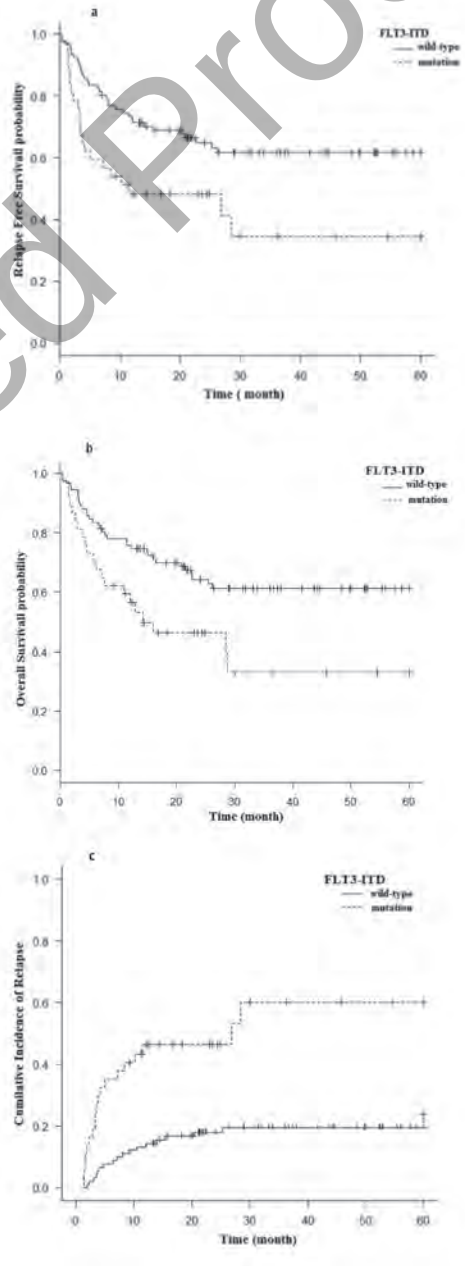

Figure 2. Survival curves of acute myeloid leukemia patients according to mutational status of FLT3-ITD: a) relapse-free survival, b) overall survival, c) cumulative incidence of relapse.

Table 2. Clinical characteristics of acute myeloid leukemia patients according to DNMT3A status.

Clinical characteristics	DNMT3A mutation	DNMT3A wild-type	p-value
Age, years (median ± SD)	39±11	32±12	
(Minimum - maximum)	21-57	16-67	0.1
Sex			
Male	12	72	0. 4
Female	8	36	
FAB subtypes			
M0/M1/M2/M3/M4/M5/M6/M7	0/3/6/0/5/4/2/0	3/16/38/1/36/12/2/0	0.75
WBC count at diagnosis			
Median	6200	13,000	0.65
(Minimum - maximum)	700-151,000	240-257,000	
PLT count at diagnosis			
Median	30,500	61,000	0.12
(Minimum - maximum)	6000-712,000	6000-340,000	
Molecular abnormalities			
NPM1 wild-type	11	93	
NPM1 mutation	7	17	0.014*
FLT3-ITDneg	13	79	
FLT3-ITDpos	5	31	0.28
Interval from CR1 to transplantation, months			
Median	3.1	4.6	
(Minimum - maximum)	1-13.4	0.3-35.4	0.2
Donor type			
Matched sibling donor	15	85	
Others	3	25	0.4
Acute GVHD			
Yes	12	95	
No	6	15	0.07
Chronic GVHD			
Yes	13	93	
No	5	17	0.19

*p<0.05 is considered significant.

SD: Standard deviation, CR: complete remission, WBC: white blood cell, PLT: platelet, GVHD: graft-versus-host disease.

Discussion

Although DNMT3A mutations were recognized as driver gene mutations in adults with AML, their roles in leukemogenesis remain poorly understood. In the past decade, however, DNMT3A mutations have been attracting much attention as markers for risk stratification in AML patients [15].

The present study showed that DNMT3A mutations occur in 15.6% (20/128) of AML patients, predominantly in patients with NPM1 aberrations. The dominant mutation in the study population being located at hotspot region R882 is in agreement with previous studies [16,17].

The present study finds that DNMT3A R882 mutations are not related to inferior survival in AML patients after allogeneic HSCT. It could be argued that allogeneic HSCT ameliorates the clinical consequences of AML cases with DNMT3A R882 mutations. In the present study, no significant difference was found in OS, RFS, or RI between cases with DNMT3A mutations and cases with wild-type DNMT3A.

Several studies with controversial results have been conducted on the prognostic impact of DNMT3A mutations in AML patients. Some studies revealed a statistically significant difference in OS between mutated and unmutated DNMT3A patients, with worse OS in the mutated cases [16,18,19,20].

Metzeler et al. [21] showed that DNMT3A mutations are on one hand related to inferior survival in AML patients and on the other hand modify the prognostic effect of mutated NPM1. They also found that different types of DNMT3A mutations had no effect on patient outcomes. Yuan et al. [22], in a meta-analysis of DNMT3A R882 mutations in AML patients consisting of eight studies with 4474 AML cases with 694 AML patients with DNMT3A R882 mutations, verified

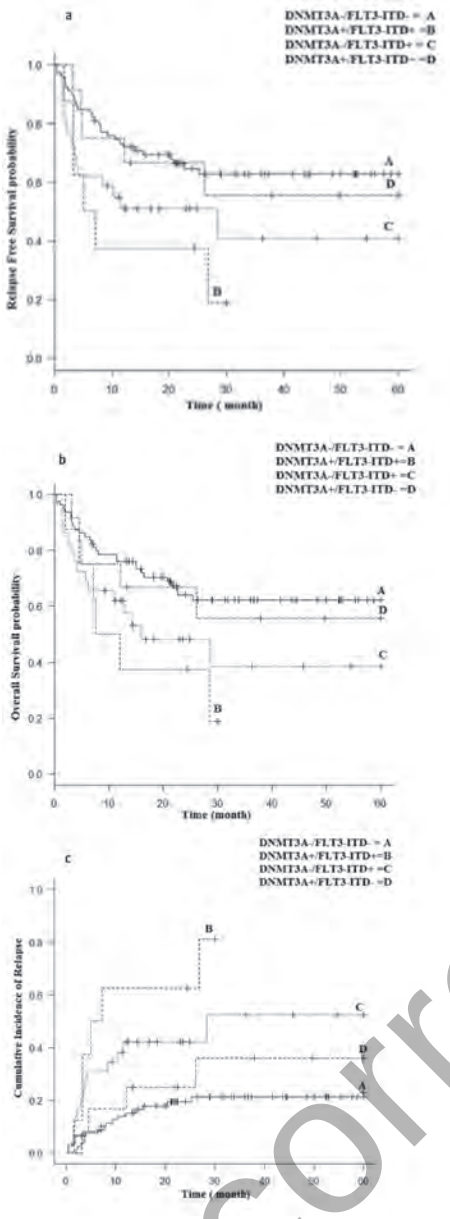

Figure 3. Survival curves of acute myeloid leukemia patients according to mutational status of DNMT3A/FLT3-ITD: a) relapse-free survival, b) overall survival, c) cumulative incidence of relapse.

significantly reduced RFS and OS in AML patients with DNMT3A R882 mutations.

Focusing on the characteristics and effects of DNMT3A R882 mutation in AML patients with or without NPM1 and FLT3 mutations, Dushyant et al. analyzed 174 AML patients with normal cytogenetics. They noticed that DNMT3A mutations in the cytogenetically normal AML patients compared to NPM1- and FLT3-mutated patients (p=0.067 and p=0.065, respectively) were related to remarkably shorter OS and progression-free survival [23].

However, few studies, as mentioned in the following text, have been designed to determine the prognostic effect of DNMT3A mutations in AML patients treated with allogeneic HSCT.

Consistent with our results, Xu et al. [24] compared the outcomes of 55 DNMT3A (mut) patients who underwent allogeneic HSCT (23 cases) or received chemotherapy (32 cases) for consolidation. They observed a significant difference in 3-year OS and DFS between the chemotherapy group and the allogeneic HSCT group. The authors concluded that DNMT3A mutations act as an unfavorable prognostic factor in AML patients with normal cytogenetics and allogeneic HSCT improves survival in DNMT3A mutation-positive AML patients. The median OS in wild-type DNMT3A patients was greater compared to mutated patients but the difference was not statistically significant (p=0.151). No significant difference was seen in DFS between these groups of patients (p=0.304) [24].

Ahn et al. [25] described DNMT3A R882mut and FLT3-ITDpos as both unfavorable prognostic markers for OS and significant risk factors for relapse and event-free survival. They declared that patients with coexistence of DNMT3A R882 and FLT3-ITD mutations had worse OS, worse event-free survival, and higher relapse rates compared with other mutations. Indeed, DNMT3A R882mut/FLT3-ITDpos status was a significant prognostic marker for poor clinical outcome with increasing RI rates even after HSCT.

Tang et al. [26] verified that DNMT3A R882 mutations confer inferior survival in AML patients. Their results also indicated that coexistence of FLT3-ITD and DNMT3A R882 mutations was an independent factor for adverse prognosis after allogeneic HSCT.

Contrary to our results, the findings of Ahn et al. [25] and Tang et al. [26] considered DNMT3A R882 as an unfavorable prognostic indicator in AML patients treated with allogeneic HSCT. The reason for this discrepancy between the results is not clear, but unknown cooperating genetic aberrations may be involved. Several studies have shown that allogeneic HSCT cannot abrogate the unfavorable effect of FLT3-ITD in AML patients [27,28]. Therefore, in the next step, we analyzed the surveillance factors of OS, RFS, and RI in FLT3-ITDpos/DNMT3A R882mut AML patients and encountered the worst condition compared with other groups. These results are consistent with the findings of the above-mentioned studies [25,26].

Our data also highlighted that CR status prior to transplantation was more obvious than other risk factors in delineating risk of relapse. In more advanced disease stages (CR2, CR3), more RI occurs. From this point of view, our data are in accordance with previous studies [29,30].

Study Limitations

The limitations of this research include the following: first, a relatively small sample size was used; second, the analysis was limited to exon 23 of the DNMT3A gene; and third, there is an absence of cytogenetic findings. Hence, caution should be taken in the interpretation of the results of the present study.

The initial goal of allogeneic HSCT is to improve hematological disorders while minimizing residual disease as much as possible. To achieve this goal, the patient should be supported through a

Table 3. Clinical characteristics of acute myeloid leukemia patients according to DNMT3A R882/FLT3-ITD groups.

Risk factors	DNMT3A$^+$/FLT3-ITD$^+$(n=8)	DNMT3A$^-$/FLT3-ITD$^+$(n=29)	DNMT3A$^+$/FLT3-ITD$^-$(n=12)	DNMT3A$^-$/FLT3-ITD$^-$(n=79)	p-value
WBC count	11,200 (3100-152,000)	26,000 (2600-226,000)	5400 (700-100,000)	6700 (240-257,000)	0.003
CR					
First CR	5	15	11	61	
≥Second CR	3	14	1	18	0.022
Interval from CR1 to HSCT, median (months)					
>Median	7	12	7	40	
<Median	1	17	5	39	0.13
NPM1 mutation					
Mutated	3	19	9	73	
Wild-type	5	10	3	6	0.008
Acute GVHD					
No	5	25	8	68	
Yes	3	4	4	11	0.14
Chronic GVHD					
No	6	17	8	65	
Yes	2	12	4	14	0.3

GVHD: Graft-versus-host disease, HSCT: hematopoietic stem cell transplantation, CR: complete remission.

Table 4. Univariate and multivariate analysis of surveillance factors according to DNMT3A R882/FLT3-ITD groups.

Risk factor status	OS					RFS			
	Univariate			Multivariate		Univariate		Multivariate	
	n (%)	Rate at 5 years (%)	p-value	p-value	HR (95% CI)	Rate at 5 years (%)	p-value	p-value	HR (95% CI)
DNMT3A+/FLT3-ITD+	8 (6.25)	0	0.025*	0.29	2.68 (1.10-6.94)	0	0.011*	0.18	2.91 (1.20-7.06)
DNMT3A+/FLT3-ITD-	12 (9.4)	57±0.15	0.72	0.71	1.2 (0.46-3.08)	55±0.15	0.76	0.76	1.16 (0.44-2.9)
DNMT3A-/FLT3-ITD+	29 (22.6)	38±0.11	0.038*	0.36	1.94 (1.03-3.65)	41±0.12	0.037*	0.35	1.97 (1.05-3.7)
DNMT3A-/FLT3-ITD-	79 (61.75)	62±0.6				63±0.6			

Risk factor status	RI			NRM	
	Univariate			Univariate	
	n (%)	Rate at 5 years (%)	p-value	Rate at 5 years (%)	p-value
DNMT3A+/FLT3-ITD+	8 (6.25)	81.2 (9.1-98.2)	0.0002*	-	0.18
DNMT3A+/FLT3-ITD-	12 (9.4)	8.3 (0.4-32.6)	0.33	36.1 (9.4-64.5)	-
DNMT3A-/FLT3-ITD+	29 (22.6)	52.3 (25.5-73.5)	0.003*	6.9 (1.2-20.1)	-
DNMT3A-/FLT3-ITD-	79 (61.75)	21.3 (12.7-31.3)	-	22.9 (9.6-39.7)	-

*p<0.05 is considered significant.
OS: Overall survival, RFS: relapse-free survival, RI: relapse incidence, NRM: non-relapse mortality, HR: hazard ratio, CI: confidence interval.

conditioning regimen and its associated complications such as GVHD while avoiding relapse. ATG can reduce the risk of GVHD, although ATG formulation (dose and type), donor type, and other medications used for GVHD prophylaxis affect the outcome of allogeneic HSCT [31]. In the present study, in order to avoid reducing the number of patients, the role of ATG and donor source were ignored.

Allogeneic HSCT is a pragmatic treatment option for relapsed and/or refractory AML patients; however, it seems that allogeneic HSCT cannot override the inferior outcomes conferred by the coexistence of DNMT3Amut/FLT3-ITDpos. Our increased knowledge of genetic and epigenetic alterations in AML has triggered the emergence of new medicines such as CPX-351, FLT3 inhibitors, and epigenetic modifiers. Indeed, it is necessary to accompany molecularly targeted therapy with allogeneic HSCT for poor prognosis in AML patients.

Table 5. Univariate and multivariate analysis of surveillance factors according to risk factor status.

Risk factor status	OS					RFS			
	Univariate			Multivariate		Univariate		Multivariate	
	n (%)	Rate at 5 years (%)	p-value	p-value	HR (95% CI)	Rate at 5 years (%)	p-value	p-value	HR (95% CI)
Age, years									
>34	66 (51.6)	53.2±6.9	0.54			53.6±6.8	0.59		
≤34	62 (48.4)	55±6.6				56±6.6			
WBC count			0.52				0.49		
CR									
First CR	92 (71.8)	60.3±5.6	0.011*	0.04	1.78 (1.02-3.13)	60.9±5.5	0.007*	0.027*	1.87 (1.07-3.27)
≥Second CR	36 (28.2)	38.6±8.5				39.7±8.5			
Interval from CR1 to HSCT, median (months)									
>Median	66 (51.6)	61±6.6	0.094*	0.24		62±6.5	0.09		
<Median	62 (48.4)	45.9±6.9				46.7±6.8			
Donor type									
HLA identical sibling	100 (78.1)	55±5.4	0.45			55.9±5.4	0.5		
Others	28 (21.9)	48±10.4				49.8±10.2			
DNMT3A exon 23									
Mutated	20 (15.6)	40±12.2	0.29			40±11.9	0.287		
Wild-type	108 (84.4)	56.7±5				57±5			
FLT3-ITD mutation									
Positive	37 (28.9)	31.6±10.8	0.015*	0.03	1.84 (1.05-3.24)	34±10.9	0.012*	0.022*	1.97 (1.094-3.36)
Negative	91 (71.1)	60.4±5.4				61.1±5.3			
NPM1 mutation									
Mutated	24 (18.75)	58±10	0.83			58.3±10	0.71		
Wild-type	104 (81.25)	53±5.4				53.9±5.3			
Acute GVHD									
No	22 (17.2)	51.5±5.3	0.4			52.1±5.3	0.37		
Yes	106 (82.8)	68±9.9				68.2±5.4			
Chronic GVHD	0.5								
No	21 (16.4)		0.5			57.1±12.5	0.484		
Yes	92 (83.6)					63.2±5.4			

Risk factor status	RI					NRM			
	Univariate			Multivariate		Univariate		Multivariate	
	n (%)	Rate at 5 years (%)	p-value	p-value	HR (95% CI)	Rate at 5 years (%)	p-value	p-value	HR (95% CI)
Age, years									
≥34	66 (51.6)	33.8 (21.2-46.8)	0.45			13 (6-22)	0.59		
<34	62 (48.4)	28.1 (17.2-40.1)				26 (9-46.7)			
CR									
First CR	92 (71.8)	23.4 (14.6-33.4)	0.001*	0.0049	2.52 (1.32-4.8)	21.7 (10.3-35.7)	0.48		
≥Second CR	36 (28.2)	29.6 (31.5-65.4)				11.8 (3.6-25.5)			

Table 5. Continued.									
Interval from CR1 to HSCT, median (months)									
>Median	66 (51.6)	28.8 (17.4-41.2)	0.53			9.5 (3.8-18.3)	0.76		
<Median	62 (48.4)	33.2 (21.1-45.8)				27.4 (12.9-44.2)			
Donor type									
HLA identical sibling	100 (78.1)	31.7 (22.1-41.7)	0.7			17.6 (8.1-30)	0.2		
Others	28 (21.9)	28.6 (12-47.7)				23.2 (8.8-41.6)			
DNMT3A exon 23									
Mutated	20 (15.6)	29.6 (20.6-39)	0.5			19.3 (8.5-33.4)	0.6		
Wild-type	108 (84.4)	36.9 (15.3-58.8)				21.9 (6-43.7)			
FLT3-ITD mutation									
Positive	37 (28.9)		0.00003	0.00015*	3.49 (1.83-6.68)	23.8 (12.4-37.2)	0.11		
Negative	91 (71.1)					5.4 (0.9-16.1)			
NPM1 mutation									
Mutated	24 (18.75)		0.04			24.4 (12.2-38.8)	0.02		
Wild-type	104 (81.25)					0			
Acute GVHD							0.7		
No	22 (17.2)		0.12			21.7 (8.5-38.8)			
Yes	106 (82.8)					18.2 (5.5-36.8)			
Chronic GVHD									
No	21 (16.4)	30.7 (20.9-41)	0.12			12.4 (3-28.8)	0.009	0.007*	5.23 (1.66-16.5)
Yes	92 (83.6)					30 (8.9-55)			

*p<0.05 is considered significant.

CR: Complete remission, WBC: white blood cell, GVHD: graft-versus-host disease, OS: overall survival, RFS: relapse-free survival, RI: relapse incidence, NRM: non-relapse mortality, HR: hazard ratio, CI: confidence interval.

Conclusion

Based on the findings of the present study, DNMT3A R882 mutations seem not to affect the clinical outcomes of AML patients undergoing allogeneic HSCT. In contrast, allogeneic HSCT probably improves the clinical outcomes of AML patients with DNMT3A R882 mutations. When DNMT3A R882 mutations were accompanied by FLT3-ITD mutations (DNMT3A R882mut/FLT3-ITDpos), OS was significantly reduced, even after allogeneic HSCT. Indeed, FLT3-ITD is a significant negative prognostic indicator compared with the DNMT3A R882 mutation. Further studies are required to better explain a rationale for the integration of DNMT3A R882mut/FLT3-ITDpos status in the treatment decisions of AML patients.

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Ethics

Ethics Committee Approval: This study was approved by the local ethics committee of Iran University of Medical Sciences (approval number: 1394.26066).

Informed Consent: Approval consent forms were obtained from all patients in compliance with the Declaration of Helsinki.

Authorship Contributions

Surgical and Medical Practices: K.A., A.G., M.V.; Concept: M.T.A., A.K., B.C.; Design: M.T.A., S.R., S.M., M.N.; Data Collection or Processing: M.T.A., S.R.; Analysis or Interpretation: S.R., M.N., M.J.; Literature Search: M.T.A., A.K., B.C.; Writing: M.T.A., A.K., B.C., S.M.

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RESEARCH ARTICLE

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Cryptochrome-1 Gene Expression is a Reliable Prognostic Indicator in Egyptian Patients with Chronic Lymphocytic Leukemia: A Prospective Cohort Study

Kriptokrom-1 Gen Ekspresyonu Mısırlı Kronik Lenfositik Lösemili Hastalarda Güvenilir Bir Prognostik Göstergedir

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Abstract

Objective: Traditional prognostic factors have proved insufficient to account for heterogeneity in the clinical behavior of chronic lymphocytic leukemia (CLL). *Cryptochrome-1 (CRY-1)* is a circadian clock gene essential in maintaining the circadian rhythm and regulating cell proliferation. We evaluated *CRY-1* gene expression in CLL and addressed its putative role as a prognostic indicator for the clinical course of CLL.

Materials and Methods: A total of 100 CLL patients at diagnosis were studied for *CRY-1* gene expression by real-time reverse-transcription polymerase chain reaction and were followed for assessment of time to first treatment (TFT).

Results: *CRY-1* was expressed in 94% of the CLL patients at diagnosis. The median *CRY-1* relative gene expression level (0.006) stratified patients into high and low expression groups. Forty of 100 (40%) CLL patients showed high *CRY-1*, 54/100 (54%) showed low *CRY-1*, and 6/100 (6%) had undetectable *CRY-1* gene expression. High *CRY-1* gene expression was concordant with $CD38^+$, $Zap\text{-}70^+$, and double $CD38^+Zap\text{-}70^+$ expression; unfavorable/intermediate cytogenetics; unmutated *immunoglobulin heavy-chain variable-region* gene; and diffuse marrow infiltration. The high *CRY-1* gene expression patient group exhibited shorter TFT than the patients with low *CRY-1* gene expression. A Cox proportional hazard regression model identified *CRY-1* gene expression to be independently predictive for TFT.

Conclusion: *CRY-1* is differentially expressed among CLL patients, stratifying them into low-risk and high-risk groups. *CRY-1* gene expression could constitute a reliable prognostic indicator for CLL progression, complementing the role of standard well-established prognostic factors. *CRY-1* gene expression could be employed as a prognostic indicator for disease progression during the initial prognostic work-up and follow-up for CLL patients.

Keywords: Chronic lymphocytic leukemia, *Cryptochrome-1*, Circadian genes, Time to first treatment, Prognosis, Real-time polymerase chain reaction, CD38, Zap-70

Öz

Amaç: Geleneksel prognostik faktörler, kronik lenfositik löseminin (KLL) klinik davranışındaki heterojenitenin nedenini açıklamak için yetersiz bulunmaktadır. *Kriptokrom-1* (*CRY-1*) sirkadiyen ritmin sağlanması ve hücre proliferasyonu regülasyonunda gerekli olan bir sirkadiyen saat genidir. Biz, KLL'de *CRY-1* gen ekspresyonunu değerlendirdik ve KLL'nin klinik seyri için prognostik gösterge olarak varsayılan rolünü irdeledik.

Gereç ve Yöntemler: Toplam 100 KLL hastası tanı sırasında kantitatif gerçek zamanlı ters transkripsiyon polimeraz zincir reaksiyonu ile *CRY-1* gen ekspresyonu için incelendi ve ilk tedaviye kadar geçen zamanın (TFT) değerlendirilmesi için takip edildi.

Bulgular: KLL hastalarının %94'ünde tanı anında *CRY-1* ekspresyonu mevcuttu. Medyan *CRF-1* rölatif gen ekspresyon düzeyi (0,006) hastaları yüksek ve düşük ekspresyon gruplarına ayırmaktadır. Yüz hastanın 40'ı (%40) yüksek *CRY-1*, 54/100'ü (%54) düşük *CRY-1*, ve 6/100'ü (%6) tespit edilemez *CRY-1* gen ekspresyonu gösterdi. Yüksek *CRY-1* gen ekspresyonu $CD38^+$, $Zap\text{-}70^+$ ve çift $CD38^+Zap\text{-}70^+$ ekspresyonu; olumsuz/orta sitogenetik; mutasyona uğramamış *immünoglobulin ağır zincir değişken bölge* geni ve diffüz kemik iliği infiltrasyonu ile uyumlu idi. Yüksek *CRY-1* gen ekspresyonu olan hasta grubu, düşük *CRY-1* gen ekspresyonu olan hastalardan daha kısa TFT gösterdi. Cox oransal hazard regresyon modeli *CRY-1* gen ekspresyonunu TFT için bağımsız prediktif olarak tanımlamıştır.

