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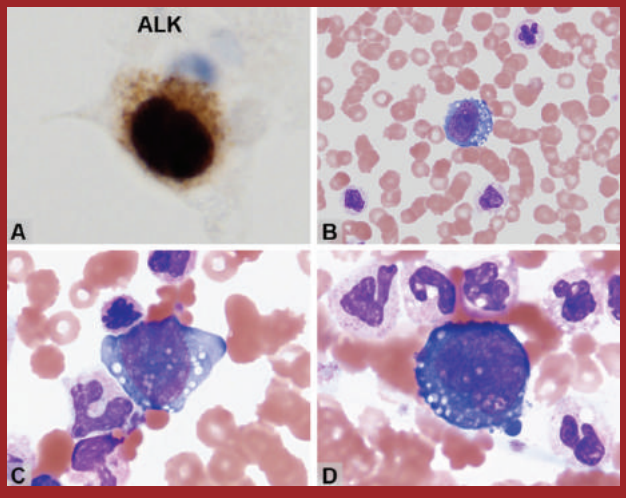
Turkish Journal of Hematology

The Official Journal of the Turkish Society of Hematology

Review

Research Articles



Cover Picture:
Shih-Sung Chuang, Yen-Chuan Hsieh, Hung-Chang Wu, Tainan, Taipei, Taiwan

ALK+ Anaplastic Large Cell Lymphoma of Null Cell Phenotype with Leukemic Transformation and Leukemoid Reaction

4

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Hemofili A Tedavisinde
DÖNÜM NOKTASI[1-3]

Tüm Hemofili A hastaları için
ŞİMDİ TÜRKİYE'DE![4]

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▼Bu ilaç ek izlemeye tabidir. Bu üçgen yeni güvenlilik bilgisinin hızlı olarak belirlenmesini sağlayacaktır. Sağlık mesleği mensuplarının şüpheli advers reaksiyonları bildirmeleri beklenmektedir. Herhangi bir şüpheli advers reaksiyonu Türkiye Farmakovijilans Merkezi'ne (TUFAM) (www.titck.gov.tr; e-posta: tufam@titck.gov.tr; tel: 0800 314 00 08; faks: 0312 218 35 99) ve/veya Roche Müstahzarları San. A.Ş.'ye e-posta (istanbul.adversolay@roche.com) veya telefon aracılığı ile (0212 366 90 00) bildirmeniz gerekmektedir.

Beşeri Tıbbi Ürünün Adı: HEMLIBRA 60 mg/0,4 mL **Etkin madde:** 0,4 mL'lik her bir flakon, (150 mg/mL konsantrasyonda) 60 mg emicizumab içerir. **Ambalajın içeriği:** Her kartonda 1 flakon bulunmaktadır. **Terapötik endikasyonları:** HEMLIBRA, faktör VIII inhibitörlü ya da inhibitörsüz hemofili A (konjenital faktör VIII eksikliği) hastalarında rutin profilakside endikedir. HEMLIBRA tüm yaş gruplarında kullanılabilir. **Pozoloji ve uygulama şekli:** Önerilen doz, subkutan enjeksiyon yoluyla, ilk 4 hafta boyunca haftada 1 kez 3 mg/kg (yükleme dozu) ve bunu takiben idame dozu olarak haftada 1 kez 1,5 mg/kg, 2 haftada 1 kez 3 mg/kg veya 4 haftada 1 kez 6 mg/kg'dır ve tüm dozlar subkutan enjeksiyon olarak verilir. Yükleme dozu, idame dozundan bağımsız olarak ayrıdır. **İstenmeyen etkiler:** HEMLIBRA ile gerçekleştirilen klinik çalışmalardan bildirilen en ciddi advers ilaç reaksiyonları (AİR'ler), kavernöz sinüs trombozu (CST) ve deri nekrozu ile eşzamanlı olarak gerçekleşen yüzeyel ven trombozu dahil olmak üzere trombotik olaylar ve trombotik mikroanjiyopatidir (TMA). En az bir doz HEMLIBRA ile tedavi edilmiş hastaların ≥%10'unda bildirilen en yaygın AİR'ler: enjeksiyon yeri reaksiyonları (%20), artralji (%15) ve baş ağrısıdır (%14). HEMLIBRA profilaksisi uygulanan klinik çalışmalarda toplamda üç hasta (%0,8) AİR'ler nedeniyle tedaviden çekilmiştir; bunlar TMA, yüzeyel tromboflebit ile eş zamanlı deri nekrozu ve baş ağrısıdır. **Diğer tıbbi ürünler ile etkileşimler:** HEMLIBRA ile yeterli ya da iyi kontrollü ilaç-ilaç etkileşim çalışmaları yürütülmemiştir. Klinik deneyim, HEMLIBRA ve aPCC arasında bir ilaç etkileşimi olduğuna işaret etmektedir. Klinik öncesi deneylere dayalı olarak, HEMLIBRA ile rFVIIa veya FVIII için bir heterokoagülabilite olasılığı mevcuttur. HEMLIBRA koagülasyon potansiyelini arttırmaktadır, bu nedenle hemostaza erişmek için gerekli rFVIIa veya FVIII dozu, HEMLIBRA profilaksisi kullanılmadığı zamana göre daha düşük olabilir. **Kontrendikasyonlar:** Etkin maddeye veya yardımcı maddelerden herhangi birine karşı bilinen aşırı duyarlılığı olan hastalarda kontrendikedir. **Özel popülasyonlara ilişkin ek bilgiler:** Gebelik kategorisi: C HEMLIBRA kullanmakta olan ve çocuk doğurma potansiyeline sahip kadınlar HEMLIBRA tedavisi süresince ve tedavinin kesilmesini takiben en az 6 ay boyunca etkili doğum kontrol yöntemleri kullanmalıdırlar. HEMLIBRA gerekli olmadıkça gebelik döneminde kullanılmamalıdır. **Saklama koşulları:** Flakonları 2°C-8°C'de buzdolabında saklayınız. Dondurmayınız. Çalkalamayınız. Flakonu, ışıktan korumak için kutusunda saklayınız. **Raf Ömrü:** 30 ay. Buzdolabından çıkarıldığında, açılmamış flakonlar 7 güne kadar oda sıcaklığında (30°C'nin altında) saklanabilir. **Ruhsat Sahibi:** Roche Müstahzarları Sanayi Anonim Şirketi, Umiq İstanbul, Ayazağa Cad. No:4, D:101 Maslak 34396, Sarıyer- İstanbul. **Ruhsat Numarası:** 2019/424 **İlk Ruhsat Tarihi:** 23.08.2019 **KÜB'ün Yenilenme Tarihi:** 23.08.2019. HEMLIBRA 60 mg/0,4 mL S.C. enjeksiyonluk çözelti.**%8 KDV Dahil Perakende Fiyatı ve Onay Tarihi:** 15.157,82 TL Fiyat Tarihi 01.10.2019. Daha geniş bilgi için firmamıza başvurunuz.

Referanslar: 1. Shaz B, Hillyer C ve Gil G (2018). Transfusion Medicine and Hemostasis (3rd edition). Elsevier Science. **2.** Knight T ve Callaghan MU. Ther Adv Hematol. 2018 Oct 10;9(10):319-334. **3.** Mahlangu JN. BioDrugs. 2018 Dec;32(6):561-570. **4.** Hemlibra® Kısa Ürün Bilgisi.

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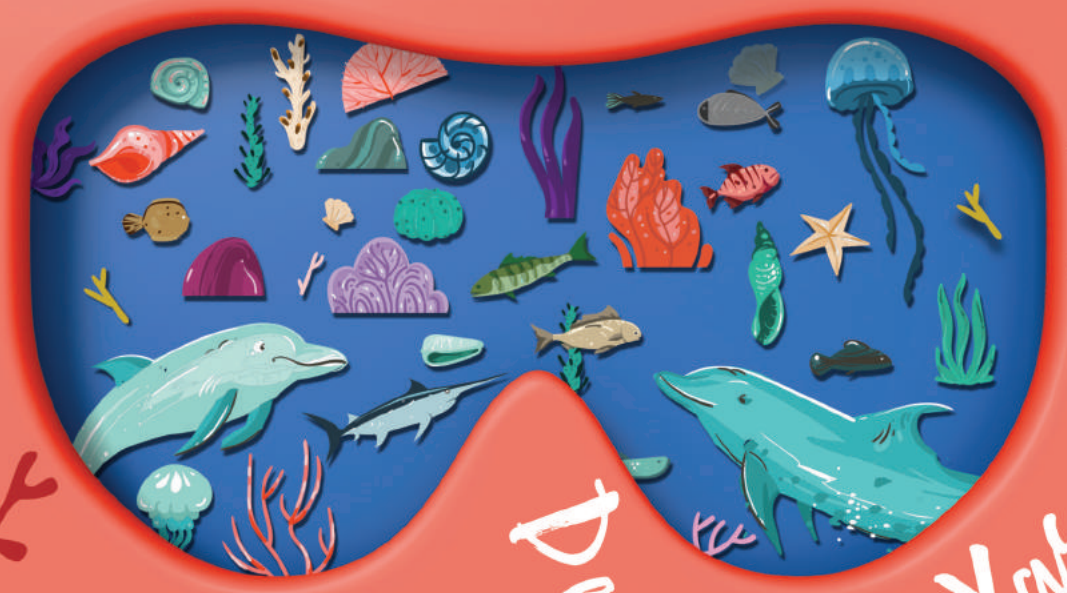

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Shih-Sung Chuang, Yen-Chuan Hsieh, Hung-Chang Wu, Tainan, Taipei, Taiwan

ALK+ Anaplastic Large Cell Lymphoma of Null Cell Phenotype with Leukemic Transformation and Leukemoid Reaction

A) ALK immunostaining revealed singly scattered positive cells in addition to those in small aggregates; B-D) leukemic cells were large with vesicular nuclei, irregular nuclear contours, and vacuolated basophilic cytoplasm.

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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.

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TURKISH JOURNAL OF HEMATOLOGY INSTRUCTIONS FOR AUTHORS

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

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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.

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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.

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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).

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Statistics: Describe the statistical methods used in enough detail to enable a knowledgeable reader with access to the original data to verify the reported 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.

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:

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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.

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Abstract length: Not to exceed 150 words.

Article length: Not to exceed 1200 words.

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

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

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

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

Invited Review Articles

Abstract length: Not to exceed 300 words.

Article length: Not to exceed 4000 words.

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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"Perspectives" are articles discussing significant topics relevant to hematology. They are more personal than a Review Article. Authors wishing to submit a Perspective in Hematology article should contact the Editor in Chief prior to submission in order to screen the proposed topic for relevance and priority. Articles submitted for "Perspectives in Hematology" must advance the hot subjects of experimental and/or clinical hematology beyond the articles previously published or in press in TJH. Perspective papers should meet the restrictive criteria of TJH regarding unique scientific and/or educational value, which will impact and enhance clinical hematology practice or the diagnostic understanding of blood diseases. Priority will be assigned to such manuscripts based upon the prominence, significance, and timeliness of the content. The submitting author must already be an expert with a recognized significant published scientific experience in the specific field related to the "Perspectives" article.

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Authors can submit for consideration illustrations or photos that are interesting, instructive, and visually attractive, along with a few lines of explanatory text and references. Images in Hematology can include no more than 200 words of text, 5 references, and 3 figures or tables. No abstract, discussion, or conclusion is required, but please include a brief title.

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Letters can include no more than 500 words of text, 5-10 references, and 1 figure or table. No abstract is required, but please include a brief title. The total number is usually limited to a maximum of five authors for a letter to the editor.

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Supply each table in a separate file. Number tables according to the order in which they appear in the text, and supply a brief caption for each. Give each column a short or abbreviated heading. Write explanatory statistical measures of variation, such as standard deviation or standard error of mean. Be sure that each table is cited in the text.

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Figures should be professionally drawn and/or photographed. Authors should number figures according to the order in which they appear in the text. Figures include graphs, charts, photographs, and illustrations. Each figure should be accompanied by a legend that does not exceed 50 words. Use abbreviations only if they have been introduced in the text. Authors are also required to provide the level of magnification for histological slides. Explain the internal scale and identify the staining method used. Figures should be submitted as separate files, not in the text file. High-resolution image files are not preferred for initial submission as the file sizes may be too large. The total file size of the PDF for peer review should not exceed 5 MB.

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Each author should have participated sufficiently in the work to assume public responsibility for the content. Any portion of a manuscript that is critical to its main conclusions must be the responsibility of at least one author.

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All submissions should contain a contributor's statement page. Each statement should contain substantial contributions to idea and design, acquisition of data, and analysis and interpretation of findings. All persons designated as an author should qualify for authorship, and all those that qualify should be listed. Each author should have participated sufficiently in the work to take responsibility for appropriate portions of the text.

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OBITUARY

In Memoriam- Prof.Dr. Ayhan Okçuoğlu Çavdar (1930-2019)

Prof. Dr. Ayhan Okçuoğlu Çavdar passed away on the 24th of June in 2019 in Ankara. She served as a researcher and mentor in the Department of Pediatric Hematology and Oncology at Ankara University School of Medicine for over forty years. Prof. Çavdar was graduated from Ankara University School of Medicine in 1953 after her education at Erenköy Kız Lisesi and completed pediatric residency in 1958. She got a pediatric hematology and oncology fellowship at Washington University. After her return to Turkey in 1961, she set up pediatric oncology and hematology units at Ankara University. Pediatric Oncology was first established in Turkey by Çavdar in 1961. She made many contributions to oncology in the fields of leukemias, mainly orbital granuloycytic sarcoma, Hodgkin's disease, and Burkitt's lymphoma in Turkish children. Additionally, she researched pica in Turkey, thalassemias, hemoglobinopathies and zinc deficiency in several conditions especially in pregnancies and newborn children with congenital abnormalities.

Dr.Çavdar wrote numerous research papers on these subjects published in international and national periodicals and books. She was elected as the first Turkish member of the SIOP (Société International d'Oncologie Pediatrique- International Society of Pediatric Oncology). Çavdar became the first Turkish member of the American Pediatric Academy after having received the Pediatric Board Certificate in 1962. She was named as the first Turkish pediatric hematologist in the *"Hematology, the Blossming of a Science"* written by Prof. Dr. Maxwell Myer Wintrobe in 1985. She established three research units namely, *"Pediatric Hematology and Oncology Research Center"*, *"Zinc Deficiency Unit"* and *"Pediatric Leukemias and Lymphomas Unit"* supported by theScientific and Technological Research Council of Turkey (TÜBİTAK). She also constituted *UNESCO Satellite Trace Elements Center* in 1998. Prof. Çavdar received many awards from professional societies including *TÜBİTAK Science Award* (1976), *Pediatric Oncology Service Award* (1984), *Prof. Dr. Nusret Fişek Public Health Service Promotion Award* (1998) and *the International Network for Cancer Treatment and Research (INCTR) Award* (2007). She published over 475 articles (about 197 in Turkish) and wrote three books.

Dr. Çavdar defended the idea that the primary role of universities was to educate young generations and conduct research. After attending international congresses, she used to share scientific knowledge with her colleagues. She was prominent figure in the Department giving us her full support over years in bad and good times. It was an honor and privilege for me to work with her.

Çavdar will be always remembered as having established Pediatric Oncology in Turkey (1961) and as a founding member of the Turkish Society of Hematology (1967), Mediterranean Blood Club (1975) and The Turkish Academy of Sciences, TUBA (1993). Her contributions will be greatly appreciated and will continue to evolve after she is gone.

Prof. Dr. Sevgi Gözdaşoğlu

Retired Professor of Pediatrics, Hematology and Oncology, Ankara University, Ankara, Turkey

REVIEW

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Diagnostic Testing for Differential Diagnosis in Thrombotic Microangiopathies

Trombotik Mikroanjiyopatilerde Ayırıcı Tanı İçin Tanı Testi

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Abstract

Thrombotic microangiopathies (TMAs) are multiple disease entities with different etiopathogeneses, characterized by thrombocytopenia, microangiopathic hemolytic anemia (MAHA) with schistocytosis, variable symptoms including fever, and multi-organ failure such as mild renal impairment and neurological deficits. The two paradigms of TMAs are represented on one hand by acquired thrombotic thrombocytopenic purpura (TTP) and on the other by hemolytic uremic syndrome (HUS). The differential diagnosis between these two paradigmatic forms of TMA is based on the presence of either frank renal failure in HUS or a severe deficiency (<10%) of the zinc-protease ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) in TTP. ADAMTS13 is an enzyme involved in the proteolytic processing of von Willebrand factor (vWF), and its deficiency results in formation of high-molecular-weight vWF-rich microthrombi in the environment of the microvasculature. The presence of these ultra-large vWF multimers in the microcirculation can recruit platelets, promoting multi-organ ischemic lesions. The presence of ADAMTS13 activity at >10% could rule out the presence of a TTP form. However, it is often difficult to differentiate either a TTP or HUS clinical scenario presenting with typical symptoms of TMA. There are in fact several additional diagnoses that should be considered in patients with ADAMTS13 activity of >10%. Widespread inflammation with endothelial damage and adverse reactions to drugs play a central role in the pathogenesis of several forms of TMA, and in these cases, the differential diagnosis should be directed at the underlying disease. Hence, a correct etiologic diagnosis of TMA should involve a critical illness, cancer-associated TMA, drug-induced TMA, and hematopoietic transplant-associated TMA. A complete assessment of all the possible etiologies for TMA symptoms, including acquired or congenital TTP, will allow for a more accurate diagnosis and application of a more appropriate treatment.

Keywords: Microangiopathic hemolytic anemia, Thrombotic microangiopathies, Anemia

Öz

Trombotik mikroanjiyopatiler (TMA), farklı etiyopatogenezleri olan; trombositopeni, şistositlerin eşlik ettiği mikroanjiyopatik hemolitik anemi (MAHA), ateş, hafif böbrek yetmezliği ve nörolojik defisitler gibi çoklu organ tutulumlarıyla karakterize bir hastalıklar grubudur. TMA'ların iki paradigması bir yandan edinsel trombotik trombositopenik purpura (TTP) ve diğer yandan hemolitik üremik sendrom (HUS) ile temsil edilir. TMA'nın bu iki paradigmatik formu arasındaki ayırıcı tanı, HUS'de belirgin böbrek yetmezliği veya TTP'de çinko-proteaz ADAMTS13'ün (bir disintegrin ve metalloproteinaz trombospondin tip 1, üye 3) ciddi eksikliğinin (<%10) varlığına dayanmaktadır. ADAMTS13, von Willebrand faktörünün (vWF) proteolitik işleminde yer alan bir enzimdir ve eksikliği, mikrovasküler ortamda yüksek molekül ağırlıklı vWF bakımından zengin mikrotrombüs oluşumuna yol açar. Bu ultra-büyük vWF multimerlerinin mikro dolaşımdaki varlığı, trombositleri toplayarak çok organlı iskemik lezyonları teşvik eder. ADAMTS13 aktivitesinin %10'dan büyük oluşu, bir TTP formunun varlığını dışlatabilir. Bununla birlikte, tipik TMA semptomları gösteren bir TTP veya HUS klinik senaryosunu ayırt etmek genellikle zordur. Aslında ADAMTS13 aktivitesi %10'dan büyük olan hastalarda göz önünde bulundurulması gereken birkaç ek tanı vardır. Endotel hasarı ve ilaçlara verilen yan etkilerle birlikte görülen yaygın yangı, birçok TMA formunun patogenezinde merkezi bir rol oynamaktadır ve bu durumlarda, ayırıcı tanı altta yatan hastalığa yönlendirilmelidir. Bu nedenle, TMA'nın doğru bir etiyolojik tanısı, kritik bir hastalık, kansere bağlı TMA, ilaca bağlı TMA ve hematopoetik transplant ile ilişkili TMA'yı içermelidir. Edinilmiş veya konjenital TTP dahil olmak üzere TMA semptomları için olası tüm etiyolojilerin tam bir değerlendirmesi, daha uygun bir tedavinin daha doğru bir şekilde teşhis edilmesine ve uygulanmasına olanak sağlayacaktır.

Anahtar Sözcükler: Mikroanjiyopatik hemolitik anemi, Trombotik mikroanjiyopatiler, Anemi

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Introduction

The name thrombotic microangiopathy (TMA) refers to rare multisystem diseases characterized by damage of endothelial walls of arterioles and capillaries, which leads to massive occlusion and formation of platelet-rich thrombi and microangiopathic hemolytic anemia (MAHA). By definition, TMA indicates neither a specific diagnosis nor a specific etiology; it is just a pathologic diagnosis made by tissue biopsy [1,2]. TMAs are medical emergencies requiring rapid diagnosis and appropriate treatment.

The term MAHA refers to nonimmune hemolytic anemia caused by red blood cell (RBC) intravascular fragmentation. This is combined with:

- schistocytosis, with a confidence threshold of 1% in peripheral blood to support a clinical diagnosis of TMA [3,4];

- consumption thrombocytopenia with platelets of $<150x10^9$ or a decrease from baseline of >25%.

- negative direct antiglobulin test (DAT);

- indirect indicators of hemolysis, such as increased plasma lactate dehydrogenase (LDH), and/or decreased hemoglobin and/or haptoglobin;

- fever and organ involvement, including renal impairment and/or neurological, gastrointestinal, cardiovascular, pulmonary, or visual symptoms.

Not all cases of MAHA are caused by a TMA, but all TMAs cause MAHA and thrombocytopenia.

History

Moschcowitz in 1924 described for the first time a case of abrupt onset and progression of petechial bleeding, pallor, fever, paralysis, hematuria, and coma [5], with disseminated microvascular hyaline thrombi in arterioles and capillaries. In 1947 Singer et al. [6] first introduced the term "thrombotic thrombocytopenic purpura" (TTP). The name TMA was introduced by Symmers in 1952 to describe the vascular lesions observed in TTP [7]. In 1955 Gasser et al. [8] described the symptoms of a child with thrombocytopenia, hemolytic anemia, and renal failure with bilateral diffuse cortical necrosis: this was called hemolytic uremic syndrome (HUS). In 1982 Moake et al. [9] suggested a defective processing of ultra-large von Willebrand factor (vWF) multimers produced by endothelial cells. In 1983, Karmali et al. [10] associated HUS with infections with *Escherichia coli* producing Shiga toxin (ST). According to Furlan et al. [11], increased proteolytic cleavage of vWF is observed in a number of cases with type 2A von Willebrand disease. Large vWF multimers, which are hemostatically active, are degraded to form smaller and less active molecules. In particular, the peptide bond between 842Tyr and 843Met is cleaved in the polypeptide subunits of vWF. The increased frequency of platelet thrombosis in TTP patients is related to a deficiency of such proteolytic activity [12,13]. The key vWF-cleaving protease, on the basis of partial amino acid sequencing, was a large zinc-containing metalloprotease, identified as "a disintegrin and metalloproteinase with thrombospondin type 1", member 13 (ADAMTS13) of the ADAMTS protease family [14,15].

Epidemiology and Pathogenesis of TMA

TMAs are rare diseases: five to ten cases/year per million cases of TTP are acquired, with a male:female ratio of 1:2 and a peak of incidence during the 4th decade of life. Hereditary TTP represents one or fewer cases/year per million [19,20].

The most prominent diagnoses associated with TMA are thrombotic TTP and HUS. They usually occur, respectively, in adults and in children. As discussed below, their pathogenesis is different: TTP results from a severe ADAMTS13 deficiency, which can be caused by circulating autoantibodies or ADAMTS13 mutations, while HUS is correlated to infection with ST-producing bacteria or gene mutations causing an excess of activation of the alternative pathway [16]. According to recent observations in TTP/HUS registries, emerging features of these disorders are the diagnostic value of ADAMTS13 measurement, efficacy of plasma exchange (PEX), and frequency of relapses after remission [17,18].

Many different disorders can cause TMA (i.e. secondary TMA; see below).

Other clinical TMA presentations are:

- HELLP syndrome (hemolysis, elevated liver enzymes, low platelet count), which is observed in a proportion of 0.5%-0.9% of pregnancies, as well as in 10%-20% of severe preeclampsia cases [21];

- catastrophic antiphospholipid syndrome, which is rarely observed patients with acute multi-organ thrombosis (less than 1%);

- malignant hypertension, in about 2.6 cases/year per 100,000 cases with a higher incidence among people of African descent;

- cancer: about 5% of patients with disseminated malignancy;

- transplant-associated TMA following a) non-renal solid organ transplantation (incidence 5%, 4.0% in liver, 2.3% in lungs) [22,23], b) renal transplantation, with 5.6/1000/year with a 50% mortality rate at three years [24], and c) hematopoietic progenitor cell transplantation, with variable ranges from 0% to 74% and median incidence of 7.9% [2,25].

Finally, TMAs are also part of the pathology of disseminated intravascular coagulation (DIC), in which it results from the deposition of fibrin or platelets within the microvasculature [26], and scleroderma renal crisis [27]. In Table 1 the TMAs are listed according to cause.

This review mainly deals with diagnostic aspects of MAHA and TMAs. A number of clinical problems await solutions in TMA, such as the positioning of rituximab in the treatment sequence of primary TTP, management of ST-producing *Escherichia coli*-HUS complicated by encephalopathy, the efficacy and long-term safety of eculizumab in atypical HUS, and elucidation of the pathogenesis of secondary TMA [28,29,30].

Clinical Forms of TMA

TTP is a clinical emergency with a mortality rate of up to 90% if not promptly treated [31]. African-Caribbean ancestry [32] and obesity [33] are risk factors. It is caused by a lack or deficiency of ADAMTS13. In normal individuals, endothelial cells produce vWF multimers from the Weibel-Palade bodies and the metalloprotease enzyme ADAMTS13 cleaves the unusually large multimers, avoiding platelet adhesion [34]. When the vWF multimers are not cleaved, platelets adhere and the endothelial layers of small vessels are damaged, causing platelet aggregation and fibrin deposition in microcirculation. Infections, drugs, and pregnancy/delivery [35,36] may act as triggers in predisposed individuals. ADAMTS13 activity may be absent or highly inhibited by circulating autoantibodies, which represent the most frequent cause of acquired TTP. Up to 75% of patients in the acute phase show the presence of IgG immunoglobulins with anti-ADAMTS13 activity, which inhibit its proteolytic activity towards vWF. Such autoantibodies circulate in the form of immuno-complexes (IC) and are the cause of the deficiency of ADAMTS13. In 20%-25% of patients anti-ADAMTS13 autoantibodies are not detectable, so that the mechanisms that underlie ADAMTS13 deficiency are not fully clarified. Less than 5% of TTP cases are due to *ADAMTS13* gene mutation (congenital TTP, Upshaw-Schulman syndrome (USS), an autosomal recessive disease presenting with early onset in childhood) [37,38]. More than 150 different *ADAMTS13* gene mutations have been described to date: 70% of these mutations are missense, while the remaining 30% are truncating [37]. In the suspicion of a congenital form of HUS, the ADAMTS13 level should be evaluated by measuring both its activity with a fluorogenic assay [39] and its antigen level to differentiate between type 1 (both activity and antigen decreased) and type 2 deficiency (severe activity defect associated with subnormal antigen level).

ST-mediated HUS is associated with the microbiological finding of *Escherichia coli*, mainly O157:H7 and O104:H4 serotypes, and/or *Shigella dysenteriae* type 1 infection: the production of the ST leads to endothelial and glomerular damage with an acute clinical picture. It is usually caused by food, with a seasonal distribution with a summer peak, and it represents the main cause of acute renal impairment in children less than 3 years old. Enterohemorrhagic diarrhea self-resolves in most cases, but in 5%-7% of them, HUS develops a few days afterwards. ST, a pentamer of B subunits, causes endothelial cell damage through binding to a globotriaosylceramide receptor expressed on the membrane of endothelial cells: after internalization by endocytosis, ST inhibits protein synthesis, causing cell apoptosis and death [40] and exposure of the extracellular matrix with platelet aggregation, fibrin deposition, and mechanical hemolysis. The kidneys, gastrointestinal tract, and central nervous system (CNS) are the key target organs. ST-mediated HUS, which can be as severe as acute HUS, reaches a mortality rate of up to 5% [41].

Complement-mediated TMA presents with thrombocytopenia, mechanical hemolysis, and acute renal failure, with severe arterial hypertension and ischemic damage due to activation and/or abnormal regulation of the alternative pathway of the complement system on cell surfaces: mutations in C3 and factor B; autoantibodies against factor H interfering with regulation; disturbed recognition by factor H, factor I, or CD46 of C3b; and disturbed recognition by factor H of self-cell surface molecules,

Table 1. Thrombotic microangiopathies listed according to causes.

▶ Thrombotic thrombocytopenic purpura, ADAMTS13 deficiency-mediated:
- *Genetic*: <10% ADAMTS13 activity
- *Acquired*: due to antibodies to ADAMTS13
▶ Shiga toxin-mediated hemolytic uremic syndrome, sustained by enteropathogenic microorganisms (*Shigella dysenteriae* and some serotypes of *Escherichia coli*, such as O157:H7 and O104:H4)
▶ Complement-mediated TMA, due to mutations in complement regulatory genes and/or antibodies blocking the complement functions
▶ Coagulation mediated TMA, due to mutations involving *DGKE*, *PLG*, and *THBD* genes
▶ Metabolism-mediated TMA due to mutations in *MMACHC* gene (methylmalonic aciduria and homocystinuria type C)
▶ Drug-mediated TMA via immunologic pathway (antibodies) and/or toxicity (quinine, ticlopidine, clopidogrel, interferon, contraceptives, etc.)
▶ Secondary TMAs: initiated by a coexisting disease or condition such as infection (*Streptococcus pneumoniae* infection, influenza virus), transplantation (solid organ or bone marrow), autoimmune disease, cancer, pregnancy, certain cytotoxic drugs (anticancer drugs, immunosuppressives), radiotherapy, malignant hypertension, disseminated intravascular coagulation, severe vitamin B12 deficiency, pancreatitis

TMA: Thrombotic microangiopathy.

such as sialic acid or glycosaminoglycans [42]. About 20% of cases show a subclinical onset, with slow disease progression [43].

Coagulation-mediated TMA is caused by mutations of genes encoding for thrombomodulin (*THBD*), plasminogen (*PLGx*), and diacylglycerol kinase epsilon (*DGKE*), inducing upregulation of prothrombotic factors [44,45].

Metabolism-mediated TMA, usually seen in infants, is caused by mutations in different genes that cause methylmalonic and aciduria homocystinuria type C (*MMACHC*) [46].

Drug-mediated TMA [47] can be caused by:

- immune-mediated mechanisms with antibodies formation (quinine) [48].

- dose-dependent/toxicity mechanisms (cyclosporine, tacrolimus, clopidogrel, interferon, vascular endothelial growth factor inhibitor, mitomycin C).

- induction of drug-independent antibodies (ticlopidine).

New observations are not rare, such as TMA associated with the intravenous injection of adulterated Opana ER tablets [49].

Secondary TMAs are caused by different coexisting disorders, such as systemic infections [50]. In particular, infections due to *Streptococcus pneumoniae* and influenza viruses are considered true etiological factors, instead of simple triggers, of TMA. Cancer [51], transplantation of bone marrow or solid organs [52], autoimmune disease [53], pregnancy [54], cytotoxic drugs, DIC, severe deficiency of vitamin B_{12} [55], and pancreatitis can be responsible for the development of secondary TMA. A common feature of the above-mentioned conditions is the generation of direct cell damage, with general activation of the complement system or enhanced activation of the complement on cell membranes [42].

Diagnostic Tests

Almost all cases of TMA are associated with MAHA. It is extremely important to exclude at a clinical level any possible cause of MAHA alternative to TMA. In particular, occasionally patients with paroxysmal nocturnal hemoglobinuria, intravascular and/or heart devices, heparin-induced thrombocytopenia, and systemic disorders such as systemic infections can present with MAHA in association with or without TMA. The main causes of secondary TMAs were mentioned above; the patient's history and physical examination are fundamental steps for the most appropriate diagnostic pathway. Diagnosis of MAHA is confirmed by negativity of DAT, increased LDH, and/or decreased haptoglobin. Organ involvement should be investigated. Complete blood count in MAHA shows normocytic anemia, reticulocytosis, and severe thrombocytopenia, while in the peripheral blood smear schistocytes, microspherocytes, and polychromatophilic RBCs, identifiable as immature reticulocytes by vital stains, are detected. Schistocytes are fragmented red cells appearing in a variety of shapes: rectangular, crescent, or helmet-shaped. Traditionally they are identified and counted by microscopic observation by trained laboratory scientists, with a large margin of error [3]. In TMA, RBCs are physically sheared by fibrin networks in the peripheral circulation: the appearance of schistocytes may be one of the earliest signs of TMA and its detection and quantitation are of primary importance. In 2012 the International Council for Standardization in Haematology published specific recommendations to standardize schistocyte identification, enumeration, and reporting [3], including morphological criteria for the identification of specific schistocyte types. Reference values are ≤0.1% in adults, 0.3%-1.9% in newborns, and ≤5.5% in preterms. Schistocytes should be evaluated on smears at medium microscope magnification as a percentage after counting at least 1000 red blood cells (Figure 1). Schistocyte count has definite clinical value for diagnosis of TMA in the absence of additional severe red cell shape abnormalities, with a confidence threshold value of 1%. Fragmented RBC enumeration by automated counters is a complement to microscopy, providing rapid results with high predictive value for negative samples [3,4]. Increased megakaryocytes in bone marrow (Figure 2), usually with left shift, associated with thrombocytopenia testify to the presence of peripheral platelet consumption. Bone marrow aspiration is not mandatory but can facilitate the differential diagnosis (versus promyelocytic leukemias with DIC or other hypoplastic/aplastic marrow diseases, including hemophagocytic syndrome).

Once primary TMA is confirmed, the type should be determined to provide the patient with the specific treatment: PEX in TTP and eculizumab in complement-mediated TMA. The patient's sample for assay of ADAMTS13 functional levels should be investigated.

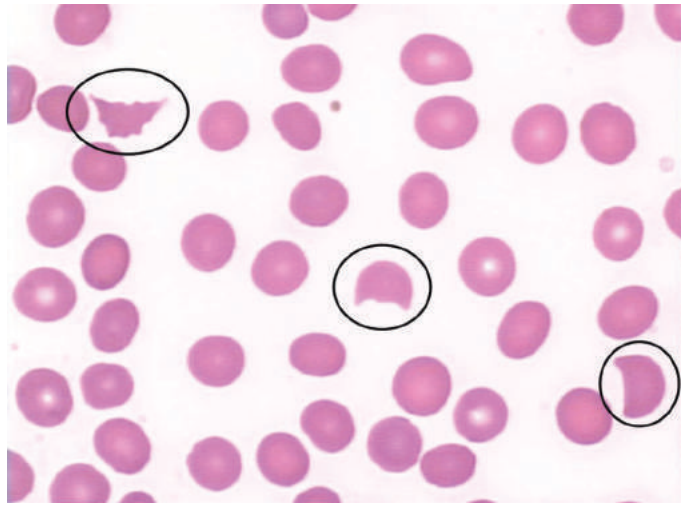

Figure 1. Schistocytes should be evaluated on smears at medium microscope magnification.