Sonuç: *CRY-1*, KLL hastalarında farklı olarak eksprese edilerek onları düşük-risk ve yüksek-risk gruplarına ayırmaktadır. *CRY-1* gen ekspresyonu, KLL progresyonu için iyi-belirlenmiş standart prognostik planlamada ve KLL hastalarının takibinde hastalık progresyonu için prognostik bir gösterge olarak kullanılabilir.

Anahtar Sözcükler: Kronik lenfositik lösemi, *Kriptokrom-1*, Sirkadiyen genler, İlk tedaviye kadar geçen zaman, Prognoz, Gerçek zamanlı polimeraz zincir reaksiyonu, CD38, Zap-70

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Introduction

Chronic lymphocytic leukemia (CLL) is a lymphoproliferative neoplasm defined by proliferation and accumulation of morphologically mature, immunologically dysfunctional monoclonal B cells in the peripheral blood (PB), bone marrow (BM), and lymphatic tissues [1].

The clinical course of CLL is heterogeneous and difficult to predict; some patients may exhibit rapid disease progression while others may live for decades without requiring treatment [2]. Early treatment of the latter group would risk the development of therapy-related complications that might affect the quality of life and/or survival. Assigning markers that reliably stratify patients into good-risk or poor-risk disease groups could help in evaluating the potential benefit of early treatment and assist risk-adapted treatment strategies [3]. Traditional prognostic factors have proved insufficient to account for heterogeneity in the clinical behavior of CLL, indicating a need for further prognostic indicators that can better correlate with patients' clinical outcome and survival [4,5].

The *immunoglobulin heavy-chain variable-region* (*IgHV*) gene mutational status correlates with clinical behavior and represents a robust prognostic indicator for CLL. However, *IgHV* gene sequencing is complicated and time-consuming for routine laboratories; hence, exploring an alternative for *IgHV* gene mutations is a priority [6].

The circadian machinery comprises various genes that show functional interplays with cell cycle regulators. Aberrant expression of circadian clock genes can therefore lead to aberrant expression of downstream target genes responsible for cell proliferation and apoptosis, resulting in the emergence of different types of cancers, including CLL [6,7,8,9].

The *Cryptochrome-1* (*CRY-1*) gene (12q23-q24.1) is a member of the circadian clock essential to the maintenance of circadian rhythm. In addition to its circadian function, it also acts as a transcriptional regulator for several genes with roles in cell metabolism and proliferation [10].

Accordingly, we aimed to evaluate *CRY-1* gene expression in CLL patients and address the gene's putative role as a prognostic indicator for the clinical course of CLL.

Materials and Methods

This prospective cohort study included 100 newly diagnosed and untreated CLL patients who attended the Hematology/Oncology Clinic of the Ain Shams University Hospitals. Patients were selected on the basis of standard clinical, hematologic, and immunophenotypic characteristics for the diagnosis of CLL [11]. These CLL patients comprised 64 males and 36 females; the male to female ratio was 1.8:1 and median age was 61 years (interquartile range (IQR): 55-68 years). All patients were followed after diagnosis for assessment of the time to first treatment (TFT) (median: 20 months; range: 10-24 months).

TFT is the time from diagnosis to the start of therapy or the last follow-up. The criteria for starting treatment are: (i) the existence of constitutional symptoms; (ii) progressive marrow failure indicated by anemia and/or thrombocythemia; (iii) autoimmune anemia and/or thrombocytopenia not responding to steroids; (iv) massive (more than 6 cm below the costal margin) or progressive splenomegaly; (v) massive nodes or nodal clusters (more than 10 cm in the longest diameter) or progressive lymphadenopathy; (vi) progressive lymphocytosis by more than 50% over 2 months or a doubling time of less than 6 months [12]. Informed consent was provided prior to patient enrollment. The study protocol was endorsed by the Ethical Committee for Human Research of Ain Shams University and complied with the Helsinki Declaration of 1975, as revised in 2002. The clinicopathologic characteristics of CLL patients at diagnosis are presented in Table 1.

Sampling

PB and BM samples were collected in K-EDTA (1.5 mg/mL) for morphologic, immunophenotypic, and molecular analyses and in lithium heparin for cytogenetic analysis. PB samples were used for flow cytometric immunophenotyping and real-time reverse-transcription polymerase chain reaction (qRT-PCR) for quantification of *CRY-1* gene expression, while PB or BM samples, when available, were used for the cytogenetic analysis.

Flow Cytometric Immunophenotyping

Immunophenotyping using the standard panel for chronic lymphoproliferative disease (CD5, CD19, CD23, FMC7, CD20, CD38, CD79b, CD10, CD25, CD103, CD123, kappa, lambda, surface immunoglobulin G) (Beckman Coulter, Miami, FL, USA), along with ZAP-70 (BioLegend, San Diego, CA, USA), was performed with the EPICS XL Flow Cytometer (Coulter Electronics, Hialeah, FL, USA). The positivity threshold was defined as the expression of the marker by ≥30% of the B lymphocytes [13]; however, for CD38 and Zap-70 a cut-off value of ≥20% and ≥10% of the B lymphocytes was considered positive, respectively [14].

Fluorescence In Situ Hybridization

Probes for 13q-, 17p-, 11q-, and +12 (Vysis, Downers Grove, IL, USA) were used. Patients were stratified into cytogenetic-based risk groups as follows: favorable, 13q- or normal karyotype; intermediate, +12; and unfavorable, 17p-, 11q-, or complex karyotype (≥3 chromosomal aberrations) [13].

qRT-PCR for Quantification of *CRY-1* Gene Expression

qRT-PCR amplification was done using gene expression sets

for the *CRY-1* gene (*Homo sapiens*; Hs00172734-m1 TaqMan® Gene Expression Assays), TaqMan β-*actin* control reagents for the β-*actin* reference gene, and TaqMan Universal PCR Master Mix (all from Applied Biosystems, Foster City, CA, USA). Total RNA was extracted from PB samples using a QIAamp® RNA blood kit (QIAGEN, Valencia, CA, USA), while cDNA was synthesized using a QuantiTectReverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. The cDNA was stored at -20 °C until it was used.

cDNA samples were employed in synthesizing PCR products using sequence-specific primers and TaqMan oligonucleotide fluorescence-labeled probes: FAM for the *CRY-1* gene (Hs_*CRY-1*_1_FAM Quantifast Probe Assay, QIAGEN) and MAX for the β-*actin* gene (Hs_β–*actin*_1_MAX Quantifast Probe Assay, QIAGEN). Both probes were labeled with Iowa Black Fluorescent Quencher. Each PCR included all the necessary reagents and 50 ng of cDNA in a final volume of 25 µL. A negative control (cDNA replaced by nuclease-free water) was included in each assay. The reaction protocol comprised 40 cycles of heating at 95 °C for 5 min (hot-start PCR), then heating at 95 °C for 30 s (denaturation), and then heating at 60 °C for 30 s (annealing/extension). PCR and data analysis were carried out with Stratagene Mx3000P (Stratagene Inc., La Jolla, CA, USA). Undetectable *CRY-1* was noted for cases in which cycle threshold (CT) values exceeded the 40th cycle.

CRY-1 expression levels in unknown samples were calculated by relative quantification using the ΔΔCT method, which relies on comparison of CT values of *CRY-1* (target gene) to β-*actin* (reference gene) in unknown and normal calibrator samples. The results were expressed as the fold change in gene expression normalized to the reference gene and relative to the calibrator [15].

Statistical Analysis

Analysis of data was done using SPSS 17 for Windows 7 (SPSS Inc., Chicago, IL, USA). Categorical variables are presented as frequency and percentage, compared using the chi-square (χ^2) test. Continuous variables are presented as mean ± standard deviation or median and IQR for parametric and nonparametric variables, respectively. Student and Mann-Whitney U tests were employed for comparing continuous parametric and nonparametric variables between two groups, respectively. Kaplan-Meier curves for TFT were plotted and the log-rank test was used to compare TFT distributions between high and low *CRY-1* gene expression groups. Multivariate Cox proportional hazard regression analysis (hazard ratio, HR) was employed to identify the independent association of *CRY-1* gene expression with TFT. Values of $p<0.05$ and $p<0.01$ were taken to be significant and highly significant, respectively.

Results

CRY-1 Gene Expression in CLL Patients at Diagnosis

The *CRY-1* gene was expressed in 94 of 100 (94%) CLL patients at diagnosis (median: 0.006; IQR: 0.000008-0.32) (Table 1).

Table 1. Clinicopathologic characteristics of chronic lymphocytic leukemia patients at diagnosis.

Parameters	CLL patients (n=100)
Age, years, median (IQR)	61 (55-68)
Sex, male: female	1.8:1
Lymphadenopathy, n (%)	76 (76)
Splenomegaly, n (%)	38 (38)
Hepatomegaly, n (%)	22 (22)
Binet stage, n (%)	
A (low-risk)	36 (36)
B (intermediate-risk)	12 (12)
C (high-risk)	52 (52)
TLC, x10^9/L, median (IQR)	38.5 (19.8-90)
Hemoglobin, g/dL, mean ± SD	10.73±2.5
Platelets, x10^9/L, mean ± SD	157±82
PB lymphocytes, x10^9/L, median (IQR)	34 (15-72)
BM lymphocytes, %, median (IQR)	85 (78-90)
CD38-positive, n (%)	44 (44)
Zap-70-positive, n (%)	22 (22)
Cytogenetic abnormalities, n (%)*	
Favorable	42 (42)
Intermediate	20 (20)
Unfavorable	22 (22)
Not available	16 (16)
IgHV gene, n (%)	
Mutated	37 (37)
Unmutated	32 (32)
Not available	31 (31)
Pattern of BM infiltration, n (%)**	
Diffuse	40 (40)
Nondiffuse	50 (50)
Not available	10 (10)
CRY-1 gene expression, median (IQR)	
All CLL patients	0.006 (0.000008-0.32)
High *CRY-1* group, n=40 (40%)	0.295 (0.034-3.618)
Low *CRY-1* group, n=54 (54%)	0.000008 (0.0000005-0.0002)
Undetectable *CRY-1*, n=6 (6%)	-
Time to first treatment, months, median (range)	20 (10-24)

*Favorable: 13q-, normal karyotype; Intermediate: +12; Unfavorable: 17p-, 11q-, complex karyotype (≥3 chromosomal aberrations) [13].

**Nondiffuse infiltration included nodular, interstitial, or mixed nodular/interstitial infiltrations.

BM: Bone marrow, CLL: chronic lymphocytic leukemia, *CRY-1: Cryptochrome-1, IgHV: immunoglobulin heavy-chain variable-region*, IQR: interquartile range, PB: peripheral blood, SD: standard deviation, TLC: total leukocytic count.

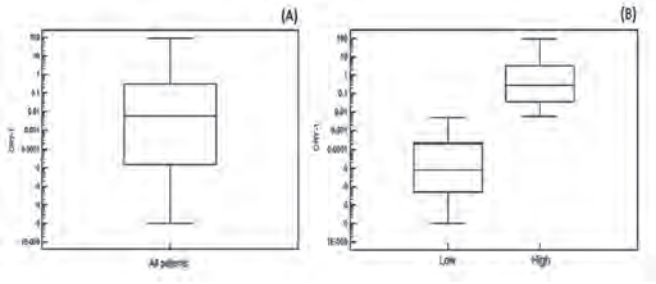

Figure 1. *Cryptochrome-1* gene expression in chronic lymphocytic leukemia patients at diagnosis: (A) all chronic lymphocytic leukemia patients, (B) low and high *Cryptochrome-1* gene expression groups.

CRY-1: Cryptochrome-1.

The median *CRY-1* relative gene expression level (0.006) was employed as the cut-off value for stratifying high and low *CRY-1* gene expression groups. Accordingly, 40 of 100 (40%) CLL patients showed high *CRY-1* gene expression (≥0.006) (median: 0.295; IQR: 0.034-3.618) and 54 of 100 (54%) CLL patients showed low *CRY-1* gene expression (<0.006) (median: 0.000008; IQR: 0.0000005-0.0002). Six of 100 (6%) CLL patients had undetectable *CRY-1* expression (Table 1, Figure 1).

High and Low *CRY-1* Gene Expression and Clinicopathologic Characteristics of CLL Patients at Diagnosis

High *CRY-1* gene expression was significantly related to $CD38^+$, $Zap\text{-}70^+$, and double $CD38^+Zap\text{-}70^+$ expression; unfavorable/intermediate cytogenetics; unmutated *IgHV* gene; and diffuse BM infiltration in trephine biopsy ($p<0.05$). On the contrary, low *CRY-1* gene expression was significantly related to $CD38^-$, $Zap\text{-}70^-$, and double $CD38^-Zap\text{-}70^-$ expression and favorable cytogenetics ($p<0.05$). No further significance was found between high and low *CRY-1* groups regarding other studied clinicopathologic parameters ($p>0.05$) (Table 2).

CRY-1 Gene Expression and TFT of CLL Patients

Using the Kaplan-Meier method, the TFT for the high *CRY-1* gene expression group (median: 16.89 months; 95% confidence interval (CI): 15.38-18.41) was found to be significantly shorter than the TFT for the low *CRY-1* gene expression group (median: 23.69 months; 95% CI: 23.44-23.95) ($p<0.001$) (Table 2, Figure 2).

Multivariate Cox hazard regression analysis denoted *CRY-1* gene expression to be independently predictive for TFT (HR: 3.99; 95% CI: 2.12-6.19; p=0.001). The same was also shown for *IgHV* mutational status ($p<0.001$), Binet stage, Zap-70, and cytogenetic-based risk groups ($p<0.05$) (Table 3).

Discussion

Diverse circadian genes are known to be correlated with poor prognosis when aberrantly overexpressed in several cancers, including *CRY-1* [16]. In this work, the *CRY-1* gene was expressed

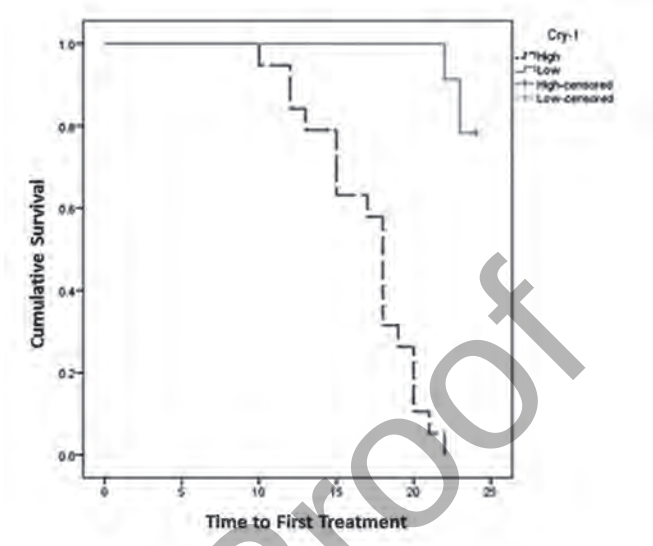

Figure 2. Kaplan-Meier curve showing the time to first treatment for chronic lymphocytic leukemia patients based on *Cryptochrome-1* gene expression levels.

CRY-1: Cryptochrome-1.

in 94% of the studied CLL patients at diagnosis. The median relative *CRY-1* gene expression level (0.006) was employed to stratify patients into high and low expression groups. High *CRY-1* was related to $CD38^+$, $Zap\text{-}70^+$, and double $CD38^+Zap\text{-}70^+$ expression while low *CRY-1* was associated with $CD38^-$, $Zap\text{-}70^-$, and double $CD38^-Zap\text{-}70^-$ expression and discordant CD38/Zap-70 was comparable between the high and low *CRY-1* gene expression groups. Similarly, a significant concordance was previously shown between elevated *CRY-1* transcripts and high-risk patients, as defined by $CD38^+$ and/or unmutated *IgHV* genes, compared with their low-risk counterparts, as defined by $CD38^-$ and/or mutated *IgHV* genes [6,10,17]. Moreover, elevated *CRY-1* transcripts in high-risk patients were found to be associated with $Zap\text{-}70^+$ and double $CD38^+Zap\text{-}70^+$ expression [6,17].

Unlike our findings, cases of discordant CD38/Zap-70 expression (intermediate-risk) showed *CRY-1* gene expression levels comparable to those of the high-risk group in a previous study [17]. This discrepancy might be attributed to two major pitfalls that could hamper the prognostic use of CD38: first, the debate about the threshold that indicates $CD38^+$ expression for defining patient prognosis, and second, the probability that CD38 expression may be unstable and differ over time, provoking the concern that CD38 may be an unreliable marker in CLL [18]. Another possible explanation is the variation in the positivity threshold for Zap-70 in the literature [6,14,17]. In accordance with Deaglio et al. [14], we used a cut-off value of ≥10% to indicate $Zap\text{-}70^+$ expression. The threshold values for CD38 and Zap-70 used by Deaglio et al. [14] were functional thresholds, below which CD38-mediated Zap-70 tyrosine phosphorylation was undetectable.

Table 2. High and low *Cryptochrome-1* gene expression and clinicopathologic characteristics of chronic lymphocytic leukemia patients at diagnosis.

Parameters	High *CRY-1* (n=40)	Low *CRY-1* (n=54)	p-value
Age, years, median (IQR)	58.5 (54.3-64.8)	62 (55-67)	0.892
Sex, n (%)			
Male	22 (55)	40 (74.1)	
Female	18 (45)	14 (25.9)	0.172
Lymphadenopathy, n (%)			
Present	30 (75)	42 (77.8)	
Absent	10 (25)	12 (22.2)	1.000
Splenomegaly, n (%)			
Present	16 (40)	22 (40.7)	
Absent	24 (60)	32 (59.3)	0.959
Hepatomegaly, n (%)			
Present	12 (30)	10 (18.5)	
Absent	28 (70)	44 (81.5)	0.489
Binet stage, n (%)			
A (low-risk)	12 (30)	20 (37)	
B (intermediate-risk)	8 (20)	4 (7.4)	
C (high-risk)	20 (50)	30 (55.6)	0.436
TLC, $x10^9$/L, median (IQR)	21.9 (10.8-66)	46 (25-125)	0.100
Hemoglobin, g/dL, mean ± SD	10.7±2.9	10.8±2.3	0.856
Platelets, $x10^9$/L, mean ± SD	145±69	158±89	0.572
PB lymphocytes, $x10^9$/L, median (IQR)	19.8 (7.6-52)	38.5 (21-95)	0.114
BM lymphocytes, %, median (IQR)	84.5 (78-90)	85 (78-90)	0.367
CD38 expression, n (%)			
Positive	24 (60)	16 (29.6)	
Negative	16 (40)	38 (70.4)	0.031
Zap-70 expression, n (%)			
Positive	16 (40)	6 (11.1)	
Negative	24 (60)	48 (88.9)	0.021
CD38 and Zap-70 expression, n (%)			
Double-positive	12 (30)	0 (0)	
Double-negative	12 (30)	32 (59.3)	
Discordant	16 (40)	22 (40.7)	0.002
Cytogenetic abnormalities, n (%)*			
Favorable	6 (15)	32 (59.3)	
Intermediate	14 (35)	10 (18.5)	
Unfavorable	18 (45)	4 (7.4)	0.005
IgHV gene, n (%)			
Mutated	14 (35)	23 (42.6)	
Unmutated	19 (47.5)	13 (24.1)	0.037
Pattern of BM infiltration, n (%)**			
Diffuse	23 (57.5)	15 (27.8)	
Nondiffuse	19 (47.5)	27 (50)	0.041
Time to first treatment, months, median (95% confidence interval)	16.89 (15.38-18.41)	23.69 (23.44-23.95)	<0.001

*Favorable: 13q-, normal karyotype; Intermediate: +12; Unfavorable: 17p-, 11q-, complex karyotype (≥ 3 chromosomal aberrations) [13].

**Nondiffuse infiltration included nodular, interstitial, or mixed nodular/interstitial infiltrations.

BM: Bone marrow, CLL: chronic lymphocytic leukemia, *CRY-1: Cryptochrome-1*, *IgHV: immunoglobulin heavy-chain variable-region*, IQR: interquartile range, PB: peripheral blood, SD: standard deviation, TLC: total leukocytic count.

Table 3. Predictors for earlier time to first treatment according to Cox proportional hazard regression model.

Predictors	Hazard rati (95% confidence interval)	p-value
Binet stage	2.80 (1.32-4.96)	0.036
CD38 expression	0.38 (0.08-1.84)	0.232
Zap-70 expression	2.41 (0.26-7.65)	0.003
Cytogenetic-based risk groups	1.26 (0.90-2.79)	0.017
IgHV gene mutational status	4.23 (2.52-6.98)	<0.001
Pattern of BM infiltration	0.98 (0.17-1.78)	0.360
CRY-1 gene expression	3.99 (2.12-6.19)	0.001

BM: Bone marrow, *CRY-1: Cyptochrome-1, IgHV: immunoglobulin heavy-chain variable-region.*

We found an association between high *CRY-1* gene expression and both unfavorable/intermediate cytogenetic abnormalities and diffuse BM infiltration. In accordance, high *CRY-1* gene expression was detected in association with 17p- and +12, while low *CRY-1* gene expression was found in a case with 13q- as the sole abnormality [6]. Interestingly, since the *CRY-1* gene is located at chromosome 12q23-q24.1, an increased copy number was detected in patients with +12 [10]. On the other hand, we could not find further association between *CRY-1* gene expression levels and other studied clinicopathologic parameters. Yu et al. [19] also stated that neither age nor sex was associated with *CRY-1* gene expression level.

In our study, *CRY-1* was differentially expressed among CLL patients with mutated and unmutated *IgHV* genes, being overexpressed in the unmutated group. Other authors advocated the association between high *CRY-1* gene expression and the unmutated *IgHV* gene [6,10,17]. A cut-off value of 0.090 for *CRY-1* gene expression in $CD19^+$ B cells of CLL patients was previously determined [6] and considered the best cut-off for segregating patients with mutated and unmutated *IgHV* genes (sensitivity: 95%; specificity: 92%; area under the curve: 0.963). The data of that study showed 92.8% concordance between *CRY-1* expression and *IgHV* mutational status.

We observed a shorter median TFT in the group with high *CRY-1* expression (16.89 months) compared with that with low *CRY-1* gene expression (23.69 months). Lewintre et al. [6] reported that high *CRY-1* gene expression was significantly related to shorter median progression-free survival of 63.2 months (95% CI: 48.2-78.2), compared to a median of 139 months (95% CI: 133.1-146.4) for the low *CRY-1* gene expression group (p<0.0001). Using multivariate Cox hazard regression, we found that *CRY-1* gene expression was independently predictive for TFT (p=0.001). Eisele et al. [17] employed univariate Cox hazard regression and reported that *CRY-1* could predict the clinical outcome of CLL patients as measured by TFT.

Of note, Hanoun et al. [10] reported that the methylation status of the *CRY-1* promoter revealed a considerable prognostic influence in CLL, whereby patients with hypermethylated *CRY-1* promoters showed significantly longer treatment-free survivals compared with their hypomethylated counterparts. Unexpectedly, they reported comparable levels of *CRY-1* mRNA in high-risk CLL and normal donor B cells. Thus, they postulated that expression differences of the *CRY-1* gene in CLL could be attributed to an underexpression of *CRY-1* in low-risk cases of CLL rather than an overexpression in the high-risk group.

Conclusion

The circadian clock gene *CRY-1* is differentially expressed among CLL patients, stratifying them into low-risk and high-risk groups. *CRY-1* gene expression could constitute a reliable prognostic indicator for CLL progression, complementing the role of standard well-established prognostic factors. Accordingly, *CRY-1* gene expression could be employed as a prognostic indicator for disease progression during the initial prognostic work-up and follow-up for CLL patients.

Evaluation of *CRY-1* expression with respect to the overall survival of CLL patients is warranted. Study of *CRY-1* gene methylation status and stability of expression at different time points throughout the course of CLL represents an interesting area for future research. Clinical trials for assessment of therapeutic modalities targeting the *CRY-1* gene in larger cohorts of CLL patients are worthwhile.

Ethics

Ethics Committee Approval: The study protocol was endorsed by the Ethical Committee for Human Research of Ain Shams University.

Informed Consent: Informed consent was provided prior to patient enrollment.

Authorship Contributions

Surgical and Medical Practices: D.M.H., D.S.E., M.M.A.; Concept: D.M.H., D.S.E.; Design: D.M.H., D.S.E.; Data Collection or Processing: M.M.A.; Analysis or Interpretation: D.M.H., D.S.E., M.M.A.; Literature Search: D.S.E., M.M.A.; Writing: D.M.H., D.S.E., M.M.A.

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.

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RESEARCH ARTICLE

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Correlation Between Baseline 18F-FDG PET/CT Findings and CD38- and CD138-Expressing Myeloma Cells in Bone Marrow and Clinical Parameters in Patients with Multiple Myeloma

Multipl Myelom Hastalarında Kemik İliği 18F-FDG PET/BT Bulguları ile Myelom Hücrelerinde CD38, CD138 Ekspresyonu ve Hematolojik Parametreler Arasındaki Korelasyon

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Abstract

Objective: The aim of this study was to evaluate the relation between the rate of fluorine-18 (18F) fludeoxyglucose (FDG) uptake and CD38 and CD138 expression in myeloma cells in bone marrow and other clinical parameters in patients with multiple myeloma (MM).

Materials and Methods: Patients with the diagnosis of MM who underwent 18F-FDG positron emission tomography/computed tomography (PET/CT) for initial staging were evaluated retrospectively. We analyzed a total of 42 patients (43-83 years old, mean: 64.4±9.9). Hematological and biochemical tests including hemoglobin, hematocrit, C-reactive protein, β2-microglobulin, creatinine, albumin, calcium, lactate dehydrogenase, and erythrocyte sedimentation rate were recorded. In bone marrow samples, plasma cell ratio and CD38 and CD138 immunohistochemical staining were evaluated. On PET/CT images, mean standardized uptake values (SUV_{mean}) of the right anterior and posterior iliac crest and right proximal femora were calculated. The correlations between the average SUV_{mean} of bone marrow and CD38- and CD138-expressing myeloma cells and other parameters were analyzed by Spearman's correlation test. Values of $p<0.05$ were considered statistically significant.

Results: Types of MM were IgG_K (45%), IgG_L (21%), IgA_K (7%), IgA_L (10%), and others (17%). Thirty-two (76%) patients were at stage III according to the Salmon-Durie staging system. There was a statistically significant positive correlation between bone marrow FDG uptake and percentage of plasma cells in bone marrow and CD38 and CD138 expression in plasma cells (r=0.403, r=0.339, and r=0.409) and β2-microglobulin and C-reactive protein levels (r=0.676, r=0.541). There was a negative correlation between bone marrow FDG uptake

Öz

Amaç: Bu çalışmanın amacı multipl myelom (MM) hastalarında kemik iliği florin-18 (18F) fluorodeoxyglucose (FDG) tutulumu ile plazma hücrelerinde CD38, CD138 ekspresyonu oranı ve diğer klinik parametreler arasındaki ilişkiyi değerlendirmektir.

Gereç ve Yöntemler: MM tanısı almış, ilk evreleme amacıyla 18F-FDG positron emisyon tomografi/bilgisayarlı tomografi (PT/BT) yapılan hastalar geriye dönük olarak değerlendirildi. Toplam 42 hasta analiz edildi (43-83 yaş, ortalaması: 64,4±9,9). Hematolojik ve biyokimyasal testlerden hemoglobin, hematokrit, C reaktif protein, beta 2-mikroglobulin, kreatinin, albumin, kalsiyum, laktat dehidrogenaz, sedimentasyon düzeyleri kayıt altına alındı. Kemik iliğinde plazma hücre düzeyi ve immünhistokimya ile CD38 ve CD138 boyanma oranına bakıldı. 18F-FDG PET/BT görüntülerinde sağ proksimal femur, sağ ön ve arka ilyak krest ortalama standart tutulum oranı ($SUV_{ort.}$) kaydedildi. Kemik iliği $SUV_{ort.}$ ve myelom hücrelerinde CD38, CD138 ekspresyonu ve diğer klinik parametreler arasındaki korelasyon Spearman korelasyon testi ile analiz edildi. P değerleri <0,05 olduğunda anlamlı kabul edildi.

Bulgular: Hastaların %45'ini IgG_K, %21'ini IgG_L, %7'sini IgA_K, %10'unu IgA_L ve %17'sini diğer myeloma tipleri oluşturuyordu. Otuz iki hasta (%76) Salmon-Durie sınıflamasına göre evre 3 idi. Kemik iliği FDG tutulumu ile plazma hücre oranı ve CD38, CD138 ekspresyonu arasında (r=0,403, r=0,339 ve r=0,409) ve beta 2-mikroglobulin ve C reaktif protein düzeyi arasında (r=0,676, r=0,541) istatistiksel anlamlı pozitif korelasyon vardı. Kemik iliği FDG uptake ile hemoglobin ve hematokrit değerleri arasında negatif korelasyon bulundu (r=-0,377 ve r=-0,368). Diğer hematolojik paremetreler kemik iliği FDG tutulumu arasında bir korelasyon saptanmadı.

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and hemoglobin and hematocrit values (r=-0.377 and r=-0.368). Other hematological parameters were not correlated with FDG uptake in bone marrow.

Conclusion: Increased FDG uptake is correlated with the percentage of CD38 and CD138 expression in plasma cells in bone marrow. In addition to initial staging, 18F-FDG PET/CT is useful in treatment planning and prognostic evaluation in MM patients.

Keywords: Multiple myeloma, CD38/CD138 antigen, Positron emission tomography/computed tomography, PET/CT

Sonuç: Kemik iliğinde artmış FDG tutulumu, plazma hücre oranı ve CD38, CD38 ekspresyonu ile ilişkilidir. MM hastalarında 18F-FDG PET/BT, ilk evreleme yanında tedavi planlaması ve prognostik değerlendirmede de yararlıdır.

Anahtar Sözcükler: Multipl myelom, CD38/CD138 antijen, Pozitron-emisyon tomografi/bilgisayarlı tomografi, PET/BT

Introduction

Multiple myeloma (MM) is a plasma cell neoplasm and the second most common hematologic neoplasm, accounting for 1% of all cancers and 13% of hematologic malignancies [1].

Positron emission tomography/computed tomography (PET/CT) with fluorine-18 (18F) fluorodeoxyglucose (FDG) is a whole-body imaging method that provides anatomical and metabolic information and is a useful technique for staging and therapy monitoring in patients with hematologic malignancies [2,3]. It is very useful in myeloma for detecting skeletal and extramedullary lesions with a sensitivity of approximately 80%-90% and a specificity of 80%-100% [4]. It can contribute to prognostic evaluation of MM patients. In a previous study, the authors showed that the number of focal lesions detected by PET/CT is a predictor of worse disease prognosis and death in these patients [5]. Recently, a guideline for imaging techniques in the management of MM patients stated that FDG PET/CT is an efficient imaging method for consecutive monitoring of disease burden of patients with nonsecretory myeloma, oligosecretory myeloma, and extramedullary disease. It was also reported that 18F-FDG PET/CT can define more lesions than plain X-rays in 40%-60% of cases and can be used for initial diagnosis of MM and assessment of suspected solitary plasmacytoma [6]. Some authors reported that elevated uptake of 18F-FDG by tumor cells is related to the metabolic activity of the tumor in MM [7,8,9].

CD38 is a membrane antigen expressed in the course of early B-cell growth. It is not expressed on mature activated B cells, while it is significantly re-expressed on plasma cells [10]. CD138 is transiently expressed by immature B-lymphocyte precursors, is lacking on mature circulating B cells, and is significantly expressed again after the differentiation to plasma cells [11]. Plasma cells in MM are positive for CD38 and CD138 compared to usual plasma cells [12]. The rate of infiltrating plasma cells expressing CD38 and CD138 in the bone marrow of MM patients may be related to disease activity.

Some laboratory parameters such as anemia, hypoalbuminemia, hypercalcemia, and high β2-microglobulin (β_2M), C-reactive protein (CRP), creatinine (Cr), and lactate dehydrogenase (LDH) are also prognostically relevant in patients with MM [13,14,15,16,17].

In this study, we aimed to evaluate the relation between the 18F-FDG uptake of bone marrow and the expression of CD38 and CD138 in plasma cells and clinical parameters in patients with MM retrospectively.

Materials and Methods

Study Design and Patient Population

From March 2013 to December 2016, all patients with newly diagnosed MM who underwent 18F-FDG PET/CT for initial staging were evaluated retrospectively. A total of 42 patients (20 males, 22 females) between 43 and 83 years old (mean ± SD: 64.4±9.9 years) were included in the study. Demographic and clinical data of the patients are shown in Table 1.

The myeloma diagnosis was made based on the updated criteria of the International Myeloma Working Group [18]. Patients who were treated with chemotherapy, radiation therapy, or hematopoietic growth factor previously or who had a history of another malignancy or rheumatological disease were excluded. 18F-FDG PET/CT imaging was performed for all patients within 2 weeks of the initial diagnosis. Hematological and biochemical

Table 1. Demographic and clinical properties of patients.

n	42 (100%)
Age, mean, years	64.4±9.9
Sex, M/F	20/22 (48%/52%)
Monoclonality type	
IgG_K	19 (45%)
IgG_L	9 (21%)
IgA_K	3 (7%)
IgA_L	4 (10%)
Other	7 (17%)
Stage	
I	3 (7%)
II	7 (17%)
III	32 (76%)

M: Male, F: female.

test results including levels of hemoglobin (Hb), hematocrit (Htc), CRP, Cr, albumin, calcium (Ca), LDH, and erythrocyte sedimentation rate were obtained for all patients within 7 days of PET/CT imaging. β_2M levels were evaluated in fifteen patients. In the bone marrow specimens, the ratios of plasma cells to CD38 and CD138 immunohistochemical staining were evaluated. Conventional radiographic skeletal surveys of the skull, ribs, spine, pelvis, humerus, and femur were examined in all patients. The flowchart of the study design is shown in Figure 1.

The local ethics committee of Adnan Menderes University approved the study.

18F-FDG PET/CT Imaging

All patients' fasting blood sugar levels were less than 180 mg/dL prior to imaging. After intravenous injection of 270-370 MBq of 18F-FDG, patients rested in a quiet room. Oral contrast was given to all patients. Whole-body imaging was performed after a resting period of 60 min using a Siemens Biograph mCT PET/CT scanner. The CT scan data were collected at 120 kV and 50 mAs. The PET acquisition was obtained from head to foot at a rate of 2 min/frame.

All FDG PET/CT images were evaluated visually and semiquantitatively by two nuclear medicine physicians. For semiquantitative evaluation, the mean standardized uptake value (SUV_{mean}) of the right anterior and posterior iliac crests and the right proximal femur was calculated with a semiautomatic image registration software package. Femurs and iliac bones were chosen to standardize the calculation of bone marrow FDG uptake concordant with previous studies and bone marrow sampling [9,19]. The average SUV_{mean} was used for statistical analysis. To reduce the effect of an inhomogeneous distribution of tracer, SUV_{mean} was preferred to SUV_{max} for calculation of bone marrow FDG uptake.

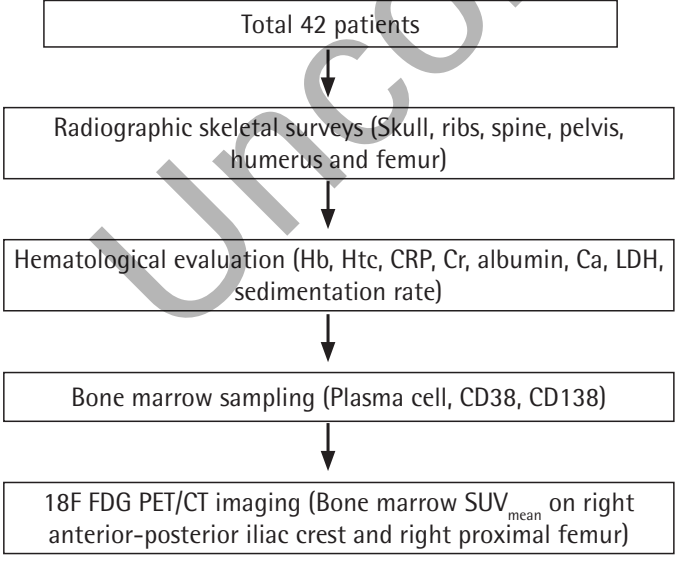

Figure 1. Flowchart of the study design.

Hb: Hemoglobin, Htc: hematocrit, CRP: C-reactive protein; Cr: creatinine, Ca: calcium, LDH: lactate dehydrogenase.

Immunohistochemical Staining

Histopathological features in tissue preparations of patients with MM were evaluated. Plasma cell ratios in bone marrow were confirmed by Giemsa-stained aspirations. CD38 and CD138 immunohistochemical staining was applied (Santa Cruz Biotechnology, USA; Sc-7325, 200 µg/mL, 1/500 dilution). Immunohistochemical staining was done with an avidin-biotin complex system. All examinations were done with a light microscope (Olympus BX51, Japan). Cytoplasmic and membranous staining was taken into account. Staining was scored by counting at least 200 tumor cells in neighboring tumor areas where the staining was the most intense, and by measuring the ratio of stained cells to those not stained. Immunohistochemical staining in two cases is shown in Figures 2A and 2B.

Statistical Analysis

Statistical assessment was done using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). The correlations between semiquantitative values of bone marrow FDG uptake and CD38- and CD138-expressing myeloma cells and other clinical parameters were analyzed by Spearman's rank correlation test. Values of $p<0.05$ were considered to be statistically significant.

Results

Average SUV_{mean} was between 0.73 and 13.84 (mean: 2.46±1.99). In bone marrow, the percentage of CD38-expressing myeloma cells ranged from 5% to 90% (mean: 38.9±22.82%) and that of CD138-expressing cells ranged from 5% to 90% (mean: 36.4±22.56%). There was a statistically significant positive correlation between bone marrow FDG uptake and CD38 and CD138 expression in plasma cells ($p=0.030$, $r=0.339$ and $p=0.008$, $r=0.409$). The ratio of plasma cells in bone marrow ranged from 5% to 80% (mean: 33.73±20.73%) and there was

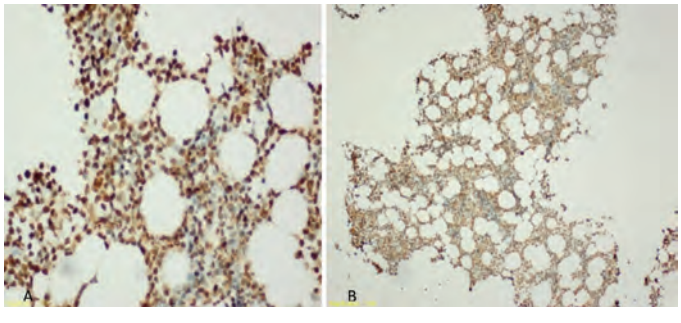

Figure 2. CD38 and CD138 expression detected by immunohistochemistry. A case with >90% CD38 positivity (A) and a case with >50% CD138 positivity (B).

Table 2. Hematological and biochemical parameters and plasma cell and CD38/CD138 ratios in patients.

	Hb, g/dL	Htc, %	Sed, mm	Alb, g/dL	Cr, mg/dL	LDH, U/L	Ca, mg/dL	CRP, mg/dL	β_2M, mg/L	Plasma cell %	CD38 %	CD138 %
Mean value	10.4±1.9	31.9±5.1	75.3±34.7	3.2±0.7	1.2±1.3	229±111.9	8.9±1.7	39.8±56.4	7.7±6.1	33.7±20.8	38.9±22.8	36.4±22.5
r	0.377*	0.368*	0.013	0.125	0.194	0.064	0.198	0.541**	0.676*	0.043**	0.339*	0.409**
p	0.023	0.027	0.935	0.436	0.223	0.691	0.246	0.001	0.011	0.009	0.030	0.008

Results of Spearman's analysis with SUV_{mean} correlation coefficients: *p<0.05, **p<0.01.

Hb: Hemoglobin, Hct: hematocrit, Sed: erythrocyte sedimentation rate, Alb: Albumin, Cr: creatinine, LDH, lactate dehydrogenase, Ca: calcium, CRP: C-reactive protein, β_2M: β2-microglobulin.

also a positive correlation between the ratio of plasma cells and FDG uptake of bone marrow (p=0.009, r=0.403). There was a negative correlation between SUV_{mean} of bone marrow and Hb and Htc values (p=0.023, r=-0.377 and p=0.027, r=-0.368). Positive correlations between bone marrow FDG uptake and β_2M (p=0.011, r=0.676) and CRP levels (p=0.001, r=0.541) were also detected. There were no correlations between bone marrow FDG uptake and albumin, Cr, Ca, sedimentation rate, or LDH levels. The mean hematological values and detailed statistical results are shown in Table 2.

Discussion

18F-FDG PET/CT is an imaging procedure that can be used for initial evaluation of MM patients. This imaging method may aid to better specify osteolytic lesions, allowing for earlier detection of the disease. It can also define lesions in patients with negative results from conventional imaging methods. FDG PET/CT can define both medullary and extramedullary disease with reasonable success in one session in patients with MM, but this method may be suboptimal when there are diffuse bone marrow plasma cell infiltrations and lytic lesions in the skull [18]. 18F-FDG PET/CT can also be used for the estimation of prognosis in MM patients. Fonti et al. [5] reported that the numbers of focal lesions in 18F-FDG PET/CT or 99mTc-MIBI imaging and diffuse 99mTc-MIBI uptake are independent predictors for progression-free survival (PFS) and overall survival (OS) in patients with MM, but neither focal nor diffuse MRI pattern was an independent predictor of PFS or OS in a comparative study. They also concluded that 18F-FDG PET/CT or 99mTc-MIBI imaging must be performed at the time of initial diagnosis for specifying patients with worse outcomes who may be helped by more aggressive therapies [5].

CD38 is a type II transmembrane glycoprotein expressed on lymphoid and myeloid cells and also in nonhematopoietic tissues. It is highly expressed especially on MM cells. CD138 is a transmembrane heparan sulfate proteoglycan that provides some cellular functions including cell-cell adhesion and cell-matrix adhesion [10,11,12]. The presence of CD38 and CD138 revealed by immunohistochemical staining is a good indicator of plasma cells in bone marrow biopsy and CD38 and CD138 expressions have a diagnostic role in MM [20,21].

Anti-CD38 monoclonal antibodies such as daratumumab are important components of myeloma treatment [22,23,24]. Immunohistochemical studies have shown that CD138 is suitable for the identification and quantitation of normal and neoplastic plasma cells and thus helpful for the classification and assessment of malignant hematologic neoplasms. In addition, CD138 is an important marker in quantitation of the plasma cell population. Recently, anti-CD138 chimeric antigen receptor-modified T-cell treatment for MM has been reported [25].

There are only two studies related to PET/CT and plasma cell infiltration of bone marrow in MM patients. Ak and Gulbas [9] investigated 18F-FDG uptake and CD38/138 expression in the bone marrow of patients with MM. They reported that the FDG uptake of bone marrow was significantly related to the ratio of CD38/138-expressing plasma cell infiltration of bone marrow in patients with MM. In another study, Sager et al. [19] reported that there were significant correlations between bone marrow biopsy cellularity and plasma cell ratio and SUV_{max} values. The sensitivity of FDG PET in defining bone marrow involvement at initial diagnosis was 90% in this study.

In our study, we analyzed the association between FDG uptake of bone marrow and CD38- and CD138-expressing plasma cell infiltration ratio in bone marrow in patients with MM. Our study revealed that there was a statistically significant positive correlation between the percentage of CD38- and CD138-expressing plasma cells in bone marrow and FDG uptake of bone marrow (p=0.030 and p=0.008). This result suggests that increased FDG uptake of bone marrow is connected to the percentage of plasma cell infiltration of bone marrow in patients with MM. In addition, increased FDG uptake of bone marrow may be a marker for CD38 expression, which offers a possible therapeutic Ab target for the therapy of MM and thus may contribute to the selection of patients for immunotherapy. Additionally, after CD38 monoclonal antibody therapy, plasma cells that express CD38/138 are decreased. Thus, posttreatment FDG PET/CT imaging can also be used for estimation of monoclonal treatment. Further studies are required to validate the relationship between bone marrow FDG uptake and therapy with monoclonal antibodies.

In this study there was a negative correlation between SUV_{mean} of bone marrow and Hb and Htc rates (p=0.023, r=-0.377 and p=0.027, r=-0.368). It is known that FDG-18 uptake of bone marrow is increased in patients with anemia. It was also shown that hematopoietic growth factors may cause high FDG uptake of bone marrow [26]. To exclude the effect of these therapeutic agents, we included only pretreatment patients in this study.

In MM patients, some laboratory parameters including anemia, hypoalbuminemia, hypercalcemia, and elevated β_2M, CRP, creatinine, and LDH are related to prognosis [13,14,15,16,17]. The correlation of pretreatment bone marrow FDG uptake with these prognostic factors may indicate a metabolic marker for poor prognosis in patients with MM. Park et al. [27] reported that SUV_{max} and number of hypermetabolic focal lesions on PET/CT images were positively correlated with prognostically relevant clinical factors. Ak and Gulbas [9] also showed a positive correlation between β_2M and bone marrow FDG uptake values.

While there were positive correlations between bone marrow FDG uptake and β_2M and CRP values, there were no correlations between FDG uptake value and albumin, Cr, Ca, sedimentation, or LDH values in our study. Although this study comprised a limited number of patients, these results showed that FDG-18 PET/CT may contribute to the identification of prognostically relevant clinical parameters, especially in the initial assessment of MM patients.

Our study has some limitations. Because it was designed as a retrospective study, we could not obtain the medical records of all patients. In addition, β_2M levels were evaluated for only 15 of 42 patients. Due to incomplete data, we could not evaluate the relation between our results and the prognosis of the patients. Another limitation of the study was the relatively small number of cases.

Conclusion

Increased FDG uptake is related to the percentage of plasma cell infiltration and CD38 and CD138 expression in plasma cells in the bone marrow. In addition to initial staging, 18F-FDG PET/CT is beneficial in therapy planning and prognostic assessment in patients with MM. Further studies with larger patient populations are required to validate the relation between bone marrow FDG uptake and CD38 and CD138 expression in plasma cells and other hematological parameters.

Ethics

Ethics Committee Approval: Adnan Menderes University (approval number: 2016/890).

Informed Consent: Retrospective study.

Authorship Contributions

Surgical and Medical Practices: H.Ü.A., İ.Y., A.Z.B.; Concept: A.C., A.Z.B.; Design: A.C., A.Z.B.; Data Collection or Processing: A.C., H.Ü.A., F.D., Y.Y.; Analysis or Interpretation: A.C., A.Z.B.; Literature Search: A.C., A.Z.B.; Writing: A.C., Y.Y., A.Z.B.

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.

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RESEARCH ARTICLE

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Anemia Associated with Worse Outcome in Diffuse Large B-Cell Lymphoma Patients: A Single-Center Retrospective Study

Anemi Diffüz büyük B-hücreli lenfoma Hastalarında Kötü Prognozla İlişkilidir: Tek Merkezli Retrospektif Bir Çalışma

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Abstract

Objective: Useful prognostic biomarkers for diffuse large B-cell lymphoma (DLBCL) patients have been reported. To determine the prognostic value of hemoglobin (Hb) level in DLBCL patients, we performed a retrospective study.

Materials and Methods: We evaluated disease outcome, progression-free survival (PFS), overall survival as the endpoint, and clinical and laboratory factors affecting the outcome of 185 DLBCL patients who had received rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone therapy during 2004-2014.

Results: The study group included 121 men and 64 women with a median age of 66 years minimum-maximum: 21-83 years. In univariate analysis, factors independently associated with worse PFS were Eastern Cooperative Oncology Group performance status ≥2, Ann Arbor stage III or IV, anemia with Hb levels of <10 g/dL, and serum albumin of <3.5 g/dL. In multivariate analysis, anemia with Hb levels of <10 g/dL and Ann Arbor stage III or IV were found to be international index-independent prognostic factors (hazard ratio: 2.4; p=0.04).

Conclusion: Anemia is an independent prognostic marker of poor outcome in DLBCL patients. Hb can be an easily available prognostic marker for risk stratification in these patients.

Keywords: Anemia, Diffuse large B-cell lymphoma, Hemoglobin

Öz

Amaç: Diffüz büyük B hücreli lenfoma (DBBHL) hastaları için yararlı prognostik belirteçler bildirilmiştir. DBBHL hastalarında hemoglobin (Hb) düzeyleri ile prognoz ilişkisini araştırmak amacıyla retrospektif bir çalışma yaptık.

Gereç ve Yöntemler: 2004-2014 yılları arasında rituksimab, siklofosfamid, doksorubisin, vinkristin ve prednizolon tedavisi almış olan 185 DBBHL hastasının klinik ve laboratuvar bulguları değerlendirildi ve bunların hastalık sonuçlarına, ilerlemesiz sağkalım (PFS) ve toplam sağkalıma (TS) etkileri araştırıldı.

Bulgular: Araştırma grubunda 121 erkek ve 64 kadın vardı, ortanca yaş 66 yıldı (minimum-maksimum: 21-83 yıl). Tek değişkenli analizde Eastern Cooperative Oncology Group performans durumunun ≥2 olması, Ann Arbor evre III veya IV olması ve Hb değerinin <10 g/dL ile anemi olması PFS ve TS'yi etkileyen bağımsız değişkenler olarak bulundu. Çok değişkenli analizde ise Hb değerinin <10 g/dL ile anemi ve Ann Arbor evre III veya IV olması uluslararası indeks-bağımsız prognostik faktörler olarak bulundu (Hazard ratio: 2,4; p=0,04).