ADAMTS13 activity measurements (degradation of a vWF substrate) are currently based on different methods [56]: fluorescence resonance energy transfer (FRET) [57], chromogenic enzyme-linked immunosorbent assay (ELISA) [58], mass spectrometry [59], and simplified methods based on coagulation analyzers [60].

Results of ADAMTS13 measurements are reported as a percentage of ADAMTS13 activity in pools of plasma from healthy donors, with a threshold of <10%. It is possible, however, in the opinion of these authors, that a lower threshold should be considered, given the increased sensitivity of new-generation methodologies. An international World Health Organization standard plasma method for the measurement of ADAMTS13 has recently become available [61]. DNA testing for ADAMTS13 genes has also been developed [62].

Clinical interpretation is fundamental because of possible false low results due to hemolysis or increased bilirubin, especially in FRETS-based assays. Moreover, unfortunately results of the diagnostic tests are not immediately available, while patients with acute MAHA and thrombocytopenia usually require immediate treatment. In this scenario the PLASMIC score [63] does represent immediate help in calculating the diagnostic probability of TTP, evaluating very simple parameters/information. One point is assigned to each of the following:

i) platelet count of $<30x10^9$/L;

ii) plasma or serum indirect bilirubin >2 mg/dL, *or* reticulocyte count >2.5%, *or* undetectable plasma haptoglobin,

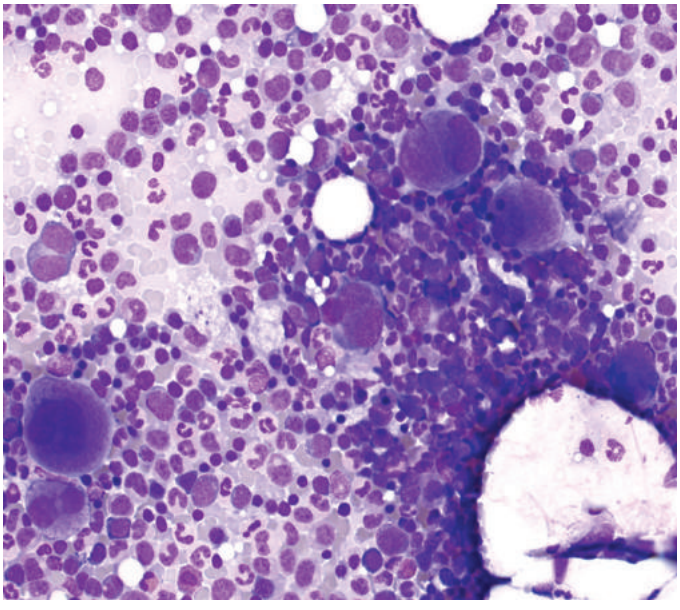

Figure 2. Increased megakaryocytes in bone marrow associated with thrombocytopenia testify to the presence of peripheral platelet consumption.

iii) absence of active cancer,

iv) absence of solid organ or stem cell transplant in the medical history,

v) mean corpuscular volume (MCV) of <90 fL,

vi) international normalized ratio (INR) <1.5,

vii) plasma or serum creatinine <2.0 mg/L.

The PLASMIC risk score for severe ADAMTS13 deficiency can be low (<5), intermediate (5), or high (>5). ST-HUS acute onset is characterized by abdominal pain, associated with vomiting and bloody diarrhea, which can anticipate by several days other clinical and laboratory signs of MAHA associated with thrombocytopenia. Stool cultures for enteric pathogens do confirm the correct diagnosis. In complement-mediated TMA, symptoms are less typical, more insidious, and generic (acute renal failure, edema); up to 20% of cases present with multi-organ failure (CNS, cardiac, pulmonary, intestinal). It is reported as familial and sporadic, presenting in up to 80% of children and 50% of adults [64,65]. Quantitative, genetic, and functional complement assessment will lead to the diagnosis, and while waiting for lab test results it is mandatory to start treatment with PEX, moving to anti-complement therapy after obtaining the results. In drug-mediated TMA, supportive therapy and drug discontinuation are indicated, while in metabolism-mediated TMA and coagulation-mediated TMA the role of molecular testing is fundamental. Figure 3 displays an algorithm for differential laboratory diagnosis in patients with clinical suspicion of TMA.

Conclusion

The differential diagnosis of TTP, HUS forms, and TMA from other etiologies can be challenging. Diagnosis has to be primarily based on clinical history (underlying disease, medications). In intensive care patients, TMA is more probably associated with the underlying illnesses. In patients presenting with TMA signs, clinical antecedents of metastatic malignancy, hypertension, polychemotherapy or immunosuppressive treatment, HELLP syndrome, or allogeneic stem cell transplant should be considered as possible causes for the TMA presentation. In the great majority of such patients, a serum level of ADAMTS13 activity lower than 10% is a useful element for the differential diagnosis. Finally, not infrequently diagnostic assessment has to be extended after treatment and recovery of patients, especially when biochemical and molecular biology studies, including mutation analysis of complement factors, may add useful elements.

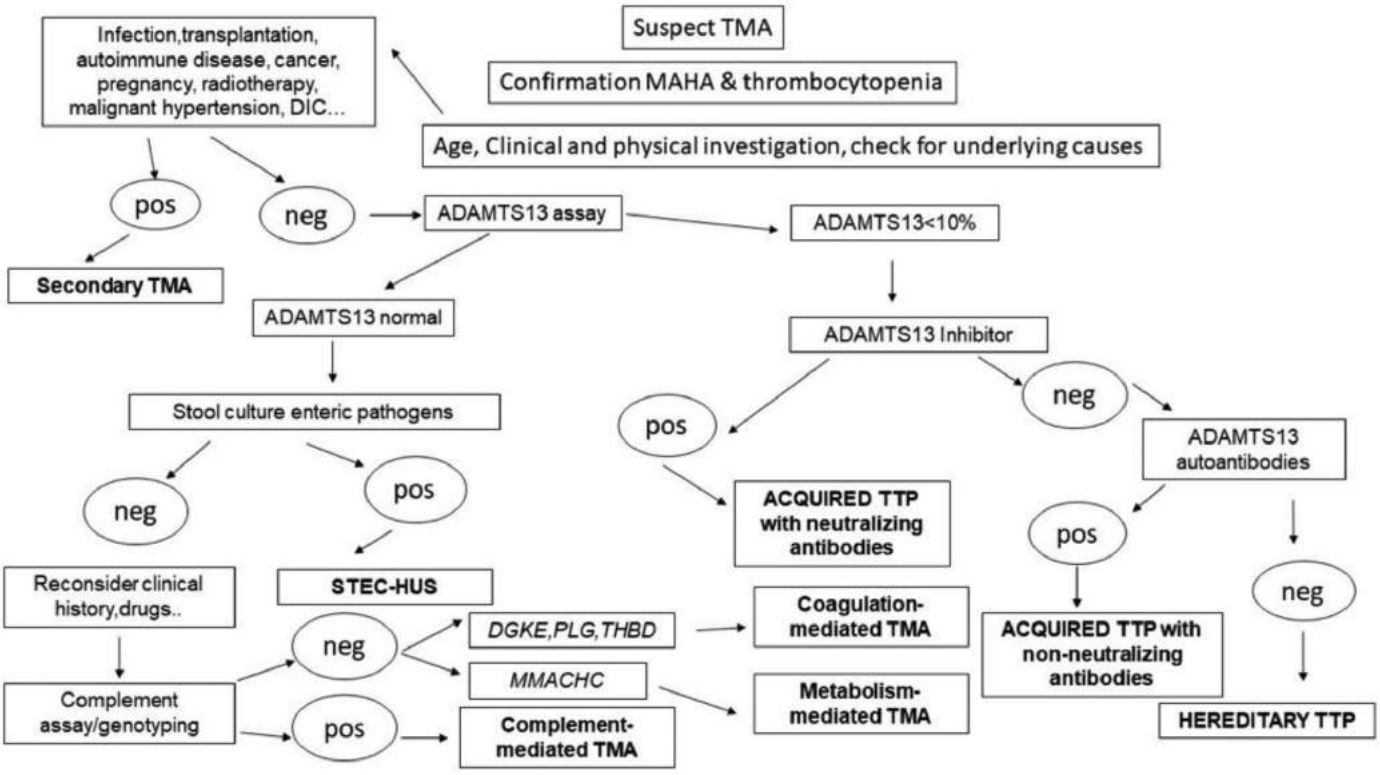

Figure 3. Algorithm for differential laboratory diagnosis in patients with clinical suspicion of thrombotic microangiopathie.

Ethics

Ethics Committee Approval: Not appliable to a review.

Authorship Contributions

Surgical and Medical Practices: G.Z., R.D.C.; Concept: G.Z., R.D.C.; Design: G.Z., R.D.C.; Literature Search: G.Z.; Writing: G.Z.

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RESEARCH ARTICLE

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A Multi-Center Study on the Efficacy of Eltrombopag in Management of Refractory Chronic Immune Thrombocytopenia: A Real-Life Experience

Refrakter Kronik İmmün Trombositopeni Tedavisinde Eltrombopagın Etkinliğine İlişkin Çok Merkezli Bir Çalışma: Gerçek Yaşam Deneyimi

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Abstract

Objective: The aim of the present study was to evaluate the efficacy and safety of eltrombopag, an oral thrombopoietin receptor agonist, in patients with chronic immune thrombocytopenia (ITP).

Materials and Methods: A total of 285 chronic ITP patients (187 women, 65.6%; 98 men, 34.4%) followed in 55 centers were enrolled in this retrospective cohort. Response to treatment was assessed according to platelet count (/mm^3) and defined as complete (platelet count of >100,000/mm^3), partial (30,000-100,000/mm^3 or doubling of platelet count after treatment), or unresponsive (<30,000/mm^3). Clinical findings, descriptive features, response to treatment, and side effects were recorded. Correlations between descriptive, clinical, and hematological parameters were analyzed.

Results: The median age at diagnosis was 43.9±20.6 (range: 3-95) years and the duration of follow-up was 18.0±6.4 (range: 6-28.2) months. Overall response rate was 86.7% (n=247). Complete and partial responses were observed in 182 (63.8%) and 65 (22.8%) patients, respectively. Thirty-eight patients (13.4%) did not respond to eltrombopag treatment. For patients above 60 years old (n=68), overall response rate was 89.7% (n=61), and for those above 80

Öz

Amaç: Bu çalışmanın amacı kronik immün trombositopeni (ITP) hastalarında bir oral trombopoietin reseptör agonisti olan eltrombopagın etkinlik ve güvenirliliğini değerlendirmektir.

Gereç ve Yöntemler: Elli beş merkezde izlem altındaki toplam 285 kronik ITP hastası (187 kadın, %65,6) bu geriye dönük küme çalışmasına alınmıştır. Tedaviye yanıt trombosit sayısına göre değerlendirilmiş ve tam yanıt (>100.000/mm^3), kısmi yanıt (30.000-100.000/mm^3 veya tedaviden sonra trombosit sayısının bir kat artmış olması) ve yanıtsızlık (<30.000/mm^3) olarak tanımlanmıştır. Hastaların klinik bulguları, tanımlayıcı özellikleri, tedaviye yanıt ve yan etki bilgileri toplanmış ve aralarındaki ilişki incelenmiştir.

Bulgular: Tanı anında yaş ortalaması 43,9±20,6 (3-95) yıl olan hastalar ortalama 18,0±6,4 (6-28,2) ay izlenmiştir. Tam ve kısmi yanıtı içeren toplam yanıt %86,7 (n=247) bulundu. Sırasıyla 182 (%63,8) ve 65 (%22,8) hastada tam ve parsiyel tedavi yanıtları gözlenmiştir. Otuz sekiz hasta (%13,4) eltrombopag tedavisine yanıt vermemiştir. Altmış yaş üzerindeki hastalarda (n=68) toplam yanıt %89,7 (n=61) bulunurken, bu oran 80 yaş üzerindeki (n=12) hastalarda %83 (n=10) olmuştur. Tedavi öncesi trombosit sayısı göz önüne alındığında, eltrombopag,

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years old (n=12), overall response rate was 83% (n=10). Considering thrombocyte count before treatment, eltrombopag significantly increased platelet count at the 1st, 2nd, 3rd, 4th, and 8th weeks of treatment. As the time required for partial or complete response increased, response to treatment was significantly reduced. The time to reach the maximum platelet levels after treatment was quite variable (1-202 weeks). Notably, the higher the maximum platelet count after eltrombopag treatment, the more likely that side effects would occur. The most common side effects were headache (21.6%), weakness (13.7%), hepatotoxicity (11.8%), and thrombosis (5.9%).

Conclusion: Results of the current study imply that eltrombopag is an effective therapeutic option even in elderly patients with chronic ITP. However, patients must be closely monitored for response and side effects during treatment. Since both response and side effects may be variable throughout the follow-up period, patients should be evaluated dynamically, especially in terms of thrombotic risk factors.

Keywords: Thrombocytopenia, Immune thrombocytopenic, Eltrombopag

Öz

tedavinin 1., 2., 3., 4. ve 8. haftalarında trombosit sayısını anlamlı şekilde artırmıştır. Kısmi veya tam cevap için gereken süre arttıkça, tedaviye cevap önemli ölçüde azaldığı saptanmıştır. Eltrombopag tedavisinden sonra maksimum trombosit sayısı ne kadar yüksekse, yan etkilerin oluşabilme ihtimalinin o kadar yüksek olabildiği dikkati çekmiştir. En sık görülen yan etkiler baş ağrısı (%21,6), güçsüzlük (%13,7) ve hepatotoksisite (%11,8) ve trombozdur (%5,9).

Sonuç: Mevcut çalışmanın sonuçları, eltrombopag tedavisinin kronik ITP'de, yaşlı hastalar dahil olmak üzere, etkili bir tedavi seçeneği olduğunu göstermektedir. Bununla birlikte, hastalar tedavi sırasında yanıt ve yan etkiler açısından yakından izlenmelidir. Hem cevap hem de yan etkiler, takip süresi boyunca değişken olabileceğinden, hastalar özellikle tromboz risk faktörleri açısından dinamik olarak değerlendirilmelidir.

Anahtar Sözcükler: Trombositopeni, İdiyopatik trombositopenik purpura, Eltrombopag

Introduction

Immune thrombocytopenia (ITP) is an acquired disorder characterized by a transient or persistent decrease in platelets accompanied with an increased risk of bleeding [1,2,3]. The estimated incidence of ITP is 100 cases per 1 million people annually [4]. Clinical presentation varies in a wide spectrum ranging from asymptomatic or mild cases with bruising and petechiae to severe mucocutaneous bleeding that could be life-threatening [5,6]. Immune thrombocytopenia has been linked to an increased rate of immune-mediated platelet destruction; however, the exact pathophysiological mechanism is still unclear [3].

In chronic ITP, antiplatelet antibodies facilitate platelet destruction and prevent the release of platelets from megakaryocytes, thus resulting in mild to serious thrombocytopenia. Therapeutic strategies for first- or second-line treatment such as corticosteroids, intravenous immunoglobulin, and splenectomy can reduce the destruction of antibody-coated platelets, but the efficacy is limited and serious adverse effects can be seen [7]. Use of immunosuppressive drugs has been restricted because of serious adverse events and splenectomy has been linked to important drawbacks such as infection and thrombosis. Monitoring patients for the effectiveness of the treatment and for side effects is an important issue in the improvement of therapeutic outcomes.

Another treatment strategy is to use thrombopoietin receptor agonists (TPO-RAs) for stimulating platelet production through interaction with the TPO receptors present on megakaryocytes. One such example is eltrombopag, an oral, non-peptide thrombopoietin receptor agonist [8]. Since eltrombopag does not compete with endogenous TPO binding at the extracellular TPO-R domain, it may possess an additive effect to thrombopoietin [9]. As a consequence, the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway stimulates megakaryocytopoiesis, while autoantibody generation is not detected [10]. Furthermore, eltrombopag does not influence agonist-induced platelet aggregation or activation [1]. Eltrombopag produces a quick and sustainable increase in platelet counts and is generally well tolerated in patients with chronic ITP.

The present study aimed to analyze the outcomes of eltrombopag treatment in patients with chronic ITP in clinical practice in Turkey and to estimate the demographic, clinical, and hematological variables that may have implications for therapeutic response.

Materials and Methods

Patients and Study Design

This retrospective study (2011-2017) was conducted in 55 tertiary care centers of Turkey. Data were collected from medical files of 285 chronic ITP patients, of whom 187 were women (65.6%) and 98 were men (34.4%). Patients with a diagnosis of chronic ITP according to the international consensus report [11] irrespective of their age at diagnosis were eligible for inclusion if they received eltrombopag at any time in their treatment schedule. The exclusion criteria for treatment with eltrombopag consisted of HIV, hepatitis B, or hepatitis C infections; cardiovascular diseases; malignancy; chemotherapy or radiotherapy; prior

diagnosis of myelodysplastic syndrome or aplastic anemia; and presence of two or more risk factors for thrombosis, such as smoking, diabetes mellitus, hypercholesterolemia, or hereditary thrombophilic disorders. Patients with a history of thrombosis were also excluded from the study because of the contraindication for these patients as a policy of the Ministry Health of Turkey.

The study was performed in accordance with the Declaration of Helsinki and conducted after the approval of the local institutional review board. Written informed consent was provided for all patients enrolled in this study. Chronic ITP is defined as persistent thrombocytopenia despite conventional initial management [12]. Eltrombopag was administered at doses of 50 mg as a starting dose as approved by the Turkish Ministry of Health. After 2 weeks of treatment, if the platelet levels were <30,000/μL, the dose was increased up to a maximal daily dose of 75 mg in increments of 25 mg. Achieving a platelet level between 150,000/μL and ≤250,000/μL, the daily dose was tapered by 25 mg. If platelet levels reached above 250,000/μL, eltrombopag was stopped, and after decreasing to <100,000/μL the treatment was restarted by reducing the last daily dose by 25 mg. Descriptive data (age at diagnosis of chronic ITP, sex), therapeutic response (none, partial, or complete), side effects (absent or present), and severity of findings linked with bleeding (none, mild, moderate, severe, or life-threatening) were recorded. Platelet counts at first admission, before treatment, and at the 1st, 2nd, 3rd, 4th, and 8th weeks; the number of days with platelet count >30,000/μL; maximum platelet counts after treatment; time to reach maximum platelet counts after treatment; and duration of follow-up were documented. Correlations between these descriptive, clinical, and hematological parameters were analyzed.

Severe or life-threatening bleeding was defined as either intracranial hemorrhage or bleeding that caused hemodynamic compromise and required intervention. Moderate bleeding was defined as bleeding that required blood transfusion but did not result in hemodynamic compromise. Minor bleeding was defined as bleeding that did not meet the criteria for either severe or moderate bleeding.

Response to treatment was defined as none (platelet count <30,000/μL), partial (platelet count between 30,000/μL and 100,000/μL or platelet count double the initial value), or complete (platelet count >100,000/μL).

Statistical Analysis

Data were analyzed using IBM SPSS 20.0 software for Windows (IBM Corp., Armonk, NY, USA). The normal distribution of continuous variables was evaluated with the Kolmogorov-Smirnov test. Parametric tests were used for variables distributed normally, while non-parametric tests were utilized for variables without normal distribution. Correlation between variables was tested with Spearman's rho test. Categorical variables were compared with Pearson chi-square and Fisher exact tests, while two independent groups were compared using t-tests and Mann-Whitney U tests. Quantitative variables are demonstrated as mean ± standard deviation or median-interquartile range. The confidence interval was 95% and differences associated with a p-value of less than 0.05 were considered as statistically significant.

Results

The average age at diagnosis was 43.9±20.6 (range: 3 to 95) years. An outline of the demographic and clinical data of the present series is shown in Table 1. Before starting the eltrombopag treatment, clinical findings associated with bleeding were as follows: mild bleeding in 110 (38.6%) patients, moderate in 78 (27.4%), severe or life-threatening in 20 (7%), and no bleeding in 77 (27%). The numbers of chronic ITP patients with no response, partial response, or complete therapeutic response to eltrombopag treatment were 38 (13.4%), 65 (22.8%), and 182 (63.8%), respectively. Using a platelet level cut-off of >30,000/μL, overall response rate was 86.7% (n=247). Considering patients above 60 years old (n=68), overall response rate was 89.7% (n=61), and above 80 years old (n=12), overall response rate was 83% (n=10). The findings of the older patients above 60 and 80 years are listed in Table 2.

Platelet counts at first admission and before and after treatment (1st, 2nd, 3rd, 4th, and 8th weeks) as well as maximum platelet count, number of days with platelet count >30,000/μL, and interval (weeks) needed to achieve maximal platelet counts are presented in Table 3.

Table 1. Descriptive and clinical data (n=285).

Variable		n	%
Sex	Female	187	65.6
	Male	98	34.4
Response to treatment	None	38	13.4
	Partial	65	22.8
	Complete	182	63.8
Side effects	No	223	78.2
	Yes	62	21.8
Findings associated with bleeding	None	77	27.0
	Mild	110	38.6
	Moderate	78	27.4
	Severe	19	6.7
	Life-threatening	1	0.3
Bone marrow aspiration biopsy result (n=138)*	Normal	75	54.4
	ITP	63	45.6

ITP: Idiopathic thrombocytopenic purpura; *: data could be gathered from 138 patients only.

The median number of days required to achieve a platelet count of >30,000/μL was 14 (range: 3-210). Median maximal platelet counts were 275,000-346,000/μL (range: 5150 to 2,068,000) and time interval until achievement of maximal platelet count was 8-18 weeks (range: 1-202).

Notably, there was a significant positive correlation between treatment response and number of days to achieve platelet count of >30,000/μL (p=0.009, r=0.180). In contrast, age (p=0.129, r=0.764), platelet count at diagnosis (p=0.764, r=-0.020), and maximum platelet count after eltrombopag treatment (p=0.133, r=0.107) did not exhibit any correlation with treatment response.

Correlation analysis demonstrated that the higher the maximum platelet count was after eltrombopag treatment, the more likely side effects were to occur (p=0.004, r=0.215). Table 4 demonstrates the results of correlation analysis seeking the association between clinical variables, platelet counts.

Sex (p=0.594) and age (≤40 years and >40 years) (p=0.218) did not have a remarkable effect on treatment response. Similarly, platelet count at diagnosis did not seem to have a significant impact on treatment response (p=0.214).

Patients with platelet count of >30,000/μL in the 1st, 2nd, 3rd, 4th, and 8th weeks after eltrombopag treatment exhibited a better response to treatment (p>0.001 for all). Pearson chi-square test results indicated that treatment response was similar among patients who had any degree of bleeding (p=0.089). Treatment response was statistically significantly associated with number of days with platelet count of >30,000/μL (p=0.010), maximal platelet count (p<0.001), and duration of follow-up (p<0.001). On the contrary, treatment response was not affected by the week in which the highest platelet count was observed (p=0.121).

Our results demonstrated that the occurrence of side effects was not affected by sex (p=0.079), age (≤40 years and >40 years) (p=0.079), or platelet count at diagnosis (p=0.586) or in the 1st week (p=0.636), 2nd week (p=0.761), 3rd week (p=0.850), 4th week (p=0.485), and 8th week (p=0.527) after eltrombopag treatment. No association was noted between occurrence of side effects and number of days with platelet count of >50,000/μL (p=0.206), the week in which maximal platelet count was achieved (p=0.231), or duration of follow-up (p=0.685).

Side effects were observed in 62 (21.8%) cases (Table 1). The most common side effects were headache (21.6%), weakness (13.7%), hepatotoxicity (11.8%), venous thrombosis (4.2%), and arterial thrombosis (1.7%). Itching, erythromelalgia, transient ischemic attack, myalgia, and neuropathy were observed in 2 patients (3.9%) each.

Table 2. Results of the older population.

	Age >60 years	Age >80 years
Total number	68	12
Sex (M/F)	31/37	5/7
Median age	70	82
Median thrombocyte level (before treatment)	10,000/mm^3	11,000/mm^3
Median thrombocyte level (after treatment)	216,000/mm^3	176,000/mm^3
Median follow-up	13	7
Complete response (n)	48 (70%)	7 (58%)
Partial response (n)	17 (25%)	4 (33%)
Unresponsive (n)	3 (5%)	1 (9%)
Side effects (n)	13 (19%)	3 (25%)
Thrombosis (n)	3 (4%)	2 (16%)

M: Male, F: female.

Table 3. Data related to platelet count during the course of eltrombopag treatment.

Platelet count	Median	IQR	Percentile		Minimum	Maximum
			25%	75%		
Initial (/μL)	8000	10,000	5000	15,000	0	70,000
Before treatment (/μL)	11,000	13,000	5000	18,000	0	45,000
1st week (/μL)	35,000	58,500	18,000	76,500	1000	1,600,000
2nd week (/μL)	49,600	127,750	21,000	148,750	1000	2,068,000
3rd week (/μL)	61,000	133,250	24,250	157,500	1000	2,500,000
4th week (/μL)	75,000	134,300	30,700	165,000	3000	1,164,000
8th week (/μL)	112,500	163,500	46,250	209,750	1550	1,035,000
Days to achieve platelet count of >50,000/μL	14	22	8	30	3	210
Maximum platelet count after treatment (/μL)	275,000	346,000	126,000	472,000	5150	2,068,000
Time to achieve maximum platelet count (weeks)	8	18	4	22	1	202
Duration of follow-up (weeks)	17.5	23.75	6	29.75	1	84

IQR: Interquartile range.

The overall thrombosis rate including arterial (n=5) and venous thrombosis (n=12) was 5.9%. Thromboses presented clinically mostly as deep vein thrombosis. Pulmonary embolism was recorded in 3 patients. For arterial thrombosis, the main presentation was a transient ischemic attack (n=3). One patient suffered from ischemic stroke and one patient suffered from sudden death clinically attributed to a cardiac event. The thrombosis rate was found to be 2% in patients over 60 years of age and 16% in patients over 80 years of age. Clinical features and management of patients with thrombosis are summarized in Table 5.

Table 4. Correlations between clinical variables, platelet counts, treatment response, and side effects.

Variable	Treatment response		Side effects	
	r-value	p-value	r-value	p-value
Age at diagnosis (years)	0.093	0.129	0.092	0.174
Plt levels, initial (/µL)	-0.020	0.764	-0.002	0.979
Plt levels before treatment (/µL)	0.128	0.042*	-0.055	0.435
Plt levels, 1st week (/µL)	0.442	<0.001*	-0.036	0.649
Plt levels, 2nd week (/µL)	0.530	<0.001*	0.076	0.297
Plt levels, 3rd week (/µL)	0.562	<0.001*	-0.006	0.948
Plt levels, 4th week (/µL)	0.552	<0.001*	-0.086	0.234
Plt levels, 8th week (/µL)	0.605	<0.001*	-0.010	0.893
Days to Plt levels >30,000/µL	0.180	<0.001*	0.098	0.207
Maximum Plt levels after eltrombopag treatment (/µL)	0.536	<0.001*	0.215	0.004*
Weeks to achieve maximum Plt levels	0.107	0.133	0.091	0.232
Duration of follow-up (weeks)	0.263	<0.001*	-0.029	0.686

Plt: Platelet, *: statistically significant.

Table 5. Characteristic features of patients with thrombosis.

Findings	Age/sex	Platelet levels at diagnosis	Week of treatment	Management*
TIA	32/M	69,000	8th	Antiaggregation
DVT	79/F	190,000	8	Anticoagulation
DVT	28/F	250,000	8	Anticoagulation
DVT+PE	49/M	592,000	20	Anticoagulation
DVT	25/F	1,236,000	16	Anticoagulation
DVT	51/M	570,000	16	Anticoagulation
MI	21/F	653,000	16	Anticoagulation tPA
DVT	82/F	160,000	24	Anticoagulation
DVT	80/F	1,598,000	12	Anticoagulation
DVT+PE	45/M	780,000	2	Anticoagulation
PE	46/F	881,000	2	Anticoagulation
Sudden death	51/M	22,000	3	-
TIA	18/M	475,000	65	Antiaggregation
DVT	43/F	550,000	8	Anticoagulation
DVT	38/F	85,000	30	Anticoagulation
CVA	68/M	300,000	4	Anticoagulation Antiaggregation
DVT	57/F	657,000	16	Anticoagulation
Total	Venous thrombosis: n=12 Arterial thrombosis: n=5		Female: n=10 Male: n=7	

TIA: Transient ischemic attack, DVT: deep vein thrombosis, PE: pulmonary embolism, MI: myocardial infarction, CVA: cerebrovascular accident, M: male, F: female, *: Eltrombopag was discontinued in every patient with thrombosis.

Other side effects observed in only one patient each were as follows: hair loss, maculation, thrombocytosis, erythrocytosis, frequent tonsillitis, frequent pneumonia, diarrhea, and ileus. Side effects of any kind of grades 3-4, mainly thromboembolic events, were found at a rate of 6.3%.

Discussion

The present study was performed to investigate the variables that may be associated with treatment response and side effects after eltrombopag treatment for chronic ITP in daily practice. The overall response to eltrombopag in this cohort was 86.3%. This finding is consistent with previous prospective and retrospective studies [13,14,15]. Our data have shown that platelet counts before, during, and after treatment as well as maximal platelet counts, duration of follow-up, and number of days to achieve platelet count of >30,000/μL could have predictive potential for therapeutic response. Side effects were found to be significantly more common in patients with higher platelet counts after treatment. In our ITP cohort, the time to reach maximum platelet levels during treatment with eltrombopag was quite variable (1-202 weeks). Platelet counts during different periods in the course of eltrombopag treatment for chronic ITP may possess important implications in terms of therapeutic response and side effect profile. In general, the response to treatment and side effects were similar in the elderly population, whereas thrombosis was more common in patients over 80 years of age, although the number of cases was small.

In chronic ITP, the goal of treatment is to provide sufficient platelet levels to avoid major bleeding and to minimize treatment-related toxicity. Patients with platelet counts of ≥30,000/μL are supposed to have adequate hemostasis and generally do not require treatment in the absence of a history of bleeding [12]. Patients with ITP who have platelet counts above the normal minimum-maximum may have a risk of thrombotic or thromboembolic complications [16]. Efforts must be made to improve functional capacity and maturation of platelets as well as platelet count to overcome bleeding problems in patients with chronic ITP while decreasing the side effects, especially serious thrombotic complications.

Our results suggest that platelet counts obtained at different intervals in the course of eltrombopag treatment can serve as important predictors for treatment response and occurrence of side effects. Patients with insufficient responses to treatments such as corticosteroids, immunoglobulins, or rituximab may also be appropriate candidates for eltrombopag treatment. Regular platelet counts and close follow-up are mandatory for monitoring the effectivity of treatment and potential safety issues.

Patients in eltrombopag clinical trials experienced both arterial and venous thrombosis. Of 135 patients receiving eltrombopag in the RAISE study, three (2.2%) developed venous thrombosis [8]. The EXTEND extension study followed 299 patients for up to 5 years and reported nine patients with venous thrombosis and seven patients with arterial thrombosis (5.4%) [17]. In the present study, venous thrombosis was observed in 12 patients (5.9%) and arterial thrombosis in 5 patients (1.7%). Although eltrombopag was generally well tolerated during treatment in RAISE, transient increases of alanine aminotransferase and indirect bilirubin concentrations were reported, perhaps related to the metabolism of both eltrombopag and bilirubin by UGT1A1 [8]. All aminotransferase abnormalities were resolved; however, aminotransferase and bilirubin levels must be monitored before initiation of and during eltrombopag treatment, and treatment should be stopped if necessary. In the present study, none of the patients experienced increases in liver tests that required permanently discontinuing the drug. Of 135 patients in the RAISE study, 30% experienced headaches and 10% experienced fatigue, while in the present study, 4.2% of the study group reported headaches and 1.8% reported fatigue. The patient who died suddenly during follow-up had normal platelets at the last visit and the exact cause of death was clinically attributed to a cardiac event.

The main limitations of the current trial include the retrospective design, lack of a control group, and possible impacts of social, genetic, environmental, metabolic, and ethnic factors on treatment outcomes and side effects.

Conclusion

The results of the current study indicate that eltrombopag can be a safe and effective therapeutic option in refractory and chronic ITP, even in older populations. However, patients must be closely monitored for therapeutic response and side effects during treatment. Since both responses and side effects may be variable throughout the follow-up period, patients should be evaluated dynamically, especially in terms of thrombosis risk factors.

Ethics

Ethics Committee Approval: Sakarya University, approval number: 71522473/050.01.04/151.

Authorship Contributions

Surgical and Medical Practices: D.Ç., S.G., R.D.K.; Concept: D.Ç., S.G., R.D.K.; Design: D.Ç., S.G., R.D.K.; Data Collection or Processing: All Authors; Analysis or Interpretation: D.Ç., S.G., R.D.K.; Literature Search: D.Ç., S.G., R.D.K.; Writing: D.Ç., S.G., R.D.K.

Informed Consent: Written informed consent was provided for all patients enrolled in this study.

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

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RESEARCH ARTICLE

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Certain Killer Immunoglobulin-Like Receptor (KIR)/KIR HLA Class I Ligand Genotypes Influence Natural Killer Antitumor Activity in Myelogenous Leukemia but Not in Acute Lymphoblastic Leukemia: A Case Control Leukemia Association Study

Bazı Öldürücü İmmünoglobulin-Benzeri Reseptör (KIR)/KIR HLA Sınıf I Ligand Genotipleri Akut Lenfoblastik Lösemide Değil ama Akut Myeloid Lösemide Doğal Öldürücü Antitümör Aktivitesini Etkilemektedir: Lösemi Birliğinin Olgu Kontrol Çalışması

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Abstract

Objective: Natural killers (NK) cell function is mainly controlled by the expression of killer immunoglobulin-like receptors (KIRs) and their ligation with the corresponding ligands. The objective of this study was to investigate the putative association of KIRs, HLA class I ligands, and KIR/ligand combinations with rates of development of acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), and chronic myeloid leukemia (CML).

Materials and Methods: The KIR/HLA I genotypes of 82 patients with leukemia (ALL, n=52; AML, n=17; and CML, n=13) were determined by PCR-SSP method and compared with genotypes of healthy controls (n=126).