Sonuç: Anemi, DLBCL hastalarında kötü sonuçların bağımsız prognostik bir göstergesidir. Hb, bu hastalarda risk sınıflandırması için kolayca bulunabilen bir prognostik belirteç olabilir.

Anahtar Sözcükler: Anemi, Diffüz büyük B-hücreli lenfoma, Hemoglobin

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common non-Hodgkin lymphoma subtype, which accounts for 30%-40% of newly diagnosed malignant lymphoma cases [1]. Standard immunochemotherapy, such as R-CHOP containing rituximab, cyclophosphamide, hydroxydaunorubicin, vincristine, and prednisolone, has been proven to be beneficial for the outcome of DLBCL patients; however, approximately one-third of patients with advanced-stage disease still experience relapse or are refractory to therapy [2,3]. There are many well-known established prognostic models to identify patients at high risk of

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disease progression or relapse, or refractory to therapy, because it is an urgent necessity to improve outcomes. The International Prognostic Index (IPI), a well-known and useful tool for predicting the clinical outcomes of DLBCL patients, includes age, serum lactate dehydrogenase (LDH) level, performance status, Ann Arbor stage, and number of extranodal lesions [4]. Variants are reported for elderly patients (age-adjusted IPI) and patients treated with rituximab (R-IPI) [5]. Recently, the National Comprehensive Cancer Network (NCCN) published a reformed IPI, which weighted the scoring for increasing age and LDH levels [6]. Unfortunately, clinical risk stratification models, such as IPI, R-IPI, and NCCN-IPI, are not completely accurate in identifying patients who will not be sufficiently cured by first-line R-CHOP therapy. There is an urgent necessity for new prognostic biomarkers. In particular, anemia is commonly associated with lymphoma, even in chemotherapy-naive patients, and in the absence of bone marrow (BM) involvement [7]. The pathogenesis of lymphoma-associated anemia is multifactorial and may include BM dysfunction, problems with iron reutilization, and an inadequate erythropoietin response. In this study, we evaluated the significance of pretreatment hemoglobin (Hb) levels.

Materials and Methods

This study was approved by the Yokohama City University Medical Center Clinical Research Ethics Board. All procedures used in this study were in accordance with the Declaration of Helsinki.

Patients

We reviewed the records of 226 patients diagnosed with DLBCL at Yokohama City University Medical Center during 2004-2014. Forty-one patients were excluded due to the following reasons: transfer to another hospital after diagnosis (n=12), other regimens (n=18), radiation only (n=5), received supportive care only because of poor performance status (n=3), double cancer (n=1), early death within 30 days (n=1), and lack of information (n=1). The following clinical data on 185 patients were collected from medical records and pathology reports: histological confirmation of diagnosis, sex, age, Ann Arbor clinical stage, presence of B symptoms, LDH levels, serum albumin levels, Eastern Cooperative Oncology Group (ECOG) performance status, and BM involvement data. Hematology data, including full blood count, were obtained at diagnosis, 1-7 days before initiating treatment. The institutional lower limit of normal (LLN) of hemoglobin (Hb) in complete blood count was set as 13.8 g/dL for male patients and 11.3 g/dL for female patients. Severity of anemia was graded by Hb levels according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.0, as follows: G1, <LLN to 10.0 g/dL; G2, <10.0 to ≤8.0 g/dL; G3, <8.0 g/dL or transfusion indicated; and G4, life-threatening. BM involvement was detected by either aspiration or biopsy of BM, except for positron emission tomography imaging.

Patients were treated with a standard immunochemotherapy regimen containing rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone (R-CHOP). The R-CHOP regimen comprised 6-8 cycles of 750 mg/m^2 cyclophosphamide, 50 mg/m^2 doxorubicin, and 1.4 mg/m^2 (maximum 2.0 mg/kg body weight) vincristine on day 1; 100 mg/body prednisolone on days 1-5; and 375 mg/m^2 rituximab per cycle for 21 days.

Progression-free survival (PFS) was defined as the time from R-CHOP therapy initiation to lymphoma progression, death from any cause, or last follow-up. Overall survival (OS) was defined as the time from diagnosis to death from any cause or until the time of last follow-up for patients who remained alive.

Statistical Analysis

Kaplan-Meier analysis was used to calculate PFS. The log-rank test was used to assess univariate associations between PFS and prognostic variables. A forward-backward stepwise variable selection for the Cox proportional hazards model was used for multivariate analysis. A value of $p<0.05$ was considered statistically significant. All statistical analyses were performed with EZR (version 1.10) [8], which is a graphical user interface for R (R Foundation for Statistical Computing, version 2.13.0), using the modified version of the R commander (version 1.6-3) designed to add statistical functions frequently used in biostatistics.

Results

Patient Characteristics

Among the 226 patients diagnosed with DLBCL at Yokohama City University Faculty of Medicine during 2004-2014, 185 patients satisfied the inclusion criteria. The characteristics of the study participants are summarized in Table 1. The study included 121 males and 64 females, with a median age of 66 years (minimum-maximum: 21-83 years).

Patient Outcomes and Prognostic Factors

The median observation period in surviving patients was 55.3 months (minimum-maximum: 4.8-117 months). The estimated PFS rate of all patients at 3 and 5 years was 76.1% and 72.0%, respectively. The OS rate at 3 and 5 years was 85.0% and 80.1%, respectively (Figure 1).

The mean baseline Hb (±standard deviation) was 12.1±2.2 g/dL in male patients and 11.4±1.8 g/dL in female patients. Eight-seven patients (47%) had G≥1 and 33 patients (18%) had G≥2 baseline anemia. Patients with G≥2 anemia showed inferior PFS compared with those with no (G0) or G1 anemia ($p<0.0029$; Figure 2).

On univariate analysis, factors associated with worse PFS included ECOG performance status ≥2 (vs. ≤1: p=0.041), Ann Arbor clinical stage (CS) ≥3 (vs. ≤2: $p<0.001$), G≥2 anemia (vs. G≤1: p=0.001), serum albumin <3.5 g/dL (vs. ≥3.5 g/dL: p=0.008), and BM involvement (vs. negative: $p<0.001$).

Multivariate analysis showed that Ann Arbor CS ≥3 [hazard risk (HR): 3.0; 95% confidence interval (CI): 1.4-6.4; p=0.005), G≥2 anemia (HR: 2.3; 95% CI: 1.2-4.3; p=0.012), and BM involvement (HR: 1.9; 95% CI: 1.0-3.6; p=0.037) were identified as risk factors (Table 2). Because the CS IV criteria include BM involvement, CS≥3

and G≥2 anemia remained as independent determinants. These factors were each assigned a score and the sum was tested as a prognostic index for PFS. The 3-year PFS of patients on an R-CHOP regimen with score 0 (n=79), score 1 (n=81), and score 2 (n=27) was 89.1%, 73.9%, and 35.5%, respectively (p<0.001), and the 3-year OS of patients on an R-CHOP regimen with score 0 (n=79), score 1 (n=81), and score 2 (n=27) was 94.6%, 82.0%, and 61.4%, respectively (p<0.001; Figures 3A and 3B).

Discussion

Anemia is commonly encountered in patients with malignant lymphoma or lymphoproliferative disorders. The incidence was reported as approximately 39% previously [7]. The purpose of the current study was to determine whether anemia has a prognostic value in DLBCL, for which a number of prognostic tools have recently been devised. Because Hb status is a standard laboratory parameter, easy to measure, cheap, and highly reproducible in the clinical setting, Hb level could be readily incorporated into a newly differentiated prognostic index.

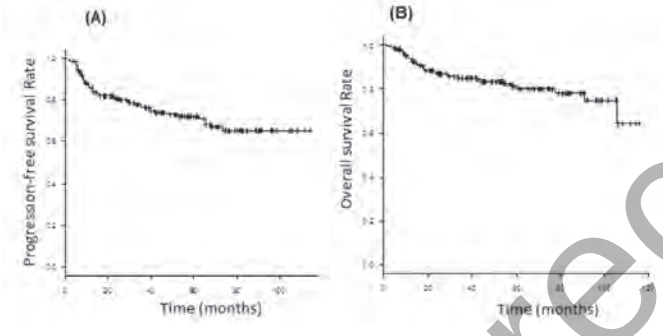

Figure 1. A) Progression-free survival (PFS) and B) overall survival (OS) in 185 patients with diffuse large B-cell lymphoma. The median observation period in surviving patients was 55 months (minimum-maximum: 4.8-117 months). The 3-year PFS rate was 76.1% and the 3-year OS rate was 80.1%.

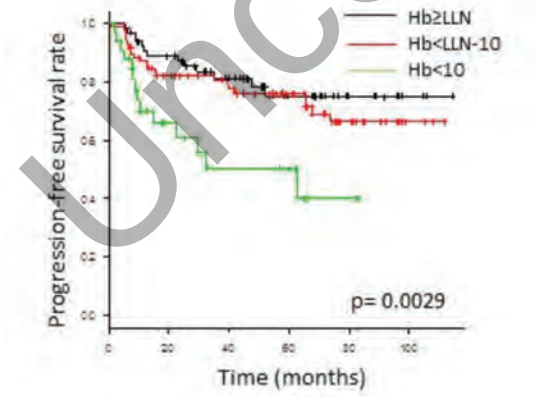

Figure 2. Analysis of the impact of anemia on treatment outcomes in patients with diffuse large B-cell lymphoma treated with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone therapy (n=185). Kaplan-Meier plots for progression-free survival according to the grade of baseline anemia.

Hb: Hemoglobin, LLN: Lower limit of normal.

In follicular lymphoma, Hb of <12 g/dL was known to be prognostic [9,10], but Chen et al. [11] showed that Hb of <12 g/dL did not show a significant association with inferior PFS

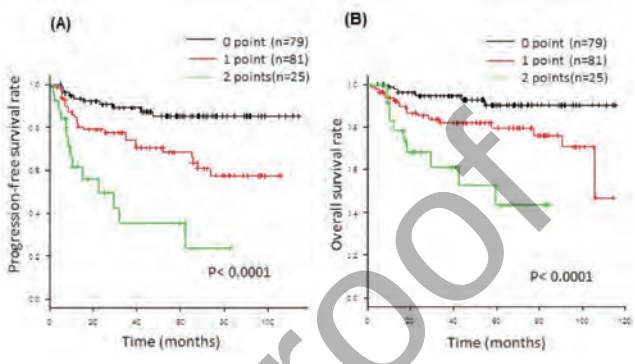

Figure 3. A) Progression-free survival and B) overall survival in 185 patients with diffuse large B-cell lymphoma according to prognostic index. Kaplan-Meier plots for event-free survival according to the grade of baseline anemia and clinical stage.

Table 1. Clinicopathological characteristics of patients with diffuse large B-cell lymphoma.

	Total
Sex	
Male	121.65%
Age at diagnosis, years	
Median	66
Minimum-maximum	21-83
>60 years	140.75%
Performance status	
≥2	49.26%
LDH	
Elevated	136.73%
Clinical stage (Ann Arbor)	
≥3	104.56%
Extranodal sites	
≥2	54.29%
B symptoms	
Present	56.30%
BM involvement	
Present	37.20%
Hemoglobin	
>LLN (grade 0)	65.35%
LLN-10 (grade 1)	87.47%
<10g/dL (grade 2)	33.18%
Serum albumin	
>3.5 g/dL	121.65%

DLBCL: Diffuse large B-cell lymphoma, LDH: lactate dehydrogenase, BM: bone marrow.

Table 2. Univariate and multivariate analysis for progression-free survival in all patients with diffuse B-cell lymphoma (n=185).

Parameters	HR (95% CI)	p-value
Univariate analysis		
Male	0.89 (0.49-1.51)	0.606
Age >60	1.16 (0.62-2.15)	0.643
ECOG performance status ≥2	1.84 (1.03-3.30)	0.041
Elevated lactose dehydrogenase	1.84 (0.92-3.68)	0.084
Clinical stage ≥3	4.31 (2.15-8.63)	<0.001
Extranodal sites ≥2	1.27 (0.70-2.30)	0.432
Presence of B symptom	1.44 (0.80-2.60)	0.226
Grade ≥2 anemia	2.78 (1.49-5.19)	0.001
Serum albumin <3.5	1.64 (0.94-2.87)	0.008
BM involvement	3.27 (1.84-5.79)	<0.001
Multivariate analysis		
Clinical stage ≥3	3.00 (1.40-6.42)	0.005
Grade ≥2 anemia	2.27 (1.19-4.30)	0.012
BM involvement	1.94 (1.04-3.63)	0.037

CI: Confidence interval, ECOG: Eastern Cooperative Oncology Group, HR: hazard ratio.

or OS in DLBCL patients treated with rituximab-containing immunochemotherapy. In a recent study by Hong et al. [12], G>2 anemia showed an association with inferior event-free survival , which was the same as our result.

Baseline anemia may not be an independent factor but rather a consequence of bone marrow involvement, which in turn may actually just be a part of various factors causing anemia. Tisi et al. [13] showed that lymphomatous BM involvement is independent of the occurrence of anemia, with no difference in Hb levels observed according to the BM status (median: 11.8 g/dL for patients without BM infiltration vs. 10.9 g/dL for those with BM infiltration, p=0.27). They also concluded that an elevated level of interleukin-6, a proinflammatory cytokine, was the dominant factor affecting anemia, and reduced erythropoietin synthesis may result in anemia.

Study Limitations

This study had some limitations, including its observational retrospective design and the analysis of a small number of patients.

Conclusion

In conclusion, anemia assessed by pretreatment Hb of <10.0 g/dL was an overall prognostic factor, and Hb is very easy to analyze in clinical settings, with almost no additional cost. For patients with DLBCL treated with R-CHOP, our new prognostic index, which consists of Hb and clinical stages, may be helpful for selecting the treatment strategy, including investigational salvage therapy, although the effectiveness of our index should be validated in a larger cohort.

Ethics

Ethics Committee Approval: Yokohama City University, approval number: D1503038.

Informed Consent: Retrospective study.

Authorship Contributions

Surgical and Medical Practices: K.M., S.F., T.A., M.K., S.K., Y.I., A.N., W.Y., K.M., M.H.; Concept: K.M., S.F.; Design: K.M.; Data Collection or Processing: K.M.; Analysis or Interpretation: K.M.; Literature Search: K.M.; Writing: K.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.

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RESEARCH ARTICLE

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Bringing Packed Red Blood Cells to the Point of Combat Injury: Are We There Yet?

Eritrosit Konsantrelerini Yaralanma Noktasına Götürmek: O Noktaya Ulaşabildik mi?

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Abstract

Objective: Hemorrhage is the leading cause of injury-related prehospital mortality. We investigated worst-case scenarios and possible requirements of the Turkish military. As we plan to use blood resources during casualty transport, the impact of transport-related mechanical stress on packed red blood cells (PRBCs) was analyzed.

Materials and Methods: The in vitro experiment was performed in the environmental test laboratories of ASELSAN®. Operational vibrations of potential casualty transport mediums such as Sikorsky helicopters, Kirpi® armored vehicles, and the NATO vibration standard MIL-STD-810G software program were recorded. The most powerful mechanical stress, which was created by the NATO standard, was applied to 15 units of fresh (≤7 days) and 10 units of old (>7 days) PRBCs in a blood cooler box. The vibrations were simulated with a TDS v895 Medium-Force Shaker Device. On-site blood samples were analyzed at 0, 6, and 24 h for biochemical and biomechanical analyses.

Results: The mean (±standard deviation) age of fresh and old PRBCs was 4.9±2.2 and 32.8±11.8 days, respectively. Six-hour mechanical damage of fresh PRBCs was demonstrated by increased erythrocyte fragmentation rates (p=0.015), hemolysis rates (p=0.003), and supernatant potassium levels (p=0.003) and decreased hematocrit levels (p=0.015). Old PRBC hemolysis rates (p=0.015), supernatant potassium levels (p=0.015), and supernatant hemoglobin (p=0.015) were increased and hematocrit levels were decreased (p=0.015) within 6 h. Two (13%) units of fresh PRBCs and none of the old PRBCs were eligible for transfusion after 6 h of mechanical stress.

Öz

Amaç: Kan kayıpları, hastane öncesi dönemdeki yaralanmalara bağlı ölümlerin en sık sebebidir. Türk ordusu için en kötü senaryoları ve olası ihtiyaçları araştırdık. Çatışma alanından nakil esnasında kan kaynaklarını kullanmayı planladığımız için nakil işleminden kaynaklı mekanik stresin eritrosit konsantreleri üzerine etkisini analiz edilmiştir.

Gereç ve Yöntemler: İn vitro çalışmalar ASELSAN®'ın dış ortam test laboratuvarlarında gerçekleştirildi. Çatışma alanında kan taşıma işleminde kullanılması muhtemel olan Sikorsky helikopteri ve Kirpi® araçlarının operasyonel vibrasyonları ve NATO MIL-STD-810G titreşim standart yazılımı kayıt altına alındı. NATO standardının en güçlü titreşime neden olduğu hesaplandı. Kan saklama çantası içindeki 15 ünite taze (≤7 gün) ve 10 ünite taze olmayan eritrosit konsantresi (>7 gün), NATO standardı olan mekanik strese maruz bırakıldı. Titreşim TDS v895 Medium-Force Shaker cihazı tarafından simüle edildi. Simülasyonun 0., 6. ve 24. saatinde biyokimyasal ve biyomekanik analiz için kan örnekleri alındı.

Bulgular: Taze ve taze olmayan eritrosit konsantreleri sırasıyla ortalama 4,9 [standart deviasyon (SD) ±2,2] ve 32,8 (SD ±11,8) günlüktü. Taze eritrosit konsantrelerinde 6. saatte gelişen mekanik hasar; artmış eritrosit fragmentasyonu (p=0,015), hemoliz oranı (p=0,003) ve supernatant potasyum (p=0,015) düzeyleri ile gösterildi. Taze olmayan eritrosit konsantrelerinin 6. saatte hemoliz oranı (p=0,015) ve supernatant potasyum düzeyi (p=0,015) yükselirken, hematokrit değerleri (p=0,015) düştü. İlk 6 saat içerisinde taze eritrosit konsantrelerinin 2'si (%13) transfüze edilebilir kalitede kalırken, taze olmayanların ise hiçbirisi uygun değildi.

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Conclusion: When an austere combat environment was simulated for 24 h, fresh and old PRBC hemolysis rates were above the quality criteria. Currently, the technology to overcome this mechanical damage does not seem to exist. In light of the above data, a new national project is being performed.

Keywords: Combat trauma, Blood transport, Prehospital transfusion, Hemolysis

Sonuç: Taze ve taze olmayan eritrosit konsantrelerinin hemoliz oranları, bu ürünleri muharebe sahasında taşıdığımızda, çoğunun kullanılamaz hale geleceğini göstermektedir. Halihazırda eritrositler üzerinde oluşan mekanik hasarlanmanın önüne geçebilecek bir teknoloji bulunmamaktadır. Yukarıdaki verilerin ışığı altında yeni bir ulusal proje gerçekleştirilmektedir.

Anahtar Sözcükler: Harp yaralanmaları, Kan taşınması, Hastane öncesi transfüzyon, Hemoliz

Introduction

During the last century, 90% of combat-related deaths occurred in the prehospital period (PHP), which only decreased to 75%-87% in recent military conflicts [1,2,3,4,5,6,7]. PHP mortality may be stratified as nonsurvivable (75%) and potentially survivable (25%) from a medical perspective [5,6,7]. The majority of potentially survivable PHP deaths (90%) were attributed to hemorrhage [7].

The Department of War Surgery of the University of Health Sciences has regarded the above data from recent military conflicts as an essential field of medical research. In order to decrease preventable injury-related deaths in the PHP, acquiring the capability of en route blood transfusion by transporting packed red blood cells (PRBCs) to the point of injury has proved valuable [8].

In a worst-case scenario, prolonged transportation of the already limited PRBC resources by army tactical ambulances and helicopters may be required for casualty evacuation missions. However, movement of these vehicles creates mechanical vibrations with different amplitudes and frequencies, which exert mechanical stress on PRBCs. We investigated the biochemical and biomechanical parameters of PRBCs exposed to vibration for 24 h.

Materials and Methods

The study was designed as a within-subjects, in vitro experiment and was approved by the Yeditepe University Clinical Research Ethics Committee (24/11/2016-682). The study was conducted at the Environmental Test Laboratory of ASELSAN Company (Macunköy, Ankara, Turkey).

Preparation of PRBCs

All consenting volunteers that met the criteria of the 2016 Turkish National Blood and Blood Products Guide for blood donations were eligible for the study. PRBCs were prepared by the Gülhane Regional Blood and Training Center from whole blood as described by Cetinkaya et al. [9]. The mean volume of PRBCs was 220±40 mL. We refer to blood of ≤7 days as fresh and blood of >7 days as old PRBCs. The mean age of fresh and old PRBCs was 4.9 (SD: ±2.19) and 32.8 (SD: ±11.8), respectively. All PRBCs were placed in an MT-25E cooler box and transported to ASELSAN Laboratories in 15 min. A Tinytag View 2 (TV-4510, UK) temperature data logger was placed in the cooler box and records showed that the temperature remained between 3.2 °C and 3.9 °C.

Vibration Analysis and Simulation of Vibration

The PRBCs were exposed to 24 h of simulated shaking by the LDS v895 Medium-Force Shaker Device (Brüel & Kjær Inc., Denmark). The first step was recording vibration profiles of potential PRBC carriers such as Sikorsky Blackhawk helicopters (S70) (routine flight) (Lockheed Martin, USA) and Kirpi multipurpose armored vehicles (rough terrain, 30 km/h speed) (BMC Inc., Turkey). Vibrations were recorded by the G-Sensor Pro v3.0.5 application for Android devices. Currently, the durability of all military equipment against vibrations is tested using the Test Method 514.6 (MIL-STD-810G, US Army Test and Evaluation Command, 2008) software program. Vibrations of Test Method 514.6 were also recorded. Root mean square acceleration values of three different vibration environments were calculated. Test Method 514.6 had the highest value and was chosen for testing PRBCs for 24 h (Table 1).

Table 1. Acceleration (a) root mean square (rms) calculations in x, y, and z directions of three different vibration recordings.

Vibration medium	Vibration movement on X axis	Vibration movement on Y axis	Vibration movement on Z axis	At,rms*
Blackhawk helicopter (S70)	1.007	2.032	9.621	9.885
Kirpi Armored Vehicle	7.622	4.510	5.605	10.481
MIL-STD-810G test method 514.6	12.187	9.402	15.554	21.883

*Total acceleration root mean square.

Blood Sample Analyses

Hematocrit, pH, supernatant hemoglobin, supernatant osmolality, supernatant potassium, 2,3-diphosphoglycerate (2,3-DPG), ATP (adenosine 5′-triphosphate), osmotic fragility, erythrocyte deformability, and erythrocyte fragmentation were measured at 0, 6, and 24 h. One milliliter of blood was collected and immediately analyzed for hematocrit and pH measurements via the IRMA TruPoint Blood Gas Analyzer (ITC, System Version 7.1, USA). Supernatant osmolality, potassium (mmol/L), and hemoglobin (g/dL) were measured using a Radiometer ABL 800 (Radiometer Trading, Denmark). ATP was assayed using an ATP assay kit (ab83366; Abcam, UK) and 2,3-DPG was assayed using a human 2,3-DPG enzyme-linked immunosorbent assay kit (CK-E11265, Eastbiopharm, China). Osmotic fragility was calculated using the Parpart method [10]. Presence of hemolysis at 0.45%-0.55%, >0.55%, and ≤0.30% NaCl concentrations was defined as normal, increased, and decreased osmotic fragility, respectively. Supernatant hemoglobin values were measured using Drabkin's method [11]. Erythrocyte deformability was determined using a laser-assisted optical rotational cell analyzer (LORCA; RR Mechatronics, the Netherlands). Elongation index (EI) was calculated during the application of 10 steps of shear stress (SS) in the range of 0.3 to 50 Pa; RBC deformability was expressed as EI-SS curves. These EI-SS data were characterized by the maximum EI at infinite SS (EI_{max}) and the SS needed to achieve one-half of this maximum ($SS_{1/2}$); the $SS_{1/2}/EI_{max}$ ratio was calculated as a normalized measure of $SS_{1/2}$. $SS_{1/2}/EI_{max}$ is inversely related to RBC deformability such that a lower value indicates better deformability. Red blood cell fragmentation was determined using a Multisizer 3 cell counter system (Beckman Coulter, USA).