Results: KIR genotype frequency differed significantly between myelogenous leukemia patients and healthy controls for KIR2DL5A (17.6% vs. 47.7%, p=0.02), KIR3DS1 (17.6% vs. 47.6%, p=0.02), and KIR2DS4*001 (36.6% vs. 20.2%, p=0.017). The incidence of homozygous HLA-BBw4 (31.0% vs. 12.5%, p=0.042) and HLA-Bw4Thr80 Thr80 (13.0% vs. 1.2%, p=0.01) was significantly elevated in myeloid leukemia patients compared to healthy controls. KIR/HLA class I ligand profile KIR3DS1(+)/L (-) was decreased and KIR3DL2(+)/HLA-A3/11(-) was increased among myeloid leukemia cases compared to controls.

Conclusion: These data suggest that the activity of NK cells as determined by inherited KIR/HLA class I ligand polymorphisms influences the susceptibility to myelogenous leukemia, but not to lymphoblastic leukemia. Additionally, the KIR genotype characterized by the absence of the inhibitory KIR2DL2 and the activating KIR2DS2 and KIR2DS3 (ID2) was found at a lower frequency in patients compared

Öz

Amaç: Doğal öldürücü (NK) hücre fonksiyonu temel olarak öldürücü immünoglobulin-benzeri reseptör (KIR) yüzey ifadesi ve bunların ilgili liganda bağlanması ile ilişkilidir. Bu çalışmanın amacı KIR, HLA sınıf I ligandlar ve KIR/ligand ilişkisinin akut lenfoblastik lösemi (ALL), akut myeloid lösemi (AML) ve kronik myeloid lösemi (KML) oluşumu ile ilişkisini araştırmaktır.

Gereç ve Yöntemler: Seksen iki lösemi hastasının (ALL, n=52; AML, n=17; ve KML, n=13) KIR/HLA I genotipleri PCR-SSP metodu ile çalışıldı ve sağlıklı kontrollerin (n=126) genotipleri ile karşılaştırıldı.

Bulgular: KIR genotip frekansı myeloid lösemi hastaları ve sağlıklı kontroller arasında KIR2DL5A (%17,6 vs. %47,7, p=0,02), KIR3DS1 (%17,6 vs. %47,6, p=0,02), ve KIR2DS4*001 (%36,6 vs. %20,2, p=0,017) açısından belirgin farklılık gösterdi. Homozigot HLA-BBw4 (%31,0 vs. %12,5, p=0,042) ve HLA-Bw4Thr80 Thr80 (%13,0 vs. %1,2, p=0,01) sıklığı da myeloid lösemi hastalarında sağlıklı kontrollere göre belirgin olarak daha yüksekti. Kontrollerle karşılaştırıldığında myeloid lösemi hastalarında KIR/HLA sınıf I ligand profili olarak KIR3DS1(+)/L(-) azalmış ve KIR3DL2(+)/HLA-A3/11(-) artmış olarak bulundu.

Sonuç: Bu bulgular, kalıtılan KIR/HLA sınıf I ligand polimorfizmleri ile belirlenen NK hücre aktivitesinin myeloid lösemiye yatkınlığı etkilediği ancak lenfoid lösemi yatkınlığını etkilemediğini düşündürmektedir. Ayrıca inhibitor KIR2DL2, aktivatör KIR2DS2 ve KIR2DS3 (ID2) ile karakterize KIR genotipi, hastalarda kontrollere oranla daha düşük bulundu, bu da bütün olası KIR/HLA sınıf I ligand polimorfizmlerine dayanan kompleks analizlerin gerekliliğini desteklemektedir.

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Abstract

to controls, which confirmed the need for complex analysis based on all possible KIR/HLA class I ligand polymorphism combinations.

Keywords: Chronic myeloid leukemia, Acute lymphoblastic leukemia, Acute myeloblastic leukemia

Öz

Anahtar Sözcükler: Kronik myeloid lösemi, Akut lenfoblastik lösemi, Akut myeloid lösemi

Introduction

In 2015, 4062 patients with leukemia were treated and/or monitored in the Republic of Bulgaria [1]. Similarly to other neoplastic diseases, the precise etiopathogenetic mechanism that leads to the transformation of "normal" hematopoietic cells into blast cells has not yet been elucidated. The idea of a combined influence of external environmental factors and "internal" factors in the onset and development of malignancies is increasingly being discussed.

NK cells play an important role in antitumor immune defense [2,3]. Their function is controlled by the interaction of cell surface receptors with appropriate ligands, among which the most important and best studied are killer immunoglobulin-like receptors (KIRs) and their HLA class I ligands [4,5,6,7,8]. Seventeen KIR genes and pseudogenes have been described so far [4]. KIRs are named based on the number of their extracellular Ig-like domains (2D or 3D) and by the length of their cytoplasmic tail (long [L], short [S], or pseudogene [P]) [9]. Ligands for most KIRs are specific patterns of the HLA class I molecules [6,7,8,9,10]. HLA C molecules with amino acid residues Ser and Asn at positions 70 and 80, respectively (Ser77 and Asn80), form the HLA-C1 KIR ligand group, which specifically binds KIR2DL1 and KIR2DS2. The HLA-C2 KIR ligand group (Asn77 and Lys80) interacts with KIR2DL2/2DL3 and KIR2DS1 [5,6,10,11,12,13]. HLA-B class I molecules with the Bw4 epitope are ligands for KIR3DL1 and KIR3DS1 [7,8,14,15]. The strength of the KIR/ligand binding is determined by the amino acid residue at position 80 in the Bw4 molecule ($Bw4^{Ile80}$ are stronger ligands for their specific KIRs than $Bw4^{Thr80}$) [7,8]. Data suggest that HLA-A alleles with Bw4 epitopes may be ligands for KIR3DL1 [11]. KIR3DL2 specifically recognizes HLA-A3 and HLA-A11 specific to certain peptides such as Epstein-Barr virus peptides [16,17].

The first immunogenic studies investigating the effect of KIRs and their HLA class I ligands and their relation to the development of oncohematological diseases were conducted by Demanet et al. [18] and Verheyden et al. [19] in 2004. There are increasing data on the association of genetic polymorphisms of KIRs and their HLA ligands with a predisposition to various hematological malignancies, although a specific polymorphism clearly associated with leukemia development has not been identified [20]. Indirect evidence for the role of NK cells in leukemia defense includes the proved decreased incidence of relapse and increased leukemia-free surveillance in the setting of allogenic KIR HLA class I ligand donor-recipient incompatibility hematopoietic stem cell transplantation [21,22]. These data additionally suggest that NK cells may play a major role in the control and clearance of leukemia. It seems that the interplay between the inhibitory KIR signals and/or the predominance of activating ones is critical for the NK-mediated anti-leukemic effect. Therefore, in the present study, we studied the polymorphism of NK-cell receptors, namely KIRs and their HLA class I ligands, in patients with leukemia and healthy controls and investigated the possible association of these immunogenic factors with different leukemias in the Bulgarian population.

Materials and Methods

Study Groups

Eighty-two patients with primary leukemia and 126 healthy controls were included after they provided signed informed consent. The healthy individuals were randomly selected from unrelated volunteers from the Bulgarian population, without chronic diseases and with negative family history of hereditary diseases, autoimmune diseases, or malignancies (46.1% male and 53.9% female, mean age 46.6±11.9 years). The patient group consisted of patients with acute lymphoblastic leukemia (ALL) (n=52, 65.4% male and 34.6% female, mean age 20.8±12.5 years), acute myeloid leukemia (AML) (n=17, 64.7% male and 35.3% female, mean age 36.9±11.3 years), and chronic myeloid leukemia (CML) (n=13, 84.6% male and 15.4% female, mean age 36.8±13.4 years).

Methods

DNA was extracted from peripheral venous blood with the iPrep PureLink® gDNA™ Blood Kit (Invitrogen, USA) and iPrep™ purification instrument (Invitrogen, USA).

KIR genotyping was performed by PCR-SSP methods (Olerup SSP™ KIR and KIR/HLA Ligand Kit, Sweden) according to the manufacturer's instructions. In brief, 24 locus-specific primer sets in the KIR genotyping kit allow detection of 16 KIR genes and pseudogenes and discrimination of KIR2DL5A, KIR2DL5B, and KIR3DL1*004 alleles and both groups of KIR2DS4 alleles (KIR2DS4*001 from KIR2DS4*003/004/006/007). The typing was interpreted with the worksheet provided with the kit. KIR/HLA ligands were determined as previously described [9].

Statistical Analysis

Individual KIR genes, KIR HLA class I ligands, and KIR/HLA class I ligand combination frequencies were determined by direct counting. KIR haplotypes and genotypes were defined in accordance with the Allele Frequency Net Database [20]. Subsequently, individual KIR genotype frequencies were also determined by direct counting. The established frequencies of each of the factors studied were compared between patients and healthy controls using the Pearson chi-square test and Fisher exact test. Odds ratios (ORs) with 95% confidence interval (CIs) were assigned to variables with significant differences determined at a threshold of $p<0.05$. All statistical analyses were performed with SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

KIR Gene/Pseudogene Frequencies

Overall, no differences in KIR gene/pseudogene frequencies were found between the patients and the healthy individuals, except for a higher incidence of KIR2DS4*001 in the leukemia group (36.6% vs. 20.2%, p=0.017) and in the AML subgroup compared to the control group (52.9% vs. 20.2%, p=0.01) (Table 1). AML patients differed from healthy individuals in the distribution of two other KIR alleles: KIR2DL5A (17.6% vs. 47.7%, p=0.02) and KIR3DS1 (17.6% vs. 47.6%, p=0.02).

The frequency of individual KIR genes and the comparison between the patients and healthy controls are presented in Table 1.

To determine and analyze whether there were findings specific to a particular leukemia type, the KIR frequencies were compared between patients with ALL, AML, and CML. The applied intragroup analysis did not show appreciable differences in the distribution of any KIRs (data not shown).

KIR Profiles

Thirty-seven different genotypes were determined according to the presence/absence of individual KIRs among the patients

Table 1. KIR gene frequencies.

KIR	Healthy (n=126) n (%)	Patients (n=82) n (%)	ALL (n=52) n (%)	AML (n=17) n (%)	CML (n=13) n (%)	ML (n=30) n (%)
3DL2; 3DL3; 3DP1	126 (100)	82 (100)	52 (100)	17 (100)	13 (100)	30 (100)
2DL4	125 (99.2)	82 (100)	52 (100)	17 (100)	13 (100)	30 (100)
2DP1	122 (96.8)	79 (96.5)	49 (94.1)	17 (100)	13 (100)	30 (100)
2DL1	118 (93.7)	78 (95.1)	48 (92.2)	17 (100)	13 (100)	30 (100)
2DL2	76 (60.3)	53 (64.6)	34 (64.7)	11 (64.7)	8 (61.5)	19 (63.3)
2DL3	108 (85.7)	70 (85.4)	42 (80.4)	16 (94.1)	12 (92.3)	28 (93.3)
2DL5	82 (65.1)	49 (59.8)	30 (56.9)	9 (52.9)	101 (76.9)	19 (63.3)
2DL5A**	##42 (47.7)	30 (36.6)	22 (41.2)	**3 (17.6)* p=0.02 OR 0.2 [0.06-0.9]**	5 (38.5)	8 (26.7)
2DL5B	##40 (45.5)	37 (45.1)	21 (39.2)	9 (52.9)	7 (53.8)	16 (53.3)
2DS1	57 (45.2)	32 (39.0)	21 (39.2)	5 (29.4)	6 (46.2)	11 (36.7)
2DS2	82 (65.1)	54 (65.9)	34 (64.7)	12 (70.6)	8 (61.5)	20 (66.7)
2DS3	48 (38.1)	35 (42.7)	22 (41.2)	7 (41.2)	6 (46.2)	13 (43.3)
2DS4	116 (92.1)	77 (93.9)	48 (92.2)	16 (94.1)	13 (100)	29 (96.7)
2DS4norm	#18 (20.2)	**30 (36.6)* p=0.02 OR 2.3 [1.1-4.5]**	15 (29.4)	**9 (52.9)* p=0.01 OR 4.4 [1.5-13.1]**	6 (46.2) p=0.07 OR 3.3[1.0-113]	**15 (50.0)* p=0.004 OR 3.9 [1.6-9.5]**
2DS4del	#70 (78.7)	66 (80.5)	41 (78.4)	14 (82.4)	11 (84.6)	25 (83.3)
2DS5	48 (38.1)	29 (35.4)	18 (35.3)	5 (29.4)	6 (46.2)	11 (36.7)
3DS1**	60 (47.6)	32 (39.0)	23 (43.1)	**3 (17.6)* p=0.02 OR 0.2 [0.07-0.9]**	6 (46.2)	9 (30.0)
3DL1	114 (90.5)	76 (92.7)	47 (90.2)	16 (94.1)	13 (100)	29 (96.7)
3DL1*004	'13 (17.6)	"8(16.0)	'"3 (11.1)	""2 (16.7)	"""3 (30.0)	""""5 (22.7)

n=88, # n=89, 'n=74, "n= 49, '"n=28, ""n=12, """n=10, """"n=22, *statistically significant difference comparing patient group to healthy controls, [ ]: 95% confidence interval.

CML: Chronic myeloid leukemia, ALL: acute lymphoblastic leukemia, ML: myeloid leukemia, AML: acute myeloid leukemia.

and healthy controls, whose characteristics and frequency are presented in Table 2. The comparison between the two groups showed a tendency for a lower frequency of KIR genotype ID2 (6.1% vs. 13.5%, p=0.09; OR 0.4 [95% CI: 0.1-1.3]) in the patient group.

KIR HLA Class I Ligands

KIR HLA-C ligands were determined in 124 healthy controls: HLA-BBw4 in 113, HLA-ABw4 in 95, and HLA-A3/11 in 83 (Table 3). No significant differences were observed, except for the more frequent presence of homozygous HLA-BBw4 (two HLA-B

Table 2. KIR genotype frequencies in patients (n=82) and healthy controls (n=126).

Patients n (%)	Healthy controls n (%)	ID*	3DL1	2DL1	2DL3	2DS4	2DL2	2DL5	3DS1	2DS1	2DS2	2DS3	2DS5	2DL4	2DP1	3DP1 3DL2, 3DL3
20 (24.3)	22 (17.5)	1	■	■	■	■	□	□	□	□	□	□	□	■	■	■
10 (12.2)	17 (13.5)	5	■	■	■	■	■	■	□	□	■	■	□	■	■	■
10 (12.2)	14 (11.1)	4	■	■	■	■	■	□	□	□	■	□	□	■	■	■
8 (9.8)	6 (4.76)	6	■	■	■	■	■	■	■	■	■	■	■	■	■	■
5 (6.1)	17 (13.5)	2	■	■	■	■	□	■	■	■	□	□	■	■	■	■
4 (4.9)	6 (4.76)	3	■	■	■	■	■	■	■	■	■	□	■	■	■	■
4 (4.9)	1 (0.79)	73	■	■	□	■	■	■	■	■	■	■	■	■	■	■
3 (3.7)	5 (3.97)	71	■	■	□	■	■	■	□	□	■	■	□	■	■	■
3 (3.7)	1 (0.79)	72	■	□	□	■	■	□	□	□	■	□	□	■	□	■
2 (2.4)	1 (0.79)	9	■	■	■	■	■	■	□	■	■	□	■	■	■	■
2 (2.4)	1 (0.79)	13	■	■	■	■	■	■	■	□	■	■	□	■	■	■
2 (2.4)	3 (2.38)	69	□	■	■	□	□	■	■	■	□	□	■	■	■	■
1 (1.22)	4 (3.2)	7	■	■	■	■	■	■	■	■	■	■	□	■	■	■
1 (1.22)	1 (0.79)	11	■	■	■	■	■	■	□	■	■	■	□	■	■	■
1 (1.22)	2 (1.58)	70	□	■	■	□	■	■	■	■	■	■	■	■	■	■
1 (1.22)	1 (0.79)	81	□	■	□	□	■	■	■	■	■	■	■	■	■	■
1 (1.22)	2 (1.58)	94	■	■	□	■	■	■	■	□	■	■	□	■	■	■
1 (1.22)	0	48	■	■	■	■	□	■	□	□	■	□	■	■	■	■
1 (1.22)	0	362	■	□	■	■	■	■	■	■	■	■	□	■	■	■
1 (1.22)	0	150	□	■	■	■	□	■	■	■	□	■	■	■	■	■
1 (1.22)	0	159	□	■	■	□	■	■	■	■	■	■	□	■	■	■
0	2 (1.58)	8	■	■	■	■	□	■	■	■	□	■	□	■	■	■
0	2 (1.58)	43	■	■	■	■	□	□	■	□	■	□	□	■	■	■
0	2 (1.58)	68	□	■	■	□	■	■	■	■	■	□	■	■	■	■
0	2 (1.58)	76	■	□	□	■	■	■	■	■	■	□	■	■	□	■
0	2 (1.58)	104	■	□	□	■	■	□	□	□	■	□	□	■	■	■
0	2 (1.58)	293	■	□	■	■	■	□	□	□	■	□	□	■	■	■
0	1 (0.79)	12	■	■	■	■	□	■	■	■	■	□	■	■	■	■
0	1 (0.79)	36	■	■	■	■	□	■	□	□	■	■	□	■	■	■
0	1 (0.79)	87	□	■	■	■	■	■	■	■	■	■	■	■	■	■
0	1 (0.79)	90	■	■	□	■	■	■	■	■	■	■	□	■	■	■
0	1 (0.79)	91	■	■	□	■	■	■	□	■	■	■	■	■	■	■
0	1 (0.79)	118	■	■	□	■	■	■	■	■	■	□	■	■	■	■
0	1 (0.79)	188	■	■	■	■	■	□	■	□	■	□	□	■	■	■
0	1 (0.79)	151	□	■	■	■	■	■	■	■	■	■	□	■	■	■
0	1 (0.79)	377	□	□	■	□	■	■	■	■	■	□	■	■	■	■
0	1 (0.79)	440	■	■	□	■	■	■	■	■	■	■	■	□	□	■

The filled squares correspond to the presence of the KIR gene and empty ones to the lack of the corresponding KIR gene; *genotype identification number according to http://www.allelefrequencies.net/kir6001a.asp.

Table 3. Frequencies of KIR HLA class I ligands and KIR HLA class I ligands combinations.

	Controls n (%)	ALL (n=52) n (%)	ML (n=30) n (%)	AML (n=17) n (%)	CML (n=13) n (%)
HLA-C1	104 (83.9)*	39 (76.5)	23 (76.7)	12 (70.6)	11 (84.6)
HLA-C2	85 (68.5)*	37 (72.5)	18 (60.0)	11 (64.7)	7 (53.8)
HLA-C1C1	39 (31.5)*	14 (27.4)	12 (40.0)	6 (35.3)	6 (46.1)
HLA- C1C2	65 (52.4)*	26 (49.1)	11 (36.7)	6 (35.3)	5 (38.5)
HLA-C2C2	20 (16.1)*	12 (23.5)	7 (23.3)	5 (29.4)	2 (15.4)
HLA- BBw4	82 (72.6)**	33 (64.7)	3 (76.7)	11 (64.7)	12 (92.3)
HLA-BBw4BBw4	14 (12.3)**	11 (21.2)	9 (30.0)	4 (23.5)	5 (38.5)
HLA- ABw4	34 (35.8)#	20 (39.2)	13 (43.3)	9 (52.9)	4 (30.8)
HLA- A3/11	29 (34.9)##	18 (35.3)	5 (16.7)	2 (11.8)	3 (23.1)

*n=124, **n=113, #n=95, ##n=83.

CML: Chronic myeloid leukemia, ALL: acute lymphoblastic leukemia, ML: myeloid leukemia, AML: acute myeloid leukemia.

alleles with Bw4 epitope) in myeloid leukemia patients compared to healthy controls (30.0% vs. 12.5%, p=0.042, OR 3.15 [95% CI: 1.08-9.16]). Considering which amino acid (isoleucine or threonine) was present at position 80 of the HLA-Bw4 molecule, the KIR HLA-B ligand genotype HLA-Bw4Thr80 Thr80 was significantly more prevalent in CML and AML compared to the control group (16.7% CML, p=0.04, OR 16.4 [95% CI: 1.0-5.07] and 13.0% AML, p=0.01 OR 12.3 [95% CI: 1.0-3.24] vs. 1.2% in healthy controls). Subgroup analysis based on leukemia type showed no differences (data not shown).

KIR/HLA Class I Ligand Combinations

The frequencies of individual KIR/HLA class I ligand combinations of inhibitory receptor with the appropriate ligand and the activating counterparts are presented in Table 4. A significantly higher incidence of the KIR3DL2(+)/HLA-A3/11(-) genotype was found in the myeloid leukemia group compared to the healthy control group (AML vs. controls: 88.2% vs. 65.9%, p=0.047, CML vs. controls: 83.3% vs. 65.9%, p=0.047). A lower frequency of KIR3DS1(+)/HLA-ABw4(-) (10.0%, p=0.009) and KIR3DS1(+)/HLA-BBw4(-) (3.3%, p=0.045) combinations among myeloid leukemia cases compared to controls (34.8% and 16.8%, respectively) was observed. In addition, the intragroup analysis between different types of leukemia showed that AML was distinguished from the immunophenotypically opposite group of ALL by the frequency of KIR3DS1(+)/HLA-ABw4(-) (AML versus ALL: 10.0% vs. 29.4%, p=0.007, data not shown).

In the next step, the KIR/HLA class I ligand combinations were investigated taking into account the ligand and the combination of its appropriate inhibitory KIR and activating KIR counterpart (inhibitory KIR/activating KIR/HLA class I ligand). Significant differences were not found between patients and healthy controls in this assay (data not shown). The subgroup analysis, depending on the cytological variant of leukemia, also showed no differences (data not shown).

Discussion

Particular KIRs and KIR HLA class I ligand polymorphisms associated with a variety of tumors have been reported but the precise disease-predisposing mechanisms have not been elucidated [20].

The KIR2DS4*001 allele was found to be significantly more frequent in the leukemia group compared to healthy controls with the difference being more prominent for AML. Two additional KIRs were identified as protective for AML: KIR2DL5A and KIR3DS1. The protective effect of KIR3DS1 that we established supports the hypothesis that genetic imbalance between activating and inhibitory KIRs in the direction of decreased activation/increased inhibition may contribute to tumorigenesis. These results are in line with the data from a similar disease-associated study in AML patients from Iran [23], as well as its reported protective effect associated with solid tumors [24] and Hodgkin's disease [25]. However, this hypothesis cannot explain the lower incidence of inhibitory KR2DL5A and the higher incidence of KIR2DS4*001 in the AML group, logically associated with decreased inhibitory and increased NK cell-activating function. KIR2DL5 has been found less frequently in patients with oncohematological diseases such as B-cell chronic lymphocyte leukemia [26] and Hodgkin's lymphoma patients [24]. Similarly, higher incidence of KIR2DS4 associated with leukemia was reported independently by Giebel et al. [27] and Zhang et al. [28] for CML and Misra et al. [29] for childhood ALL. A similar inconsistency is known for a number of other activating KIRs, which are associated with a higher risk of oncohematological diseases, such as KIR2DS1 [29,30,31], KIR2DS3 [29,31], and KIR2DS2 and KIR2DS5 [29]. On the other hand, the lack of accurate information on the ligand

Table 4. Frequencies of KIR/HLA ligand combinations.

KIR/HLA class I ligand*	Healthy n (%)	Patients n (%)	ALL n (%)	ML n (%)	AML n (%)	CML n (%)
Inhibitory KIR/HLA class I ligand						
2DL1(+)/ HLA-C2 (+)	78 (62.9)	54 (65.9)	35 (68.6)	18 (60.0)	11 (64.7)	7 (53.8)
2DL1(+)/ HLA-C2 (-)	38 (30.65)	24 (29.3)	12 (23.6)	12 (40.0)	6 (35.3)	6 (43.2)
2DL1(-)/ HLA-C2 (+)	7 (5.65)	2 (2.4)	2 (3.9)	0.0	0.0	0.0
2DL2/L3(+)/HLA-C1 (+)	104 (83.9)	63 (76.8)	39 (76.5)	23 (76.7)	12 (70.6)	11(84.6)
2DL2/L3(+)/ HLA-C1 (-)	20 (16.1)	19 (23.2)	13 (23.5)	7 (23.3)	5 (29.4)	2 (15.4)
3DL1(+)/ HLA-BBw4 (+)	76 (67.3)	54 (65.9)	30 (58.8)	23 (76.7)	11 (64.7)	12 (92.3)
3DL1(+)/HLA-BBw4 (-)	26 (23.0)	22 (26.7)	16 (31.4)	6 (20.0)	11 (29.4)	1 (7.7)
3DL1(-)/HLA-BBw4 (+)	6 (5.3)	3 (3.7)	3 (5.9)	0.0	0.0	0.0
3DL1(+)/HLA-ABw4 (+)	30 (31.6)	32 (39.0)	20 (39.2)	12 (40.0)	8 (47.1)	4 (30.8)
3DL1(+)/HLA-ABw4 (-)	55 (57.9)	44 (53.7)	26 (51.0)	17 (56.7)	8 (47.1)	9 (69.2)
3DL1(-)/HLA-ABw4 (+)	4 (4.2)	1 (1.2)	0.0	1 (3.3)	1 (5.8)	0.0
3DL2(+)/HLA-A3/11 (+)	29 (34.1)	24 (29.3)	18 (35.3)	5 (16.7)	2 (11.8)	3 (23.1)
3DL2(+)/HLA-A3/11 (-)	54 (65.9)	58 (70.7)	33 (64.7)	**25 (83.3) p=0.047 OR 3.9 [0.8-26.6]**	**15 (88.2) p=0.047 OR 1.56 [0.5-5.5]**	10 (76.9)
Activating KIR/HLA class I ligand						
2DS1(+)/ HLA-C2 (+)	37 (29.8)	22 (26.8)	14 (27.4)	7 (23.3)	4 (23.5)	3 (23.1)
2DS1(+)/ HLA-C2 (-)	18 (14.6)	10 (12.2)	6 (11.8)	4 (13.3)	1 (5.9)	3 (23.1)
2DS1(-)/ HLA-C2 (+)	48 (38.7)	34 (41.5)	23 (45.1)	11 (36.7)	7 (41.2)	4 (30.7)
2DS2(+)/ HLA-C1 (+)	67 (54.1)	39 (47.6)	24 (47.1)	14 (46.7)	7 (41.2)	7 (53.9)
2DS2(+)/ HLA-C1 (-)	14 (11.3)	15 (18.3)	9 (17.6)	6 (20.0)	5 (29.4)	1 (7.7)
2DS2(-)/ HLA-C1 (+)	37 (29.8)	24 (29.3)	15 (29.4)	9 (30.0)	5 (29.4)	4 (30.7)
3DS1(+)/ HLA-BBw4 (+)	37 (32.8)	25 (30.5)	16 (31.4)	8 (26.7)	2 (11.8)	6 (46.15)
3DS1(+)/HLA-BBw4 (-)	19 (16.8)	7 (8.5)	6 (11.8)	**1 (3.3) p=0.045 OR 0.2 [0.01-1.3]**	1 (5.9)	0.0
3DS1(-)/ HLA-BBw4 (+)	45 (39.8)	32 (39.0)	17 (33.3)	15 (50.0)	9 (52.9)	6 (46.15)
3DS1(+)/ HLA-ABw4 (+)	16 (16.8)	13 (15.9)	7 (13.7)	6 (20.0)	3 (17.6)	3 (23.1)
3DS1(+)/ HLA-ABw4 (-)	33 (34.8)	19 (23.1)	15 (29.4)	**3 (10.0) p=0.009 OR 0.2 [0.1-0.8]**	**0.0**	3 (23.1)
3DS1(-)/ HLA-ABw4 (+)	18 (18.9)	20 (24.4)	13 (25.5)	7 (23.3)	6 (35.3)	1 (7.7)

[ ]: 95% confidence interval; *KIR/HLA ligand combinations not identified in any of the study groups are not presented.

CML: Chronic myeloid leukemia, ALL: acute lymphoblastic leukemia, ML: myeloid leukemia, AML: acute myeloid leukemia.

specificity of most KIRs, such as KIR2DL5, and their importance in the regulation of NK cell function significantly impedes the interpretation of the current results. Furthermore, NK cell activity is regulated not only by the individual KIR genes but also by their individual KIR and KIR/HLA class I ligand genotype combinations.

The complex influence of the inherited KIR genes in individual KIR genotypes for development of hematological malignancies was demonstrated first by Verheyden et al. [32]. Their group showed an increased risk of leukemia associated with KIR genotypes, associated with a higher number of inhibitory KIRs. Data from more recent studies that reported predisposition to leukemia associated with KIR genotypes containing a higher number of inhibitory than activating KIRs support this hypothesis [26,33]. A tendency for higher incidence of KIR profile ID2 was found in a study comparing healthy individuals with leukemia patients. The KIR genotype ID2 is characterized by the absence of the inhibitory KIR2DL2 and its activating counterpart, KIR2DS2. The same KIRs, KIR2DL2 and KIR2DS2, are reported as risk factors

for acute leukemia [29,33]; in other words, their absence in the KIR genotype ID2 can be interpreted as absence of a genetically predetermined disease susceptibility factor and higher tumor resistance.

Analysis of disease susceptibility by testing the inherited KIRs ligands showed a higher incidence of the HLA-BBw4 (HLA-BBw4/Bw4) homozygous genotype in patients compared to healthy controls. Particularly at risk were patients in the myeloid leukemia group. HLA-BBw4 is a ligand for both KIR3DL1 and KIR3DS1, the former binding it with greater affinity than its activating counterpart. Moreover, KIR3DL1 is significantly more frequent than KIR3DS1. It can be assumed that the expression of HLA-Bw4 ligands maintains NK cells in a state of hyporesponse rather than contributing to NK activation by KIR3DS1/HLA-BBw4. Thus, HLA-BBw4/Bw4 appears to be a risk factor for leukemia development. Two independent studies by Middleton et al. [34] and de Smith et al. [35] also reported homozygous HLA-BBw4 as a risk factor for AML and CML development. de Smith et al. [35] also showed that the KIR3DL1+/HLA-BBw4/Bw4 combination was associated with ALL. Additionally, carriers of two HLA-Bw4 alleles with threonine at the 80 position (HLA-Bw4Thr80 Thr80) were found with a higher frequency in myeloid leukemia cases compared to healthy controls. Bw4Thr80 binds KIR3DL1 with a lower affinity than Bw4Iso80, which results in a lower inhibitory signal to the NK cells by HLA-Bw4Thr80 homozygous individuals. Association of the KIR HLA-Bw4 ligand according to amino acid at position 80 was also reported by Shen et al. [36], who demonstrated significantly higher frequencies of HLA-Bw4Iso80 in the prognostically "poor" AML risk group compared to those with "favorable" risk. In contrast to other studies of KIR HLA class I ligands in leukemia, no other differences were observed [36,37].

Analysis of KIR/KIR HLA class I ligand combinations first confirmed that the investigated polymorphic gene systems have the highest importance for myeloid leukemia susceptibility, whereas their role in disease predisposition to ALL could not be confirmed. The KIR3DL2(+)/HLA-A3/11(-) combination was found significantly more frequently in myeloid leukemia patients than in the healthy population. KIR3DL2 belongs to the framework of KIR genes and is present almost ubiquitously [20]. Thus, the myeloid leukemia-associated genotype KIR3DL2(+)/HLA-A3/11(-) very likely indicates lack of NK cell activity mediated by the inhibitory receptor. Another KIR/HLA class I ligand combination where the activating KIR3DS1 is expressed, but not its putative HLA-A/BBw4 ligand, KIR3DS1(+)/L(-), was found at a lower frequency in the myeloid leukemia group compared to the healthy individuals. On the contrary, La Nasa et al. reported an increased risk of Hodgkin's disease associated with genotype KIR3DS1(+)/HLA-Bw4(-) [38]. It should be noted, however, that in our study, KIR3DS1 was found to be significantly less common among patients with AML, which may be an explanation for the observed differences and logically raises the question of whether KIR3DS1 is an independent protective factor for leukemia development or the receptor-ligand combinations in which it participates are also important. There is support for both hypotheses in the available literature [23,25]. For the second possible mechanism [26,30], the discussions are only addressing the presence of the binding ligand.

In summary, it is obvious that the patient group is distinguished from healthy controls by the presence of both individual KIR genes and some KIR HLA class I ligands and KIR/HLA class I ligand combinations. These data support the hypothesis of the complex influence of various polymorphic gene systems, in particular KIRs and KIR HLA class I ligands, in the genetically regulated NK immune response. On the other hand, the differences found are valid for patients with myeloid leukemia, but not for the ALL group. These results are not unusual considering the higher susceptibility of myeloblastic cells compared to lymphoblastic cells to NK-mediated cytolysis [39,40,41] and the presumed direct involvement of NK cells in the antitumor response in hemoblastosis of myeloid origin.

Study Limitations

As a limitation of this study, most importantly, the group of patients analyzed was heterogeneous and included three different types of leukemia characterized by different clinical evolution and prognosis. When dividing patients into separate groups, depending on the type of leukemia (ALL, AML, and CML), the number of subjects analyzed in each group was limited, and highly variable factors such as KIR genotypes did not allow the comparison of each subgroup of leukemia with healthy controls.

Conclusion

The leukemia susceptibility factors we have found confirm the importance of KIR/HLA class I ligand gene systems in NK-mediated antitumor response in patients with myeloid leukemia. The understanding of the mechanisms of their influence on NK cell function remains limited. Interpretation of the results obtained in the context of the hypothesis of different NK cell activity predetermined at the genetic level depending on the inherited inhibitory/activating potential is difficult due to the poorly studied ligand specificity of the KIR genes as well as their functional activity.

Ethics

Ethics Committee Approval: Medical University, Sofia, Bulgaria, Approval number: 3-Д).

Authorship Contributions

Surgical and Medical Practices: V.V.; Concept: V.V., M.A.; Design: V.V., M.A., N.E.; Data Collection or Processing: V.V., M.A., M.S.;

Analysis or Interpretation: V.V., M.A., N.E., M.S.; Literature Search: V.V., M.A., N.E., M.S.; Writing: V.V., M.A.

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RESEARCH ARTICLE

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Stress-Induced Premature Senescence Promotes Proliferation by Activating the *SENEX* and p16^{INK4a}/Retinoblastoma (Rb) Pathway in Diffuse Large B-Cell Lymphoma

Diffüz Büyük B Hücreli Lenfomada, Stres Kaynaklı Erken Hücresel Yaşlanma (Senesens), *SENEX* ve p16$^{INK4a/-}$ Retinoblastom (Rb) Yolağını Aktive Ederek Proliferasyonu Artırır

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Abstract

Objective: Cellular senescence has been thought to be an important barrier to tumor formation. Recent studies have shown that stress-induced premature senescence (SIPS) can promote partial tumor invasion, but how SIPS affects diffuse large B-cell lymphoma (DLBCL) remains inconclusive. This study aimed to address that issue.

Materials and Methods: The immunophenotype of the LY8 cell line was measured with flow cytometry. SIPS induced by tert-butyl hydroperoxide (tBHP) was detected by senescence β-galactosidase staining. Cell proliferation was analyzed with CCK8 and expression levels of ARHGAP18 (*SENEX* gene-encoding protein), p16/p21, and Rb/pRb were measured with western blot. LY8 cells were transfected with *SENEX*-SiRNA/NC and verified by western blot.

Results: Our results suggested that the immunophenotype of the LY8 cell line is CD19-, CD20-, and CD10-positive and the immunoglobulin light chain is the kappa type. The cellular senescence model of DLBCL could be successfully induced by 30 μM tBHP. ARHGAP18, p21, p16, and Rb protein levels were significantly increased but the level of pRb expression was decreased in the SIPS group compared with other groups. Meanwhile, the proliferation rate was increased in the SIPS group more than other tBHP groups. Furthermore, the expressions of p21 and p16 were significantly decreased in the *SENEX*-SiRNA group compared with the negative control group.

Conclusion: SIPS formation activates ARHGAP18 and the p16/Rb pathway and promotes DLBCL cell proliferation. Furthermore, *SENEX* activates the p16 pathway in DLBCL. SIPS promotes proliferation by activating *SENEX* and the p16/Rb pathway in DLBCL. *SENEX*-related SIPS may serve as an important target for relapsed/refractory DLBCL therapy.

Keywords: Stress-induced premature senescence, Proliferation, *SENEX*, p16, Rb/pRb, Diffuse large B-cell lymphoma

Öz

Amaç: Hücresel yaşlanmanın tümör oluşumuna karşı önemli bir engel olduğu düşünülmektedir. Son çalışmalarda stresin tetiklediği erken yaşlanmanın (senesens) (SIPS) kısmi tümör invazyonunu kolaylaştırabileceği gösterilmiş olmakla birlikte SIPS'nin diffüz büyük B hücreli lenfomayı (DBBHL) nasıl etkilediği bilinmemektedir. Çalışmada bu konunun ele alınması amaçlanmıştır.

Gereç ve Yöntemler: LY8 hücre dizisinin immünfenotipi akış sitometri ile belirlendi. Tert-butil hidroksiperoksitin (tBHP) oluşturduğu SIPS β-galaktozidaz boyası ile tesbit edildi. Hücre proliferasyonu CCK8 ile analiz edildi ve ARGHAP18 (*SENEX* genini kodlayan protein), p16/p21 ve pRb ekspresyon seviyeleri western-blot ile ölçüldü. LY8 hücreleri *SENEX*-SiRNA/NC ile transfekte edilerek western-blot ile gösterildi.

Bulgular: Sonuçlarımıza göre LY8 hücre dizisinin immünfenotipi CD19, CD20 ve CD10 pozitiftir ve immünoglobulin hafif zinciri kappadır. DBBHL'de hücresel yaşlanma modeli 30 μM tBHP ile başarılı bir şekilde oluşturulabilir. ARHGAP18, p21,p16 ve Rb protein seviyeleri anlamlı olarak arttı fakat pRb ekspresyon seviyeleri diğer gruplarla karşılaştırıldığında SIPS grubunda azaldı. Bu arada, SIPS grubunda, diğer tBHP grubu ile karşılaştırıldığında proliferasyon hızı daha çok arttı. Ek olarak, p21 ve p16 ekspresyonları *SENEX*-SiRNA grubunda, negatif kontrol grubu ile karşılaştırıldığında anlamlı ölçüde azaldı.

Sonuç: SIPS oluşumu ARHGAP18 ve p16/Rb yolağını aktive eder ve DBBHL hücrelerinin proliferasyonunu artırır. Ek olarak *SENEX* DBBHL'da p16 yolağını aktive eder. SIPS DBBHL'de *SENEX* ve p16/Rb yolağını aktive ederek proliferasyonu destekler. *SENEX* ilişkili SIPS relaps/refrakter DBBHL tedavisinde önemli bir hedef olabilir.

Anahtar Sözcükler: Stresin tetiklediği erken yaşlanma, Proliferasyon, *SENEX*, p16, Rb/pRb, Diffüz büyük B hücreli lenfoma

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Introduction

Diffuse large B-cell lymphoma (DLBCL), representing about 30%-40% of non-Hodgkin's lymphoma (NHL), is the most common subtype [1]. The introduction of rituximab (R) in combination with standard cyclophosphamide, doxorubicin, vincristine, and prednisolone (CHOP) chemotherapy, known as "R-CHOP", has significantly improved survival outcomes [2]. However, approximately 30% of cases of advanced-stage DLBCL remain intractable and the disease could relapse in the end [3]. In recent years, cellular immunotherapy has achieved important breakthroughs, especially CD19-chimeric antigen receptor T-cells (CAR-T-CD19) for the treatment of relapsed/refractory acute B lymphoblastic leukemia with up to 70%-90% complete remission rates, but in B-cell lymphomas such as DLBCL, CAR-T treatment did not achieve similar satisfactory results, with only about 50% of the response rate [4]. Studies have suggested that this difference may be related to the specific immune escape protection mechanism of DLBCL [5]. Tumor cell immune escape is associated with the paracrine effects of cellular senescence [6,7,8]. Cellular senescence refers to a relatively stable and continuous state leading to cell detachment from the cell cycle and loss of proliferation during various non-lethal pressures from inside and outside. It is divided into replicative senescence and stress-induced premature senescence (SIPS) according to the different mechanisms [9]. SIPS is telomere-independent and occurs after stimulation by autologous oncogenes, external oxidative and genotoxic substances, or infections. When stress is relieved or the environment changes, SIPS cells may resuscitate, reentering the cell cycle and proliferating [8,10]. Cellular senescence has been thought to be an important barrier to tumor formation. Recent studies have shown that SIPS can promote partial tumor invasion [11].

SENEX is a new gene associated with SIPS that was identified as a successful clone in 2004 and was named ARHGAP18 in the RefSeq system [12]. Studies have revealed that *SENEX* can regulate $p16^{INK4a}$ and Rb protein activation in endothelial cells (ECs) under conditions of H_2O_2-mediated stress [13]. Once a senescence signal is received from the p53 and p16 pathways, the Rb protein becomes the central link in the control of the aging process. In this study, endogenous *SENEX* remains unchanged during endothelial aging in ECs, but when exposed to oxidative stress, *SENEX* levels are altered, and activated *SENEX* mediates EC SIPS formation and produces resistance through the p16 pathway. Inflammation and *SENEX* overexpression do not alter the expression of p53 or p21. This result suggests that the *SENEX* gene mediates the SIPS mechanism in ECs primarily through the p16 pathway rather than the p53/p21 pathway. However, how does the *SENEX* gene trigger the SIPS phenomenon found in vascular EC functions in tumor cells? Our previous study illustrated that *SENEX* gene expression was upregulated in regulatory T cells (Tregs) of elderly bladder cancer patients, while silencing of the *SENEX* gene by SiRNA increased Treg apoptosis and pro-apoptotic gene expression in response to tBHP-mediated stress [14]. However, the way in which SIPS affects DLBCL remains inconclusive. The present study aims to address this question.

Materials and Methods

Cell Culture

Human DLBCL cell line OCI-LY8 was cultured in RPMI (Roswell Park Memorial Institute)-1640 medium supplemented with 10% fetal bovine serum (FBS). Cell cultures were maintained and incubated at 37 °C in humidified air with 5% CO_2.

Phenotype Analysis

For analysis of the immunophenotype of the DLBCL LY8 cell line, cells were harvested for flow cytometry (FC-500, Beckman Coulter, Miami, FL, USA). Antibodies were purchased from Beckman Coulter as follows: FITC fluorescently labeled CD19, PE fluorescently labeled CD10, PE fluorescently labeled CD20, ECD fluorescently labeled CD19, FITC fluorescently labeled kappa, and PE fluorescently labeled lambda.

Induction of Senescence

A tert-butyl hydroperoxide (tBHP) stock solution (5 mol/L) was purchased from Energy Chemical (Shanghai, China). The tBHP stock solution was diluted in RPMI-1640 supplemented with 10% FBS to final concentrations of 10, 30, and 50 μM, and then LY8 cells (10^6/mL) were treated with 10, 30, or 50 μM tBHP respectively for 24 h in vitro.

Senescence Staining

According to the Senescence β-Galactosidase Staining Kit (Beyotime, Shanghai, China), LY8 cells treated with 10, 30, and 50 μM tBHP were fixed with galactosidase fixative and incubated in dyeing working fluid. Finally, stained cells were observed under a microscope (CNOPTEC, Chongqing, China). Cells that stained green-blue were evaluated as positive senescent cells.

SiRNA Synthesis and Transfection

The individual small interfering RNA target *SENEX* gene (*SENEX*-siRNA) and scrambled negative control siRNA (NC) (the sequences are listed in Table 1) were synthesized by Sangon (Shanghai, China). The final siRNA concentration was 33 nM [14]. LY8 cells (4×10^5/well) were plated in 24-well plates overnight and were then transfected with *SENEX*-SiRNA or NC for 24 h using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was extracted from LY8 cells using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA was reverse-transcribed using a Transcript RT Kit (Sangon, Shanghai, China). qRT-PCR was performed on the ABI 7500 Real-Time PCR System (Life Technologies, Grand Island, NY, USA) using SYBR Green PCR Master Mix (TaKaRa, Dalian, China). All primers were synthesized by Sangon (Shanghai, China). The relative *SENEX* expression level was calculated using the $2^{-\triangle\triangle Ct}$ method. Sequences used for qRT-PCR primers and SiRNA transfection are shown in Table 1.

Western Blot

Total proteins from cells were extracted by western blot with IP cell lysis liquid (Beyotime, Shanghai, China) according to standard procedures (Table 2). Proteins were developed using the SuperSignal West Femto Trial Kit (Thermo Fisher Scientific, Shanghai, China) as previously described [15].

Proliferation Analysis

LY8 cells were plated at a density of 5000 cells/well in 96-well plates and subsequently transfected with *SENEX*-SiRNA or NC at a final concentration of 33 nM. At 24 h or 48 h after transfection, cell proliferation was measured with the CCK-8 Kit (BestBio, Shanghai, China) [16]. Each assay was performed with 5 replicates in 3 independent experiments.

Table 1. Sequences used for quantitative real-time polymerase chain reaction primers and SiRNA transfection.

Name	Sequences (5′ to 3′)
GAPDH - forward	GTGAAGGTCGGTGTGAACGG
GAPDH - reverse	GATGCAGGGATGATGTTCTG
SENEX-Forward	TTGCTCTGTTTTCCAGATTGGA
SENEX-Reverse	GCCCCAGTGCTTGAGGCT
SiRNA-*SENEX*-homo-1189 sense	GGAGCUGCCAUUAGAAUCATT
SiRNA-*SENEX*-homo-1189 Antisense	UGAUUCUAAUGGCAGCUCCTT
NC sense	UUCUCCGAACGUGUCACGUTT
NC antisense	ACGUGACACGUUCGGAGAATT

Statistical Analysis

All statistical analysis was performed with SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The classical t-test method was used to compare the data between the two groups that conformed to normal distribution and $p<0.05$ was considered statistically significant.

Results

SIPS Model of DLBCL Is Successfully Induced by 30 μM tBHP

The immunophenotype of the DLBCL LY8 cell line was CD19-, CD20-, and CD10-positive and the immunoglobulin light chain is the kappa type (Figure 1). The LY8 cells were respectively stimulated with 10, 30, and 50 μM tBHP for 24 h in vitro and treated with senescence β-galactosidase staining (Figure 2). Compared with the control group, cell growth was obviously affected by tBHP intervention. Senescent DLBCL cells had enlarged nuclei, irregular shapes, and clumps of growth (Figure 2C). Stimulation with 50 μM tBHP led to a large amount of apoptosis in LY8 cells (Figures 2D). On the other hand, there were no senescent cells in the 10 μM and 50 μM tBHP groups (Figures 2B and 2D), but blue-green senescent cells could be obviously observed in the 30 μM tBHP group (Figure 2C). These results suggest that the SIPS model of DLBCL can be successfully induced by 30 μM tBHP for 24 h in vitro.

SIPS Activates *SENEX* and the p16/Rb Pathway

Both p21 and p16 are important markers of cellular senescence [17]. In our studies, we observed that the expression of p21 protein was significantly increased in the 30 μM group compared with the control group ($p<0.01$) (Figure 3A), and the level of p21 was also obviously upregulated in the 30 μM group compared to the control group ($p<0.05$) (Figure 3A). These results indicate that senescence promoted p16 and p21 activation.

Studies indicated that the Rb pathway inhibits transcription of genes that are necessary for the transition from the G1 to the S phase. Central to this pathway is the regulation of phosphorylation of the Rb protein [18]. In our studies, we found that the expression of the Rb protein was higher in the 30 μM group than the control group ($p<0.01$) (Figure 3B). In contrast, the level of pRb expression in the 30 μM group was downregulated

Table 2. Primary antibodies used for western blotting.

Name	Company	Item number	Dilution ratio
Anti-ARHGAP18	Abcam	ab175970	1:1000
P16 INK4A(D7C1M) Rabbit mAb	Cell signaling technology	#80772	1:1000
P21 Waf1/Cip1(12D1) Rabbit mAb	Cell signaling technology	#2947	1:1000
Rb(D20) Rabbit mAb	Cell signaling technology	#9313	1:1000
Phospho-Rb(Ser780)(D59B7) Rabbit mAb	Cell signaling technology	#8180	1:1000
Mouse Anti-β-Actin mAb	ZSGB-BIO	TA-09	1:2000

compared with the control group (Figure 3B). These results indicated that senescence inhibited the phosphorylation of Rb.

Based on these results, we further tested the expression of ARHGAP18, which is encoded by the *SENEX* gene, in the DLBCL cellular senescence model. The expression of ARHGAP18 was significantly higher in the 30 μM group than the control group ($p<0.01$) (Figure 4A). This suggests that ARHGAP18 was significantly increased in senescent DLBCL cells. The relationship between senescence, the *SENEX* gene, and the p16/Rb pathway needs further exploration.

SIPS Promotes Proliferation

In this study, the cell proliferation rate was detected respectively by CCK8 analysis in the 10 μM tBHP group, 30 μM tBHP group, and 50 μM tBHP group. We found that the cell proliferation rate in the 30 μM tBHP group was significantly upregulated compared with the 50 μM tBHP group ($p<0.01$) and was also higher than in the 10 μM tBHP group (Figure 4B). Proliferation of senescent DLBCL cells is accelerated compared with other cells under the pressure of tBHP. These results suggest that SIPS promotes proliferation.

SENEX Activates the p16 Pathway

To determine whether *SENEX* is important in the activation of the p16 pathway, LY8 cells were transfected with the individual small interfering RNA target *SENEX* gene (*SENEX*-siRNA) and scrambled negative control siRNA (NC) to silence the *SENEX* gene, and then we analyzed the expression of p16/p21. We verified the efficiency of transfection at both gene and protein levels. The level of ARHGAP18 was obviously reduced in the *SENEX*-SiRNA group compared to the control and NC groups (Figure 4C), and the expression of *SENEX* mRNA was also significantly decreased in the *SENEX*-SiRNA group compared with the control group (LY8 cells without any treatment) and the NC group (Figure 4D). These results suggest that transfection with *SENEX*-SiRNA could silence the *SENEX* gene in DLBCL cells. The expression of p16 was significantly decreased in the *SENEX*-SiRNA group compared with the NC and unprocessed groups (Figure 4C). Consistent with this, the expression of p21 was also decreased in the *SENEX*-SiRNA group compared with the NC and unprocessed groups (Figure 4C). These results suggest that *SENEX* activates the p16 pathway in DLBCL.

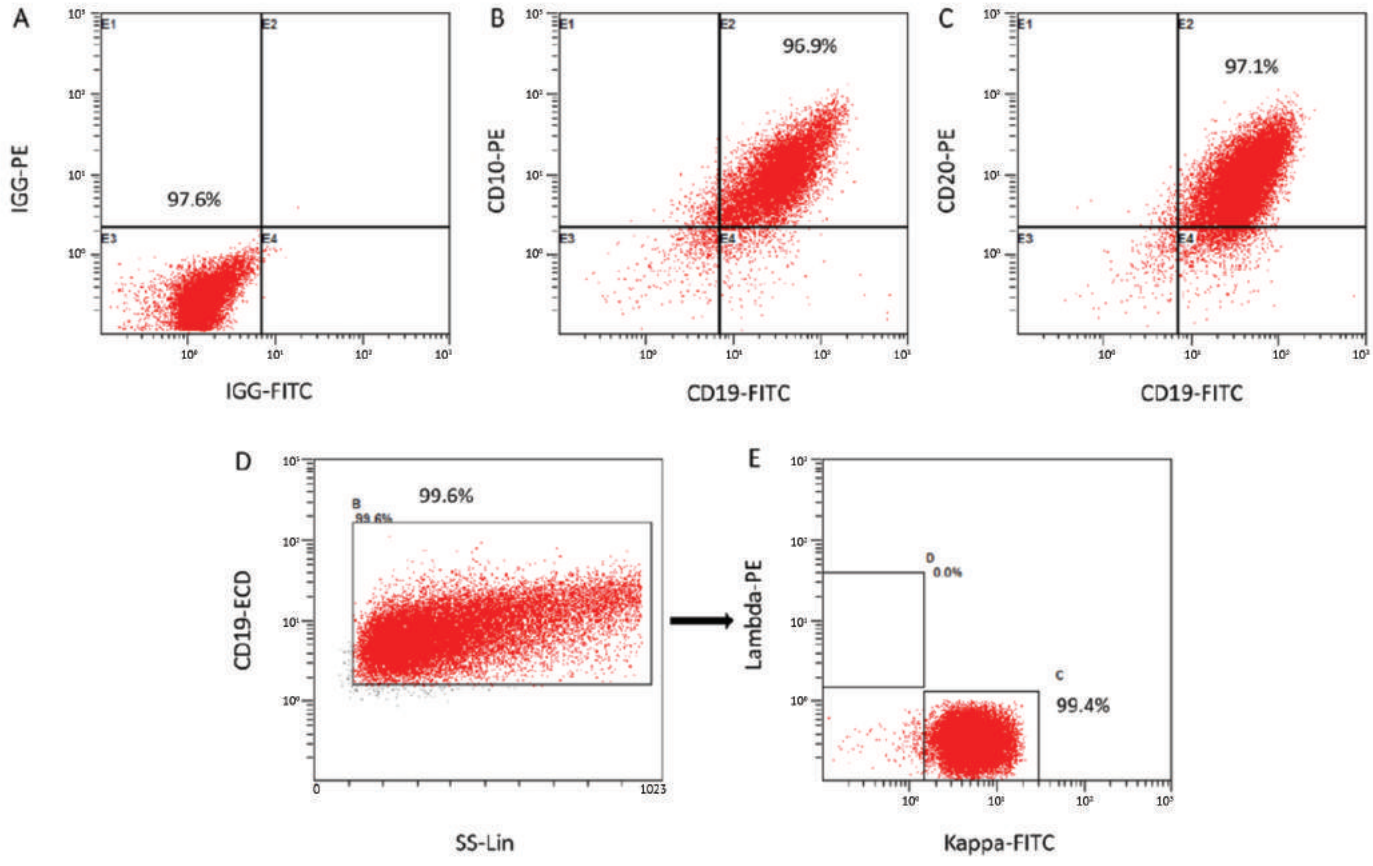

Figure 1. Immunophenotype of DLBCL LY8 cell lines. LY8 cell line was used for detecting immunophenotyping by flow cytometry (FCM). (A, B) FCM analysis of CD19, CD20, and CD10 expressions in LY8 cell line. (C) Negative control of PE and FITC molecules. (D) FCM analysis of type of immunoglobulin light chain in LY8 cell line.

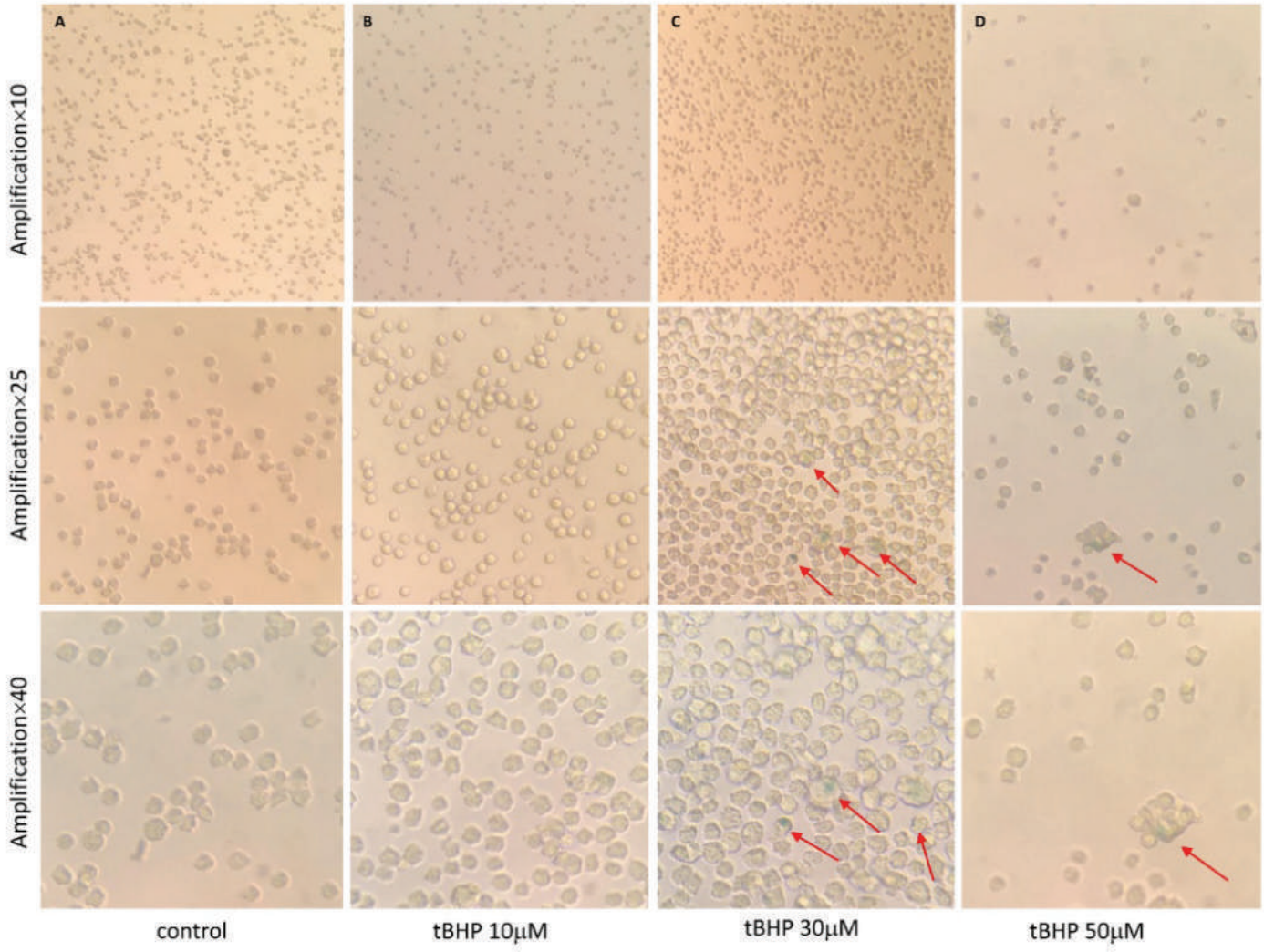

Figure 2. Stress-induced premature senescence model of DLBCL induced by 30 μM tBHP for 24 h. LY8 cells were treated with 10, 30, and 50 μM tBHP respectively for 24 h in vitro. Then cells were stained for β-galactosidase. A represents the control group, which was observed at amplifications of 10^x, 25^x, and 40^x. B represents the 10 μM tBHP group, which was observed at amplifications of 10^x, 25^x, and 40^x. C represents the 30 μM tBHP group, which was observed at amplifications of 10^x, 25^x, and 40^x. D represents the 50 μM tBHP group, which was observed at amplifications of 10^x, 25^x, and 40^x.

Discussion

Cell senescence is a state of cell cycle arrest under stress, which is an indispensable mechanism to prevent the proliferation of injured cells [19]. The permanent growth arrest caused by cell senescence suggests that the senescence response can inhibit the development of tumors. It is now thought that senescence-induced growth retardation is irreversible because no physiological stimuli have been found to enable senescent cells to reenter the cell cycle [20]. However, when cells undergo some molecular biological changes, such as the successive inactivation of certain tumor-suppressor genes, it can cause abnormal proliferation of senescent cells. Senescence-induced inhibition of proliferation is strictly irreversible. It is supported and maintained by at least two major tumor-suppressor gene pathways (p53/p21 and p16INK4a/pRb signaling pathways) [21]. In addition to growth arrest, senescent cells also exhibit a wide range of alterations in chromosome and gene expression. This will lead to some proinflammatory cytokines, chemokines, growth factors, and protease and other cell secretory factor secretion changes, and the abnormal expression of this factor is known as the senescence-related secretory phenotype or senescence-associated secretory phenotype (SASP). SASP, associated with a large number of paracrine activities, will have a wide range of effects on cells and the whole body [22]. On the one hand, it can inhibit the development of tumors and promote tissue repair and regeneration in the face of injury. On the other hand, these abnormal secreted cytokines can cause malignant transformation of normal cells and promote the occurrence and development of tumors [23].

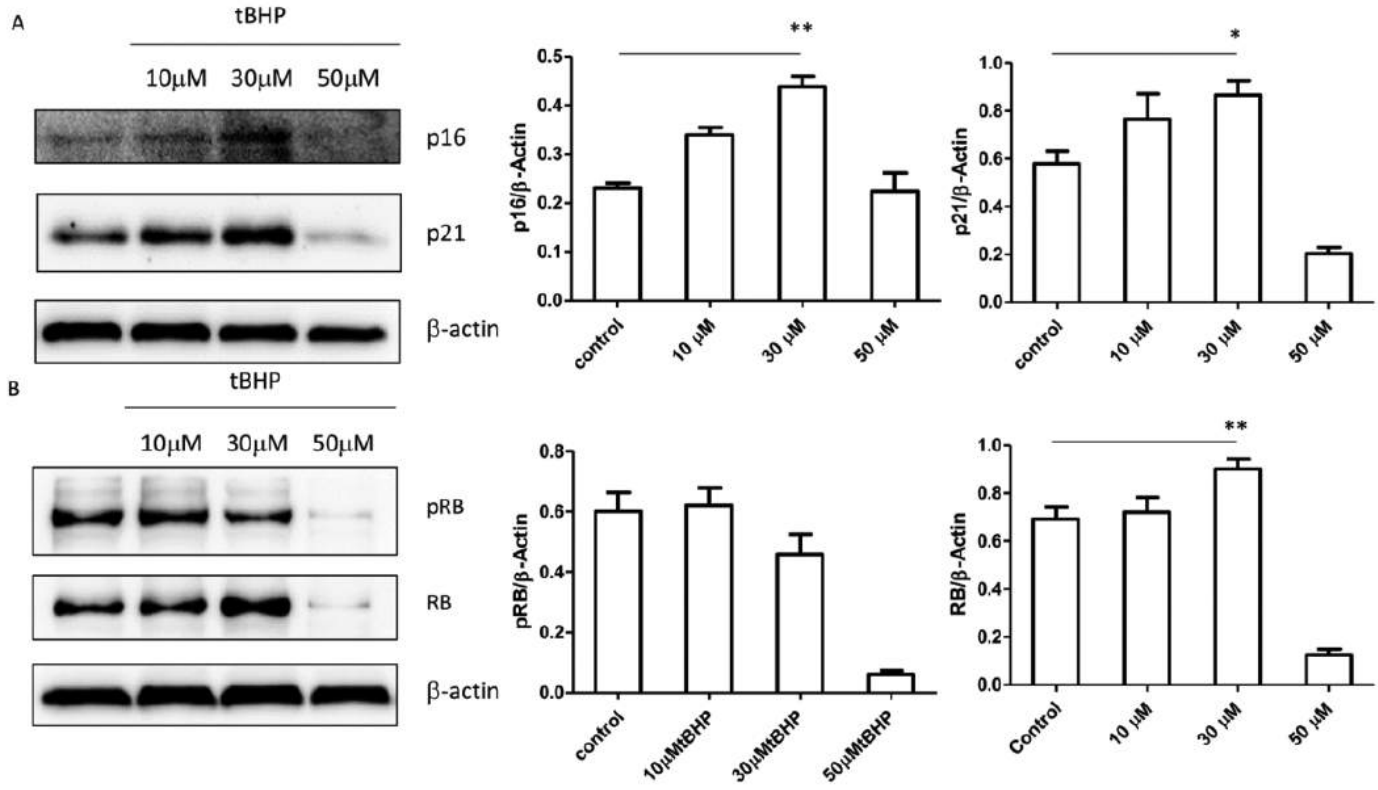

Figure 3. Stress-induced premature senescence activates the p16/Rb pathway. LY8 cells were treated with 10, 30, and 50 μM tBHP respectively for 24 h in vitro. After 48 h they were harvested for western blot analysis. (A) The expression of p16 and p21 protein in LY8 cells under the pressure of tBHP. (B) The expression of Rb and pRb in LY8 cells under the pressure of tBHP.

In this study, we investigated tBHP-induced SIPS in a DLBCL cell line. First of all, the immunophenotype of the LY8 cell line is CD19+, CD20+, and CD10+, which is a phenotype consistent with lymphocytes of germinal center origin (Figure 1). Senescent cells usually become larger in size and express β-galactosidase with high enzymatic activity at pH 6 [24]. After stimulation with tBHP, LY8 cells were treated with senescence β-galactosidase staining. Compared with the control group, we observed that cell growth was obviously affected by tBHP intervention. Senescent cells could be most obviously observed in the 30 μM tBHP group. The senescent cells had enlarged nuclei, irregular shapes, and clumps of growth (Figure 2). These results suggest that SIPS in DLBCL cells can be successfully induced by 30 μM tBHP. Secondly, the cell proliferation rate was detected by CCK8 analysis. We found that cell proliferation rate was significantly upregulated in the 30 μM tBHP group. Cell proliferation of senescent DLBCL cells is accelerated compared with other cells under the pressure of tBHP (Figure 4). These results suggested that SIPS promotes the proliferation of DLBCL cells.

Senescence is mediated through the p53 pathway, which transactivates the cyclin-dependent kinase inhibitor p21, or through the p16 pathway to inhibit cyclin-dependent kinases 2 and 4, preventing phosphorylation of the Rb protein [26,27]. In our studies, we observed significantly upregulated p21 and p16 protein in senescent cells. These studies suggest that senescence is characterized by developmental cues that converge on p21 and p16 proteins. Studies indicated that the Rb pathway inhibits the transcription of genes that are necessary for the transition from the G1 to the S phase. Central to this pathway is the regulation of phosphorylation of the Rb protein [28]. In our studies, we found significantly high expression of Rb protein. This indicates that the p53/p21/Rb and p16/Rb axes are both important signaling pathways involved in the induction of senescence.

The *SENEX* gene has been proved to provide a unique gatekeeper function in the SIPS and apoptosis pathways in ECs [13]. Furthermore, *SENEX* overexpression induced an increase in both the mRNA and protein levels for p16 and there was a decrease in the protein expression of the hyperphosphorylated Rb. These results indicated that *SENEX* activated the p16/pRb pathway [13]. In our study, the expression of *SENEX* protein was significantly higher in the 30 μM group than the control group. It is suggested that the *SENEX* protein was significantly increased in senescent DLBCL cells.

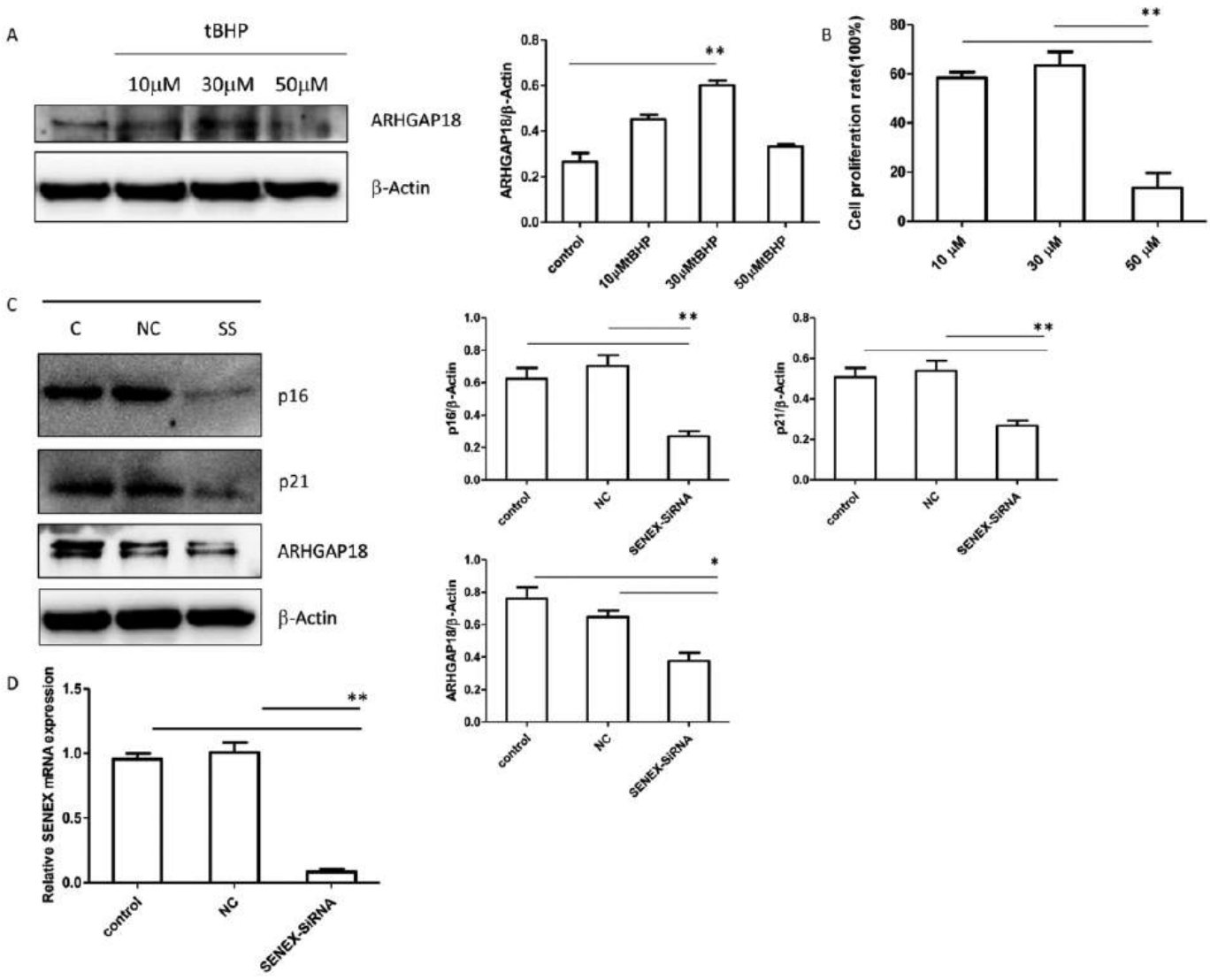

Figure 4. Stress-induced premature senescence promotes *SENEX* activation and proliferation and *SENEX* activates the p16 pathway. (A) LY8 cells were treated with 10, 30, and 50 μM tBHP respectively in vitro. After 48 h they were harvested for western blot analysis. The expression of *SENEX* in LY8 cells is shown under the pressure of tBHP. (B) LY8 cells were transfected with *SENEX*-SiRNA or NC in vitro. After 48 h they were harvested for western blot analysis. The expression of p16 and p21 in LY8 cells transfected with *SENEX*-SiRNA/NC is shown (C = unprocessed control group; NC = LY8 cells transfected with NC group; SS = LY8 cells transfected with *SENEX*-SiRNA group). (C) LY8 cells were treated with 10, 30, and 50 μM tBHP respectively for 24 h in vitro. After 48 h they were harvested for western blot analysis. The cell proliferation rates of LY8 cells induced by tBHP were measured with CCK8 analysis. **p<0.01.