Statistical Analysis

In order to determine the number of fresh and old PRBC units, we conducted a priori power analysis. PRBCs in a blood cooler box were exposed to Test Method 514.6 vibrations for 6 h and tested for hemolysis percent and potassium levels. Sample size analysis was performed using the Güç Analizi (Power Analysis) application (Savante Mobile Apps, Google Play). Sample size power was set at 80%. Analysis showed that 15 units of fresh and 10 units of old PRBCs were required for the study. All data were analyzed using SPSS 22 (IBM Corp., Armonk, NY, USA). The Friedman test was used to analyze differences within each group. Upon finding a statistically significant difference, analyses between comparison groups were performed using the Bonferroni-corrected Wilcoxon signed-ranks test. The Mann-Whitney U test was performed for analyzing the differences in biochemical and biomechanical values between the fresh and old PRBCs. As Friedman and Wilcoxon tests were performed for statistical analyses, we used median (minimum/maximum) values for comparative analyses and descriptive purposes. The level of statistical significance was set at 0.05.

Results

Analyses between 0 and 6, 6 and 24, and 0 and 24 h were defined as comparisons 1, 2, and 3, respectively.

Analysis of Fresh PRBCs

There were no statistically significant differences in the erythrocyte deformability parameters of the EI_{max} and $SS_{1/2}$ values (p=0.14 and p=0.36, respectively) (Figure 1). However, statistically significant erythrocyte fragmentation occurred in comparison 1 [1.72 (1.13/2.43 vs. 2.29 (1.36/3.15), p=0.015], which continued to increase without statistical significance in comparison 2 [2.29 (1.36/3.15) vs. 2.24 (1.69/4.96), p=0.09] (Figure 2). Similarly, hemolysis of erythrocytes was statistically significantly increased in comparison 1 [0.37 (0.19/0.80) vs. 1.49 (0.47/5.09), p=0.003], which continued to increase in comparison 2 with borderline statistical significance [1.49 (0.47/5.09) vs. 1.74 (0.78/5.21), p=0.04] (Table 2). Unsurprisingly, the hemolysis percentage was

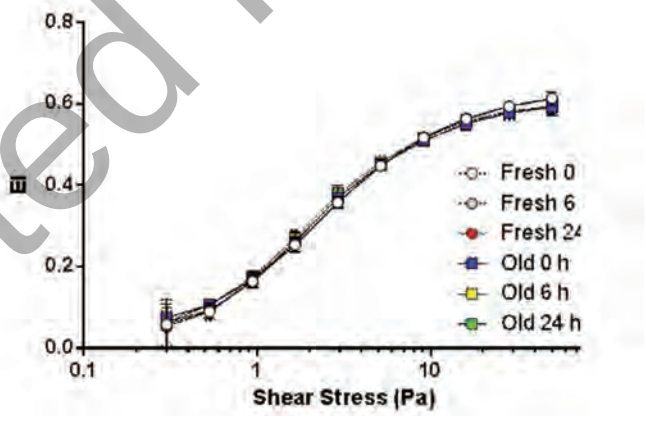

Figure 1. Elongation index of fresh and old samples measured at shear stresses between 0.3 and 50 Pa.

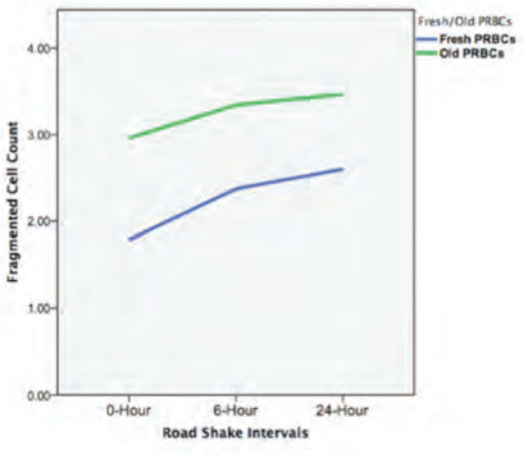

Figure 2. Cell counts between 15 and 40 fL (decreased cell volume due to fragmentation) of fresh and old samples measured with a Multisizer 3 (Beckman & Coulter, USA) (209x296 mm, 72x72 DPI).

PRBCs: Packed red blood cells.

also statistically significantly increased in comparison 3 [0.37 (0.19/0.80) vs. 1.74 (0.78/5.21), p=0.003] (Figure 3). Only two fresh PRBC units at 6 h and one PRBC unit at 24 h had hemolysis percentages less than 0.8%.

Simulated SS for 24 h was found to statistically significantly affect pH levels (p=0.001) (Table 2). However, the statistical significance was due to differences in comparison 1 [6.9 (6.9/7.08) vs. 6.8 (6.8/7.0), p=0.003], whereas comparisons 2 [6.8 (6.8/7.0 vs. 6.8 (6.7/7.1), p=1) and 3 [6.9 (6.9/7.08) vs. 6.8(6.7/7.1), p=0.05] were not statistically significantly different.

ATP levels decreased significantly in comparison 1 [90.2 (60.88/189.8) vs. 70.4 (38.2/102.9), p=0.003] and comparison 2 [70.4 (38.2/102.9) vs. 84.6 (63.9/166.8), p=0.04]. Likewise, 2,3-DPG levels also decreased statistically significantly in comparison 1 [1.06 (0.9/1.9) vs. 0.86 (0.65/1.35), p=0.003], comparison 2 [0.86 (0.65/1.35) vs. 0.64 (0.53/0.84), p=0.003], and comparison 3 [1.06 (0.9/1.9) vs. 0.64 (0.53/0.84), p=0.003]. However, despite the statistically insignificant increase in osmotic fragility in comparison 1 [0.4 (0.35/0.40) vs. 0.40 (0.4/0.45), p=0.25], continued mechanical agitation resulted in a statistically significant increase in comparison 2 [0.40 (0.4/0.45) vs. 0.45 (0.4/0.45), p<0.001] and comparison 3 [0.4 (0.35/0.40) vs. 0.45 (0.4/0.45), p<0.001]. The supernatant hemoglobin levels were not significantly increased in comparison 1 [0.08 (0.02/0.17) vs. 0.07 (0.02/0.17), p=0.08] and comparison 2 [0.07 (0.02/0.17) vs. 0.11 (0.02/0.3), p=0.09]. However, the difference was statistically significant in comparison 3 [0.08 (0.02/0.17) vs. 0.11 (0.02/0.3), p=0.009]. Supernatant osmolality was statistically significantly decreased in comparison 1 [284 (248/307) vs. 265.4 (234/292), p=0.003], comparison 2 [265.4 (234/292) vs. 259 (227.7/293), p=0.003], and comparison 3 [284 (248/307) vs. 259 (227.7/293), p=0.003]. Naturally, mechanical agitation caused a significant

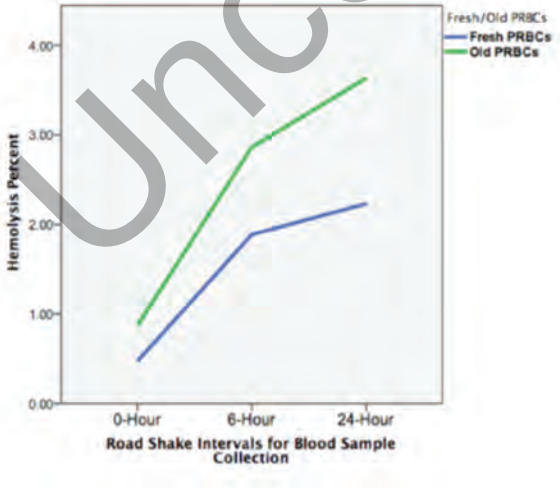

Figure 3. Differences in hemolysis percentages between fresh and old packed red blood cells during road shaking simulation.

PRBCs: Packed red blood cells.

increase in supernatant potassium in comparison 1 [21.8 (10.2/33.12) vs. 32.9 (21/40.7), p=0.003], comparison 2 [32.9 (21/40.7) vs. 37.7 (27/44.5), p=0.003], and comparison 3 [21.8 (10.2/33.12) vs. 37.7 (27/44.5), p=0.003]. As a result of fresh PRBC hemolysis, hematocrit levels were significantly decreased in comparison 1 [62.4 (50/79.1) vs. 48 (38.3/80), p=0.015] and comparison 3 [62.4 (50/79.1) vs. 46.4 (36/60.4), p=0.015) (Table 2).

Analysis of Old PRBCs

No statistically significant differences in EI_{max} and $SS_{1/2}$ values were found in any of the comparisons (p>0.05) (Figure 1). Fragmentation rates of old PRBCs continued to increase without significance in comparison 1 [3.01 (1.3/4.39) vs. 2.53 (1.62/5.69), p>0.05] and comparison 2 [2.53 (1.62/5.69) vs. 3.20 (1.3/6.71), p>0.05] (Figure 2). The median (min/max) hemolysis percentage of old PRBCs at 0, 6, and 24 h were 0.7 (0.26/0.8), 2.47 (1/6.44), and 2.95 (1.31/10.3), respectively, and the increases were statistically significant (p<0.05) (Table 2). Hemolysis percentages of all blood bags were above 0.8% after 6 h of simulation.

ATP and osmotic fragility values showed no significant changes in any of the comparisons (p>0.05). However, there were significant differences in supernatant osmolality values in comparison 1 [240.3 (191/274.1) vs. 235 (189.4/268), p=0.015], comparison 2 [235 (189.4/268) vs. 233.9 (185/266), p=0.015], and comparison 3 [240.3 (191/274.1) vs. 233.9 (185/266), p=0.015]. Supernatant potassium values in comparison 1 [38.6 (28.3/48.6) vs. 42.8 (32.9/53.5), p=0.015], comparison 2 [42.8 (32.9/53.5) vs. 43.5 (33.5/56.2)], and comparison 3 [38.6 (28.3/48.6) vs. 43.5 (33.5/56.2), p=0.015] were also statistically significant. Likewise, 2,3-DPG values in comparison 1 [1.16 (0.84/1.58) vs. 0.74 (0.6/1), p=0.015], comparison 2 [0.74 (0.6/1) vs. 0.61 (0.53/0.76), p=0.015], and comparison 3 [1.16 (0.84/1.58) vs. 0.61 (0.53/0.76), p=0.015] were statistically significantly different. Supernatant Hb levels increased significantly in comparison 2 [0.07 (0.02/0.11) vs. 0.19 (0.12/0.44), p=0.013], while differences between comparisons 1 [0.10 (0.03/0.2) vs. 0.07 (0.02/0.11), p=0.2] and 3 [0.10 (0.03/0.2) vs. 0.19 (0.12/0.44), p=0.06] were statistically insignificant. The hematocrit levels were also statistically significantly decreased in comparison 1 [58 (44.7/70.2) vs. 45.9 (34.3/56.6), p=0.015] and comparison 3 [58 (44.7/70.2) vs. 43.6 (34/49.5), p=0.015] (Table 2).

Analyses of Differences between Fresh and Old PRBCs

2,3-DPG levels between the fresh and old PRBCs were not statistically significantly different (p>0.05). The ATP levels of fresh PRBCs at 0 h were significantly higher (p<0.001), but the differences in analysis at 6 and 24 h were not statistically significant (p>0.05). Supernatant hemoglobin levels and hemolysis percentages between fresh and old PRBCs were not statistically significantly different (p>0.05). Supernatant K levels of old PRBCs were statistically significantly higher than those of

Table 2. Effect of simulated Test Method 514.6 (MIL-STD-810G) shaking on biochemical and biomechanical values of fresh and old packed red blood cell stored in blood cooler box.

Parameters	Fresh PRBCs (14 Units) [Median (Min / Max)]				Old PRBCs (10 Units) [Median (Min / Max)]				Fresh PRBCs vs Old PRBCs		
	Before shaking	After 6 h of shaking	After 24 h of shaking	p-value	Before shaking	After 6 h of shaking	After 24 h of shaking	p-value	p-value (0-hour)	p-value (6-hour)	p-value (24-hour)
pH	6.9 (6.9/7.08)	6.8 (6.8/7.0)	6.8 (6.7/7.1)	0.001	6.4 (6.32/6.72)	6.4 (6.3/6.7)	6.4 (6.2/6.7)	0.53	<0.001	<0.001	<0.001
Supernatant Osmolality (mOsm/kg)	284 (248/307)	265.4 (234/292)	259 (227.7/293)	<0.001	240.3 (191/274.1)	235 (189.4/268)	233.9 (185/266)	<0.001	<0.001	0.001	0.004
Supernatant K^+ (mmol/L)	21.8 (10.2/33.12)	32.9 (21/40.7)	37.7 (27/44.5)	<0.001	38.6 (28.3/48.6)	42.8 (32.9/53.5)	43.5 (33.5/56.2)	<0.001	<0.001	<0.001	0.005
Supernatant Hb (g/dL)	0.08 (0.02/0.17)	0.07 (0.02-0.17)	0.11 (0.02/0.3)	0.015	0.10 (0.03/0.2)	0.07 (0.02-0.11)	0.19 (0.12/0.44)	0.01	0.26	0.19	0.10
Osmotic fragility	0.4 (0.35/0.40)	0.40 (0.4/0.45)	0.45 (0.4/0.45)	<0.001	0.45 (0.4/0.5)	0.45 (0.4/0.5)	0.47 (0.4/0.55)	0.057	<0.001	<0.001	0.07
2,3 DPG (mmol/mL)**	1.06 (0.9/1.9)	0.86 (0.65/1.35)	0.64 (0.53/0.84)	<0.001	1.16 (0.84/1.58)	0.74 (0.6/1)	0.61 (0.53/0.76)	<0.001	0.6	0.1	0.2
ATP (pmol/mL)	90.2 (60.68/189.8)	70.4 (38.2/102.9)	84.6 (63.9/166.8)	0.001	60.9 (49.5/73.6)	68.4 (50.2/85.6)	76.4 (58.6/120)	0.06	<0.001	0.9	0.4
Fragmentation	1.72 (1.13/2.43)	2.29 (1.36/3.15)	2.24 (1.69/4.96)	0.008	3.01 (1.3/4.39)	2.53 (1.62/5.69)	3.20 (1.3/6.71)	0.67	0.007	0.43	0.06
Hemolysis (%)	0.37 (0.19/0.80)	1.49 (0.47/5.09)	1.74 (0.78/5.21)	<0.001	0.7 (0.26/0.8)	2.47 (1/6.44)	2.95 (1.31/10.3)	<0.001	0.06	0.08	0.08
Deformability** (EIMAX)	0.65 (0.61/0.67)	0.65 (0.6/0.7)	0.66 (0.6/0.7)	0.91	0.63 (0.6/0.7)	0.62 (0.6/0.7)	0.63 (0.61/0.65)	0.14	0.003	0.004	0.004
Deformability** ($SS_{1/12}$)	2.5 (2.12/2.97)	2.56 (2.03/3.03)	2.7 (2.13/3.04)	0.42	2.20 (1.88/2.93)	2.19 (1.89/2.94)	2.15 (1.87/2.79)	0.14	0.031	0.036	0.004
Hematocrit (%)	62.4 (50/79.1)	48 (38.3/80)	46.4 (36/60.4)	<0.001	58 (44.7/70.2)	45.9 (34.3/56.6)	43.6 (34/49.5)	<0.001	0.1	0.09	0.7

2,3 DPG: 2,3 Diphosphoglycerate, $EI_{MAX.}$ Maximum elongation index, $SS_{1/2}$: Half elongation index, PRBCs: Packed red blood cells.

the fresh PRBCs in all three successive analyses (p<0.001, p<0.001, p=0.005, respectively). The other biochemical analyses are shown in Table 2. EI_{max} (p=0.003, p=0.004, p=0.004, respectively) and $SS_{1/2}$ (p=0.031, p=0.036, p=0.004, respectively) values of fresh PRBCs were significantly better in all comparisons. Fragmentation rates of fresh erythrocytes were significantly lower at 0 h; however, the difference was not significant at 6 or 24 h (Table 2).

Discussion

In order to prevent hemorrhage-related early mortality, strategies that comprise crystalloid use, a high ratio of fresh frozen plasma, and platelets in PRBC transfusion protocols have been developed [12]. Malsby et al. [13] reported the initial military experience of en route blood product transfusion for combat trauma casualties. Transfusions were started aboard upon receiving the casualties from the point of injury. More interestingly, clinical indications for transfusion were appreciated and executed by well-trained flight medics. They concluded that flight medic-initiated transfusions were safe and effective and studies to determine the effect of PHP transfusion on outcomes were required. Brown et al. [8] performed an outcomes study and showed that PHP PRBC transfusion was associated with significant 24-h and 30-day reduction in mortality rates. They also showed that trauma-induced coagulopathy was reduced by 88%.

In order to take the transfusion capability closer to the point of injury, some practical questions about blood logistics need answers for future planning, such as: "If PRBCs could be kept at 4 °C, would their quality be maintained after prolonged transport times?" Otani et al. [14] investigated whether a helicopter flight affected the quality and shelf-life of RBCs. Seven days after donation, five units of PRBCs were packed into a blood cooler box and transported in a helicopter for 4 h. Then they were stored again and their quality was evaluated 7, 14, 21, and 42 days after donation. Only supernatant hemoglobin and hemolysis levels were slightly increased 42 days after donation. Supernatant potassium, hematocrit, pH, and 2,3-DPG levels at 42 days remained unchanged.

Boscarino et al. [15] exposed 20 units of pooled PRBCs (7 days old) in a Golden Hour container to a 30,000-foot parachute descent, followed by carrying the container in a rucksack for 12 h in an environment of 48 °C and 9% humidity. They investigated the biochemical (pH, lactate, potassium, and ATP) and biomechanical (EI_{max}, half EI_{max}, percent hemolysis, and morphology) parameters and found no significant impact on markers of RBC stress.

In our worst-case scenario, blood supplies are limited and the forward-deployed fresh and old blood products will be subject to continuous shear forces due to perpetual tactical evacuation missions. As the above-mentioned studies' simulated conditions showed no resemblance to our envisioned combat environment, we have designed a 24-h simulation study.

Our study was performed in "within limits" temperature settings, as hemolysis would increase linearly with temperature [16]. When blood is collected in a bag with limited amounts of dextrose, phosphate, and adenine to maintain ATP and 2,3-DPG levels, erythrocytes metabolize these preservatives to maintain their integrity. The lactate level increases progressively in the blood bag, which decreases the pH and 2,3-DPG levels during the storage period [17,18]. Decreased ATP levels reduce the deformability of the cells and cellular homeostasis [19]. Approximately 25% of ATP content and over 90% of 2,3-DPG is lost in a unit of PRBCs after 42 days of storage [20]. As this study lacks control groups, the decrease in erythrocyte metabolism-related parameters cannot be solely attributed to shear stress. The pH levels of fresh PRBCs decreased significantly in the first 6 h.

Storage temperatures of 1 °C to 4 °C slow the RBC metabolism and decrease the energy demand. However, storage at 4 °C impairs the ATP-dependent potassium pump, resulting in potassium leakage. The extracellular potassium concentration increases by approximately 1 mEq/L per day until the intracellular and extracellular potassium ions reach equilibrium. Potassium loading may be of clinical importance in patients receiving massive transfusions [21]. After 24 h, the supernatant potassium level of fresh and old PRBCs was significantly higher by 71% and 87% than the expected value.

According to the European Directorate for the Quality of Medicine-Healthcare of the Council of Europe (EDQM) criteria and the North American Blood Quality Licensure, the acceptable level of hemolysis has been set at 0.8% and 1%, respectively [22]. The EDQM hemolysis criterion has been approved by the Turkish National Blood and Blood Products Guide (2016) [23]. After 6 and 24 h of shear stress, only 2 (13%) and 1 (6.6%) of the fresh blood packs were eligible for transfusion, respectively. None of the old PRBCs were found eligible at the 6-h test. Mechanical agitation significantly increased hemolysis and fragmentation values of fresh and old PRBCs at 6-h and 24-h analyses. Decreases in hematocrit levels were observed in fresh and old PRBCs at the 6-h analysis.

Storage-induced red blood cell damage increases osmotic fragility, especially after 5 weeks [24]. Increased osmotic fragility was evident in old PRBCs. However, mechanical stress significantly increased osmotic fragility values of fresh PRBCs, especially after 6 h of simulation.

Erythrocytes may deform under a wide range of mechanical stresses and LORCA is capable of measuring the deformation, which is usually presented as the maximum elongation index (EI_{max}) and half maximum elongation index ($SS_{1/2}$). Boscarino et al. [15] exposed PRBCs to parachute descent and 12 h of simulated soldier patrol and found no shear stress-related differences. In our

vigorous study of longer duration, EI_{max} and $SS_{1/2}$ values showed no significant changes. Unsurprisingly, fresh erythrocyte deformability values were significantly better throughout the simulation.

The current study is not without limitations. Our primary concern was creating control groups that would not represent a real-life environment. Dividing each donor's blood into two equal volumes and blood sampling would further decrease the blood volume. The duration of the experiment was set at 24 h due for the convenience of the simulator.

Conclusion

Under the simulated conditions, we were unable to demonstrate the feasibility and safety of carrying PRBCs. Given the demonstrated benefits of transfusion in the PHP, our efforts shall not be hindered by the initial experience and new projects are underway.

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Ethics

Ethics Committee Approval: Yeditepe University Clinical Research Ethics Committee (24/11/2016-682).

Informed Consent: Participants provided informed consent forms.

Authorship Contributions

Surgical and Medical Practices: A.Ü., S..Y., M.U.; Concept: A.Ü., Ö.Y., İ.E., R.A.Ç.; Design: A.Ü., T.Ö., İ.Y.A., N.Z.; Data Collection or Processing: A.Ü., A.C.A.; Analysis or Interpretation: P.P., S.Y., M.U.; Literature Search: A.Ü., S.Y.; Writing: A.Ü., S.Y.

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.

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BRIEF REPORT

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Anemia and Its Effect on Cardiovascular Findings in Obese Adolescents

Obez Ergenlerde Anemi ve Aneminin Kardiyovasküler Bulgulara Etkisi

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Abstract

Objective: We assessed the effect of anemia on cardiovascular findings in obese adolescents.

Materials and Methods: We studied 29 anemic and 33 nonanemic obese adolescents, and 33 nonobese healthy adolescents. These three groups were investigated for clinical and laboratory features of anemia and obesity. Echocardiography was used to examine cardiac functions.

Results: The anemia was mild (mean hemoglobin: 11.67±0.79 g/dL), ferritin level was significantly low, and C-reactive protein and fibrinogen levels were significantly high in anemic obese patients. Increased cardiac pulse and echocardiographic findings, which may be indicative of early left ventricular diastolic dysfunction, were present in these patients.

Conclusion: Anemia may develop due to iron deficiency and chronic inflammation in obese adolescents. Even mild anemia may cause increased heart rate and affect left ventricular diastolic functions. Diet programs for obese children should be carefully planned to avoid iron deficiency anemia, which may worsen the cardiac events in long-term follow-up.

Keywords: Anemia, Cardiac function, Inflammation, Iron deficiency, Obesity

Öz

Amaç: Obez adölesanlarda aneminin kardiyovasküler bulgular üzerine etkisinin araştırılması amaçlanmıştır.

Gereç ve Yöntemler: Çalışmaya 29 anemik, 33 anemik olmayan obez adölesan ve 33 obez olmayan sağlıklı adölesan dahil edildi. Bu üç grup, anemi ve obesitenin klinik ve laboratuvar bulguları açısından değerlendirildi. Kardiyak fonksiyonları değerlendirmek için ekokardiyografi kullanıldı.

Bulgular: Anemik obez hastalarda anemi hafifti (ortalama hemoglobin 11,67±0,79 g/dL), ferritin seviyesi anlamlı olarak düşük, C-reaktif protein ve fibrinojen düzeyleri anlamlı olarak yüksek bulundu. Bu grupta kardiyak nabız anemik olmayan obez adölesanlarınkine göre anlamlı yüksekti ve ekokardiyografik incelemede anemik obez grupta erken ventriküler diyastolik disfonksiyon göstergesi olabilecek bulgular saptandı.

Sonuç: Obez adölesanlarda demir eksikliği ve kronik enflamasyona bağlı anemi gelişebilir. Hafif anemi varlığı bile kardiyak nabızda artışa ve sol ventrikül diyastolik fonksiyonlarında etkilenmeye neden olabilir. Bu nedenle obez çocuklarda uzun dönemde kalp fonksiyonlarının olumsuz yönde etkilenmemesi açısından diyet programları demir eksikliği anemisini önleyecek şekilde dikkatlice planlanmalıdır.

Anahtar Sözcükler: Anemi, Kardiyak fonksiyon, Enflamasyon, Demir eksikliği, Obezite

Introduction

The prevalence of childhood obesity has progressively increased in the world in the last decades due to sedentary life style and poor dietary habits [1,2]. Childhood obesity is a major risk factor for development of cardiovascular diseases in adulthood [3,4,5,6]. On the other hand, anemia is another well-defined risk factor that has a negative impact on the prognosis of cardiovascular diseases [7,8,9]. The cardiac problems in anemic obese adolescents are not well known. The purpose of this study

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was to assess the effect of anemia on cardiovascular findings in obese adolescents by means of standard, pulsed-wave Doppler (PWD), and tissue Doppler imaging (TDI) echocardiography.