Conclusion

We investigated a SIPS model of DLBCL and found that it can be successfully induced by tBHP. SIPS formation activates the *SENEX* gene and the p16/Rb pathway and promotes DLBCL cell proliferation. Furthermore, *SENEX* activates the p16 pathway in DLBCL. SIPS promotes proliferation by activating *SENEX* and the p16/Rb pathway in DLBCL. *SENEX*-related SIPS may serve as an important target for relapsed/refractory DLBCL therapy. By improving the knowledge on the molecular basis of senescence, novel strategies relying on senescence induction will reach the clinic as potential cancer therapies. *SENEX*-related senescence may serve as an important target for relapsed/refractory DLBCL therapy.

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Ethics

Ethics Committee Approval: Anhui Medical University, approval number: LLSC20160082.

Informed Consent: N/A.

Authorship Contributions

Surgical and Medical Practices: J.W., Z.W.; Concept: Z.Z.; Design: J.W., Q.T.; Data Collection or Processing: Z.W., H.W., Z.W.; Analysis or Interpretation: J.W., Y.P.; Literature Search: F.Z.; Writing: Jiyu Wang.

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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Differentiation Potential and Tumorigenic Risk of Rat Bone Marrow Stem Cells Are Affected By Long-Term In Vitro Expansion

Sıçan Kemik İliği Kök Hücrelerinin Farklılaşma Potansiyeli ve Tümörojenik Riski Uzun Süreli İn Vitro Ekspansiyondan Etkilenmektedir

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Abstract

Objective: Mesenchymal stem cells (MSCs) have the capacity for extensive expansion and adipogenic, osteogenic, chondrogenic, myogenic, and neural differentiation in vitro. The aim of our study was to determine stemness, differentiation potential, telomerase activity, and ultrastructural characteristics of long-term cultured rat bone marrow (rBM)-MSCs.

Materials and Methods: rBM-MSCs from passages 3, 50, and 100 (P3, P50, and P100) were evaluated through immunocytochemistry, reverse transcription-polymerase chain reaction, telomerase activity assays, and electron microscopy.

Results: A dramatic reduction in the levels of myogenic markers actin and myogenin was detected in P100. Osteogenic markers Coll1, osteonectin (Sparc), and osteocalcin as well as neural marker c-Fos and chondrogenic marker Coll2 were significantly reduced in P100 compared to P3 and P50. Osteogenic marker bone morphogenic protein-2 (BMP2) and adipogenic marker peroxisome proliferator-activated receptor gamma (Pparγ) expression was reduced in late passages. The expression of stemness factor Rex-1 was lower in P100, whereas Oct4 expression was decreased in P50 compared to P3 and P100. Increased telomerase activity was observed in long-term cultured cells, signifying tumorigenic risk. Electron microscopic evaluations revealed ultrastructural changes such as smaller number of organelles and increased amount of autophagic vacuoles in the cytoplasm in long-term cultured rBM-MSCs.

Conclusion: This study suggests that long-term culture of rBM-MSCs leads to changes in differentiation potential and increased tumorigenic risk.

Keywords: Bone marrow, Differentiation, Long-term culture, Mesenchymal stromal cells, Stemness, Telomerase

Öz

Amaç: Mezenkimal kök hücreler (MKH) in vitro uzun süreli ekspansiyon, adipojenik, osteojenik, kondrojenik, miyojenik ve nöral farklılaşma potansiyeline sahiptir. Çalışmamızın amacı uzun süre kültüre edilen sıçan kemik iliği (sKİ)-MKH'lerinin kök hücre niteliklerini, farklılaşma potansiyellerini, telomeraz aktivitelerini ve ultrayapısal özelliklerini belirlemektir.

Gereç ve Yöntemler: 3., 50. ve 100. pasajlardan (P3, P50 ve P100) elde edilen sKİ-MKH'leri, immünohistokimya, revers-transkriptaz polimeraz zincir reaksiyonu, telomeraz aktivite analizleri ve elektron mikroskopi ile değerlendirilmiştir.

Bulgular: P100'de miyojenik belirteçlerden aktin ve miyogenin seviyelerinde düşüş gözlemlenmiştir. Osteojenik belirteçler Coll1, osteonektin (Sparc) ve osteokalsin ile nöral belirteç c-Fos ve kondrojenik belirteç Coll2 P100'de P3 ve P50'ye kıyasla önemli ölçüde azalmıştır. Osteojenik belirteç kemik morfojenik protein-2 (BMP2) ve adipojenik belirteç peroksizom proliferatör ile aktive olan reseptör gamma (Pparγ) geç pasajlarda düşüş göstermiştir. Kök hücre belirteçlerinden Rex-1'in ekspresyonu P100'de düşüş gösterirken, Oct4 ekspresyonunun P50'de P3 ve P100'e göre düşüş gösterdiği belirlenmiştir. Uzun süre kültüre edilen hücrelerdeki artmış telomeraz aktivitesi tumorijenik riske işaret etmektedir. Elektron mikroskopik değerlendirmeler, uzun süre kültüre edilen sKİ-MKH sitoplazmasında düşük sayıda organel ve artmış oranda otofajik vakuol gibi utrayapısal değişiklikler ortaya koymuştur.

Sonuç: Bu çalışma, sKİ-MKH'lerinin uzun süreli kültüre edilmesinin bu hücrelerin farklılaşma potansiyelinde değişikliklere ve tümörijenik riskin artmasına neden olduğunu göstermiştir.

Anahtar Sözcükler: Kemik iliği, Farklılaşma, Uzun süreli kültür, Mezenkimal kök hücreler, Telomeraz

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Introduction

Mesenchymal stem cells (MSCs) have the capacity for extensive expansion in vitro and are able to undergo adipogenic, osteogenic, chondrogenic, myogenic, and neural differentiation [1,2,3]. MSCs can be obtained from several sources, such as placental tissue [4], amniotic fluid [5], cord blood [6,7], adipose tissue [8,9], and dental pulp [10]. However, bone marrow aspiration remains the source of choice for MSCs in most laboratories [11,12]. The secretion of a broad range of bioactive molecules is believed to be the main mechanism by which MSCs achieve their therapeutic effects and these can be divided into eight main categories: immunomodulation, anti-apoptosis, angiogenesis, support of the growth and differentiation of local stem and progenitor cells, anti-scarring, chemoattraction, gene transfer, and exosomes [13,14,15,16,17,18,19].

A sufficient quantity of stem cells can be obtained by in vitro expansion in order to be used in clinical applications [20]. However, during long-term cultures of stem cells, several abnormalities were recorded, such as increased telomerase activity and changes in the expression of genes regarding cell regulation, apoptosis, and senescence due to increased cell doublings and culture times [21,22,23,24]. Thus, we proposed that long-term expansion of MSCs in vitro might be associated with tumorigenic risk. MSCs were reported to promote tumor progression and metastasis in a number of studies [25,26,27,28], while other studies suggested that MSCs suppress tumor growth [29,30,31]. Spontaneous transformation was not observed during in vitro expansion of human MSCs (hMSCs) [32,33,34,35]. However, there are reports providing evidence that murine bone marrow (BM)-MSCs [36] as well as adipose-derived hMSCs [37] displayed malignant transformation in vitro. It was suggested that the tendency of hMSCs to undergo malignant transformation was caused by the genomic plasticity of undifferentiated hMSCs allowing their longevity [38]. BM-MSCs were also reported to be associated with the in vivo growth of colon cancer, lymphoma, and melanoma cells [26,39,40]. MSCs were found to transform into tumor-associated fibroblasts, constructing a fibrovascular network for the tumors [41]. On the other hand, BM-MSCs were also shown to suppress tumorigenic cells in vivo [30,42].

The aim of the current study was to evaluate long-term (18 months, 100 passages) cultured rat bone marrow (rBM)-MSCs in terms of stemness and differentiation characteristics as well as cell cycle progression and telomerase activity in order to determine their lineage differentiation potential and tumorigenic risk under in vitro conditions.

Materials and Methods

Isolation and Culture of rBM-MSCs

The animals (8-week-old male Wistar rats) were anesthetized with Ketalar (Pfizer) and killed by cervical dislocation for rBM-MSC isolation. Animal housing and experiments were approved by the local animal care committee (Kocaeli University, HAEK/33-4) according to the institutional guidelines and national animal welfare standards. rBM-MSCs were obtained from both femurs and tibias of the rats as described in our previous study [43].

For each passage the cells were plated similarly and grown to confluency of 70%. Passages were performed until 100 passages and the below-mentioned analyses were performed for passages 3, 50, and 100.

Immunocytochemistry and Immunofluorescence Staining

The streptavidin-peroxidase method (UltraVision Plus Large Volume Detection System Anti-Polyvalent, HRP Immunostaining Kit, Thermo Scientific, UK) was used for immunocytochemistry analysis as described previously [10]. Immunofluorescence staining was applied as indicated in our previous study [10]. The primary antibodies listed in Table 1 were used for immunocytochemistry and immunofluorescence stainings.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from rBM-MSCs (passages 3, 100, and 150) according to the manufacturer's instructions (QIAGEN,

Table 1. Primary antibodies used for immunocytochemistry and immunofluorescence analysis.

Antibody/Marker	Dilution	Source
Collagen Ia1 (D-13)	1:50	Santa Cruz Biotechnology
Collagen II Ab-2 (2B1.5)	Prediluted	Thermo Scientific
Myosin IIa (A4.74)	1:50	Santa Cruz Biotechnology
Myogenin Ab-1 (F5D)	Prediluted	Thermo Scientific
Osteonectin (SPARC)	1:50	Chemicon International
Osteocalcin (FL-100)	1:50	Santa Cruz Biotechnology
α-Smooth muscle actin Ab-1	1:800	Thermo Scientific
Actin (C-2)	1:50	Santa Cruz Biotechnology
c-Fos (4)	1:50	Santa Cruz Biotechnology
Tropomyosin (CH1)	1:50	Santa Cruz Biotechnology
Vimentin (C-20)	1:100	Santa Cruz Biotechnology
Cytokeratin 19 (CK 19)	1:50	Santa Cruz Biotechnology

USA). RT-PCR analysis was performed as described in our previous study [44] and bands were quantified using NIH image analysis software (ImageJ Version 1.36b, National Institutes of Health, Bethesda, MD, USA) as described previously [45].

Telomerase Activity

Telomerase activity was detected by applying a conventional telomeric repeat amplification protocol (TRAP) using the TRAP TeloTAGGG PCR enzyme-linked immunosorbent assay kit (Roche, Mannheim, Germany). The TRAP method was applied as described previously [46].

Electron Microscopy

rBM-MSCs at passages 3, 50, and 100 were prepared for electron microscopic analysis. The samples were fixed and embedded as described previously [47]. Ultrathin sections were observed with a transmission electron microscope (Carl Zeiss Libra 120).

Statistical Analysis

The data obtained from ImageJ for RT-PCR bands were analyzed with non-parametric ANOVA on ranks (Kruskal-Wallis test) and parametric one-way ANOVA (Holm-Sidak method). The values are presented as mean ± SEM. Statistical calculations were performed using Sigma Stat for Windows, version 3.0 (Jandel Scientific Corp., San Rafael, CA, USA). Statistical significance was defined as $p<0.05$.

Results

Immunolocalization of Differentiation Markers in Long-Term Cultured rBM-MSCs

Immunocytochemistry analysis in the current study showed that levels of particular myogenic markers including a-SMA and tropomyosin remained similar both in early and late passages. There was a dramatic reduction in actin, myosin IIa, and myogenin levels in passage 100 when compared to passages 3 and 50. Osteogenic markers including Coll1, osteonectin, and osteocalcin as well as neural marker c-Fos and chondrogenic marker Coll2 were reduced in passage 100 compared to passages 3 and 50 (Figure 1; Table 2).

Immunofluorescence analysis revealed that expression of epithelial marker CK-19 was increased after passage 70, while expression of mesenchymal marker vimentin was decreased after passage 70 when compared to passage 3 (Figure 2).

Gene Expression Profiles of Long-Term Cultured rBM-MSCs

The expressions of the stemness factors as well as adipogenic, chondrogenic, osteogenic, myogenic, and neural differentiation markers were detected in long-term cultured rBM-MSCs by RT-PCR analysis using specific primer sets (Table 3).

The expressions of stemness factors Rex-1 and Oct4 were identified in rBM-MSCs in all passages (P3, P50, and P100). Rex-1 expression level was increased in P50 and was decreased in P100 to a lower level than in P3. Oct4 was decreased in P50 compared to P3. Although it was found to be increased in P100, its expression in P100 was lower than in P3. Chondrogenic marker Sox9 was expressed in both early and late passages, and its expression was increased in P50 and significantly decreased in P100. The expressions of differentiation markers of precursor osteoblasts such as osteopontin (Opn/Ssp1), run-related transcription factor 2 (Runx2), and osteonectin (Sparc) were increased in P50 and were significantly reduced in P100. BMP2 was detected to be expressed at significantly lower levels both in P50 and P100 compared to P3, whereas the BMP4 level was lower only in P50.

Expression of the adipogenic marker Pparγ was decreased in P100 compared to P3 and P50. Adiponectin and monoglyceride lipase (MgLL) expressions were detected to be similar in all passages. ADFP was expressed in all three passages, with a higher level in P50. Neurofilament heavy chain (NF-H) and glial fibrillary acidic protein (GFAP) expressions were higher in P50 compared to P3 while they were decreased in P100. Neuroprogenitor cell marker β3-tubulin (TUBB3) was significantly decreased in P100 compared to P3 and P50. Another neuroprogenitor cell marker, gamma enolase (Eno2), was increased in P50 and reduced in P100 compared to P50. Precursor myoblast markers α-smooth muscle actin (Acta2) and ActB were increased in P50 and decreased in P100, whereas desmin (Des) and myogenin (Myog) expression levels were similar in all passages (Figure 3).

Telomerase Activity

Relative telomerase activities (RTAs) of rBM-MSCs (P3, P50, and P150) were measured and the calculations were normalized to 1 µg of total protein equivalent. The results for rBM-MSCs at

Table 2. Immunocytochemical properties of rBM-MSCs.

	rBM-MSCs		
Antibody/Marker	**P3**	**P50**	**P100**
α-SMA	+	+	+
Actin	+	+	Ø
Collagen type I	+	-/+	Ø
Collagen type II	+	+	Ø
c-Fos	+	+	-/+
Osteocalcin	+	+	-/+
Osteonectin (SPARC)	+	+	-/+
Myosin IIa	+	-/+	-/+
Myogenin	+	-/+	Ø
Tropomyosin	+	+	+

+: Positive marker expression. -/+: Weak marker expression. Ø: Lack of marker expression, rBM-MSCs: Rat bone marrow mesenchymal stem cells.

passages 3, 50, and 100 were found as 8.4, 19.89, and 45.09 RTA/µg total protein, respectively. According to these data, rBM-MSCs at later passages show a higher rate of telomerase activity (Figure 4).

Ultrastructural Characteristics

rBM-MSCs from both early and late passages showed pale, eccentric, irregularly shaped, and large euchromatic nuclei with one or more nucleoli located near the perinuclear cisternae. The cell cytoplasms from passage 3 had an intensely stained inner zone rich in elongated mitochondria and rough endoplasmic reticulum (rER) cisternae and a relatively peripheral zone poor in organelles. The rER cisternae were dilated and contained moderately electron-dense material. Aggregates of a few lipid droplets, granules, and glycogen were also observed. Numerous thin pseudopodia were observed on the cell surfaces. rBM-MSCs from late passages contained a smaller number of organelles and increased amount of pseudopodia on the cell surfaces. Empty vacuoles in the cytoplasm were observed to be increased in rBM-MSCs from late passages with respect to early passages. Free ribosomes were observed in the cytoplasms of cells from both early and late passages. These results constitute the first comparative and comprehensive detailed report of ultrastructural characteristics on long term cultured rBM-MSCs (Figure 5).

Discussion

There are conflicting results in the literature regarding malignant transformation of MSCs during in vitro culture. A number of reports proved the transformation of these cells [38,48,49,50], whereas certain studies found a relation with aneuploidy [51,52,53,54] and genetic mutations [55] while other studies suggested that these cells do not undergo transformation after long-term expansion [34]. In the current study we performed long-term, non-stop culture of rBM-MSCs for 18 months including 100 passages. These long-term cultured cells were examined for stemness factors as well as myogenic, chondrogenic, adipogenic, osteogenic, and neurogenic differentiation markers; epithelial and mesenchymal cell markers; telomerase activity; and ultrastructural characteristics. Interestingly, these cells showed higher expressions of CK-19 and lower expressions of vimentin after passage 70, signifying mesenchymal-to-epithelial transition in late passages. Previous studies regarding long-term culture of hMSCs also identified transformation of spindle-shaped cells into round epithelial-

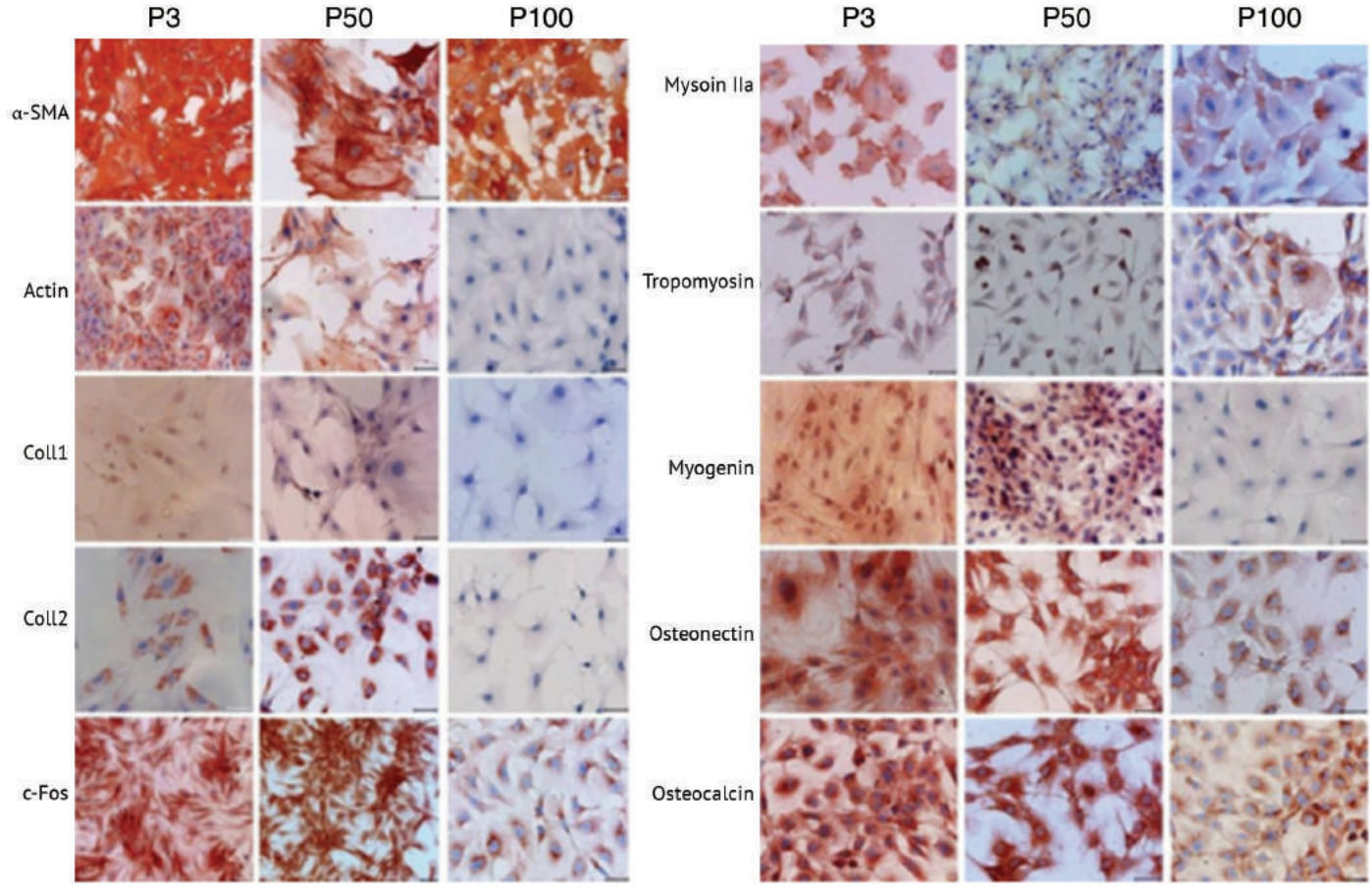

Figure 1. Lineage differentiation marker localizations in cultured rat bone marrow mesenchymal stem cells. P3: Passage 3. P50: Passage 50. P100: Passage 100. Nuclei were counterstained with hematoxylin. All experiments were repeated 3 times. Scale bars: 50 µm.

like cells that had an increased nucleus-to-cytoplasm ratio [38]. In previous studies it was shown that long-term cultures of both human [56] and rabbit [57] MSCs resulted in cellular senescence. Long-term expanded senescent cells were shown to have reduced differentiation potential, which led to restriction in MSC expansion for therapeutic applications [56,58,59].

Table 3. Primers used in polymerase chain reaction analysis.

Gene	Primer Sequence	GenBank	Product
		Acc. No.	Size (bp)
ACTA2	F: ATGAGGGCTATGCCTTGCCC	NM_001613	307
Smooth muscle actin	R: CCCGATGAAGGATGGCTGGA		
ACTB	F: TGGCACCACACCTTCTACAATGAGC	NM_001101	395
Actin beta	R: GCACAGCTTCTCCTTAATGTCACGC		
ADIPOQ	F: ATGGTCCTGTGATGCTTTGA	NM_004797	229
Adiponectin	R: GTTGAGTGCGTATGTTATTTTT		
BMP2	F: GTGCTTCTTAGACGGACTGC	NM_001200	1,232
Bone morphogenetic	R: GTACTAGCGACACCCACAAC		
protein 2			
BMP4	F: AGCCATTCCGTAGTGCCATC	NM_130851	1,374
Bone morphogenetic	R: AAGGACTGCCTGATCTCAGC		
protein 4			
DES	F: CAGGTGGAGATGGACATGTCTAAGC	NM_001927	186
Desmin	R: TCATCTCCTGCTTGGCCTGG		
ENO2	F: TTATTGGCATGGATGTTGCTGC	NM_001975	269
Enolase 2, gamma	R: CCCGCTCAATACGTTTTGGG		
GFAP	F: TCCTCAGGGGAGATGATGGT	NM_0011310	211
Glial fibrillary acidic protein	R: TTCTCGATGTAGCTGGCAAAG	19	
MGLL	F: CAATCCTGAATCTGCAACAACTTTC	NM_007283	411
Monoglyceride lipase	R: ATGTTTATTTCATGGAAGACGGAGT		
MYOG	F: TATGAGACATCCCCCTACTTCTACC	NM_002479	279
Myogenin	R: CTTCTTGAGCCTGCGCTTCT		
NEF-H	F: GAACACAGACGCTATGCGCTCAG	NM_021076	396
Neurofilament, heavy	R: CACCTTTATGTGAGTGGACACAGAG		
OCT4/POU5F1	F: TGCCGTGAAACTGAAGAAG	NM_203289	72
	R: TTTCTGCAGAGCTTTGATGTTC		
OPN/SPP1	F: CAGTGACCAGTTCATCAGATTCATC	NM_0010400	374
Osteopontin	R: CTAGGCATCACCTGTGCCATACC	58	
PLIN2	F: CGCTGTCACTGGGGCAAAAGA	NM_001122	173
Adipophilin	R: ATCCGACTCCCCAAGACTGTGTTA		
Peroxisome proliferator-activated receptor gamma	F: CAGTGGGGATGCTCATAA R: CTTTTGGCATACTCTGTGAT	NM_138711	422
REX-1/ZFP42	F: GGATCTCCCACCTTTCCAAG R: GCAGGTAGCACACCTCCTG		
	F: GGATCTCCCACCTTTCCAAG	NM_020695	104
RUNX2 Runt-related transcription factor 2	F: CAGACCAGCAGCACTCCATA R: CAGCGTCAACACCATCATTC	NM_004348	177
SOX9 SRY-box 9	F: TGAAGAAGGAGAGCGAGGAA R: GGGGCTGGTACTTGTAATCG	NM_000346	348
SPARC Osteonectin	F: TCTTCCCTGTACACTGGCAGTTC R: AGCTCGGTGTGGGAGAGGTA	NM_003118	73
TUBB3 Tubulin, beta 3	F: CATGGACAGTGTCCGCTCAG R: CAGGCAGTCGCAGTTTTCAC	NM_006086	175

Though we have found that rBM-MSCs preserve stemness factors even in late passages, they lack particular differentiation markers after long-term culture, highlighting their limited differentiation potential.

As an indication of the reduced adipogenic differentiation capacity of rBM-MSCs during long-term culture, in the current study we detected that expression of adipogenic marker PPAR-c was significantly decreased in late passages. PPAR-c is known to induce adipogenesis [60]. PPAR-c suppression was detected to cause generation of osteoblasts rather than adipocytes from BM progenitors [61]. After long-term in vitro expansion, although BM-MSCs were unable to display adipogenic differentiation, they were shown to have osteogenic differentiation potential [58].

Furthermore, it was also shown that osteogenic differentiation potential does not depend on the age of the donor [62]. In our study, expression levels of most of the osteogenic markers, including BMP-2, were significantly reduced during long-term culture. Coll1, osteonectin, osteocalcin, and Runx2 were detected to be reduced, especially in P100. Additionally, a dramatic reduction in chondrogenic marker SOX9 levels was detected, as well as a decrease in Coll2 levels in late passages. Thus, our results including in vitro expanded stem cells showed that the osteogenic and chondrogenic potential of long-term cultured stem cells might be disrupted. In previous studies, it was reported that human adipose-derived stem cells were able to differentiate into osteogenic cells, but this ability was reduced after long-term in vitro expansion [63].

The level of neural marker TUBB3 was gradually reduced in late passages and was detected to be significantly lower in P100. The c-Fos level was also decreased in P100. Although NF-H and GFAP levels were increased in P50, they were detected to be reduced in P100. Thus, the data obtained in this study indicate that the neurogenic differentiation potential of rBM-MSCs might be affected by long-term culture. Particular myogenic differentiation markers including myogenin and desmin were detected to be expressed in low levels both in early and late passages, whereas myogenin was found to be reduced during the late passages. Levels of a-SMA and tropomyosin were also detected to be similar in both early and late passages. However, myogenic markers ACTa, ACTb, and myosin IIa were detected to be reduced in P100. These results indicate that, in order to evaluate the lineage differentiation of stem cells, particular markers should be assessed in terms of both gene and protein levels.

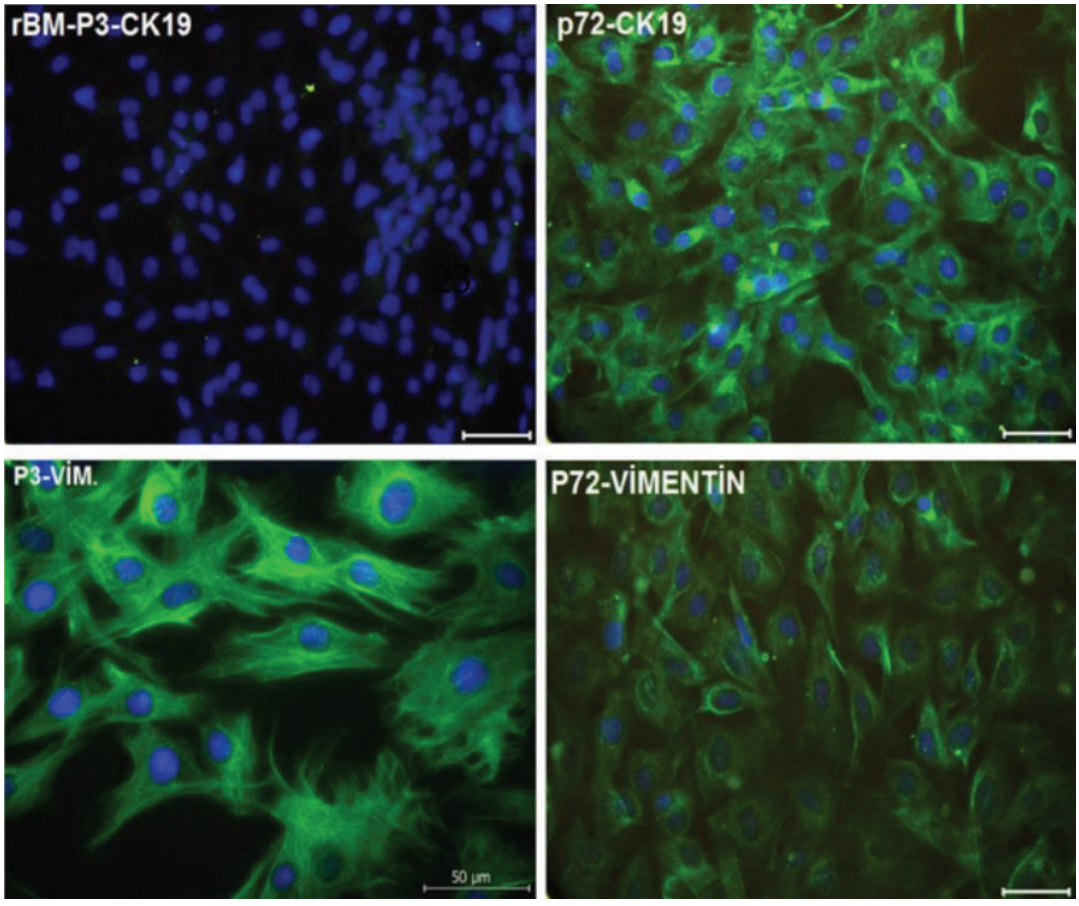

Figure 2. Localizations of cytokeratin 19 (green) and vimentin (green) in cultured rat bone marrow mesenchymal stem cells. P3: Passage 3. P72: Passage 72. Nuclei were labeled with DAPI (blue). All experiments were repeated 3 times. Scale bars: 50 µm.

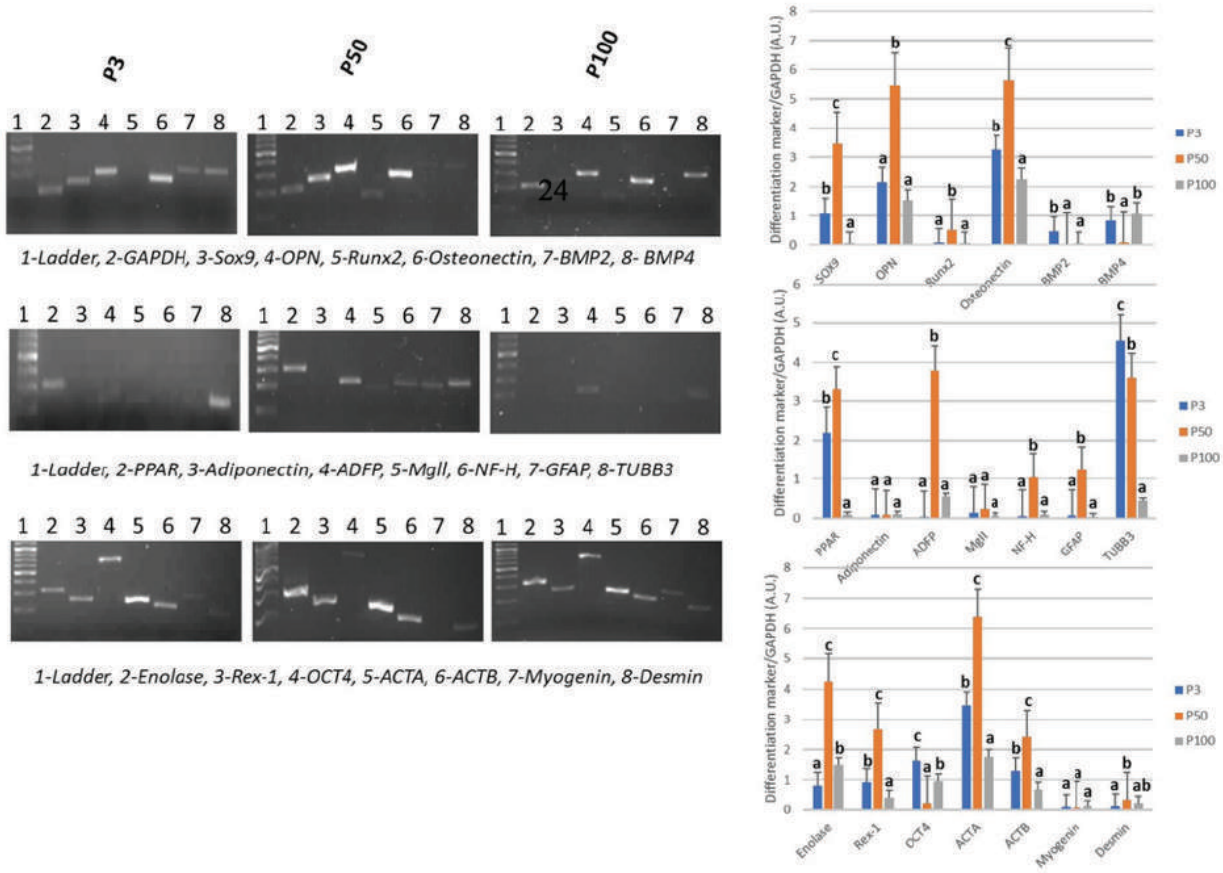

Figure 3. Reverse transcription-polymerase chain reaction bands and graphics of mathematical values of ImageJ evaluations of embryonic stem cell (Rex-1 and Oct4) and differentiation (Sox-9, osteopontin, Runx2, osteonectin [SPARC], BMP-2, BMP-4, PPAR, adiponectin, ADFP, MgII, NF-H, GFAP, TUBB3, Eno2, ACTA, ACTB, myogenin, and desmin) markers in cultured rat bone marrow mesenchymal stem cells. Values are presented as mean ± SEM. Different letters mark statistical significance ($p<0.05$) (one-way ANOVA, Holm-Sidak method). P3: Passage 3. P50: Passage 50. P100: Passage 100. All experiments were repeated 3 times.