Materials and Methods

Adolescent patients admitted to our hospital with exogenous obesity between the ages of 12 to 18 years were included. The study group was divided into two groups as anemic obese (n=29) and nonanemic obese (n=33) patients. Those who had endogenous obesity, infection, chronic use of medications, or other accompanying diseases were excluded. Healthy adolescents (n=33) whose body mass indexes (BMIs) were between the 3rd and 85th percentiles were included as the control group.

Obesity was defined as a BMI at or above the 95th percentile for children and teenagers of the same age and sex. BMI is calculated by dividing a person's weight in kilograms by the square of height in meters [10]. Anemia was defined according to the World Health Organization as hemoglobin value of ≤12 g/dL in women and ≤13 g/dL in men [11]. Hypertension was defined by a systolic and/or diastolic blood pressure at or above the 95th percentile for children and teenagers of the same age and sex [12].

Clinical data and results of laboratory measurements of patients were obtained from the hospital records, including complete blood cell count; renal, liver, and thyroid function tests; serum glucose, insulin, insulin resistance, lipid, fibrinogen, and C-reactive protein (CRP) levels; and iron parameters.

Echocardiography was performed after 15 min of resting by a pediatric cardiologist. Standardized M-mode echocardiography, PWD, and TDI echocardiography were performed to evaluate the status and functions of the heart [13]. By using M-mode echocardiography, interventricular septum diastolic diameter (IVSDD), left ventricular end-diastolic diameter, left ventricular posterior wall diastolic diameter (LVPWDD), left ventricular end-systolic diameter, ejection fraction (EF), left ventricular mass (LVM), and LVM index (LVMI) were calculated. Early diastolic mitral flow (E-wave), late diastolic mitral flow (A-wave), and early mitral to late mitral flow ratio (E/A) were found using PWD. Systolic myocardial velocity (S), late diastolic myocardial velocity (Em), early diastolic myocardial velocity (Am), ratio of early to late diastolic myocardial velocity (Em/Am), isovolumetric relaxation time, and myocardial performance index were calculated using TDI echocardiography.

All statistical analyses were performed using SPSS 15 (SPSS Inc., Chicago, IL, USA). Differences between groups for categorical variables were compared by chi-square test. The Student t-test and Mann-Whitney U test were used for the comparison of continuous variables. One-way analysis of variance (ANOVA) and Kruskal-Wallis tests were used for the comparison of more than two groups.

Results

Demographic data and clinical features of the groups are given in Table 1. The values of hemoglobin, mean corpuscular volume, red cell distribution width, iron parameters, fibrinogen, and CRP are shown in Table 2. Test results including serum glucose, insulin, insulin resistance, lipid profile, and renal, liver, and thyroid function tests did not differ among the three groups ($p>0.05$).

M-mode and TDI echocardiographic parameters are given in Table 3 and Table 4. As seen in Table 3, there were significant changes of LVM, LVMI, LVPWDD, and IVSDD in obese patients. PWD measurements demonstrated that the E-wave and A-wave showed significant differences between the three groups ($p=0.012$, $p=0.013$) and between anemic and nonanemic obese groups ($p=0.016$, $p=0.039$). The E/A ratio was not statistically significant between the three groups ($p=0.751$).

Discussion

In our study, a significant proportion of anemic obese children were found to be on a diet to lose weight. Their ferritin levels were significantly lower even though there were signs of chronic inflammation, such as high levels of fibrinogen and CRP. Anemia may be seen in the obese population due to poor dietary habits and as a result of chronic inflammatory condition [14,15,16,17,18]. In obese patients, adipose tissue secretes proinflammatory cytokines that restrict erythropoiesis [17,18]. On the other hand, obesity-associated inflammation is closely linked to iron deficiency and involves impaired duodenal iron absorption associated with low expression of duodenal ferroportin and elevated hepcidin levels [14,19]. Iron deficiency and anemia may change mitochondrial and cellular energy homeostasis and increase the inactivity and fatigue of obese patients [19].

Anemia may cause hemodynamic changes, cardiomegaly, and left ventricular hypertrophy in the long-term period [9,20]. EF is one of the most commonly used parameters in evaluation of left ventricular systolic function. EF was not impaired in our study. Studies demonstrated that EF does not decrease in the early period of obesity [21,22,23]. It has been reported that these changes correlate with the degree and duration of anemia [9,20,24]. In a recent study, Zhou et al. [24] demonstrated that LV remodeling and LV systolic dysfunction occurred in patients with iron deficiency anemia when the hemoglobin level was in the range of 6-9 g/dL. In our study, the mean hemoglobin value was 11.67 ± 0.79 g/dL; it can be concluded that mild anemia in the obese population does not deteriorate systolic dysfunction.

Tachycardia, a well-known complication of anemia, develops as a compensatory response of the heart to inadequate tissue oxygenation caused by decreased erythroid mass [9]. In our

Table 1. Demographic and clinical features of the three groups.

	Anemic obese n=29 mean ± SD/ median/(min-max)	Nonanemic obese n=33 mean ± SD/ median/(min-max)	Healthy controls n=33 mean ± SD/ median/(min-max)	p
Age (years)*	13.89±1.39/ 14/(12-17)	14.18±1.28/ 14/(12-16)	14.4±1.42/ 14/(12-17)	0.613
Male/female (n)	13/16	18/15	18/15	0.683
Fatigue (n/%)	21/72%	24/73%	12/36%	**0.033**
Diet (n/%)	8/28%	1/3%	1/3%	**0.020**
Effort intolerance (n/%)	14/48%	11/33%	5/15%	**0.019**
Weight (kg)*	84.34±17.68/ 80/(65.5-144.8)	86.69±13.1/ 87.5 (59.5-117)	56.08±(9.21) 55.5/(36.8-75)	**0.001** p1=0.326 **p2=0.001** **p3=0.001**
BMI (percentile)*	97.78±1.25/ 98/(95-99.7)	97.35±1.25/ 97/(95-99.03)	50.83±26.54 57/(3-88)	**0.001** p1=0.208 **p2=0.001** **p3=0.001**
Cardiac pulse** (beats/minute)	88.93±14.08/ 88/(60-116)	84±11.07 84/(62-109)	77.57±9.69 80/(48-88)	**0.001** p1=0.225 **p2=0.001** p3=0.070
Hypertension (n/%)	14/48%	14/42%	-	0.644
Systolic blood pressure* (mmHg)	130.55±14.35/ 129/(107-168)	128.3±10.62/ 130/(100-164)	111.36±10.62 111/(93-140)	**0.001** p1=0.719 **p2=0.001** **p3=0.001**
Diastolic blood pressure* (mmHg)	80.03±9.76 80/(68-108)	79.72±10.23/ 80/(60-104)	68.45±7.37 70/(52-80)	**0.001** p1=0.938 **p2=0.001** **p3=0.001**

p1: Anemic obese versus nonanemic obese, p2: anemic obese versus healthy controls, p3: nonanemic obese versus healthy controls, SD: standard deviation.
Statistically significant values are shown in bold.

Table 2. Hemoglobin, erythrocyte indexes, iron parameters, fibrinogen, and C-reactive protein levels of anemic and nonanemic obese adolescents.

	Anemic obese n=29 mean ± SD	Nonanemic obese n=33 mean ± SD	p
Hemoglobin (g/dL)	11.7±0.8	14.0±1.2	**0.001**
Mean corpuscular volume (fL)	76.1±3.8	83.4±4.1	**0.001**
Red cell distribution width (%)	15.5±5.8	13.6±0.6	**0.001**
Serum iron (µg/dL)	53±36	72±42	0.067
Iron binding capacity (µg/dL)	399±69	375±52	0.791
Transferrin saturation (%)	13.8±10.2	18.2±9.2	0.082
Ferritin (ng/mL)	18.9±14.9	28.18±15.8	**0.023**
Fibrinogen (g/dL)	3.9±0.7	3.5±0.7	**0.045**
C-reactive protein (mg/dL)	8.3±8.4	3.4±4.0	**0.002**

SD: Standard deviation.
Statistically significant values are shown in bold.

study, the anemic obese group was found to have significantly higher cardiac pulse rates than the nonanemic obese group, even though the anemia was mild. The changes in E- and A-waves seen in PWD might be caused by increased heart rates in our anemic obese group, which may be indicative of early subclinical ventricular diastolic dysfunction [21,23,25,26].

Regarding the cardiac geometry, an increased LVMI has been shown in obese children [6]. Sharpe et al. [27] demonstrated that BMI is directly related to LVMI. An increased LVMI results in ventricular hypertrophy, which eventually results in left ventricular diastolic dysfunction [23,25,26,27,28,29,30]. Similarly, in our study, measurements of LVM, LVMI, LVPWDD, and IVSDD were found to be increased in both obese groups compared to the healthy control group.

In this study, the number of patients was relatively low and the anemia was mild, so we recommend further studies with larger

Table 3. Comparison of M-mode echocardiographic parameters between the three groups.

	Anemic obese n=29 mean ± SD	Nonanemic obese n=33 mean ± SD	Healthy controls n=33 mean ± SD	p
EF (%)*	66.70±5.68	67±5.62	66.81±4.78	0.931
LVM (g)	143.09±33.07	145.26±38.07	107.11±25.7	**0.001 p1=0.977 p2=0.001 p3=0.001**
LVMI (g/m²)	82.71±19.12	84.12±21.84	68.24±13.76	**0.002 p1=0.933 p2=0.002 p3=0.002**
LVESD (mm)	28.4±3.6	29.8±2.7	28±5.3	0.154
LVPWDD (mm)	9.3±1.1	9.0±1.3	7.9±1.2	**0.001 p1=0.276 p2=0.001 p3=0.001**
LVEDD (mm)	45.9±38	46.8±5.8	45.1±3.8	0.337
IVSDD (mm)	8.9±1.5	9.2±2.4	7.4±1.3	**0.001 p1=0.682 p2=0.001 p3=0.001**

p1: Anemic obese versus nonanemic obese; p2: anemic obese versus healthy controls; p3: nonanemic obese versus healthy controls. Statistically significant values are shown in bold.

EF: Ejection fraction, LVM: left ventricular mass, LVMI: LVM index, LVESD: left ventricular end-systolic diameter, LVPWDD: left ventricular posterior wall diastolic diameter, LVEDD: left ventricular end diastolic diameter, IVSDD: interventricular septum diastolic diameter, SD: standard deviation.

Table 4. Comparison of the tissue Doppler image parameters between the three groups.

	Anemic obese n=29 mean ± SD	Nonanemic obese n=33 mean ± SD	Healthy controls n=33 mean ± SD	p
S (cm/s)	10.23±1.66	10.76±2.36	10.91±2.96	0.799
Em (cm/s)	16.37±3.1	16.9±3.87	16.94±3.72	0.791
Am (cm/s)	9.16±2.41	9.46±2.3	8.65±2.51	0.678
Em/Am	1.85±0.41	1.82±0.38	2.03±0.44	0.092
E/Em	5.93±1.15	5.40±1.53	5.29±1.40	0.090
IVRT (ms)	44.06±6.29	44.6±4.6	45.81±4.57	0.530
MPI	0.31±0.04	0.31±0.04	0.31±0.04	0.261

p1: Anemic obese versus-nonanemic obese; p2: anemic obese versus healthy control; p3: nonanemic obese versus healthy control.

S: Systolic myocardial velocity, Em: late diastolic myocardial velocity, Am: early diastolic myocardial velocity, Em/Am: ratio of early to late diastolic myocardial velocity, E/Em: early mitral flow to late diastolic myocardial velocity, IVRT: isovolumetric relaxation time, MPI: myocardial performance index, SD: standard deviation.

samples of obese adolescents with different stages of anemia for more accurate investigation of effects of anemia in obese adolescents. Follow-up of these adolescents is also important to provide a prompt therapeutic approach and better outcome.

Conclusion

Anemia may develop due to iron deficiency and chronic inflammation in obese adolescents. Our study suggests that blood pressure, heart rate monitoring, and echocardiographic measurements should be carefully checked in anemic obese adolescents at frequent intervals for early detection of hypertension, tachycardia, and left ventricular diastolic dysfunction. Even mild anemia may cause increased heart rate and change the left ventricular diastolic functions in obese adolescents. Diet programs of obese children should therefore be carefully planned to avoid iron deficiency anemia, which may worsen the cardiac outcome in long term follow-up.

Ethics

Ethics Committee Approval: This study was approved by the Dokuz Eylül University Drug and Clinical Investigation Ethics Committee (protocol no: 1583-GOA, decision no. 2014/23-16).

Informed Consent: Informed consent for study participation was obtained from all patients and their parents.

Authorship Contributions

Concept: H.Ö.; Design: H.Ö., N.Ü., M.K., E.B., A.A., N.A., Ş.Y., Ö.Y.; Data Collection or Processing: Ö.Y., T.D., Ö.K., P.K.; Analysis or Interpretation: H.Ö., N.Ü., A.A., M.K., Ö.T., Ö.Y.; Literature Search: H.Ö., Ö.T., Ö.Y.; Writing: H.Ö., M.K., Ö.T., Ö.Y.

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Leukemic Phase of CD5+ Diffuse Large B-Cell Lymphoma

CD5+ Diffüz Büyük B Hücreli Lenfomanın Lösemik Fazı

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Acute lymphoid leukemia and diffuse large B-cell lymphoma, though categorized as lymphoid neoplasms, have different clinical presentations, treatment protocols, and outcomes. However, the rare situation of a leukemic phase of CD5+ diffuse large B-cell lymphoma sometimes mimics acute lymphoid leukemia and requires careful differentiation. We report here a rapid and accurate diagnosis by flow cytometry.

A 55-year-old woman suffered from hemoptysis and thrombocytopenia with lymphadenopathies. Complete blood count revealed a white cell count of 10.2×10^9/L with 46% blast cells. Peripheral blood smear showed marked blastocytosis with fine nuclear chromatin and prominent nucleoli and scanty cytoplasm (Figure 1, left). Flow cytometry showed positive results for CD5, CD19, CD20, and kappa light chain but was negative for CD7, CD10, CD11b, CD13, CD33, CD34, CD56, and terminal deoxynucleotidyl transferase (TdT). Bone marrow examination revealed scattered involvement of CD20-positive and TdT-negative cells (Figures 1 and 2). Biopsy of the neck lymph nodes confirmed the diagnosis of CD5+ diffuse large B-cell lymphoma (Figure 2, lower right). Under the diagnosis of stage IV disease, she received 8 courses of R-CHOP therapy with stem cell transplantation later on. She has sustained complete response after therapy for 2 years to date.

A leukemia phase of diffuse large B-cell lymphoma is rare and mimics acute lymphoblastic leukemia [1,2]. Flow cytometry with an appropriate panel could help in differentiating lymphoma from leukemia [2,3]. In this case, having the surface light chain and TdT markers made for an accurate and rapid diagnosis.

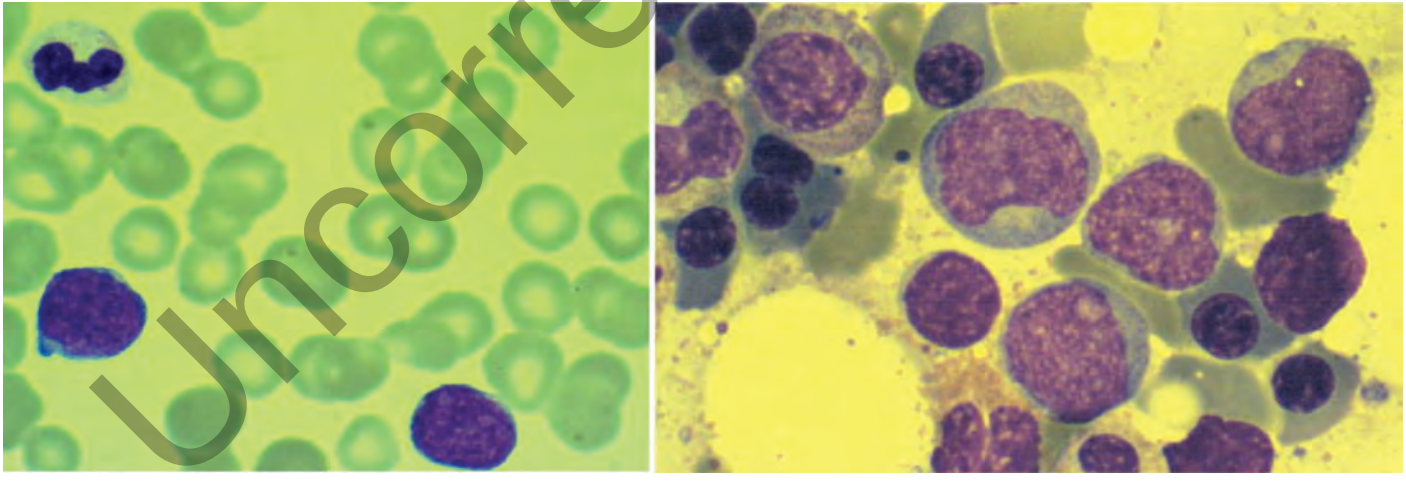

Figure 1. Peripheral blood smear showed thrombocytopenia with marked lymphoid blast-like cells of fine nuclear chromatin with prominent nucleoli and scanty cytoplasm (left: hematoxylin and eosin stain, 1000^x). Bone marrow examination revealed scattered involvement of median to large cells with prominent nucleoli (right: hematoxylin and eosin stain, 1000^x).

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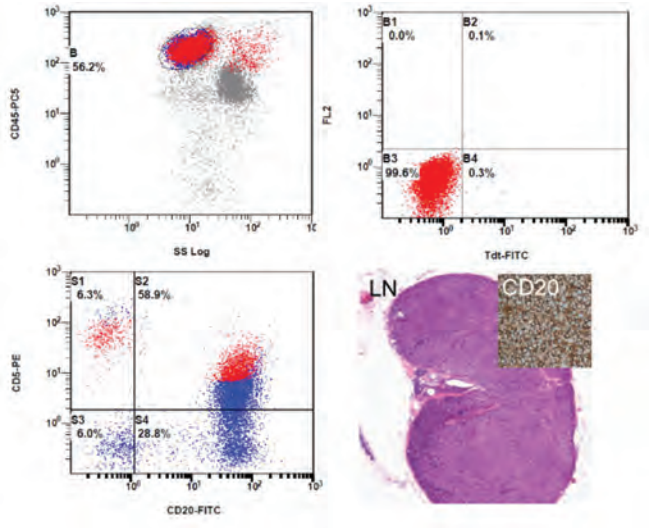

Figure 2. Flow cytometry revealed positivity for CD5 and CD20 with negativity for terminal deoxynucleotidyl transferase (upper and lower left). Lymph node biopsy showed diffuse lymphoma pattern with positivity for CD20 (lower right).

TdT: Terminal deoxynucleotidyl transferase.

Keywords: Lymphoma, Acute leukemia, Flow cytometry

Anahtar Sözcükler: Lenfoma, Akut lösemi, Akım sitometri

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.

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The Elusive Diagnosis of Primary Esophageal Lymphoma

Primer Özofagus Lenfomasının Yanıltıcı Tanısı

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A 76-year-old woman presented with a 2-month history of progressive dysphagia that was associated with weight loss. Computed tomography of the neck showed significant circumferential soft tissue thickening involving the upper esophagus with luminal narrowing (Figure 1A). Upper gastrointestinal endoscopy revealed a very tight stricture below the cricopharyngeus muscle. The stricture was traversed using a neonatal endoscope. Endoscopic ultrasonography using a miniprobe revealed marked esophageal wall thickening with diffuse hypoechoic infiltration involving the entire wall (Figure 1B). Biopsy specimens from the esophageal stricture revealed malignant non-Hodgkin lymphoma (diffuse large B-cell type) confirmed by immunohistochemistry (Figure 1C: hematoxylin and eosin staining at 100^{x} magnification of the lymphoid infiltration; Figure 1D: Ki67 (proliferation index) staining at 400^{x}, 40% tumor cells). The patient received 6 cycles of chemotherapy [anti-CD20 monoclonal antibody (rituximab) plus the CVP regimen], followed by positron emission tomography/computed tomography and upper endoscopy with a biopsy that showed no evidence of lymphoma.

Keywords: Endoscopy, Endosonography, Esophagus, Non-Hodgkin lymphoma

Anahtar Sözcükler: Endoskopi, Endosonografi, Özofagus, Non-Hodgkin lenfoma

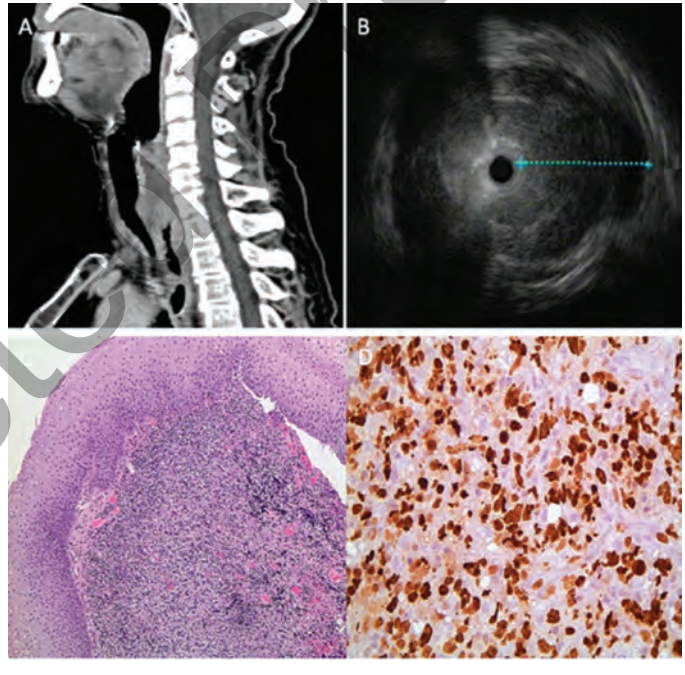

Figure 1. Diagnosis of primary esophageal lymphoma.

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First Report of an *SH2D1A* Mutation Associated with X-Linked Lymphoproliferative Disease in Turkey

Türkiye'den Bildirilen İlk X'e Bağlı Lenfoproliferatif Hastalık İlişkili *SH2D1A* Mutasyonu Olgusu

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To the Editor,

X-linked lymphoproliferative disease (XLP) is a rare disorder characterized by an extreme vulnerability to Epstein-Barr virus (EBV) infection, frequently resulting in hemophagocytic lymphohistiocytosis (HLH) [1]. XLP-1, its more common subtype, is caused by defects in the *SH2D1A* gene that encodes the signaling lymphocyte activation molecule-associated protein (SAP), which regulates the activation of T lymphocytes [2], whereas XLP-2 is caused by mutations in the *XIAP* gene, also known as *BIRC4* [3].

We present here an XLP-1 patient with a family history of the death of multiple male children, who presented with EBV-triggered fatal HLH. To our knowledge, this is the first report of an *SH2D1A* mutation from Turkey.

Case: The 19-month-old male patient, admitted with the complaints of fever and abdominal distention, had pale appearance, fever (body temperature: 39.5 °C), dyspnea, tachycardia, abdominal distention, and hepatosplenomegaly. Laboratory findings are summarized in Table 1.

In the family history, the death of a 2-year-old male sibling with the clinical diagnosis of HLH and of five young male children of unknown etiology among maternal relatives was noted (Figure 1).

The patient received intravenous immunoglobulin. However, in the follow-up, fever recurred and his general condition worsened. Bone marrow aspiration revealed hemophagocytosis. Therefore, the patient fulfilled the HLH diagnostic criteria. Plasma exchange was performed. Blood products, antimicrobials, and supportive therapeutic agents were used as indicated.

The results of EBV serologic testing and polymerase chain reaction were both reported as positive. On the 6th hospitalization day, the HLH-2004 protocol treatment was initiated, and rituximab therapy was planned. Continuous veno-venous hemodialysis was performed. However, the vital signs of the patient deteriorated further and active gastrointestinal bleeding was observed. The patient died on the 10th day of hospitalization.

In the cytotoxic lymphocyte activity analysis, low SAP expression in addition to signs of severe immunoactivation was detected (Figure 1). In the genetic analysis performed in the Clinical Genetics Unit of Karolinska University Hospital, Stockholm, Sweden, the c.163C>T (p.Arg55Ter) mutation in the *SH2D1A* gene, described previously as pathologic [4], was identified (Figure 1). Genetic counseling was provided to the family. This letter was written after receiving informed consent from the parents.