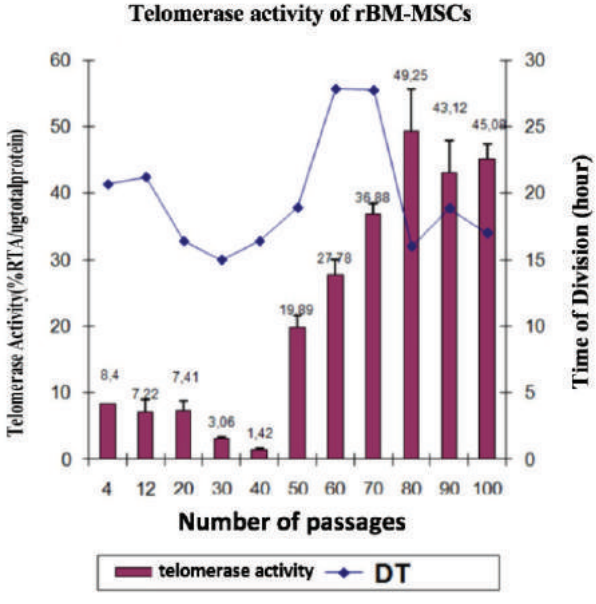

Figure 4. Telomerase activity assessment of cultured rat bone marrow mesenchymal stem cells. Values are presented as mean ± SEM. All experiments were repeated 3 times.

Autophagy has been shown to effect the inhibition of continuous growth of precancerous cells and suppression of cancer [64]. As reviewed by Kocaturk et al. [65], autophagy leads to the removal of damaged macromolecules or organelles, such as mitochondria [66], ER [67], ribosomes [68], and lipid droplets [69]. We have also revealed ultrastructural changes in BM-MSCs at late passages, including a smaller number of organelles as well as a high number of autophagic vacuoles in the cytoplasm, which might be an indication of tumorigenic cells with increased rates of autophagy. Telomere length displays the proliferative potential of somatic cells [70]. Telomerase activity levels and telomere lengths were investigated in order to examine the safety of long-term cultured hMSCs in previous studies [56,71]. There are conflicting reports regarding the telomerase activity of these cells. It was shown that telomerase activity in hMSCs during long-term culture was not altered and remained at a very low level, and telomere lengths of hMSCs were remarkably decreased at late passages [71], while other studies showed that the telomerase activity of cultured hMSCs decreased and these cells displayed telomere shortening during serial passaging

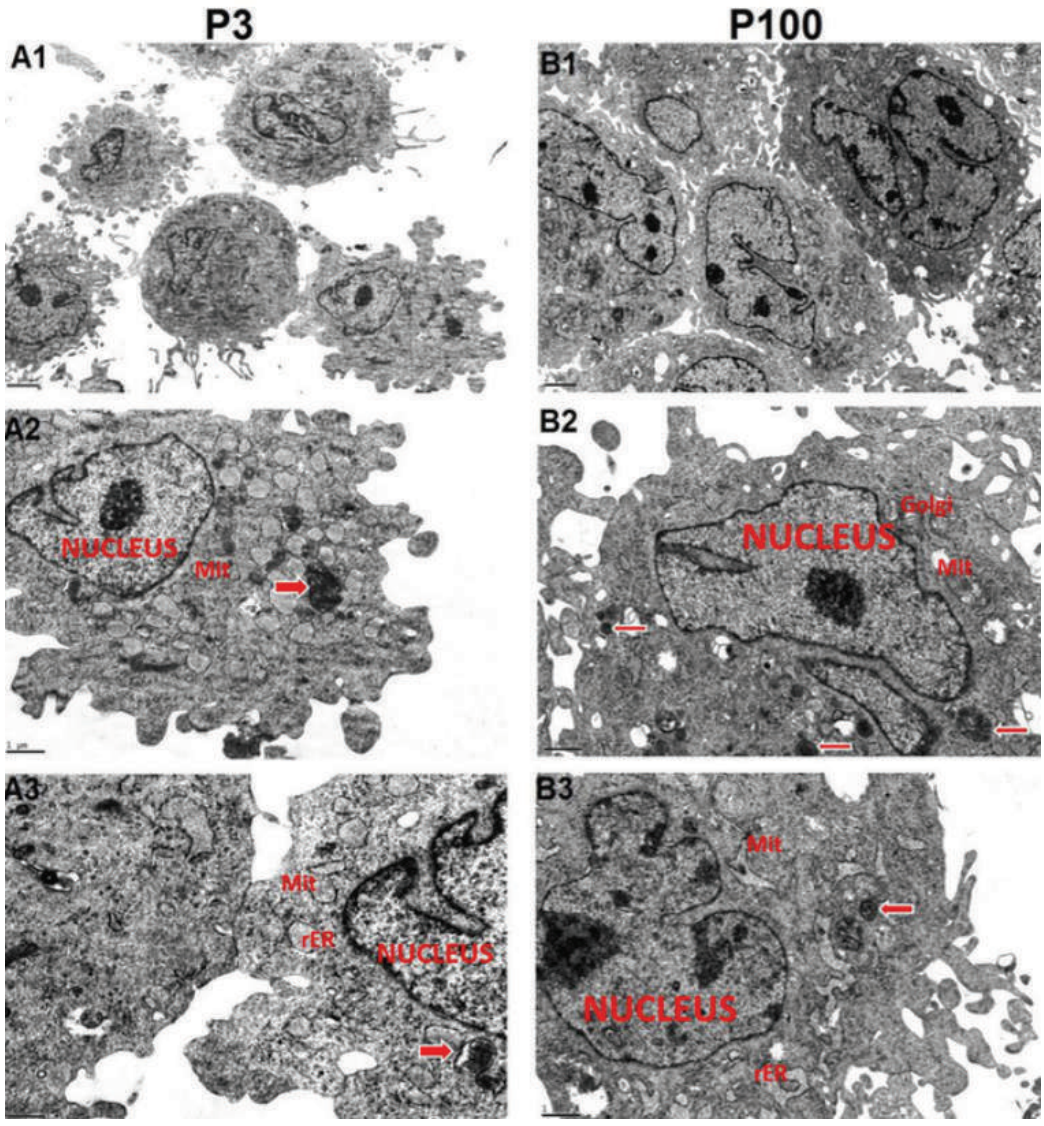

Figure 5. Electron micrographs of cultured rat bone marrow mesenchymal stem cells. P3: Passage 3. P100: Passage 100. Rough endoplasmic reticulum (rER), mitochondria (Mit), Golgi apparatus (Golgi), and autophagic vacuoles (arrows) are marked. All experiments were repeated 3 times.

[56,72,73]. Some reports revealed senescence in the culture ultimately [55]. However, in our study, we have found increased telomerase activity of BM-MSCs in late passages, consistent with particular reports revealing several abnormalities in long-term cultured MSCs including increased telomerase activity [21,22].

Rodent BM-MSCs and hMSCs have displayed some common surface antigens such as CD29, CD90, and CD105, used for MSC characterization [74]. Gene expression profiling of MSCs from rodents has revealed a high degree of concordance with hMSCs [75]. The changes in gene expressions and protein levels of rBM-MSCs during long-term culture might also be possible for hMSCs and we believe that for clinical applications following long-term culture of these cells, data obtained from both humans and rodents must be considered.

Conclusion

The data obtained from this study reveal that long-term culture of rBM-MSCs leads to changes in the MSC characteristics of these cells as well as increased tumorigenic risk via increased telomerase activity. In order to provide efficiency of differentiation potential and safety regarding tumor formation risk of cultured MSCs for cellular therapy, further phenotypic and functional investigations as well as genetic characterizations of MSCs must be conducted.

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Ethics

Ethics Committee Approval: Animal housing and experiments were approved by the local animal care committee (Kocaeli University, HAEK/33-4) according to the institutional guidelines and national animal welfare standards.

Authorship Contributions

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RESEARCH ARTICLE

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Hepatitis B Reactivation Rate and Fate Among Multiple Myeloma Patients Receiving Regimens Containing Lenalidomide and/or Bortezomib

Lenalidomid ve/veya Bortezomib İçeren Tedavi Alan Multipl Myelom Hastalarında Hepatit B Reaktivasyon Sıklığı ve Sonuçları

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Abstract

Objective: Reactivation of the hepatitis B virus (HBV) refers to an increase in HBV replication in a patient with inactive or resolved HBV. In this retrospective study, our aim is to present and compare HBV reactivation in multiple myeloma (MM) patients who received lenalidomide and/or bortezomib at any time during treatment, evaluate the factors associated with reactivation, and demonstrate the outcome of patients.

Materials and Methods: We evaluated 178 MM patients who received lenalidomide (n=102) and/or bortezomib (n=174) during their treatment schedules. The HBsAg, anti-HBc, anti-HBs, HBeAg, and anti-HBe were detected by chemiluminescence by ARCHITECT lab analyzers using commercially available kits (Abbott, USA). HBV-DNA titers were determined by quantitative PCR. The results were evaluated by IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA).

Results: HBV reactivation was diagnosed in 6 patients (3%) after bortezomib and in 8 patients (8%) after bortezomib and lenalidomide. Three of the patients in each group had HBsAg+, HBeAg+, AntiHBeAg-, AntiHBc-, and AntiHBS+ status, whereas 5 patients in the bortezomib- and lenalidomide-treated group and 3 patients in the bortezomib-treated group had HBsAg-, HBeAg-, AntiHBeAg-, AntiHBc-, and AntiHBS+ status prior to treatment. There were no statistical differences observed between HBV reactivation in the bortezomib-treated or bortezomib- and lenalidomide-treated groups in terms of age at diagnosis, sex, International Staging System subtype, frequency of extramedullary disease, dialysis requirement, or receiving of autologous stem cell transplantation. In patients who received antiviral prophylaxis, a higher incidence of HBV reactivation was detected in HBsAg-positive patients compared to HBsAg-negative patients (4/4, 100% vs. 2/7, 29%; p=0.045). The 3-year and 5-year

Öz

Amaç: Hepatit B virüs (HBV) reaktivasyonu, HBV enfeksiyonunun inaktifleştiği veya iyileştiği hastalarda virüs replikasyonunun artışıdır. Bu geriye dönük çalışmada amacımız tedavilerinin herhangi bir döneminde lenalidomid ve/veya bortezomib alan multipl myelom (MM) hastalarında HBV reaktivasyonunu göstermek, reaktivasyonla ilişkili faktörleri ve sağkalımlarını değerlendirmektir.

Gereç ve Yöntemler: Tedavileri sırasında lenalidomid (n=102) ve/veya bortezomib (n=174) alan 178 MM hastası değerlendirilmiştir. ARCHITECT lab analiz cihazlarıyla HBsAG, anti-HBc, anti-HBs, HBeAg, anti-HBe piyasada bulunan kitlerle (Abbott, ABD) kemiluminesans yoluyla, HBV-DNA titreleri kuantitative PCR ile tespit edilmiştir. Sonuçların değerlendirilmesinde IBM SPSS 20.0 (IBM Corp., Armonk, NY, ABD) kullanılmıştır.

Bulgular: HBV reaktivasyonu, bortezomib kullanan 6 hastada (%3) ile bortezomib ve lenalidomid alan 8 hastada (%8) tespit edilmiştir. Tedavi öncesi iki gruptan 3 hastada HBsAg+, HBeAg+, AntiHBeAg-, AntiHBc-, ve AntiHBS+ saptanırken, bortezomib ve lenalidomid alan 5 hastada ve sadece bortezomib alan 3 hastada HBsAg-, HBeAg-, AntiHBeAg-, AntiHBc-, ve AntiHBS+ saptanmıştır. Bortezomib veya bortezomib ve lenalidomid ile tedavi edilen gruplar arasında HBV reaktivasyonu ile tanı anındaki yaş, cinsiyet, evre, ekstramedüllar hastalık, diyaliz ihtiyacı veya otolog kök hücre nakil sıklığı arasında istatistiksel olarak fark saptanmamıştır. Antiviral profilaksi alan grupta, HBsAg pozitif olan hastalarda HBsAg negatif olan hastalara göre daha sık HBV reaktivasyonu tespit edilmiştir (4/4, %100 ile 2/7, %29; p=0,045). HBV reaktivasyonu gelişen ve gelişmeyen hastalarda 3-yıllık ve 5 yıllık sağkalımlar benzerdir (%83 ile %84, %73 ile %74, p=0,84).

Sonuç: Sadece HBsAg pozitif hastalar değil HBsAg negatif hastalar da yakından takip edilmelidir.

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Abstract

overall survival rates were similar in patients with or without HBV reactivation (83% vs. 84%, 73% vs. 74%, p=0.84).

Conclusion: Close follow-up is recommended for not only HBsAg-positive but also HBsAg-negative patients.

Keywords: Hepatitis B reactivation, Bortezomib, Lenalidomide, Multiple myeloma, Antiviral therapy

Öz

Anahtar Sözcükler: Hepatit B reaktivasyonu, Bortezomib, Lenalidomid, Multipl myelom, Antiviral terapi

Introduction

The hepatitis B virus (HBV) represents a serious health concern worldwide. HBV is intermediately endemic in Turkey, where seropositivity of the hepatitis B surface antigen (HBsAg) has been reported to range between 2% and 7% [1,2]. When there is an increase in HBV replication in a patient with inactive or resolved HBV, this is referred to as reactivation of HBV. Commonly, it occurs in HBsAg-positive cancer patients; HBsAg-negative patients with positive anti-hepatitis B core antibody (anti-HBc) and/or anti-hepatitis B surface antibody (anti-HBs) also carry an increased risk [3,4,5,6]. Cytotoxic chemotherapy, monoclonal antibody treatments, and bone marrow transplantation have been demonstrated as risk factors for HBV reactivation [7,8,9,10]. HBV infection may result in severe hepatic dysfunction and fulminant hepatitis [11,12]. In current treatment guidelines, a prophylactic nucleoside analogue is recommended to be continued for at least 6 months after discontinuation of immunosuppressive therapy [13,14].

Multiple myeloma (MM) is characterized by malignant proliferation of plasma cells. Bortezomib, a proteasome inhibitor that disrupts the cell-signaling pathways, has shown anti-myeloma activity and has been recommended as a standard treatment in patients with newly diagnosed and relapsed MM [15]. Lenalidomide is a potent oral immunomodulatory drug with direct tumoricidal, anti-angiogenic, and immunostimulatory effects [16]. Both bortezomib and lenalidomide show remarkable activity in MM patients with manageable toxicity profiles. There are several case reports and studies on MM showing HBV reactivation under bortezomib treatment [17,18,19], but the literature is scarce regarding HBV reactivation after lenalidomide treatment. In this retrospective study, our aim is to present and compare HBV reactivation in our MM patients who received lenalidomide and/or bortezomib at any time during treatment, evaluate the factors associated with reactivation, and demonstrate the outcome of patients.

Materials and Methods

We retrospectively included 178 MM patients who were diagnosed between 2002 and 2015 at the Ankara University Faculty of Medicine's Department of Hematology. Informed consent was obtained from all participants. International Staging System (ISS) scores, counts of hemoglobin and lymphocytes, extramedullary involvement, and plasma cell percentage in bone marrow were recorded at the initiation of chemotherapy. The patients' data were analyzed via electronic medical records. All patients received lenalidomide and/or bortezomib during their treatment schedules, whether for induction, relapse, or post-induction maintenance.

Hepatitis B surface antigen (HBsAg), hepatitis B core antibody (anti-HBc), hepatitis B surface antibody (anti-HBs), hepatitis B e-antigen (HBeAg), and hepatitis B e-antibody (anti-HBe) were detected by chemiluminescence by ARCHITECT lab analyzers using commercially available kits (Abbott, USA) before each line of chemotherapy. HBV DNA titers were determined by quantitative PCR. Patients with active hepatitis B prior to chemotherapy were excluded from the study. If a patient was HBsAg-positive before chemotherapy or HBsAg-negative but positive for anti-HBc, HBeAg, and/or anti-HBe, a prophylactic antiviral drug was administered during and for at least 6 months after chemotherapy. Hepatitis B serologies were closely monitored in patients who were HBsAg-negative but seropositive for anti-HBc and/or anti-HBs, both before autologous peripheral stem cell transplantation and if liver enzyme abnormality occurred, to determine reactivation. Reactivation was defined as 1) loss of anti-HBs and reoccurrence of HBsAg in HBsAg-negative and/or anti-HBs-positive patients and 2) increase of HBV DNA level by at least a factor of 10 or an absolute count of HBV DNA reaching $1x10^9$ copies/mL. Antiviral treatment was initiated as soon as reactivation was detected. None of the patients had received hepatitis B vaccinations.

Statistical Analysis

The results were evaluated by IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA). All numerical values are given as medians with distribution ranges. We used the Pearson chi-square test or the Fisher exact test to compare categorical variables. The Kaplan-Meier method was used for survival curves. In evaluating the results, $p<0.05$ was considered statistically significant.

Results

The median age of the 178 MM patients was 62 (range: 34-86). The baseline characteristics of the study population are summarized in Table 1. At diagnosis, the mean lymphocyte count and hemoglobin concentration were respectively 1936/mL (range: 200-13200) and 11.5 g/dL (range: 7-16). Subjects received a median of 3 lines of treatment (range: 1-7). First-line regimens were as follows: for 80 patients (45%), bortezomib + cyclophosphamide + dexamethasone (VCD); 40 patients (22%), vincristine + doxorubicin + dexamethasone (VAD); 21 patients (12%), cyclophosphamide + dexamethasone (Cy-Dex); 15 patients (8%), bortezomib + dexamethasone (Vel-Dex); 12 patients (7%), bortezomib + melphalan + prednisolone (VMP); 7 patients (4%), melphalan + prednisolone + thalidomide (MPT); and 3 patients (2%), lenalidomide + dexamethasone (Len-Dex). In total, 124 patients (70%) were treated with high-dose chemotherapy and underwent autologous hematopoietic stem cell transplantation (auto-HSCT). During the treatment period, 102 patients (57%) received 25 mg/day lenalidomide with dexamethasone or 10 mg/day lenalidomide as a single agent; 174 patients (98%) received 1.3 mg/m^2 bortezomib in combination with dexamethasone, cyclophosphamide plus dexamethasone, lenalidomide plus dexamethasone, or melphalan plus prednisone. Bortezomib and lenalidomide were administered to 98 patients (55%). Disease relapse was detected in 122 patients (69%). During follow-up, 41 patients (23%) had progressive disease and 37 patients (21%) died. Herpes virus reactivation (herpes zoster) was detected in 15 patients (8%), 2 of whom received lenalidomide and bortezomib.

Table 1. Study population characteristics.

Variables	
Age, n (%)	
<65 years	114 (64%)
≥65 years	64 (36%)
Sex, n (%)	
Male	102 (57%)
Female	76 (43%)
MM subtype, n (%)	
IgA kappa	28 (16%)
IgA lambda	13 (7%)
IgG kappa	76 (43%)
IgG lambda	34 (19%)
Kappa light chain	13 (7%)
Lambda light chain	6 (3%)
Others (IgD, nonsecretory, biclonal)	8 (4%)
International Staging System, n (%)	
I	50 (28%)
II	55 (31%)
III	73 (41%)
Extramedullary disease, n (%)	
Yes	87 (49%)
No	91 (51%)
Hypogammaglobulinemia, n (%)	
Yes	178 (100%)
Dialysis requirement, n (%)	
Yes	15 (8%)
No	163 (92%)

Among all subjects, HBsAg was positive in 4 patients (2%) at diagnosis. Among HBsAg-positive patients, 3 patients had HBV DNA levels of >1000 IU/mL. For prophylaxis, patients received either 100 mg of lamivudine (n=2) or 245 mg of tenofovir (n=2) daily, which continued for 6 months after termination of treatment for MM, except in 1 patient who died of infection in the second month of chemotherapy. Among HBsAg-negative patients who were positive for anti-HBc, anti-HBe, or HBeAg (n=7), 6 patients received 100 mg/daily lamivudine, and 1 patient had entecavir at 0.5 mg/daily for prophylaxis that was prolonged for 6 months after treatment of MM. All HBsAg-negative patients had HBV DNA levels of <500 IU/mL. No significant differences were observed in sex, age at diagnosis, ISS stage, subtype, frequency of extramedullary disease, or dialysis requirements between HBsAg-positive and HBsAg-negative patients.

Hepatitis B reactivation was observed in 14 patients (8%). The patients' HBV and prophylaxis statuses at diagnosis are summarized in Table 2. The median time from diagnosis to hepatitis B reactivation was 32 months (range: 2-78). Of 174 bortezomib-treated patients, 6 had HBV reactivation (3%). HBV reactivation was detected in 8 patients out of the 98 patients who received lenalidomide and bortezomib (8%). Reactivation developed in 4 patients (100%) who were HBsAg-seropositive at diagnosis, while 10 patients (6%) were initially HBsAg-negative. HBsAg-positive patients who received prophylaxis had significantly higher incidence of

Table 2. HBV and prophylaxis status at diagnosis of patients with reactivation.

	HBsAg+ HBeAg+ AntiHBeAg- AntiHBc- AntiHBS+ / Prophylaxis (+), n (%)	HBsAg- HBeAg- AntiHBeAg- AntiHBc- AntiHBS+ / Prophylaxis (-), n (%)	Total reactivation, n (%)
Bortezomib (diagnosis)	3 (21%)		3 (21%)
Bortezomib (relapse)		3(21%)	3 (21%)
Lenalidomide	3 (21%)	5 (38%)	8 (58%)
Total	6 (42%)	8 (58%)	14 (100%)

HBsAg: Hepatitis B surface antigen, HBeAg: hepatitis B e-antigen.

hepatitis B reactivation than HBsAg-negative patients (4/4, 100% vs. 2/7, 29%; p=0.045). The 3-year and 5-year overall survival (OS) was similar in patients with and without HBV reactivation (83% vs. 84%, 73% vs. 74%, p=0.84) (Figure 1). Details of patients with HBV reactivation are given in Tables 3 and 4. Patient number 5 in Table 4 had HBV reactivation under lamivudine prophylaxis and died because of bacterial infection following 2 months of chemotherapy. Chemotherapies were suspended until liver function tests and HBV DNA levels were decreased.

Baseline characteristics including MM subtype, extramedullary disease, median age, sex, ISS, incidence of herpes infection, and auto-HSCT did not differ between the bortezomib- and lenalidomide-treated vs. bortezomib-treated groups that had HBV reactivation. Lenalidomide treatment was interrupted in 4 (50%) of the patients due to progression of disease. Except for 1 patient, all patients underwent autologous stem cell transplantation (ASCT), and 1 patient who received a second ASCT for a secondary refractory disease had progression to cirrhosis following high-dose melphalan. After treatment with tenofovir, HBV DNA titers decreased in all patients and became undetectable in 4 of the 8 patients. In patients treated with only bortezomib, all patients received dexamethasone, and 4 of 6 patients underwent ASCT. Progression of disease after bortezomib was detected in 2 patients. Among these 6 patients, 4 patients were treated with tenofovir (2 achieved HBV DNA negativity), and the other 2 were treated with lamivudine. The response could not be evaluated for patient number 5, because she died of infection within 2 months of the initiation of chemotherapy (Tables 3 and 4).

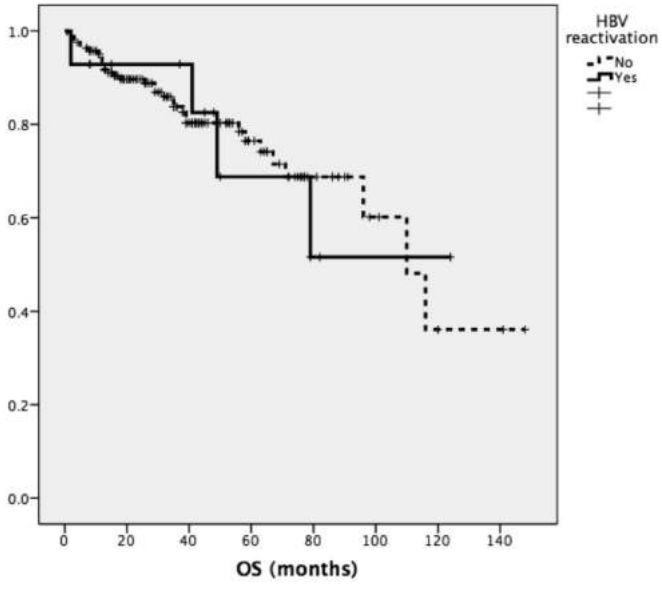

Figure 1. Comparison of overall survival in patients with or without hepatitis B virus reactivation (p=0.84).

OS: Overall survival, HBV: hepatitis B virus.

Discussion

Generally, HBV reactivation has been documented in HBsAg-positive cancer patients [20]. In one study, the rate of HBsAg seropositivity in MM cases was higher than in patients with acute leukemia [21]. Antiviral prophylaxis is the critical step in managing HBsAg-positive patients undergoing systemic chemotherapy [13,22]. Clinical studies showed a reduction of HBV activation rate, severity of hepatitis, and mortality with prophylaxis [23,24]. The American Gastroenterological Association suggests antiviral drugs with high barriers to resistance rather than lamivudine for at least 6 months in high-risk patients [14]. Previously, in our experience, because HBV reactivation in a lamivudine-untreated group occurred 12 months after the individual's chemotherapy had been discontinued, lamivudine prophylaxis was maintained for a year following discontinuation of any chemotherapy [25,26]. The choice of lamivudine or a shorter duration of prophylaxis might have caused the HBV reactivation that occurred in all HBsAg-positive patients who received prophylaxis in this cohort. One patient with HBV reactivation died under lamivudine prophylaxis within 2 months of chemotherapy. Recent data have shown HBV reactivation in HBsAg-negative lymphoma patients who received rituximab plus steroid combination chemotherapy [3,4,27]. Lee et al. [28] demonstrated HBV reactivation in 5.2% of 230 MM patients. All of these patients had HBsAg-negative/anti-HBc-positive serology. Similarly, we found that the incidence of HBV reactivation in HBsAg-negative patients was 6%. The preferred prophylaxis was lamivudine in HBsAg-negative patients.

This is the first study of the recently developed agents lenalidomide and bortezomib in MM, and we observed an incidence of HBV reactivation of 8%. HBV reactivation after bortezomib was described in previous case reports [17,18,19]. Mya et al. [29] found an incidence of HBV reactivation of 5.5% in 273 MM patients after bortezomib and dexamethasone salvage therapy; one of the HBV reactivation cases was HBsAg negative initially. Li et al. [30] conducted one of the largest retrospective studies of HBV reactivation in patients who received regimens containing bortezomib. HBV reactivation was observed in 6 HBsAg-positive and 2 HBsAg-negative cases from a total of 139 patients. OS and progression-free survival were shorter in HBsAg-positive MM patients compared to HBsAg-negative patients (p<0.01) [30]. We did not detect any survival advantage in HBsAg-negative patients in our study. Bortezomib dysregulated the cell-mediated immunity that played an important role in the suppression of varicella zoster virus reactivation [31]. HBV is another DNA virus that remains dormant in human hosts. Bortezomib may promote HBV reactivation by altering the number and functions of CD8 T cells and CD56 NK cells [29]. In addition, MM itself causes immunodeficiency that involves various parts of the immune system, including B, dendritic, T, and NK

cell dysfunction. HBV reactivation after lenalidomide has not been reported previously in the literature. König et al. [32] reported 10 varicella zoster virus or other complicated VSC/herpes simplex virus infections from 93 MM patients who received lenalidomide-based chemotherapy, which may have resulted from the immunomodulation effects of lenalidomide. Since the patients in our study were heavily pretreated, and there was no control group assigned for patients not

Table 3. Patients with lenalidomide-related HBV reactivation.

Patient No.	Sex/Age	Subtype/ISS	Treatment lines/ Response	Hepatitis B markers before treatment/ Prophylaxis	Time to reactivation after lenalidomide withdrawal (months)	Hepatitis B markers after reactivation	Antiviral treatment/ Response	OS/Outcome
1	M/56	Lambda/II	VCD, ASCT, Len-Dex/CR	HBsAg-, HBeAg-, AntiHBeAg-, AntiHBc-, AntiHBS+/-	11	HBsAg+, HBeAg+, AntiHBeAg-, AntiHBc+, AntiHBs-	Tenofovir/ HBV DNA decreased	24/Alive, Liver Bx: Ishak 4, Stage 1
2	M/75	IgGKappa/II	VMP, VP, ASCT, Len-Dex/VGPR	HBsAg-, HBeAg-, AntiHBeAg-, AntiHBc-, AntiHBS+/-	18	HBsAg+, HBeAg+, AntiHBeAg-, AntiHBc+, AntiHBs-	Tenofovir/ HBV DNA decreased	46/Alive
3	M/61	IgGKappa/III	VAD, Vel-Dex, ASCT, Lenalidomide, Benda-Dex, Pomalidomide-Dex, ASCT/ Progression	HBsAg-, HBeAg-, AntiHBeAg-, AntiHBc-, AntiHBS+/-	5	HBsAg+, HBeAg+, AntiHBeAg-, AntiHBc+, AntiHBs-	Tenofovir/ HBV DNA decreased	37/Alive
4	F/43	IgALambda/ III	Cy-Dex, ASCT, Vel-Dex, Len-Dex, Carfilzomib/ Progression	HBsAg-, HBeAg-, AntiHBeAg+, AntiHBc-, AntiHBS+/ Lamivudine	7	HBsAg+, HBeAg+, AntiHBeAg-, AntiHBc+, AntiHBs-	Tenofovir/ HBV DNA-	49/Exitus, Disease Progression
5	F/62	IgGLambda/ III	VAD, Vel-Dex, ASCT, Thalidomide, Lenalidomide, Benda-Dexa, Carfilzomib-Dexa/ VGPR	HBsAg-, HBeAg-, AntiHBeAg-, AntiHBc-, AntiHBS+/-	10	HBsAg+, HBeAg+, AntiHBeAg-, AntiHBc+, AntiHBs-	Tenofovir/ HBV DNA-	79/Exitus
6	M/69	IgGKappa/III	Vel-Dex, ASCT, Len-Dex/CR	HBsAg-, HBeAg-, AntiHBeAg-, AntiHBc-, AntiHBS+/-	13	HBsAg+, HBeAg+, AntiHBeAg-, AntiHBc+, AntiHBs-	Tenofovir/ HBV DNA-	67/Alive
7	F/61	IgGLambda/ II	VCD, ASCT, Len-Dex/CR	HBsAg-, HBeAg-, AntiHBeAg+, AntiHBc-, AntiHBS+/ Lamivudine	13	HBsAg+, HBeAg+, AntiHBeAg-, AntiHBc+, AntiHBs-	Tenofovir/ HBV DNA-	21/Alive
8	F/63	IgGKappa/III	VAD, Vel-Dex, MPT, DCEP, Len-Dex, Benda-Dex	HBsAg+, HBeAg+, AntiHBeAg-, AntiHBc-, AntiHBS+/ Lamivudine	10	HBsAg+, HBeAg+, AntiHBeAg-, AntiHBc+, AntiHBs-	Tenofovir/ HBV DNA decreased	54/Alive

ASCT: Autologous stem cell transplantation, Anti-HBc: hepatitis B core antibody, AntiHBe: hepatitis B e-antibody, Anti-HBs: hepatitis B surface antibody, Benda: bendamustine, CR: complete remission, Cy-Dex: cyclophosphamide + dexamethasone, DCEP: dexamethasone + cyclophosphamide + etoposide + cisplatin, HBsAg: hepatitis B surface antigen, HBeAg: hepatitis B e-antigen, Len-Dex: lenalidomide + dexamethasone, MPT: melphalan + prednisolone + thalidomide, VCD: bortezomib + cyclophosphamide + dexamethasone, VAD: vincristine + doxorubicin + dexamethasone, Vel-Dex: bortezomib + dexamethasone, VMP: bortezomib + melphalan + prednisolone, VGPR: very good partial remission.

receiving either bortezomib or lenalidomide, it is not clear whether the HBV reactivation was driven by bortezomib and/or lenalidomide. Multiple lines of treatment may cause severe immunosuppression that results in an increased risk of HBV reactivation [33].

Auto-HSCT was shown to be a risk factor for HBV reactivation in several reports. Uhm et al. [34] retrospectively analyzed changes in HBV serology prior to and following auto-HSCT and concluded that 6 of 129 HBsAg-negative MM patients became HBsAg-positive, possibly related to dysfunction of humoral immunity. Lee et al. [28] determined auto-HSCT to be an independent risk factor (p=0.025) for HBV reactivation and suggested that regular monitoring should be considered in patients who underwent auto-HSCT [28]. However, we did not find a significant correlation between HBV reactivation and auto-HSCT.

HBV reactivation may be variable, from mildly clinical to hepatic failure. Development of fatal hepatitis following HBV reactivation was reported in CD20-positive lymphoma patients who received rituximab and steroid combination treatment [7,27]. Yoshida et al. [35] described HBV reactivation in 2 HBsAg-seronegative MM patients resulting in liver damage. Similarly, one of our heavily pretreated patients with HBV reactivation had disease with liver damage progressing to cirrhosis following a second ASCT treatment.

Conclusion

We found that the incidence of HBV reactivation was notable in patients who received lenalidomide- and/or bortezomib-based chemotherapy. Most of the patients were heavily pretreated, which might have caused immune deficiencies. HBV reactivation was diagnosed in both HBsAg-positive and HBsAg-negative

Table 4. Patients with bortezomib-related HBV reactivation.