We report here an XLP-1 case in which the patient presented with EBV-associated HLH. Although no genetic analysis was performed among the male relatives of the patient lost previously in childhood, XLP-1 seems to be the underlying cause in those children as well.

In XLP cases, the most common clinical manifestation is fulminant infectious mononucleosis (frequency: 58%, survival: 4%). Death is generally attributable to liver failure with hepatic

Table 1. Laboratory findings of the patient.	
Hemoglobin (g/L)	76 (NR: 98-134)
White blood cells (10^3 /µL)	23.04 (NR: 5-14.8)
Platelets (10^3/µL)	169 (NR: 150-400)
Direct Coombs	Negative
Ferritin (ng/mL)	841* (NR: 12-150)
Lactate dehydrogenase (IU/L)	757 (NR: 140-304)
Albumin (g/dL)	2.6 (NR: 3.1-4.8)
Serum sodium (mEq/L)	128 (NR: 135-143)
Aspartate aminotransferase (IU/L)	354 (NR: <48)
Alanine aminotransferase (IU/L)	178 (NR: 0-39)
Bilirubin (total/direct) (mg/dL)	2.0/1.1 (NR: 0-2.0/0-0.5)
Triglyceride (mg/dL)	320 (NR: 30-100)
Prothrombin time (s)	20.3 (NR: 10.0-14.7)
Activated partial thromboplastin time (s)	38.7 (NR: 22.0-34.0)
Fibrinogen (mg/dL)	130 (NR: 170-350)
D-dimer (ng/mL)	4,658 (NR: 0-550)
Immunoglobulin M (mg/dL)	455 (NR: 72-212)
Immunoglobulin G (mg/dL)	1,620 (NR: 658-1,460)
Immunoglobulin A (mg/dL)	347 (34-89)
C-reactive protein (mg/L)	60 (NR: 0-4)
EBV VCA IgM	Positive
EBV PCR	Positive (526,736 copies/mL)

*Serum ferritin rose to 28,321 ng/mL on the 5th hospitalization day.

NR: Normal range, EBV: Epstein-Barr virus, PCR: polymerase chain reaction, VCA: viral capsid antigen, IgM: immunoglobulin M.

encephalopathy or bone marrow failure with fatal hemorrhages in various organs [5]. The only curative treatment of XLP is hematopoietic stem cell transplantation [6].

In our case, the HLH-2004 protocol, initiated on the 6th hospitalization day, did not prevent the deterioration of the patient's clinical status. Rituximab therapy has been reported to successfully induce remission in some cases of XLP [7,8]. Unfortunately, our patient was lost before we could start rituximab therapy.

Establishment of the genetic diagnosis in male children suspected to have XLP will enable valuable genetic counseling.

Keywords: Lymphoproliferative disease, Hemophagocytosis, Epstein-Barr virus

Anahtar Sözcükler: Lenfoproliferatif hastalık, Hemofagositoz, Epstein-Barr virüsü

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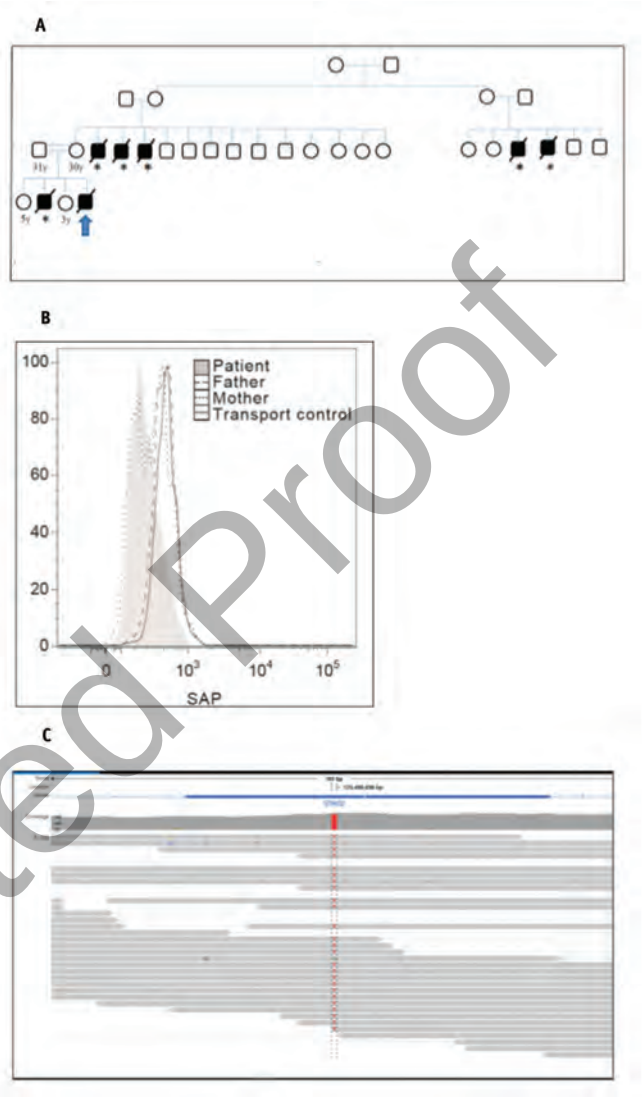

Figure 1. A) Pedigree of the family demonstrating loss of six male children, compatible with X-linked recessive inheritance of disease. *All of the designated deaths occurred between 1 and 3 years of age. The propositus is indicated with an arrow; B) The levels of signaling lymphocyte activation molecule-associated protein (SAP) expression on dim natural killer cells of the patient and the parents by intracellular SAP analysis; C) Identification of the c.163C>T (p.Arg55Ter) mutation in the *SH2D1A* gene by sequencing analysis in the index case.

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Intracranial Bleeding in a Female Hemophilia Patient: Molecular Analysis of the *Factor 8* Gene and Determination of a Novel Mutation

İntrakraniyal Kanama ile Başvuran Hemofili A Olgusu: Yeni Bir Mutasyonun Moleküler Olarak Tanımlanması

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To the Editor,

An 11-month-old female patient was admitted to the emergency department with right occipital fracture and epidural hematoma. The father had severe hemophilia A and the parents were cousins. Laboratory tests revealed normal complete blood count and prolonged activated partial thromboplastin time. Mixing test results were normalized after mixing with normal plasma. After plasma samples were collected for further diagnostic tests, fresh frozen plasma and dexamethasone were administered. The factor VIII level was 0.1%, 35%, and 0.5% for the patient, mother, and father, respectively. The patient's von Willebrand factor (VWF) level was 128 IU/mL, VWF:Ricof was 110 IU/mL, collagen ADP was 110 (reference: 71-118) s, and collagen epinephrine was 98 (reference: 85-165) s. Intron 22 inversion was investigated with the IS-PCR method and was found to be normal. Whole-genome analysis including all exonic regions of the *F8* gene (NM_000132.3) was conducted and the homozygous c.608T>C (L203P) mutation was found. This mutation was not previously reported. As this variant was not reported in any exome databases (ExAC, EVS) and as it was shown to be the cause of the disease in at least three in silico protein modeling programs, the mutation was considered as a novel mutation causing hemophilia A ("probably damaging" with 0.987 PolyPhen2 score, "disease causing" with 0.999 MutationTaster score, and "damaging" with 0 SIFT score). The mutation was also confirmed by Sanger sequencing (Figure 1). Plasma-derived FVIII at 2x500 IU/day was administered for 14 days followed by 300 IU/week prophylaxis. Inhibitor screening at the 5th and 10th exposure days was negative.

Hemophilia A is rarely seen in female patients due to skewed inactivation of the X chromosome leading to inactivation of the wild-type X chromosome, anomalies like Turner syndrome, or translocations, as well as homozygous/compound heterozygous mutations for hemophilia A [1,2,3,4,5]. The karyotype analysis of our patient revealed 46,XX. The patient and the father were hemizygous and mother was heterozygous for the c.608T>C

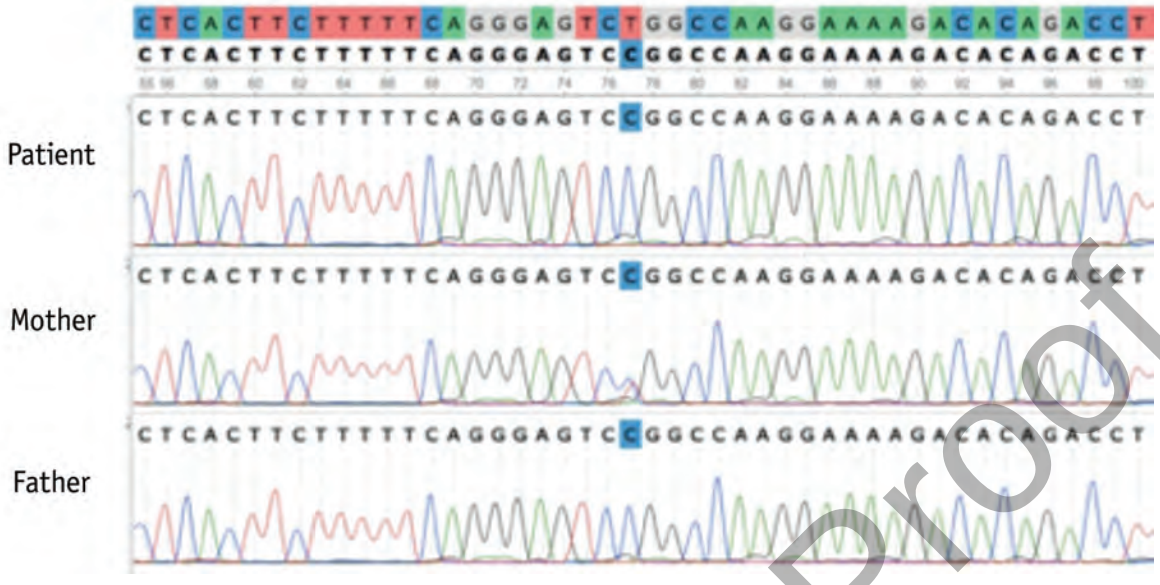

Figure 1. Sanger sequencing confirmation: (a) the mother (b), the father, and (c) the patient show heterozygous, hemizygous, and homozygous c.608T>C (L203P) mutation in the *F8* gene.

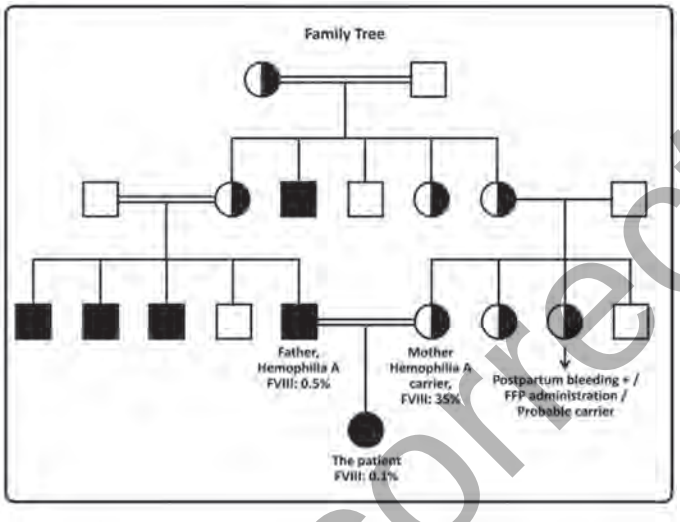

Figure 2. Family tree. The patient was homozygous, the father hemizygous, and mother heterozygous for the c.608T>C (L203P) mutation.

(L203P) mutation (Figure 2). The clinical situation of our patient as she was admitted with epidural hematoma requiring surgical intervention and the fact that the family did not apply for prenatal diagnosis before birth point out the importance of prenatal diagnosis in regions where consanguineous marriage is common.

Keywords: Hemophilia, Intracranial bleeding, Female

Anahtar Sözcükler: Hemofili, İntrakraniyal kanama, Kız cinsiyet

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Kasabach-Merritt Syndrome in an Adult

Erişkin Bir Hastada Kasabach-Merritt Sendromu

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To the Editor,

Kasabach-Merritt syndrome (KMS) is characterized by capillary hemangiomas and consumptive thrombocytopenia and coagulopathy, and may also be associated with microangiopathic hemolysis [1]. KMS is most commonly reported in infants and young children. Here we report a rare case of adult KMS in a 47-year-old woman, giving rise to severe thrombocytopenia and bleeding.

A 47-year-old woman presented with purpuric hemorrhages over her upper and lower limbs and gum bleeds for 8 days. She was found to have two large purple hemangiomas on the tongue (Figure 1A) and a few smaller cutaneous hemangiomas on the face (Figure 1B). These lesions had been present since her childhood and the tongue hemangiomas had enlarged over the past several years. Laboratory evaluation revealed hemoglobin of 89 g/L, leukocyte count of $9.48x10^9$/L (leukocyte differential: neutrophils 72%, lymphocytes 25%, eosinophils 2%, basophils 1%), platelet count of $6x10^9$/L, prothrombin time of 13.4 s (control: 13 s), activated partial thromboplastin time of 32 s (control: 29 s), serum fibrinogen of 1.36 g/L, elevated fibrin degradation products, positive D-dimer, and normal liver and renal functions. A peripheral blood smear showed normocytic normochromic erythrocytes and markedly reduced platelets. Bone marrow evaluation revealed normal erythropoiesis, myelopoiesis, and megakaryocytic hyperplasia. Contrast-enhanced computed tomography of the chest and abdomen excluded deep-seated visceral hemangiomas. She received platelet transfusions and oral tranexamic acid for control of gum bleeds, and she was also started on oral prednisolone at 1 mg/kg/day. One month after starting the steroid treatment, the bleeding had stopped and the platelet count had improved to $152x10^9$/L. However, the hemangiomas had remained the same. Considering the risk of traumatic bleeding, she was advised to have surgical excision of the tongue hemangiomas. However, she was unwilling to undergo surgery.

KMS is most commonly reported in infants and only a small percentage (~0.3%) of infants with hemangiomas develop KMS [1]. If not recognized and treated in time, KMS may be potentially fatal by causing disseminated intravascular coagulation and severe bleeding, and large hemangiomas can cause high-output cardiac failure and vital organ compression.

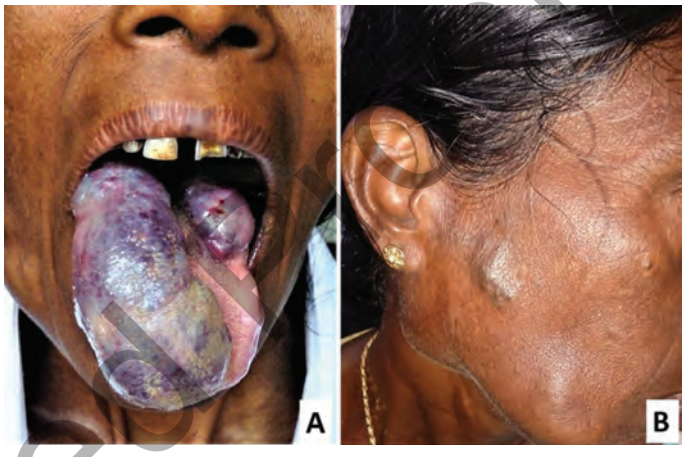

Figure 1. Two large purple hemangiomas of sizes 3x8 cm and 2x3 cm on the dorsum of the protruded tongue (A) and a smaller cutaneous hemangioma on the right side of the face (B).

Though rare, KMS has been reported among adults as well [2,3,4,5]. The pathogenesis involves activation and consumption of platelets and clotting factors inside hemangiomas, giving rise to consumption coagulopathy and bleeding. However, no correlation has been reported between site, size, and number of hemangiomas and the development of KMS [1]. Cutaneous and visceral hemangiomas have both been implicated in KMS. Work-up in the index patient revealed probable low-grade disseminated intravascular coagulation, but there was no evidence of microangiopathic hemolysis. Histologically, kaposiform hemangioendotheliomas and tufted angiomas are the most frequent lesions reported in KMS [1]. Treatment options include compression therapy for hemangiomas, surgical excision of large solitary vascular lesions (whenever feasible), or ligation/embolization of feeder vessels when lesions are inaccessible for surgery. Systemic steroids and interferon alfa have shown benefit when vascular lesions are extensive and not amenable to surgery or embolization.

Keywords: Hemangioma, Thrombocytopenia, Coagulopathy, Consumption

Anahtar Sözcükler: Hemanjiom, Trombositopeni, Koagülopati, Tüketim

Informed Consent: Informed consent has been obtained from the patient for publication of her clinical images.

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.

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Spurious Thrombocytosis in the Setting of Hemolytic Anemia and Microcytosis Secondary to Extensive Burn Injury

Yaygın Yanık Yaralanmasına Sekonder Gelişen Hemolitik Anemi ve Mikrositoz Zemininde Yalancı Trombositoz

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To the Editor,

A 57-year-old man was brought to our emergency department from a house fire. On physical examination, he was nonresponsive, hypotensive, and tachycardic with full-thickness skin burns covering the entirety of the body except the lower back (>98% of his body surface area). He was intubated and aggressively resuscitated with IV fluids and multiple pressors for circulatory support.

A complete blood count (CBC) showed normal hemoglobin (14.5 g/dL) with leukocytosis (23.6x10^9/L) and thrombocytosis (979x10^9/L). Repeat CBCs also showed thrombocytosis (815x10^9/L and 1121x10^9/L). Microscopic examination of the peripheral blood smear showed widespread red blood cell (RBC) fragmentation, budding, spherocytes, and microspherocytes (Figure 1). Manual platelet count estimates on the peripheral blood smear demonstrated a count of 173x10^9/L. The patient remained in intractable hypotension and eventually went into cardiac arrest.

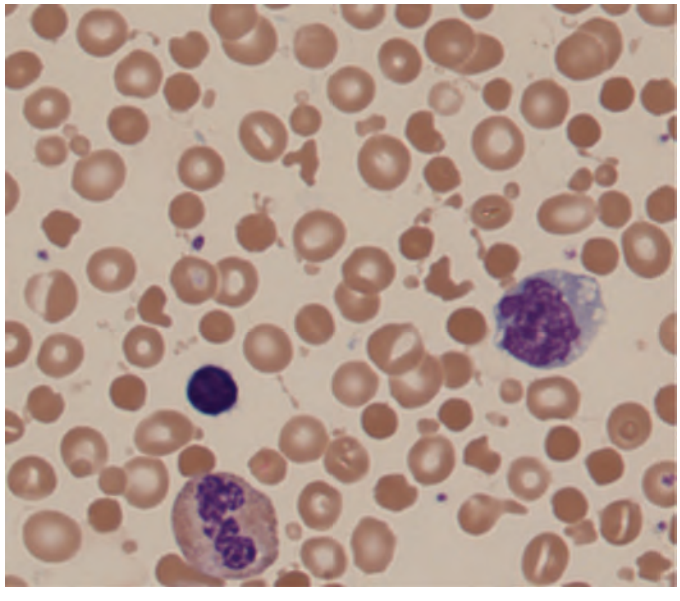

Figure 1. Widespread red blood cell fragmentation, budding, spherocytes, and microspherocytes were revealed by microscopic examination.

The aforementioned findings are seen in patients with severe burns due to direct thermal injury of RBCs circulating through the skin. Exposure to extreme heat leads to the denaturation of RBC membrane proteins, which results in hemolysis, RBC fragmentation, and vesiculation [1]. The loss of cell membrane causes the RBCs to lose their biconcavity and assume the shape of spherocytes and microspherocytes [1]. These RBC fragments and microspherocytes persist in the peripheral circulation for several days until completely removed from circulation by the reticuloendothelial system in the spleen. They are counted as platelets by aperture-based automated analyzers due to their size, leading to falsely elevated platelet counts in cases of acute burns [1,2]. Although reactive thrombocytosis can be seen in acute injury as recently reported by Sapanara et al. [2] in a similar burn case of a 48-year-old woman, such instances should always prompt a microscopic examination of the peripheral smear to confirm if in fact the platelet count is elevated. A manual count of platelets on peripheral smear from that patient (as in our case) revealed a normal platelet count. Such examples emphasize the importance of correlating the peripheral smear with automated CBC results.

Keywords: Spurious, Thrombocytosis, Burn, Platelets, Microcytosis

Anahtar Sözcükler: Yalancı, Trombositoz, Yanıklar, Trombositler, Mikrositoz

Contributors' Statement: M.F.Z. and M.S.A. both reviewed the peripheral smear and took the image. M.F.Z. wrote the first draft of the manuscript and M.S.A. revised it for intellectual content. Both authors approved the final version of the manuscript being submitted.

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.

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A Rare Cause of Paraplegia: Myeloid Sarcoma

Nadir Bir Parapleji Nedeni: Myeloid Sarkom

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To the Editor,

Myeloid sarcoma (MS), also known as granulocytic sarcoma or chloroma, is a rare extramedullary tumor consisting of myeloblasts or immature myeloid cells that disrupt the normal architecture of the involved tissue and typically occurs concurrently with acute myeloid leukemia (AML) [1,2]. It can also occur in association with accelerated-phase chronic myeloid leukemia or myelodysplastic syndrome; as an extramedullary relapse of AML, including in the post-bone marrow transplant setting; and occasionally as the first presenting manifestation, even before bone marrow involvement [3,4]. Bone, periosteum, skin, orbit, lymph nodes, the gastrointestinal tract, and the central nervous system are the most commonly involved sites in patients presenting with MS; however, skin and orbital localizations are the most often reported sites in children [4]. Here we present a 4-year-old male patient who was referred to the pediatric hematology oncology clinic due to a thoracolumbar mass and subsequently diagnosed with MS.

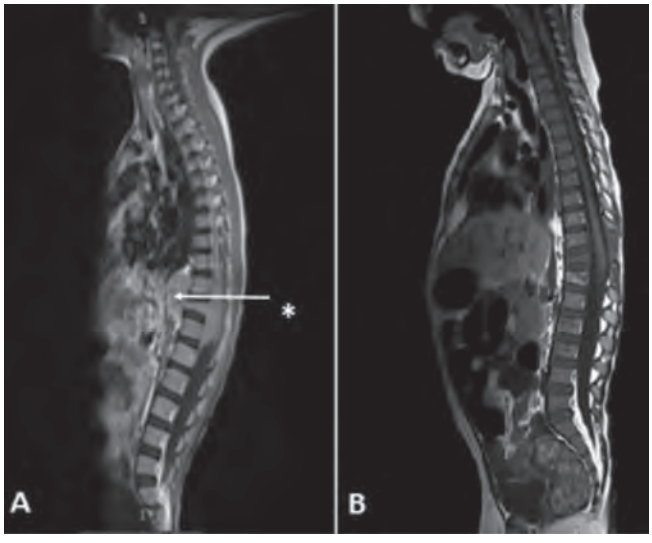

Figure 1. A) Sagittal T1-weighted MRI image showing an epidural, hypointense, craniocaudal mass of 4.5 cm in diameter compressing the spinal cord at the level of D 10-12; B) image of the mass 1 month before completion of acute myeloid leukemia maintenance therapy.

A 4-year-old boy was referred to the pediatric hematology oncology clinic with the complaint of hemiparesis and a subsequent thoracolumbar mass was detected by magnetic resonance imaging (MRI) (Figure 1A). On physical examination, bilateral lower extremity paralysis was noted and deep tendon reflexes were absent. Complete blood count and blood biochemical analysis were normal, and no blasts were detected on peripheral blood film. Bone marrow aspiration showed 30% blasts compatible with AML. The pathology of the mass revealed MS. After administration of radiotherapy, given at a dose of 18 Gy in 10 daily fractions in 2 weeks, and dexamethasone therapy, the patient achieved neurological improvement. He was treated with the AML-Berlin Frankfurt Münster 2012 protocol and achieved both remission and mass reduction following AML induction chemotherapy. The patient is still in remission without any residual tumor on follow-up MRI (Figure 1B).

MS may occur at any site of the body, and therefore clinical manifestations of MS exhibit diversity depending on the specific location and size, which leads to significant diagnostic challenges, in particular in patients without initial bone marrow involvement. Incorrect diagnosis of malignant lymphoproliferative disorders, Ewing's sarcoma, thymoma, melanoma, round blue cell tumors, or poorly differentiated carcinoma has been reported at a rate of 25%-47% in patients subsequently diagnosed with MS. In this regard, any atypical cellular infiltrate should raise the suspicion of MS to make a correct diagnosis in a timely manner and to allow for proper management [2,4,5]. Diagnostic tools for the correct diagnosis of MS are also important in this context and should include MRI and/or computed tomography scan for evaluation of the size and location of the tumor and for distinguishing the tumor from other lesions, morphological and flow cytometric analysis of bone marrow and peripheral blood, or biopsy of the tumor and immunohistochemical staining in patients without bone marrow involvement [4]. Treatment of MS includes AML-based protocols and, as in our case, surgery and/or radiotherapy may be indicated for symptomatic lesions or tumors causing local organ dysfunction [5]. Considering the most common presentation sites in children with MS, which are skin and orbital localizations, the current patient is presented to highlight a rarely encountered presenting feature of MS.