Patient No.	Sex/ Age	Subtype/ISS	Treatment lines/ Response	Hepatitis B markers before treatment/ Prophylaxis	Time to reactivation after bortezomib withdrawal (months)	Hepatitis B markers after reactivation	Antiviral treatment/ Response	OS/ Outcome
1	M/67	IgALambda/I	VAD, Vel-Dex, ASCT/PR	HBsAg-, HBeAg-, AntiHBeAg-, AntiHBc-, AntiHBS+/-	23	HBsAg+, HBeAg+, AntiHBeAg-, AntiHBc+, AntiHBs-	Tenofovir/ HBV DNA-	41/Exitus
2	M/61	IgGKappa/II	VCD, ASCT/VGPR	HBsAg+, HBeAg+, AntiHBeAg-, AntiHBc-, AntiHBS+/ Tenofovir	7	HBsAg+, HBeAg+, AntiHBeAg-, AntiHBc+, AntiHBs-	Tenofovir/ HBV DNA decreased	8/Alive
3	M/45	IgGKappa/II	VAD, Vel-Dex, ASCT, Lenalidomide, VCD, Benda-Dex, ASCT, Thalidomide/ Progression	HBsAg-, HBeAg-, AntiHBeAg-, AntiHBc-, AntiHBS+/-	5	HBsAg+, HBeAg+, AntiHBeAg-, AntiHBc+, AntiHBs-	Tenofovir/ HBV DNA-	50/Alive
4	F/66	IgKappa/II	VCD/VGPR	HBsAg+, HBeAg+, AntiHBeAg-, AntiHBc-, AntiHBS+/ Tenofovir	7	HBsAg+, HBeAg+, AntiHBeAg-, AntiHBc+, AntiHBs-	Tenofovir/ HBV DNA decreased	8/Alive
5	F/62	IgGkappa/III	VCD/PR	HBsAg+, HBeAg+, AntiHBeAg-, AntiHBc-, AntiHBS+/ Lamivudine	-	HBsAg+, HBeAg+, AntiHBeAg-, AntiHBc+, AntiHBs-	Lamivudine/ NA	2/Exitus
6	F/66	Kappa/I	VAD, ASCT, Vel-Dex, Len-Dex/ VGPR	HBsAg-, HBeAg-, AntiHBeAg-, AntiHBc-, AntiHBS+/-	5	HBsAg+, HBeAg+, AntiHBeAg-, AntiHBc-, AntiHBs-	Lamivudine/ HBV DNA decreased	15/Alive

ASCT: Autologous stem cell transplantation, Anti-HBc: hepatitis B core antibody, AntiHBe: hepatitis B e-antibody, Anti-HBs: hepatitis B surface antibody, Benda: bendamustine, CR: complete remission, Cy-Dex: cyclophosphamide + dexamethasone, DCEP: dexamethasone + cyclophosphamide + etoposide + cisplatin, HBsAg: hepatitis B surface antigen, HBeAg: hepatitis B e-antigen, Len-Dex: lenalidomide + dexamethasone, MPT: melphalan + prednisolone + thalidomide, VCD: bortezomib + cyclophosphamide + dexamethasone, VAD: vincristine + doxorubicin + dexamethasone, Vel-Dex: bortezomib + dexamethasone, VMP: bortezomib + melphalan + prednisolone, VGPR: very good partial remission.

patients. This finding suggests a close follow-up strategy in HBsAg-positive patients as well as HBsAg-negative but anti-HBc-, HBeAg-, or anti-HBe-positive MM patients, plus early initiation of active antiviral therapy.

Ethics

Ethics Committee Approval: N/A.

Informed Consent: N/A.

Authorship Contributions

Surgical and Medical Practices: P.A.A., E.A., M.B., R.İ.; Concept: P.A.A., E.A., M.B.; Design P.A.A., E.A., M.B.; Data Collection or Processing: P.A.A., E.A., M.Y.; Analysis or Interpretation: P.A.A., E.A., M.Y.; Literature Search: P.A.A., E.A.; Writing: P.A.A., E.A., M.B., R.İ.

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BRIEF REPORT

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Fertility in Patients with Thalassemia and Outcome of Pregnancies: A Turkish Experience

Talasemi Hastalarında Fertilite ve Gebelik Sonuçları: Türk Deneyimi

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Abstract

Objective: In recent years, the rates of marriage and pregnancy are increasing in patients with thalassemia major. The aim of the present study was to investigate the fertility rate of thalassemic patients and the course of pregnancies in terms of mother and infant health.

Materials and Methods: In this observational study patients with major hemoglobinopathy were evaluated regarding marital status, the need for assisted reproductive techniques, fertility rate, iron status, and pregnancy complications.

Results: Seventeen female patients gave birth to 21 healthy infants. About one-third of the patients needed assisted reproductive techniques. Thalassemia major patients showed increased serum ferritin levels from 1203±1206 µg/L at baseline to 1880±1174 µg/L at the end of pregnancy. All babies are still alive and healthy.

Conclusion: Pregnancy in patients with thalassemia can be safe for the mother and newborn with close monitoring and a multidisciplinary approach.

Keywords: Thalassemia, Fertility, Pregnancy

Öz

Amaç: Son yıllarda, talasemi majör olgularının evlilik ve gebelik oranları giderek artmaktadır. Bu çalışmanın amacı, talasemi hastalarının fertilite oranlarının araştırılması ve anne ve bebek sağlığı açısından gebelik sonuçlarının değerlendirilmesidir.

Gereç ve Yöntemler: Bu gözlemsel çalışmada; majör hemoglobinopatili hastalar; evlenme ve çocuk sahibi olma oranları, yardımcı üreme tekniklerine gereksinimleri, demir statüleri ve gebelik komplikasyonları açısından değerlendirilmiştir.

Bulgular: On yedi talasemik kadın hasta, toplam 21 sağlıklı bebek doğurmuştur. Hastaların üçte biri bebek sahibi olabilmek için yardımcı üreme tekniğine ihtiyaç duymuştur. Talasemi majör olgularının serum ferritin değerleri hamileliğin başında ortalama 1203±1206 ug/L saptanmış olup, hamileliğin sonunda 1880±1174 ug/L seviyesine yükselmiştir. Tüm bebekler halen hayatta ve sağlıklıdırlar.

Sonuç: Talasemi olgularında hamilelik süreci yakın takip ve multidisipliner yaklaşım ile beraber güvenli olarak geçirilebilecektir.

Anahtar Sözcükler: Talasemi majör, Fertilite, Gebelik

Introduction

Until the new millennium, many medical and social barriers such as limited life expectancy resulting from iron-induced cardiac disease [1,2] and significant morbidities particularly resulting from endocrine complications [1,3,4,5,6] have been main factors in the negative attitudes towards starting a family in the thalassemic population. However, therapeutic advances in the management of thalassemia have significantly improved the quality of life and life expectancy in the past two decades [7,8,9,10,11,12,13] and have consequently encouraged the thalassemic population to marry and have children. This study was conducted to assess the current tendency towards marriage among patients with thalassemia, the reproductive rate of those who wish to have children, and the course of pregnancies with respect to maternal and infant outcomes in one of the largest thalassemia centers of Turkey.

Materials and Methods

One hundred and eighty-four patients (108 females, 76 males) with thalassemia aged above 18 years old were included in this observational study. All male and female patients who wished to have children but suffered from hypogonadotropic hypogonadism (HH) were referred to an infertility clinic. Female patients were carefully assessed for the severity of iron overload by serum ferritin (SF), cardiac T2* magnetic resonance imaging (MRI), liver R2 MRI, cardiac status by echocardiography, and the

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presence of endocrine disturbances. The optimization of iron burden and normalized organ functions in the pre-conception period was strongly suggested. The overall rate of fertility and the course and outcome of the pregnancies were recorded. All pregnancies were followed in close collaboration with an obstetrician. A cardiac workup was performed at 3-month intervals throughout the pregnancies.

Results

Fertility Rate in Female and Male Thalassemia Patients

Fifty of the 184 adult patients were married. Forty-one patients (29 females and 12 males) were married to healthy partners, and nine marriages were composed of thalassemic couples. Seventeen of the 29 female patients (59%) gave birth to 21 healthy babies (three had two pregnancies, and one had twins). Conception was spontaneous in 14 (70%) and was achieved by gonadotrophin stimulation or an assisted reproductive technique (ART) in six female patients. Overall, six of 12 male patients (50%) had seven children spontaneously while the other six, who were receiving hormone replacement therapy, did not yet have a child. Although both male and female infertility was 50%, in our cohort 33% of females but none of the males with HH could have a child.

Thalassemic couples did not wish to give birth to an affected baby. However, in a couple with beta-thalassemia intermedia (TI) and S/beta-thalassemia, spontaneous fertilization occurred. Prenatal diagnosis was performed at the 12th week of gestation and genetic counseling was given to the couple, who decided to give birth to an offspring with S/beta-thalassemia.

Disease Characteristics and the Course of the Pregnancies

The baseline characteristics of pregnant patients are reported in Table 1. The average monthly red cell concentrate (RCC) consumption showed a nonsignificant increase during pregnancy compared to the pre-pregnancy period (14.5±2.4 vs. 12.7±2.4 mL/kg/month) in patients with thalassemia major (TM). Three patients with non-transfusion-dependent thalassemia (NTDT), including TI, S/beta-thalassemia, and hemoglobin H disease, received RCC transfusions of 7.7, 7.2, and 4.2 mL/kg/month, respectively, during pregnancy to maintain the pre-transfusion hemoglobin levels of ≥8 g/dL. New red cell alloantibody formation did not occur in any patients, but cross-match compatible RCC could not be provided to the patient with TI who developed multiple alloantibodies and experienced a hemolytic transfusion reaction before pregnancy. This patient was not transfused with any incompatible RCC during pregnancy. Hemoglobin levels gradually decreased to as low as 6 g/dL and were barely maintained at around 7 g/dL by erythropoietin administration during pregnancy. Ultimately, the patient delivered a healthy full-term baby.

Iron chelation therapy was immediately ceased for all pregnant patients but deferoxamine (DFO) subcutaneous infusions were initiated after the second trimester for two subjects whose SF increased over 2229 and 7199 µg/L, and one revealed a cardiac T2* of 16 ms before pregnancy.

The TM patients had slightly increased SF from baseline (1203±1206 µg/L) until the end of pregnancy (1880±1174 µg/L). None of the patients demonstrated myocardial T2* of <20 ms in the first cardiac MRI obtained after delivery.

Delivery and Outcomes in Newborns

All patients but one underwent a cesarean section following complication-free pregnancies. An ectopic pregnancy and a pregnancy with a fetus with trisomy 21 were terminated. Intrauterine growth retardation (IUGR) was observed in the full-term offspring of two patients with thalassemia major who maintained an average pre-transfusion hemoglobin level of 9.4 g/dL during pregnancy. Four of the 21 births (19%) were preterm (33- and 34-week singletons and 30-week twins).

Four infants were admitted to the neonatal intensive care unit due to prematurity, IUGR, or pneumothorax (Table 2). All infants were breastfed for at least 3 months.

Discussion

Although spontaneous fertility can occur in well-transfused and well-chelated patients with thalassemia, infertility mainly due to HH still remains one of the most common morbidities and obstacles for having children [9,10,11,12,13,14,15].

Table 1. Baseline characteristics of pregnant patients with thalassemia.

Diagnosis, n (TM, TI, HbH, S/B)	17 (12, 2, 2, 1)
Mean age at pregnancy ± SD, years (range)	28.3±4.9 (18.8-36.2)
Race, n (Caucasian, Asian, other)	17 (17, 0, 0)
Onset of puberty, years ± SD (range)	9.75±1.39 (9-14)
Onset of menarche, years ± SD (range)	14.4±1.35 (12.5-16)
Type of pregnancy, n, spontaneous vs. induced	14 vs. 6
Type of chelation prior to pregnancy, n (none, DFO, DFX, DFP)	5†, 0, 12, 1
Eusplenic vs. asplenic	9 vs. 8
Mean pre-transfusional ± SD, g/dL	9.28±0.34
Mean serum ferritin ± SD, µg/L	1203±1206
Mean LIC ± SD, mg/g dw (range)	3.7±3.9 (1.2-14.1)
Mean cardiac T2* ± SD, ms (range)	24.9±4.8 (16*-33.2)

†The patients with TI, HbH disease, and S/B thalassemia were not receiving iron chelation.

Cardiac T2 was below 20 ms in one patient.

n: Number, TM: thalassemia major, TI: thalassemia intermedia, HbH: hemoglobin H disease, S/B: S/beta-thalassemia, SD: standard deviation, DFO: deferoxamine, DFX: deferasirox, DFP: deferiprone, LIC: liver iron concentration.

Table 2. Delivery and newborn outcomes.	
Pregnancies with live births, n (1, 2, twins)	17 (13, 3, 1)
Miscarriage, n	2
Mean duration of pregnancies, weeks (range)	37.7 (33.1-40.8)
Type of delivery, spontaneous vs. cesarean	2 vs. 18
Mean birth weight, g (range)	2748 (1300-3680)
Admitted to NICU, n	4
Intrauterine growth retardation, n	2
Prematurity, n	4
Respiratory distress syndrome, n	1
Pneumothorax, n	1
n: Number, NICU: neonatal intensive care unit.	

In our cohort, male and female fertility rates were 50%. Gestation and delivery may result in an increased cardiac load and together with chronic hypoxia and myocardial iron deposition may aggravate cardiac dysfunction in female patients with thalassemia [10,16,17]. Severe anemia can also be a risk factor for gestational hypertension [18]. As suggested in previous studies [19,20], we assessed organ function in female patients who wished to conceive, and only those with normal cardiac function and well-controlled iron overload were encouraged to conceive. Under these conditions, cardiac health did not deteriorate in any patient during pregnancy, and all deliveries were safely performed.

Because of the potential teratogenicity of chelators, the use of chelation therapy during pregnancy has remained controversial. The current standard of practice is to cease any chelation therapy when pregnancy is established [21,22,23]. Only DFO chelation may be restarted after the first trimester when the benefits outweigh the risks of excess iron [24,25,26,27,28,29]. In our cohort, three pregnant patients received DFO after the second trimester and delivered healthy babies with no specific hearing or visual defects.

It is suggested to maintain the pre-transfusion hemoglobin at ≥10 g/dL during pregnancy in patients with thalassemia [16,30,31,32]. We have followed the current clinical practice in TM patients but have been cautious of potential risks of alloimmunization in patients with NTDT. In the latter group, the pre-transfusion hemoglobin was maintained at ≥8 g/dL.

In accordance with other reports, the majority of our patients delivered via cesarean section [33,34,35]. The prevalence of fetal and maternal complications including miscarriages, IUGR, premature labor, and even fetal death is reported to be higher in thalassemic females compared to the normal population [36,37,38]. In our cohort, premature birth was observed in 19% of the deliveries, which was considerably higher than the rate of premature spontaneous live births (6.9%) in the Turkish registry [39].

Conclusion

Male and female thalassemic patients may conceive spontaneously, or conception may be achieved by ART. Pregnancy in patients with thalassemia can be safely managed with remarkably positive outcomes for both the mother and infant under the supervision of a multidisciplinary team.

Ethics

Ethics Committee Approval: N/A.

Informed Consent: N/A.

Authorship Contributions

Surgical and Medical Practices: B.A., A.Ş.Y., N.Ö.K., Z.Ö.S., H.H.Ö., D.Y.K., C.B., K.K., Y.A.; Concept: B.A., A.Ş.Y., N.Ö.K., Z.Ö.S., H.H.Ö., D.Y.K., C.B., K.K., Y.A.; Design: B.A., A.Ş.Y., N.Ö.K., Z.Ö.S., H.H.Ö., D.Y.K., C.B., K.K., Y.A.; Data Collection or Processing: B.A., A.Ş.Y., N.Ö.K., Z.Ö.S., H.H.Ö., D.Y.K., C.B., K.K., Y.A.; Analysis or Interpretation: B.A., A.Ş.Y., N.Ö.K., Z.Ö.S., H.H.Ö., D.Y.K., C.B., K.K., Y.A.; Literature Search: B.A., A.Ş.Y., N.Ö.K., Z.Ö.S., H.H.Ö., D.Y.K., C.B., K.K., Y.A.; Writing: B.A., A.Ş.Y., N.Ö.K., Z.Ö.S., H.H.Ö., D.Y.K., C.B., K.K., Y.A.

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Gingival Leukemic Infiltration in Chronic Lymphocytic Leukemia

Kronik Lenfositik Lösemide Gingival Lösemik İnfiltrasyon

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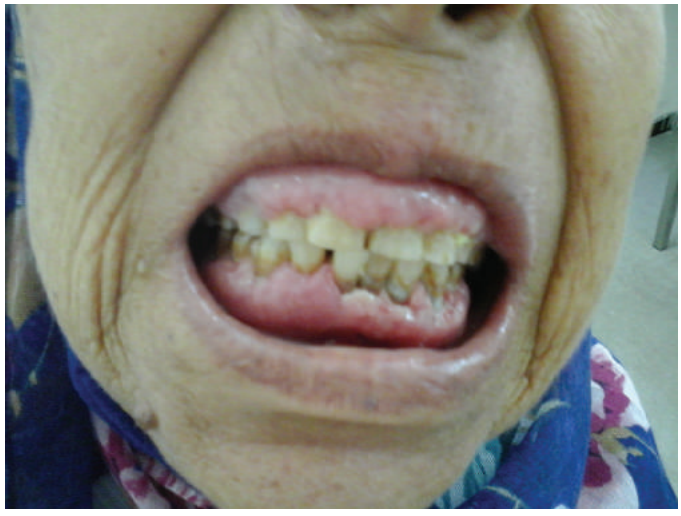

Figure 1. Physical examination revealed gingival enlargement with swollen margins and glossy texture.

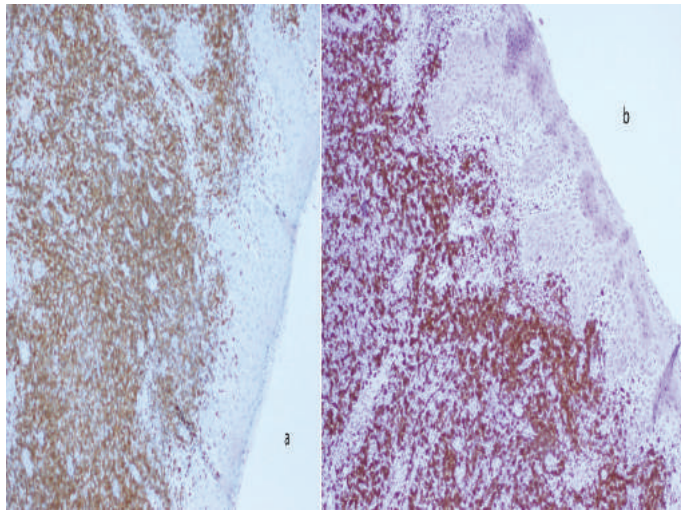

Figure 2. A biopsy of the gingiva showed infiltration by small lymphocytes expressing CD5 and CD20 positivity.

A 66-year-old female patient was referred for asymptomatic peripheral blood lymphocytosis. The blood smear showed 77% mature lymphocytes. Flow cytometry confirmed the clonality of the circulating B lymphocytes with positivity for CD19, CD5, and CD23. The patient was diagnosed with chronic lymphocytic leukemia (CLL), stage A, and a decision was made to watch and wait. Six years later, she was referred again for multiple adenopathy with splenomegaly. She reported a toothache that was worse upon biting, causing food restriction. Physical examination revealed multiple cervical lymphadenopathy, splenomegaly, and gingival enlargement with swollen margins and glossy texture (Figure 1). There were no exudates, necrosis, ulcerations, or active bleeding. Laboratory evaluation revealed a white blood cell count of 71,000/mm^3, hemoglobin of 7.2 g/dL, and platelet count of 294,000/mm^3. A biopsy of the gingiva showed infiltration by small lymphocytes expressing CD5 and CD20 positivity (Figures 2a and 2b). This typical pattern of CLL infiltration excluded a diagnosis of prolymphocytic leukemia or a transformation into aggressive lymphoma. The patient was treated with rituximab and chlorambucil for CLL, stage C. Gingival enlargement was less painful after one cycle, with reduction of the swelling. The patient was lost after the third cycle. Gingival hyperplasia due to leukemic infiltration is commonly observed in acute leukemia but is rare in CLL and represents an extranodal site. To our

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knowledge, only two prior cases were reported in the literature [1,2].

Keywords: Chronic lymphocytic leukemia, CD19, Leukemia
Anahtar Sözcükler: Kronik lenfositik lösemi, CD19, Lösemi

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Auer Rod-Like Inclusions in B-Cell Prolymphocytic Leukemia

B Hücreli Prolenfositik Lösemide Auer-Rod Benzeri İnklüzyonlar

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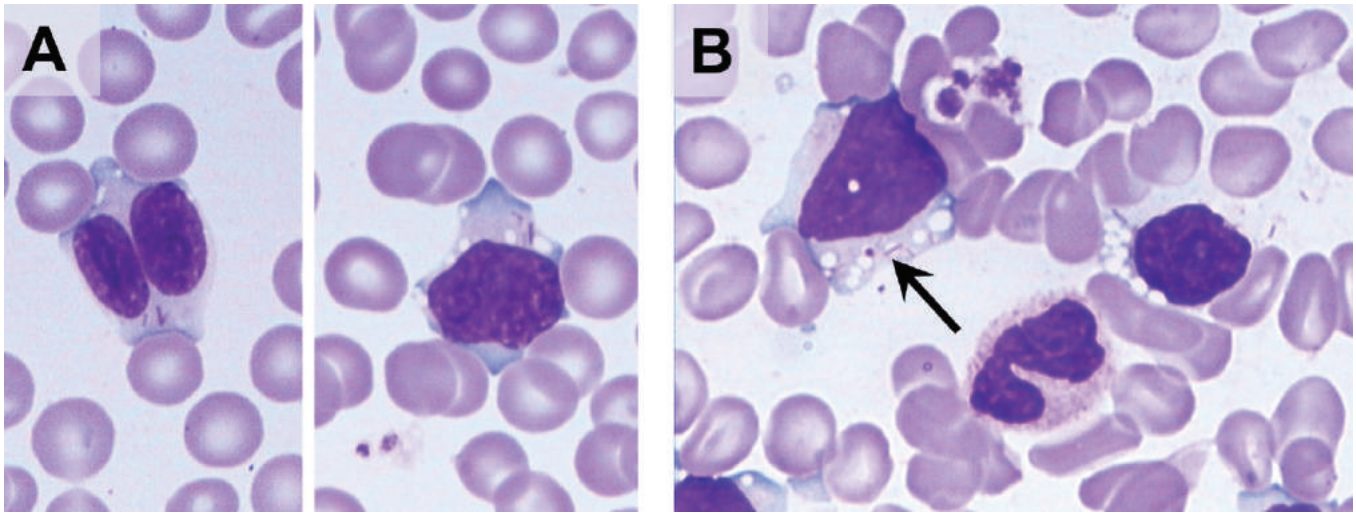

Figure 1. (A) Blood smears and (B) bone marrow smears demonstrating abnormal lymphocytes with Auer rod-like inclusions (1000x, Wright-Giemsa stain).

A 76-year-old male patient presented with increasing leukocytes in the past month. Laboratory investigation showed leukocytosis of 30.03x10^9/L (normal: 3.5-9.5x10^9/L) with absolute lymphocytosis of 20.7x10^9/L (normal: 1.1-3.2x10^9/L), with normal hemoglobin and platelet counts. Review of the peripheral blood smears (Figure 1A) and bone marrow smears (Figure 1B) demonstrated 64% and 74.5% prolymphocytes, respectively, with nucleoli, vacuoles, and Auer rod-like inclusions. The cytoplasmic inclusions were negative for myeloperoxidase by immunohistochemistry. Flow cytometry demonstrated a kappa-restricted CD19 and CD20 immunoreactive B-cell population making up to 67.1% of cells and 93.1% of lymphocytes, with partial expression of sIgM and lacking CD5, CD10, and CD23. No significant expression of CD38 was present. Although Auer rod-like inclusions were seen, there was no evidence of increased immature myeloid cells by flow cytometry or morphology. *IgVH* (FR1-FR3) mutation was not appreciable by molecular biology studies before or during this period. The patient achieved a partial response with chlorambucil treatment.

Auer rod-like inclusions have been reported in B-lineage malignancies like multiple myeloma [1,2]. Electron microscopy revealed these structures to be swollen mitochondria or immunoglobulins [3,4], while classical Auer rods are formed by aggregation and concentration of peroxide granules in myeloid blasts.

Keywords: Auer rod-like inclusions, B-cell prolymphocytic leukemia, Lymphocytes

Anahtar Sözcükler: Auer-Rod benzeri inklüzyonlar, B hücreli prolenfositik lösemi, Lenfositler

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An Update of the Definition of Transfusion-Related Acute Lung Injury

Transfüzyon İlişkili Akut Akciğer Hasarının Tanımında Güncelleme

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To the Editor,

In the past transfusion-related acute lung injury (TRALI) was regarded as a rare complication of transfusion medicine. Subsequently, TRALI has been shown to be one of the leading causes of transfusion-related morbidity and mortality. Insight into TRALI pathogenesis in the past decades has resulted in the development of preventive strategies [1]. The accumulation of clinical and basic science knowledge has provided the rationale for a recent update of the widely used 2004 Canadian Consensus Conference (CCC) definition of TRALI (Table 1) [2]. A panel of 10 international experts on TRALI, including two members with hemovigilance expertise, used the Delphi panel approach to develop a redefinition of TRALI by modifying the 2004 CCC definition [3].

The updated TRALI definition along with the rationale for the changes has now been published (Table 2) [3]. The main modifications are as follows: 1) The term "possible TRALI" has been dropped. 2) TRALI has been separated into two types: TRALI type I (without an acute respiratory distress syndrome (ARDS) risk factor) and TRALI type II (with an ARDS risk factor or with mild preexisting ARDS). Notably, the presence of either an ARDS risk factor or mild ARDS does not exclude the diagnosis of TRALI as it

Table 1. 2004 Canadian Consensus Conference definition of TRALI and possible TRALI [2].

TRALI	a.	i.	Acute onset		
		ii.	Hypoxemia	Research setting:	
					PaO_2/FiO_2 ≤300 or SpO_2 <90% on room air
				Non-research setting:	
					PaO_2/FiO_2 ≤300 or SpO_2 <90% on room air or other clinical evidence of hypoxemia
		iii.	Bilateral infiltrates on chest radiograph		
		iv.	No evidence of left atrial hypertension and/or Central venous pressure <18 mmHg		
	b.	No preexisting ALI before transfusion			
	c.	During or within 6 hours of transfusion			
	d.	No temporal relationship to an alternative risk factor for ALI			
Possible TRALI	a.	As mentioned above			
	b.	In the presence of an alternative risk factor for ALI			

Table 2. New consensus TRALI definition [3].

TRALI type I - Patients who have no risk factors for ARDS and meet the following criteria:			
a.	i.	Acute onset	
	ii.	Hypoxemia	PaO_2/FiO_2 ≤300* or SpO_2 <90% on room air
	iii.	Clear evidence of bilateral pulmonary edema on imaging (e.g., chest radiograph, chest CT, or ultrasound)	
	iv.	No evidence of left atrial hypertension** or, if LAH is present, it is judged to not be the main contributor to the hypoxemia	
b.	Onset during or within 6 hours of transfusion***		
c.	No temporal relationship to an alternative risk factor for ARDS		
TRALI type II - Patients who have risk factors for ARDS (but who have not been diagnosed with ARDS) or who have preexisting mild ARDS (PaO_2/FiO_2 of 200-300), but whose respiratory status deteriorates**** and is judged to be due to transfusion based on:			
a. Findings as described in categories *a and b* of TRALI type I, and			
b. Stable respiratory status in the 12 hours prior to transfusion			

*If altitude is higher than 1000 m, the correction factor should be calculated as follows: [(PaO_2/FiO_2) x (barometric pressure/760)].

**Use objective evaluation when LAH is suspected (imaging, e.g., echocardiography, or invasive measurement using, e.g., pulmonary artery catheter).

***Onset of pulmonary symptoms (e.g., hypoxemia - lower P/F ratio or SpO_2) should be within 6 hours of end of transfusion. The additional findings needed to diagnose TRALI (pulmonary edema on a lung imaging study and determination of lack of substantial LAH) would ideally be available at the same time but could be documented up to 24 hours after TRALI onset.

****Use PaO_2/FiO_2 ratio deterioration along with other respiratory parameters and clinical judgement to determine progression from mild to moderate or severe ARDS. See the conversion table in Appendix S2 of the original report to convert nasal O_2 supplementation to FiO_2 [3].

did under the old definition. 3) Cases with an ARDS risk factor that meet ARDS diagnostic criteria and where respiratory deterioration over the 12 hours prior to transfusion implicates the risk factor as causative should be classified as ARDS rather than TRALI type II. 4) The 2012 updated ARDS consensus definition (referred to as the BERLIN definition) has been evaluated for its relevance to TRALI and essential updates (including guidance in diagnosing hydrostatic pulmonary edema) have been incorporated into the new TRALI definition.

More broadly, the Delphi panel recommended that all pulmonary complications after blood transfusion should be reported to the transfusion service and then categorized (either by the transfusion service, a hospital transfusion committee, or a hemovigilance system) into one of several categories: TRALI (type I or type II), ARDS, transfusion-associated circulatory overload (TACO), TRALI/TACO - cannot distinguish, or an alternate diagnosis. Importantly, the panel reaffirmed that TRALI remains a clinical diagnosis and does not require detection of cognate leukocyte antibodies, though it did recommend that these data be captured through a hemovigilance reporting system. Future research directions have been identified and include identifying the mechanism behind the onset of TRALI in the absence of cognate leukocyte antibodies. Furthermore, the panel is working on developing a universal reporting form for posttransfusion pulmonary complications including suspected TRALI.

We believe that the TRALI definition update is such an important change for transfusion medicine that it needs to be widely disseminated and discussed. To this end, the panel has submitted this letter to the editors of several important transfusion and hemovigilance journals [4,5]. We hope that the new definition contributes to an enhanced level of reporting and a more accurate classification of respiratory complications associated with blood transfusion.

Keywords: TRALI, Definition, Delphi

Anahtar Sözcükler: TRALI, Tanım, Delphi

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Overwhelming Asplenic Sepsis due to Babesiosis

Babesiyozis İlişkili Ağır Asplenik Sepsis

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To the Editor,

A 70-year-old female from southern Massachusetts, USA, was admitted to the intensive care unit with septic shock and acute respiratory distress syndrome (ARDS) after 3 days of acute febrile illness. She had undergone splenectomy at the age of 5 related to trauma from a traffic accident. Laboratory studies reveled pancytopenia, acute renal insufficiency, increased lactate dehydrogenase, depressed haptoglobin, and elevated liver enzymes with indirect hyperbilirubinemia. Prothrombin time and activated partial thromboplastin time were both elevated and fibrinogen level was low, consistent with disseminated intravascular coagulation (DIC). A direct anti-globulin test was negative. A thin blood smear with oil immersion showed intraerythrocytic polymorphic ring forms (Figure 1, arrows) morphologically consistent with *Babesia* species and the presence of Howell-Jolly bodies (Figure 1, arrowhead), confirming the history of splenectomy. Real-time DNA-PCR confirmed *Babesia microti* as the offending parasite. The patient was started on treatment for babesiosis with quinine, azithromycin, and atovaquone. She also received red cell exchange transfusion due to the high level of parasitemia (14% of the erythrocytes) and completely recovered in the next few weeks.

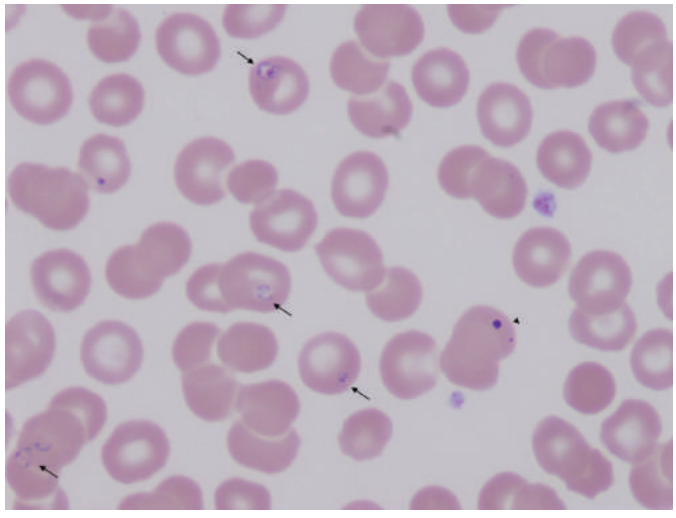

Figure 1. Thin blood smear with oil immersion showed intraerythrocytic polymorphic ring forms (arrows) morphologically consistent with *Babesia* species and the presence of Howell-Jolly bodies (arrowhead), confirming the history of splenectomy.

Human babesiosis is a malaria-like tick-borne illness caused by the protozoan parasite *Babesia microti*, endemic in the Midwest and Northeast USA; it has also been reported in parts of Europe, Asia, and Australia [1]. It has also been reported after transfusion of contaminated blood products [2]. Infection is usually mild to moderate in an immunocompetent host but a severe infection requiring hospitalization can occur in patients with a history of splenectomy or immunodeficiency such as cancer, human immunodeficiency virus infection, or hemoglobinopathy and in the elderly with co-morbidities and allogeneic hematopoietic stem cell transplant recipients [1,3]. Severe babesiosis with ARDS and DIC can occur in immunocompromised or asplenic individuals, which can be fatal [4,5]. Milder illness in immunocompetent hosts manifests with malaise, fever, headache, myalgia, and nausea. Laboratory findings typically show non-immune hemolytic anemia and thrombocytopenia, but immune hemolytic anemia has also been reported. A rapid diagnosis can be made by identification of *Babesia* organisms on thin blood smears under oil immersion. The diagnosis can be confirmed by using DNA-PCR to identify the DNA of the parasite. Serology is available, but it is difficult to distinguish current from recent or past infection in a patient coming from an endemic area. The most commonly used agents for treatment of severe babesiosis include azithromycin, atovaquone, quinine, and clindamycin. Patients with severe infection with high-grade parasitemia, severe hemolysis, or compromised organ functions (pulmonary, liver, or renal impairment) may benefit from red cell exchange transfusion.

This case confirms that a severe form of babesiosis can occur in patients who have undergone splenectomy. A high index of suspicion and a timely review of blood smears in asplenic patients presenting with febrile illness and hemolytic anemia from endemic areas can aid in rapid diagnosis and prompt treatment, which can be lifesaving. This case illustrates that an early diagnosis and aggressive treatment can be lifesaving even with a fulminant and severe infection with babesiosis. The key is to quickly decrease the parasitic burden for a good clinical outcome.

Keywords: Babesiosis, Splenectomy, Sepsis

Anahtar Sözcükler: Babesiyozis, Splenektomi, Sepsis

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Isolated Mediastinal Myeloid Sarcoma after *NPM1*-Positive Pediatric Acute Myeloid Leukemia

NPM1-Pozitif Pediatrik Akut Myeloid Lösemi Sonrası İzole Mediastinal Myeloid Sarkom

Özlem Tüfekçi, Şebnem Yılmaz, Melek Erdem, Birsen Baysal, Hale Ören

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To the Editor,

Myeloid sarcoma (MS) is a rare extramedullary mass that consists of immature myeloid cells. The most common locations are the soft tissue, bone, periosteum, orbit, and lymph nodes [1,2]. Mediastinal involvement is very rare and most commonly reported with concurrent bone marrow involvement [3]. Herein we present a previously treated nucleophosmin (*NPM1*)-positive acute myeloid leukemia (AML) patient who later presented with isolated mediastinal MS.