Keywords: Myeloid sarcoma, Children, Paraplegia

Anahtar Sözcükler: Myeloid sarkom, Çocuk, Parapleji

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.

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Recognizing Pinch Purpura as the First Manifestation of Light-chain Amyloidosis

Hafif Zincir Amiloidozun İlk Bulgu Olarak İzole Purpuradan Tanınması

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A 74-year-old female presented with a 6-month history of easy bruising as manifested by purpura after minor trauma to her face. Her physical examination was unremarkable except for the presence of pinch purpura scattered on her face (Figure 1). Laboratory tests showed leukocytes of 8100/µL, hemoglobin of 11.2 g/dL, platelets of 208,000/µL, prothrombin time of 11 s (normal range: 11.2-13.0 s), activated partial thromboplastin time of 25 s (normal range: 23.0-33.0 s), and erythrocyte sedimentation rate of 72 mm/h. Upon further workup, the presence of IgG lambda monoclonal gammopathy of the serum and lambda monoclonal light chain was found in urine immunofixation electrophoresis. Bone marrow biopsy revealed 7% lambda-restricted plasma cell infiltration, showing green birefringence with Congo red stain and vascular amyloid P deposition (Figure 2). There were no CRAB symptoms, organ dysfunction, or organomegaly. Echocardiography and pro-B-type natriuretic peptide results were normal. A diagnosis of amyloid light-chain (AL) amyloidosis initially presenting with purpura was made and a chemotherapy regimen of bortezomib and dexamethasone was started. Complete remission was achieved after six courses of chemotherapy and the purpuric lesions disappeared.

Figure 1. Purpura scattered on face (temporal region).

Cutaneous manifestations are reported in 30%-40% of AL amyloidosis cases [1]. The lesions usually reflect capillary

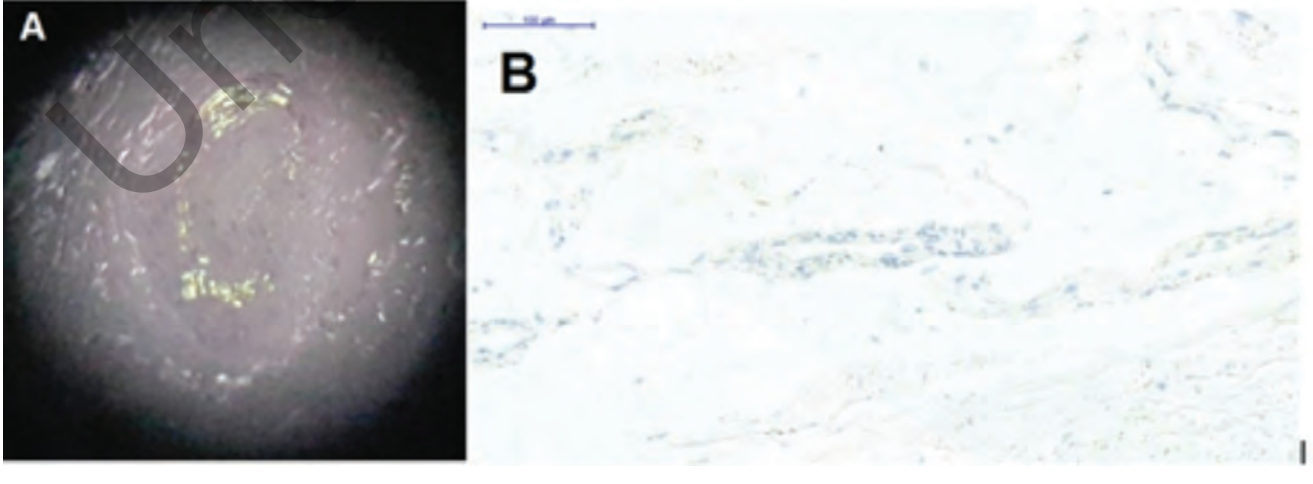

Figure 2. A) Microscopic section of the bone marrow stained with Congo red shows green birefringence under polarized light microscopy. B) Amyloid P with light microscopy.

infiltration and fragility with petechiae and purpura, characteristically affecting the eyelids, beard area, and upper chest [2]. Purpura as the initial manifestation leading to the diagnosis of AL amyloidosis is relatively rare [3,4]. Therefore, cutaneous findings are valuable in making a diagnosis of this challenging disorder since early diagnosis before development of organ failure is essential for improving the prognosis of AL amyloidosis patients.

Keywords: Amyloidosis, Purpura, Congo red

Anahtar Sözcükler: Amiloidoz, Purpura, Kongo kırmızısı

Informed Consent: Informed consent was obtained.

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Sclerosing Extramedullary Hematopoietic Tumor

Sklerozan Ekstramedüller Hematopoetik Tümör

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To the Editor,

Sclerosing extramedullary hematopoietic tumor (SEMHT) is a very rare disease. It was first described by Remstein et al. [1] in 2000 and few cases have been reported since then. It is associated with chronic myeloproliferative disorders. We describe a patient with SEMHT with bilateral renal involvement.

A 72-year-old man was admitted to the emergency clinic with dyspnea and abdominal pain. Physical examination revealed decreased breath sound over the right and left lower lung areas and severe edema in both lower extremities. He was clinically overhydrated. The rest of the physical examination was normal. Routine laboratory tests showed the following: serum levels of hemoglobin, 9.8 g/dL; white blood cell count, $31x10^9$/L; platelet count, $100x10^9$/L; blood urea nitrogen, 36 mg/dL; serum creatinine, 1.9 mg/dL; serum albumin, 2.2 g/dL. The peripheral blood smear was normal. A bone marrow biopsy was not performed. Abdominal ultrasound showed a perirenal hypoechoic mass compressing the bilateral kidneys (Figure 1a). Magnetic resonance imaging revealed heterogeneous renal capsular involvement with maximum thickness of 30 mm (Figure 1b). Renal Tru-cut biopsies were performed from both kidneys. Microscopically, both lesions showed myxoid and sclerotic stroma intermixed with large atypical cells (Figures 1c and 1d). Immunohistochemistry revealed positivity for factor-8 (Figure 1e) and CD41 in atypical cells (Figure 1f). Scattered mature myeloid cells were positive for myeloperoxidase, while CD34, CD117, S100, CD3, CD30, CD20, glycophorin, MDM2, keratin, EMA, desmin, and myogenin were all negative. The presence of CD41-positive atypical megakaryocytes within the tumor suggested the diagnosis of SEMHT. His previous history of primary myelofibrosis and splenectomy was learned after the histologic diagnosis of the renal tumor.

SEMHT is an uncommon lesion formerly known as fibrous hematopoietic tumor or myelosclerosis [1]. It is associated with

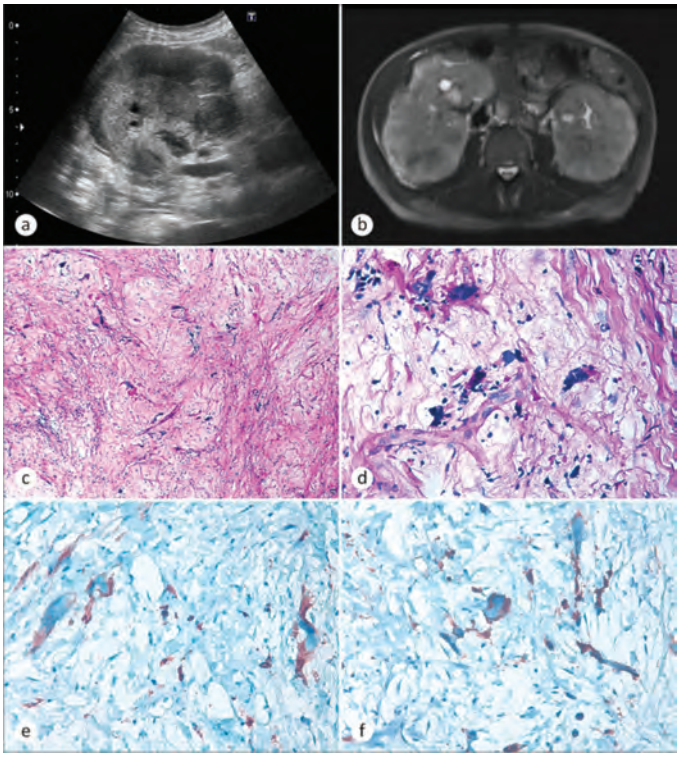

Figure 1. a) Abdominal ultrasonographic image and b) magnetic resonance image showing heterogeneous renal capsular mass. c) Photomicrograph showing large atypical megakaryocytes in a myxoid to collagenous background (hematoxylin and eosin, 100x). d) Megakaryocytes at high power view (hematoxylin and eosin, 400x), e) showing factor-8 positivity (400x) and f) CD41 positivity (400x).

chronic myeloproliferative disorders, mainly chronic idiopathic myelofibrosis in older age groups. SEMHT has a predilection for the mesentery and retroperitoneum. However, tumors involving the skin, liver, kidneys, and lacrimal glands were described as single reports [1,2,3]. SEMHT has usually presented as multiple nodules with varying sizes. Our case showed the diffuse infiltrative nature of the tumor, compressing the bilateral kidneys. Bilateral renal involvement is an unreported radiologic finding. Microscopically, these tumors were characterized by myxoid to sclerotic stroma with thick collagen bundles, intermixed with large atypical megakaryocytes. Occasional foci of mature hematopoietic cells were encountered [1,2]. It is believed that sclerosis within the tumor was produced by fibroblasts, induced by cytokines released from clonal megakaryocytes. The presence of the *JAK2 V617F* mutation may also suggest the clonal nature of the lesion [4].

The differential diagnosis includes sclerosing liposarcoma, malignant fibrous histiocytoma/pleomorphic sarcoma, sarcomatoid/anaplastic carcinoma, and Hodgkin lymphoma [1,3]. The presence of dysplastic megakaryocytes with "ink blot-like" nuclei and eosinophilic cytoplasm is in favor of SEMHT. Factor-8, CD41, and CD61 are helpful markers for the confirmation of the diagnosis. Pathologists should keep this rare entity in mind for the differential diagnosis of tumors with anaplastic morphology. High cellular pleomorphism may lead to inaccurate diagnosis of sarcoma or carcinoma and a subsequent unnecessary surgery.

Keywords: Sclerosing extramedullary hematopoietic tumor, Myelofibrosis, Kidney

Anahtar Sözcükler: Sklerozan ekstramedüller hematopoetik tümör, Myelofibrozis, Böbrek

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Report on Three Patients with Blastic Plasmacytoid Dendritic Cell Neoplasm

Blastik Plazmasitoid Dentritik Hücreli Neoplazmlı Üç Olgu Sunumu

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To the Editor,

Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is a rare, clinically aggressive tumor that was classified as a distinct entity among myeloid neoplasms in the World Health Organization's 2016 revision of the classification of acute myeloid leukemia and related neoplasms. Most patients present with cutaneous lesions with or without bone marrow involvement and leukemic dissemination. The tumor cells express CD4, CD56, CD123, and TCL1 [1]. In general, acute lymphocytic leukemia (ALL)/lymphoma-type regimens were reported to show better survival outcomes than acute myeloid leukemia (AML)-type regimens. Complete remissions were registered for 7 of 26 patients after AML-type regimens and 10 of 15 patients after ALL/lymphoma-type regimens, with a significant advantage for the ALL/lymphoma-type approach [2,3]. Patients who were treated with hyper-CVAD showed an objective response, but the duration of response was short so hematopoietic stem cell transplantation (HSCT) should also be considered [4]. Recent interest has been directed towards SL-401, a novel immunotherapy directed at IL-3R, notably overexpressed in BPDCN as well as other myeloid malignancies. This led to the development of SL-401 as an IL-3-diphtheria toxin conjugate that has demonstrated promise for BPDCN in early-phase trials [5,6,7,8]. We aim to share our experience with BPDCN due to its rareness and the lack of a consensus about treatment.

All three patients were male, aged 19, 55, and 65 years, and were admitted to the hospital with fever, weight loss, weakness, and lymphadenopathy. Physical examination revealed that all of them had lymphadenopathies, one of them had hepatosplenomegaly, and two of them had skin lesions. Skin lesions were bruise-like brown to violaceous infiltrated plaques on the back and extremities. One patient had a brown-purple tumoral mass and also brown-purple nodular lesions of the head region (Figure 1). Bone marrow and lymph node biopsies showed diffuse infiltration by medium-sized blasts with irregular nuclear contour, slightly large cytoplasm, high mitotic index, and immunohistochemical expression of CD4+, CD56+, CD123+, and TCL1+. Skin biopsies revealed diffuse infiltration by similar cells. One patient had central nervous system involvement that was pathologically proven by cerebrospinal fluid cytology. In one patient's bone marrow results, 36% *TCF3* and 35% *TEL* gene deletions were detected by hybridization. A hyper-CVAD regimen was initiated for all patients. After one cycle of chemotherapy, two patients achieved complete remission (CR). One patient who achieved CR and the patient who could not achieve CR died of sepsis. The other patient who achieved CR after one course of chemotherapy was treated with three cycles of the hyper-CVAD regimen as maintenance and afterwards he underwent transplantation with peripheral blood progenitor cells from a related mismatched donor. BuCy was administered for the conditioning regimen before transplantation.

Two patients achieved CR with the hyper-CVAD regimen and one of them who underwent allogenic transplantation is still in CR 18 months after diagnosis. BPDCN can go into durable remission with HSCT regardless of the type of the induction regimen. In particular, auto-HSCT in first CR appears to be a reasonable treatment option and may play an important role in improving the outcomes of BPDCN [9]. On the other hand, high-dose therapy followed by allo-HSCT can provide durable disease control in up to 50% of patients and allo-HSCT should be administered in first CR if possible [10]. Allogeneic stem cell transplantation seems to improve the prognosis, but further studies are needed to confirm the place and the indication of this treatment strategy.

Figure 1. Brown-purple tumoral mass of 3x3 cm in diameter on the right temporal region (A). After a single cycle of chemotherapy, skin lesions regressed (B).

Keywords: Blastic plazmositoid dentritic cell neoplasm, HyperCVAD regimen, Stem cell transplantation

Anahtar Sözcükler: Blastik plazmositoid dentritik hücreli neoplazi, HyperCVAD rejimi, Kök hücre nakli

Informed Consent: In this case presentation, two of the three patients had died. Informed consent was received from the third living patient.

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.

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Lymphomatoid Granulomatosis with Isolated Cutaneous Lesions: Prolonged Remission After DA-EPOCH Protocol

İzole Deri Lezyonları ile Lenfomatoid Granülomatozis: DA-EPOCH Protokolü Sonrası Uzun Süreli Remisyon

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To the Editor,

Lymphomatoid granulomatosis (LG) is a rare Epstein-Barr virus (EBV)-related neoplasm that typically presents with pulmonary symptoms and radiographic lung nodules in the majority of patients [1,2]. Frequent skin involvement underscores the importance of a careful cutaneous examination. However, it is rare for a patient to present with isolated skin involvement of this disease. Here we present a case of LG with isolated extensive skin involvement.

A 39-year-old man with no prior known illnesses presented to our oncology clinic located in North India with chief complaints of swellings present over his back over the last 10 years. He had noted the presence of these swellings but had not sought specific therapy as they were essentially asymptomatic except for the cosmetic disfigurement. However, over the last 6 months, there had been a sudden rapid increase in the size of these lesions, associated with ulceration. An associated history of low-grade fever was also reported. No history of significant weight loss or night sweats was described.

Upon examination, the patient's Eastern Cooperative Oncology Group performance status score was 1. A large, hard, lobulated swelling measuring 20 cm in largest diameter with overlying ulceration was present over his left lower back (Figure 1b). The skin surrounding the swelling was indurated. Another swelling with similar morphology measuring 7 cm in largest diameter was noted over the right lower back. A firm, non-tender left axillary lymph node of 2 cm was palpated. The rest of his general and systemic examination was unremarkable.

Based on his presentation, a clinical suspicion of cutaneous lymphoma was entertained and a skin biopsy was performed. On light microscopy, the epidermis was unremarkable. The dermis showed an inflammatory infiltrate of lymphocytes and histiocytes with scattered large atypical cells with areas of necrosis (Figure 1a). Immunohistochemistry demonstrated that the large cells were positive for CD20, CD30, and EBV-latent membrane protein while negative for CD3, ALK1, and EMA. The background population was an admixture of CD3- and CD20-positive lymphocytes. A histological diagnosis of LG was made.

Further staging investigations (contrast-enhanced computed tomography of neck to pelvis, diagnostic lumbar puncture, bone marrow aspiration, and biopsy) did not reveal involvement of any other site. Interestingly, bilateral lung fields were completely

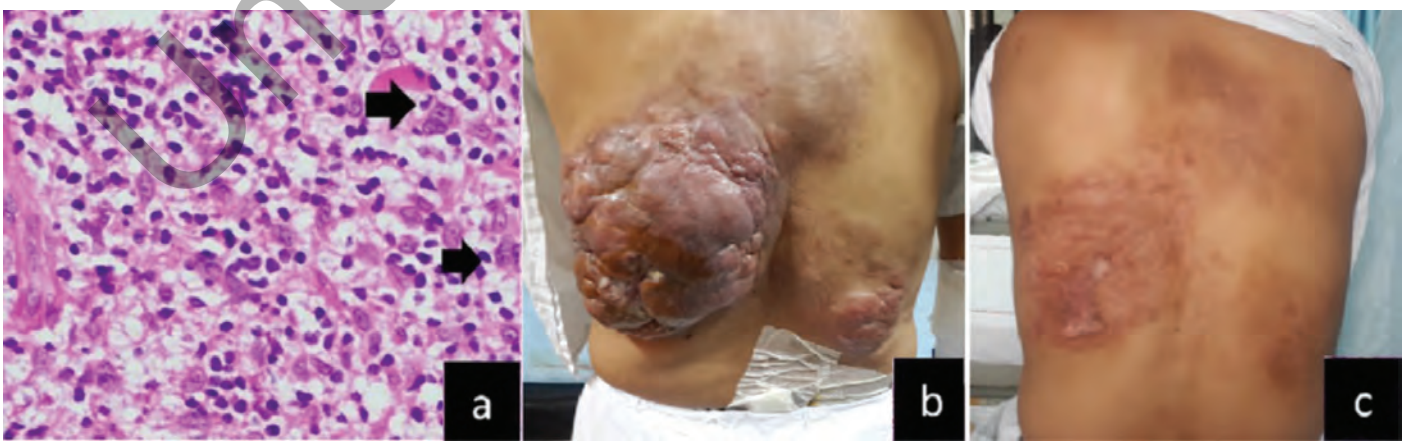

Figure 1. a) Histopathological examination: dermis showing inflammatory infiltrate of lymphocytes and histiocytes along scattered large atypical cells [hematoxylin and eosin, 400^{x} (arrows)]; b) clinical image at baseline presentation; c) clinical image after 4 cycles of chemotherapy.

normal radiologically. Serum lactate dehydrogenase level was highly elevated at 1516 U/L (normal range: 240-420 U/L). Complete blood counts, liver and kidney function tests, serology for human immunodeficiency and hepatitis B and C viruses, and immunoglobulin levels were within normal limits. Keeping in mind the rapid growth of the disease in a relatively short period of 6 months, a decision was made to treat the patient with six cycles of infusional dose-adjusted etoposide, prednisolone, vincristine, doxorubicin, and cyclophosphamide (DA-EPOCH), drawing from the good experience at the US National Cancer Institute (NCI) with this regimen [1,3,4]. The option of adding rituximab to each cycle was suggested to the patient but could not be done due to affordability issues. Chemotherapy was well tolerated. A clinicoradiological complete remission was documented after 4 cycles (Figure 1c) and reconfirmed after 6 cycles. The patient is now disease-free at 2 years from the end of therapy.

The lung is the predominant site of involvement in LG, with extrapulmonary sites variably involved [1,2]. Skin involvement is seen in 25%-50% of patients and is typically diagnosed concurrently with lung lesions. Isolated skin involvement in the absence of pulmonary disease is unusual, seen in 10%-15% of patients. Skin lesions in LG are variable and include papules, macules, nodules, vesicles, ulcers, alopecia, and ichthyosis [1,5,6]. However, large skin masses as seen in the current case have been seldom described. The unusual presentation in our case could be attributed to neglecting the disease process for a long duration (10 years) on the part of the patient. Occasionally, LG may also transform to an aggressive non-Hodgkin lymphoma, although this was not noted in our histology specimen. There is no consensus on the optimal treatment of LG. Any underlying immunosuppression should be corrected, but no such condition was found in our patient. The treatment of LG is not standardized, and no comparative studies exist for this rare disease. The NCI has demonstrated good outcomes based on DA-EPOCH [1,4] and we decided to follow their protocol with gratifying results.

To conclude, our case demonstrates an unusual isolated cutaneous presentation of LG and is indicative of the clinical heterogeneity of this rare disease. Histopathology remains the key to diagnosis of LG and effective curative therapy is available.

Keywords: Lymphomatoid granulomatosis, Remission, Chemotherapy

Anahtar Sözcükler: Lenfomatoid granülomatozis, Remisyon, Kemoterapi

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.

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Results of the Hematology Laboratory Survey: What has Changed in Eight Years?

Hematoloji Laboratuvarı Anket Sonuçları: Sekiz Yılda Neler Değişti?

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To the Editor,

The Scientific Subcommittee on Laboratory Standards of the Turkish Society of Hematology (TSH) conducted two surveys, in 2009 and 2017, evaluating the tests, devices, and systems used in hematology laboratories (or other laboratories where hematological analyses are performed) in Turkey. The survey was shared online with TSH members as an informational message. Results from the 2017 survey were compared with those obtained in 2009 [1].

The survey was completed by 18 laboratories (14 university hospitals, 2 Ministry of Health education and research hospitals, 1 research institute, 1 private hospital) in 2009 and by 20 laboratories (12 university hospitals, 6 Ministry of Health hospitals, 2 foundation universities) in 2017 (Table 1).

In 2009, 11 (61%) laboratories were independent and 2 (11%) were part of a central laboratory. In 2017, 3 (15%) were independent and 12 (60%) were part of a central laboratory.

Regarding employed personnel, respondents in 2009 indicated that 24 medical doctors, 71 biologists, and 75 technicians worked in the laboratories. Respondents in 2017 indicated that 12 medical doctors, 12 biologists, and 16 technicians were employed.

In 2009, only three laboratories conducted internal quality control analyses for all tests. In 2017, internal quality control was conducted for all tests in seven laboratories, flow cytometry in two laboratories, coagulation in two laboratories, and electrophoresis in one laboratory. External quality control programs were utilized in 15 laboratories in 2009 and 9 in 2017. A written hematology laboratory manual was used by 13 (72.2%) and 11 (55%) laboratories in 2009 and 2017, respectively.

Performance of molecular studies, flow cytometry analyses, and minimal residual disease tests increased over the 8-year period. Additionally, 12 (60%) laboratories surveyed in 2017 had automation systems for peripheral blood smears, while none had automation systems in 2009.

Table 1. Changes of the hematology laboratories in Turkey between the 2009 and 2017 surveys.

	2009	2017
Number of independent laboratories	11	3
Number of personnel	24 doctors 71 biologists 75 technicians	12 doctors 12 biologists 16 technicians
Number of laboratories using internal quality control	3	12
Number of laboratories using external quality control	15	9
Existence of a laboratory manual	13	11
Test variety in hematology laboratories	14	18
Survey was completed by	14 university hospitals 2 MoH hospitals 1 research institute 1 private hospital Total: 18	12 university hospitals 6 MoH hospitals 2 foundation universities Total: 20

MoH: Ministry of Health.

In the 2017 survey, eight laboratories responded to the question "What are your expectations from the Laboratory Subcommittee?" Three respondents expressed their views on efforts to develop regulations pertaining to existing legislation,

two indicated a desire for more active training, and three discussed efforts to prepare laboratory guidelines.

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. This has led to the closure of many hematology laboratories and/or their inclusion into a central laboratory system.

Between 2009 and 2017, the number of personnel working in hematology laboratories in Turkey decreased. The hardware and infrastructure are in a position to match the developing technology, but not the standardization [2]. The TSH and the Scientific Subcommittee on Laboratory Standards are closely monitoring the current legislation and efforts are continuing to improve the existing legal situation.

Keywords: Hematology laboratory, Survey, Turkish Society of Hematology and Laboratory Subcommittee

Anahtar Sözcükler: Hematoloji laboratuvarı, Anket, Türk Hematoloji Derneği ve Laboratuvar Alt Komitesi

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