A 9-year-old female patient presented with fatigue and weakness. Physical examination revealed no pathological findings. Blood tests demonstrated hemoglobin of 12.2 g/dL, hyperleukocytosis (100,500/µL), and thrombocytopenia (43,000/µL) with 88% blasts in the peripheral blood smear. Bone marrow aspirate revealed 90% blasts with M1 subtype. Treatment was started according to the AML-BFM 2012 protocol. Conventional cytogenetic analysis failed due to lack of spontaneous mitosis and fluorescent in situ (FISH) analysis for t(8;21), inv(16), t(15;17), and t(9,22) from bone marrow samples revealed negative results. Molecular genetic analysis in the peripheral blood showed *NPM1* positivity and *FLT3-ITD* negativity. Morphologic and molecular remission was obtained at the end of the first induction block. She presented with back pain and fever seven months after cessation of maintenance treatment. Computed tomography (CT) of the thorax showed a solid mass of 84x75x41 mm in the anterior mediastinum (Figure 1). Bone marrow examination was normal; however, peripheral blood showed *NPM1* positivity. Conventional cytogenetic analysis from the bone marrow was within normal limits, while *NPM1* could not be studied from bone marrow. Her previous CT scans that were performed for investigation of invasive pulmonary aspergillosis were all normal. Fine-needle aspiration biopsy of the mass was performed; histopathological examination revealed myeloblasts that were positive for myeloperoxidase, CD15, and CD33. Microscopic examination of the imprint of the biopsy also revealed myeloblasts of M1 subtype (Wright stain). Major reduction in tumor mass (7 mm residual tumor) and *NPM1* negativity were achieved after one block of FLAG (fludarabine, cytarabine, filgrastim) and two blocks of FLAG-mitoxantrone. The patient underwent successful bone marrow transplantation from a matched unrelated donor and has been in remission for one year.

MS of the mediastinum is very rare; most of the cases have been reported as initial presentation with concurrent bone marrow involvement [3,4,5]. MS as a relapse has been more frequently reported in post-transplant patients compared to those treated without allogeneic hematopoietic stem cell transplantation [6,7]. Our patient is unique as she presented with isolated mediastinal MS after chemotherapy treatment. Another important point about our patient is that the *NPM1* positivity was detected at

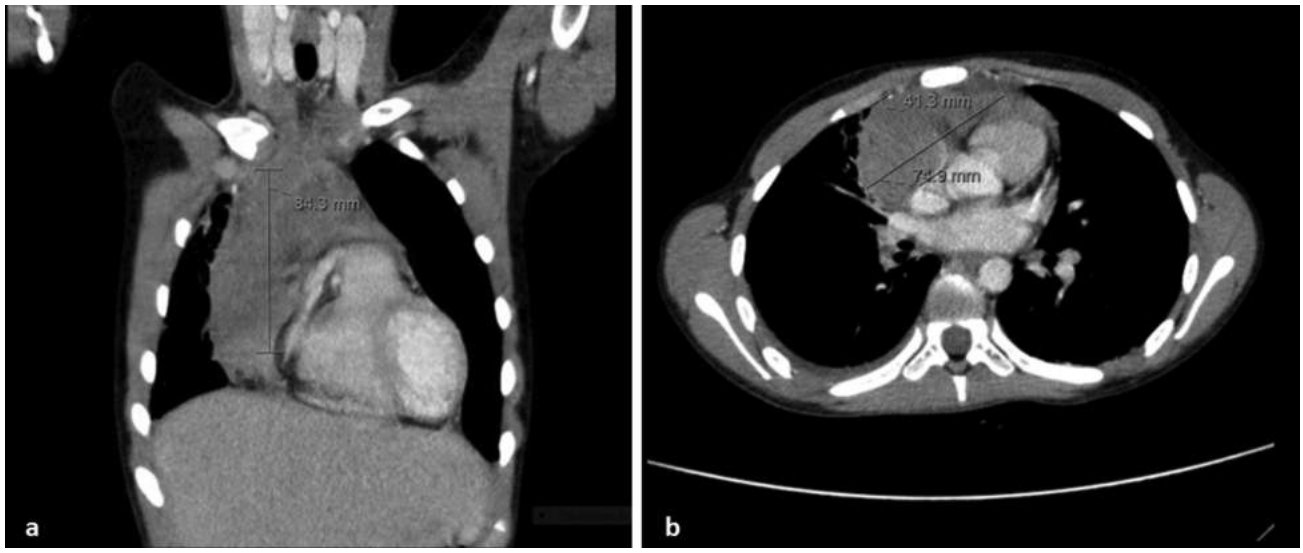

Figure 1. Computed tomography of the thorax showing anterior mediastinal mass in coronal (a) and axial (b) sections.

the same time as MS. The incidence of MS has been known to be higher in certain cytogenetic abnormalities, in particular t(8,21) [1,6]. Falini et al. [8], in their study with 181 MS samples, identified *NPM1* mutations as the most frequent molecular lesion in MS, defining the molecular status in 15% of cases. Our patient was negative for t(8:21) but had *NPM1* positivity.

In conclusion, even though *NPM1* is not a poor prognostic factor for AML, it should be kept in mind that patients with *NPM1* positivity may later present with MS, as in the case of our patient, who presented with isolated MS of the mediastinum months after cessation of chemotherapy.

Keywords: Acute myeloid leukemia, Myeloid sarcoma, Mediastinal mass, *NPM1*

Anahtar Sözcükler: Akut myeloid lösemi, Myeloid sarkom, Mediastinal kitle, *NPM1*

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Acute B Lymphoblastic Leukemia Developing in Patients with Multiple Myeloma: Presentation of Two Cases

Multipl Myelom Hastalarında Akut B Lenfoblastik Lösemi Gelişimi: İki Olgu Sunumu

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**Jiang Mei and Li Na contributed equally to this study.*

To the Editor,

Therapy-related acute myeloid leukemias (t-AMLs) following therapy are well described in the literature, but only rare cases of therapy-related acute lymphoblastic leukemia (t-ALL) have been reported previously. Cases of multiple myeloma (MM) terminating in ALL are even rarer. Herein, we report the clinicopathological, immunological, cytogenetic, and molecular features of two patients diagnosed with B-cell acute lymphoblastic leukemia (B-ALL) and MM who presented with MM at the initial diagnosis.

Patient 1, a 68-year-old male, was diagnosed with MM in 2015. He received 2 cycles of PD (bortezomib and dexamethasone) with a good response, and then maintenance with thalidomide.

Patient 2, a 65-year-old female, was diagnosed with MM in 2012. She received 4 cycles of VAD (vincristine, epirubicin, and dexamethasone) with a partial response. She then relapsed and received treatment with one cycle of TAD (thalidomide, epirubicin, and dexamethasone). After that, she achieved complete remission. In 2016, the patient relapsed again. She continued treatment with BTD (bortezomib, dexamethasone, and thalidomide) and achieved partial response.

In 2017, the two patients both presented with leukopenia. Immunofixation electrophoresis showed monoclonal IgG and K light chain. The bone marrow was heavily infiltrated by lymphoblasts and a few malignant plasma cells. Flow cytometry analysis demonstrated that malignant plasma cells with CD38, CD138, and monoclonal K chain and B-cell lymphoblasts expressed CD10, CD19, CD34, HLA-DR, cCD79a, and CD33. No other aberrant expression of myeloid or T lymphocyte-associated antigens was identified (Figures 1A-1C). The female patient's G-banding cytogenetic results revealed a hypodiploid

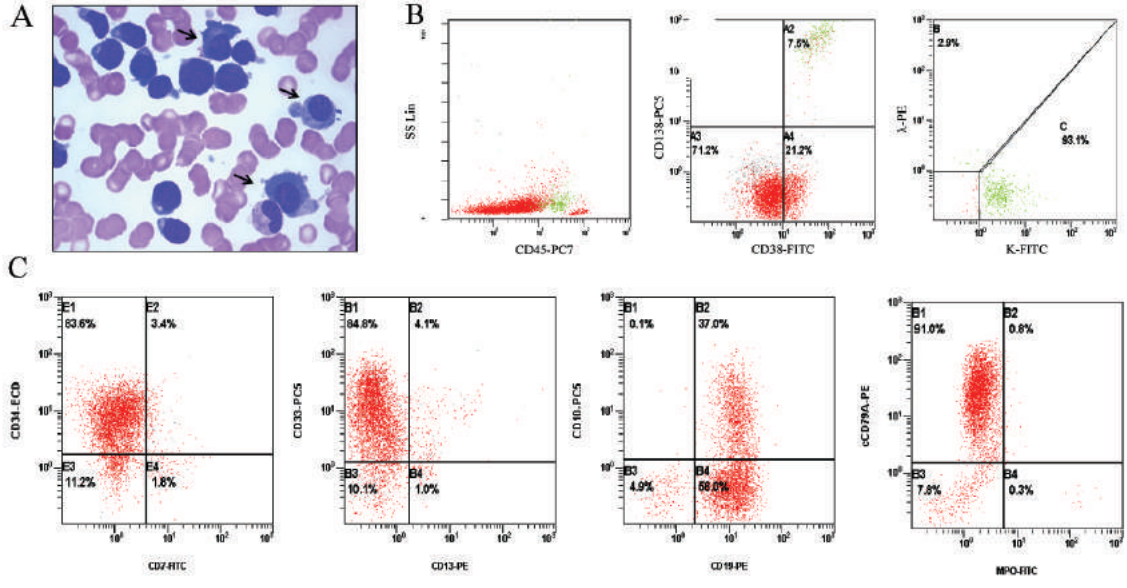

Figure 1. Patient 1: A) Black arrows point at malignant plasma cells, which are very different from other lymphoblasts (Wright-Giemsa staining, 100×). B) Malignant plasma cells were positive for CD38, CD138, and monoclonal kappa (green region of the scatter plot). C) The lymphoblasts were immunophenotyped as B-cell and expressed CD10, CD19, CD34, and cCD79a with aberrant coexpression of CD33. The morphologic and immunological characteristics of Patient 2 were similar.

Table 1. Clinical features of acute lymphoblastic leukemia in patients with previously treated multiple myeloma (MM) from our institution.

	Patient 1	Patient 2
Sex/age (years) at the time of MM diagnosis	Male/68	Female/65
Time interval between last therapy for MM and the ALL diagnosis	26 months	3 months
Laboratory findings		
WBC, $x10^9$/L	0.87	0.8
% Blasts, peripheral blood	0	0
Hb, g/L	79	99
Platelets, $x10^9$/L	56	105
Immunofixation electrophoresis	IgG/kappa	IgG/kappa
Serum free κ light chain (mg/L)	25.4	>163
Serum free λ light chain (mg/L)	17.1	5.76
Bone marrow findings		
BM cellularity	Hypercellular	Hypercellular
Immunophenotype	95% blasts (CD10, CD19, CD33, CD34, HLA-DR, cCD79a), 0.3% plasma cells (CD38, 138, monoclonal kappa)	86.3% blasts (CD10, CD19, CD33, CD34, HLA-DR, cCD79a), 7.5% plasma cells (CD38, 138, monoclonal kappa)
Cytogenetics	Not detected	MLL rearrangement (-), bcr-abl (-)
PCR detection	(-)*	(-)*
Clinical diagnosis	MM with B-ALL	MM with B-ALL
Therapy for original disease (MM)	Bortezomib, dexamethasone	Vincristine, epirubicin, dexamethasone, thalidomide, epirubicin, dexamethasone, bortezomib-dexamethasone, thalidomide
Therapy for MM with B-ALL	Declined any treatment due to poor performance status and died four months later	Treated with low-dose chemotherapy (vincristine, epirubicin, dexamethasone, bortezomib); did not respond well and died one month later

(-)*: RT-PCR for detection of 30 fusion genes was negative (including *MLL-AF9, MLL-AF4, MLL-ENL, MLL-AF10, MLL-SEPT6, MLL-ELL, MLL-AF17, MLL-AF1q, MLL-AF1p, MLL-AF6, PML-RARA, NPM-RARA, PLZF-RARA, AML1-ETO, AML1-MDS1/EVI1, AML1-MTG16, AML1-EAP, TEL-AML1, TEL-PDGFRB, TEL-ABL, E2A-PBX1, E2A-HLF, BCR-ABL, CBFβ-MYH11, SIL-TAL1, FIP1L1-PDGFRA, DEK-CAN, SET-CAN, TLS-ERG,* and *NPM-MLF*).

RT-PCR: Reverse transcription polymerase chain reaction, MM: multiple myeloma, ALL: acute lymphoblastic leukemia.

and complex karyotype. Reverse transcription-polymerase chain reaction for detection of fusion genes in the two patients was negative. Both patients were diagnosed with B-ALL with MM. The male patient declined any treatment due to poor performance status and died four months later. The female patient was treated with low-dose chemotherapy (vincristine, epirubicin, dexamethasone, and bortezomib) and did not respond well; she died one month later. The clinical features of the two patients are summarized in Table 1.

It has been reported in the literature that therapy-related acute leukemia comprises 2 major types: alkylating agent/radiotherapy-related and topoisomerase II inhibitor-related [1]. Alkylating agent-related acute leukemia is associated with abnormalities of chromosomes 5 and/or 7, while topoisomerase II inhibitor-related acute leukemia has been linked to 11q23 [2,3]. The female patient received a topoisomerase II inhibitor while the male did not, and neither of them showed specific genetic abnormalities. Intriguingly, the clinical, morphologic, and immunological characteristics of the two patients were similar. MM is a plasma cell neoplasm derived from mature B-lymphocytes, whereas B-ALL is a B-cell neoplasm derived from early B-precursors. It is possible that MM and B-ALL may derive from the same stem cell clones, or MM cells dedifferentiate into immature B cells that develop B-ALL [4]. They may have identical karyotypes and immunophenotyping, and they may share some cytogenetic abnormalities [5,6]. The possibility of MM and B-ALL deriving from two independent clones cannot be excluded, either. Future studies such as molecular and cytogenetic studies to explore their relationship would be intriguing.

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Keywords: Acute lymphoblastic leukemia, Multiple myeloma, Therapy-related, Genetics, Immunophenotyping

Anahtar Sözcükler: Akut lenfoblastik lösemi, Multipl myelom, Terapi ilişkili, Genetik, İmmünfenotipleme

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ALK+ Anaplastic Large Cell Lymphoma of Null Cell Phenotype with Leukemic Transformation and Leukemoid Reaction

Lösemi Transformasyonu ve Lökomoid Reaksiyon ile Giden "Null" Hücre Fenotipli ALK+ Anaplastik Büyük Hücreli Lenfoma

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To the Editor,

Anaplastic large cell lymphoma (ALCL) frequently involves both nodal and extranodal sites and is rarely leukemic. A 21-year-old male presented with abdominal pain. His complete blood count, which had been normal four months ago, showed increasing white cell counts from $14.9x10^9$/L to $95.5x10^9$/L in a month, with neutrophils ranging from 81.6% to 89.6%. Blood cultures were negative. Laparoscopic nodal biopsy showed sheets of medium-sized lymphocytes diffusely expressing CD30, TIA-1, granzyme B, and ALK, but not T-cell markers including CD2, CD3, CD4, CD5, CD7, CD8, and βF1, indicating ALK+ ALCL of null cell phenotype. Bone marrow biopsy showed two small aggregates of tumor cells in a background of normal tri-lineage hematopoiesis. ALK immunostaining revealed singly scattered positive cells (Figure 1A) in addition to those in small aggregates. The staining pattern was both nuclear and cytoplasmic, indicating translocation t(2;5)(p23;q35). We retrospectively reviewed the blood smear and found that 4.5% of the last peripheral smear were tumor cells, which were overlooked by the clinical laboratory. The leukemic cells were large with vesicular nuclei, irregular nuclear contours, and

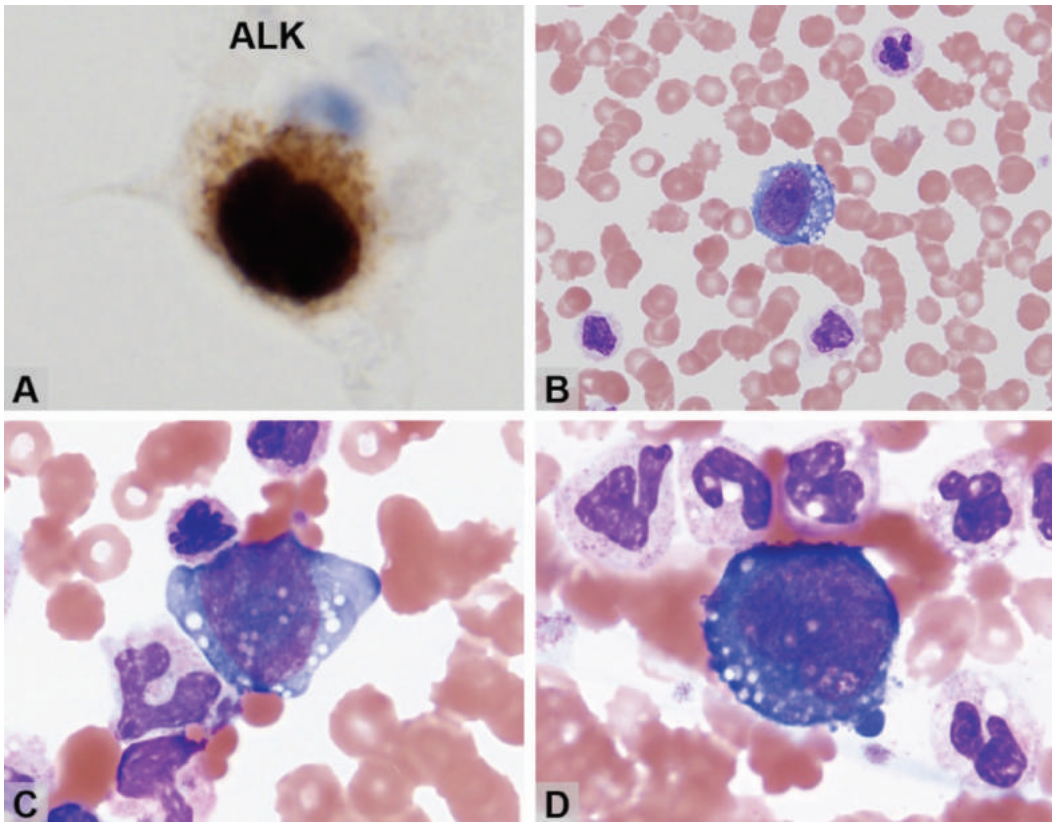

Figure 1. A) ALK immunostaining revealed singly scattered positive cells in addition to those in small aggregates; B-D) leukemic cells were large with vesicular nuclei, irregular nuclear contours, and vacuolated basophilic cytoplasm.

vacuolated basophilic cytoplasm (Figures 1B-1D). The disease progressed rapidly, and the patient passed away shortly after the first cycle of CEOP chemotherapy. In advanced diseases, ALK-positive ALCL may rarely be associated with leukemoid reaction and leukemic transformation.

Keywords: ALK, Anaplastic lymphoma kinase, Anaplastic large cell lymphoma, CD30, Leukemoid reaction, Leukemic phase, Leukemic transformation

Anahtar Sözcükler: ALK, Anaplastik lenfoma kinaz, Anaplastic büyük hücreli lenfoma, CD30, Lökomoid reaksiyon, Lösemik faz, Lösemik transformasyon

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Thirty-Two Case Reports of Synchronous Hematological Malignancy and Solid Tumor

Eş Zamanlı Hematolojik Malignite ve Solid Tümörü Olan Otuz İki Olgunun Analizi

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To the Editor,

Synchronous multiple primary cancer (SMPC) is defined as two or more malignancies diagnosed within 6 months of each other [1]. Its incidence is low, while the simultaneous occurrence of a hematological malignancy and a solid tumor is even less common with only cases reports provided [2,3,4,5,6]. We analyzed 32 patients with a synchronous hematologic malignancy and solid tumor at The Affiliated Cancer Hospital of Zhengzhou University from June 2012 to June 2018.

Patients and disease characteristics are shown in Table 1. These 32 patients included 17 males and 15 females. The median age at diagnosis was 58.5 years (range: 30-81 years). The incidence of SMPC in our center was approximately 0.05%, while this rate was reported as 0.5% in the literature [5]. The difference in this incidence might be attributable to differences in geography, environment, race, or various diagnostic criteria or, more importantly, the experience of the clinicians or the examination methods between studies.

The median interval between the diagnoses of these 2 primary malignancy types was 0.2 months (range: 0-5.3 months). Of the 32 cases, 2 patients were lost to follow-up while the other 30 patients completed the treatment: 3 cases with complete remission (CR), 9 cases with stable disease (SD), recurrence of gastric cancer in 1 case, 1 case of lymphoma recurrence, and 16 cases of death. The median overall survival (OS) of the 32 patients was 17.7 months (range: 1.3-68 months). Among the 16 deceased patients, there were 8 patients with a median age of 60.5 years (range: 44-78 years) who survived less than 10 months, and 4 of them had reported a family history of cancer. Eight patients were diagnosed with hematologic malignancies or solid tumors of stage III or IV. Among these 8 patients, 3 patients died early after surgery, 3 patients died of pulmonary infection after radiotherapy and chemotherapy, and 2 patients died of primary disease progression.

The pathogenesis of SMPC is not completely clear. Tabor et al. [7] found that tumors of different types and different tissues might originate from identical precancerous lesions. An Argentine study group found that 32% of multiple primary cancer patients reported a family history of cancer [8]. Genetic instability may play an important role in the development of multiple primary cancers. Based on the detection of replication errors on microsatellite loci, Horii et al. [9] found that genetic defects in the mismatch repair system represent a high-risk factor for multiple primary cancer patients. We identified 8 patients whose first-degree relatives had experienced malignant tumors in our study.

No standard treatment options are available for synchronous hematological malignancies and solid tumors. The degree of malignancy of each tumor, the response of each tumor to therapy, the therapy indications, and the general condition of the patient should be considered simultaneously. For patients who were diagnosed with a solid tumor and indolent lymphoma such as mucosa-associated lymphoid tissue lymphoma or marginal zone lymphoma, chemotherapy or I-131 radiotherapy was performed first to treat the solid tumor. However, for patients who were diagnosed with an early-stage solid tumor and highly aggressive lymphoma such as diffuse large B-cell lymphoma or anaplastic large-cell lymphoma, after surgical removal of the solid tumor, chemotherapy and sequential hematopoietic stem cell transplantation were administered to treat the lymphoma and at the same time regular postoperative follow-up for the solid tumor was performed.

Keywords: Synchronous multiple primary cancer, Hematological malignancy, Solid tumor

Anahtar Sözcükler: Senkron çoklu primer kanser, Hematolojik malignite, Solid tümör

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Table 1. Clinical characteristics of 32 synchronous multiple primary cancer patients.

No.	Sex	Age, years	Family history	Hematological malignancy			Interval, months	Solid tumor			Outcome	OS, months
				Diagnosis	Primary site	Treatment		Diagnosis	Primary site	Treatment		
1	M	61	Liver cancer	HL (stage IVS)	Lymph node	ABVD×6, radiotherapy	3	Adenocarcinoma (stage I)	Stomach	Operation	Death	59
2	M	57		DLBCL (stage II)	Stomach	CHOP×2, CHOPE×2	0	Esophageal cancer	Esophageal	Radiotherapy	Death	28
3	F	78		DLBCL (stage IV)	Colon	R-EPOCH×2	0	Adenocarcinoma (stage I)	Colon	Operation	Death	3.4
4	F	53		DLBCL (stage IIIA)	Thyroid	CHOP×3, EPOCH×3, DICE×2	0	Microscopic papillary carcinoma (stage I)	Thyroid	Operation	CR	19.3
5	F	74		MZL (stage I)	Lymph node	Operation	0	Papillary carcinoma (stage IVA)	Thyroid	Operation + I-131	Death	19.5
6	M	61		MZL (stage IIIA)	Lymph node	R-CHOP×2, CHOP×6	-5.3	Microscopic papillary carcinoma (stage I)	Thyroid	Operation	SD	26.9
7	F	70		MZL (stage IS)	Spleen	Operation	0	Squamous carcinoma (stage IB)	Esophageal	Operation	SD	52.8
8	F	48		Nasal NK/T-cell lymphomas	Nose	DDGP-L×5, radiotherapy	0	Lung cancer	Lung	PC×2, DN×1, DIED×2, crizotinib	Death	24
9	F	54		NK/T-cell lymphomas (stage IVE)	Nose	Operation	0	Papillary carcinoma (stage I)	Thyroid	Operation,radiotherapy	Lost	
10	M	30		Nasal NK/T-cell lymphomas (stage IVE)	Nose	DICE-L×5, P-Gemox-VP16×1, radiotherapy, HSCT	0	Neuroendocrine neoplasm G3 (stage I)	Rectum	Operation	CR	31.4
11	F	51		MALT (stage IVE)	Stomach	Operation	0	Adenocarcinoma (stage IV)	Stomach	Operation, TP×4	Relapse	16
12	F	73		MALT (stage IE)	Thyroid	FC×2	0	Microscopic papillary carcinoma (stage I)	Thyroid	Operation	CR	42
13	M	67	Gastric cancer	MALT (stage IE)	Stomach	Operation	0	Adenocarcinoma (stage IIIB)	Stomach	Operation, SOX×1	Death	25
14	F	68	AML	FL (stage IIIA)	Lymph node	R-COP×4	5.2	Adenocarcinoma (stage IV)	Lung	Chemotherapy	Death	7.1
15	M	43		ALK-ALCL (stage IB)	Lymph node	EPOCH×4, auto-HSCT, radiotherapy	0	Microscopic papillary carcinoma (stage I)	Thyroid	Operation	Relapse	44

16	M	46		MCL	Lymph node	Operation	0	Papillary carcinoma (stage I)	Thyroid	Operation	Lost	
17	M	57		B-NHL	Submandibular gland	Operation	-4	Squamous carcinoma (stage IIIA)	Esophageal	Operation, TP×3	SD	68
18	M	44	Liver cancer	B-NHL	Stomach	Operation	0	Liver cancer (stage IIIB)	Liver	Operation	Death	2
19	M	65	-	CLL		Monitoring	-3.6	Adenocarcinoma (stage IIIB)	Stomach	Operation, DP×4	SD	46
20	M	60	-	Plasmacytoma		Operation	1.2	Adenocarcinoma (stage I)	Stomach	Operation	SD	54
21	F	65	Lung cancer	MDS-RA		Stanozolol, EPO	0.4	Adenocarcinoma (stage II)	Breast	Operation, FEC×6	SD	14.2
22	M	55	Colon cancer	MDS RAEB-2		Decitabine + CAG	0.3	Squamous carcinoma (stage IIIB)	Esophageal	-	Death	3.4
23	F	67	-	MDS-RCMD		G-CSF, EPO	0.3	Squamous carcinoma (stage IIIA)	Esophageal	Operation, TP×3	SD	8.4
24	M	66	-	MDS-RCMD		EPO, danazol	4.2	Adenocarcinoma (stage IV)	Prostate	Endocrinotherapy	Death	6.2
25	M	76	Esophageal cancer	CML CP		Hydroxyurea and imatinib	0.3	Adenocarcinoma (stage IIB)	Stomach	Operation, mFOLFOX6×4	SD	14.5
26	F	46	-	CML CP		Hydroxyurea and imatinib	0.2	Adenocarcinoma (stage IIIB)	Stomach	Operation	Death	1.3
27	M	52	-	CML CP		Hydroxyurea and imatinib	0.1	Adenocarcinoma (stage IIIB)	Lung	PC×4, S-1	SD	8.2
28	F	81	-	CML CP		Hydroxyurea	0.2	Squamous carcinoma	Scalp	Operation	Death	13.6
29	F	45	-	AML-M5		HAA	-4.6	Invasive mole (stage III)	Uterus	EMA/CO×4	Death	6.4
30	F	77	Pancreatic cancer	AML-M2		CAG	1.2	Adenocarcinoma (stage IV)	Colon	-	Death	3.8
31	M	47		AML-M2		IA, D-Ara-c	2.9	Adenocarcinoma (stage IIIA)	Colon	Operation, oxaliplatin-5-FU×4	Death	11.4
32	M	56	-	APL		Arsenic trioxide and retinoic acid	4.8	Squamous carcinoma (stage IIIB)	Esophageal	Operation, cisplatin-5-FU×4, Radiotherapy after recurrence	Death	20.2

A negative interval represents a hematological malignancy that was diagnosed after the diagnosis of a solid tumor; all intervals between the 2 primary tumors were less than 6 months.

M: Male, F: female, OS: overall survival, CR: complete response, SD: stable disease, HL: Hodgkin lymphoma, DLBCL: diffuse large B-cell lymphoma, MZL: marginal zone lymphoma, MALT: mucosa-associated lymphoid tissue lymphoma, FL: follicular lymphoma, ALCL: anaplastic large-cell lymphoma, MCL: mantle cell lymphoma, B-NHL: B-cell non-Hodgkin lymphoma, CLL: chronic lymphocytic leukemia, MDS: myelodysplastic syndrome, RA: refractory anemia, RCMD: refractory cytopenia with multilineage dysplasia, RAEB-2: refractory anemia with excess blasts 2, CML CP: chronic myeloid leukemia in the chronic phase, AML: acute myeloid leukemia, APL: acute promyelocytic leukemia, auto-HSCT: autologous hematopoietic stem cell transplantation, ABVD: adriamycin, bleomycin, vincristine, dacarbazine, R-EPOCH: rituximab, etoposide, vincristine, pirarubicin, cyclophosphamide, prednisone, R-CHOP: rituximab, pirarubicin, cyclophosphamide, vincristine, prednisone; CHOPE: pirarubicin, cyclophosphamide, vincristine, prednisone, etoposide; DICE: dexamethasone, ifosfamide, cisplatin, etoposide; DDGP: cisplatin, dexamethasone, gemcitabine, pegaspargase, P-Gemox-VP16: gemcitabine, oxaliplatin, etoposide, dexamethasone, asparaginase, R-COP: rituximab, cyclophosphamide, vincristine, prednisone; FC: fludarabine, cyclophosphamide; EPO: erythropoietin; G-CSF: recombinant human granulocyte colony-stimulating factor; HAA: homoharringtonine, aclacinomycin, cytarabine, CAG: cytarabine, aclacinomycin, G-CSF; IA: idarubicin, cytarabine, ID-Ara-c: intermediate-dose cytarabine, PC: pemetrexed, carboplatin, DN: docetaxel, nedaplatin, DIED: vinorelbine, ifosfamide, epirubicin, dexamethasone, TP: taxol, oxaliplatin; S-1: tegafur-gimeracil-oteracil potassium capsule, SOX: oxaliplatin, S-1; DP: cisplatin, docetaxel, FEC: fluorouracil, epirubicin, cyclophosphamide, mFOLFOX6: fluorouracil, oxaliplatin, folic acid calcium, EMA/CO: etoposide, actinomycin D, methotrexate, vincristine, cyclophosphamide, 5-FU: 5-fluorouracil.

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Successful Outcome of a Case of Acute Myeloid Leukemia with t(8;21)/AML-ETO Following Langerhans Cell Histiocytosis

Langerhans Hücreli Histiositozunu Takiben Gelişen t(8;21) Akut Myeloid Lösemi Olgusunun Başarılı Tedavisi

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To the Editor,

The occurrence of Langerhans cell histiocytosis (LCH) and acute myeloid leukemia (AML) in the same case has been reported occasionally. We report a new case of AML with t(8;21)/AML-ETO in an adolescent after LCH. To our knowledge, this is the first description of AML with t(8;21)/AML-ETO after LCH diagnosis and therapy.

A 15-year-old boy was diagnosed with LCH in October 2010. He presented with a 1-year history of a skull mass. After 9 cycles of ifosfamide, vincristine, etoposide, and prednisone, the skull mass disappeared. Two years later, the patient presented to the Hematology Department of Beijing Friendship Hospital with progression of his disease in the form of lumber fracture. The mutation BRAF V600E was negative. After relapse of LCH, he received 6 cycles of etoposide and prednisone and 1 cycle of etoposide, prednisone, cyclophosphamide, and vincristine. On 12 March 2013, he received an autologous hematopoietic stem cell transplant. When he came to the clinic with complaints of dizziness on 20 November 2017, a routine blood examination was performed with the following results: white blood cell count, $6.3x10^9$/L; hemoglobin, 60 g/L; and platelet count, $12x10^9$/L. Bone marrow biopsy showed 69% myeloblasts, and Auer rods were found. The immunophenotype profile of the blast cells was CD34 (++), CD13 (+), CD33 (++), CD117 (++), CD38 (+), CD15 (+), HLA-DR (++), MPO(+). Cytogenetic analysis revealed 46, XY, t(8;21)(q22;q22)[20]. The *AML-ETO* and *WT1* genes were positive. The patient responded well to induction chemotherapy. Standard DA chemotherapy (daunorubicin and cytarabine) was given and the boy achieved complete response (CR) after one cycle. After an additional cycle of DA consolidation chemotherapy, he received an HLA-identical sibling allogeneic hematopoietic stem cell transplant (HSCT). He received a conditioning protocol composed of busulphan and cyclophosphamide, and he was given fluconazole and acyclovir as infection prophylaxis and cyclosporine and mycophenolate mofetil as graft-versus-host disease prophylaxis. Up to 30 March 2019, the patient was in a state of persistent CR for 16 months after the diagnosis of the AML, and the *AML-ETO* and *WT1* genes were negative.

There is an association between LCH treatment and subsequent development of AML; however, the reason why AML develops in patients treated for LCH is not entirely understood. The chemotherapy for LCH and genetic predisposition may be explanations. Previously, 27 such cases have been reported [1], and lymphomas, solid tumors, and other hematologic malignancies have been associated with chemotherapy for LCH [2]. Most patients develop AML at least 2 years (mean: 5.5 years) after LCH treatment [3]. LCH treatment agents, together with the genetic predisposition of the patient, might therefore be the reason for AML development. Etoposide, a DNA-topoisomerase II inhibitor, is commonly employed in LCH treatment and is primarily related to therapy-related AML (t-AML) [4]. A safe dose of etoposide does not truly exist; between 2 and 20 years after exposure to etoposide, 1%-5% of patients may develop t-AML [5]. Etoposide-associated AML has been described after its usage for a wide spectrum of diseases, including non-Hodgkin lymphoma [6], acute lymphoblastic leukemia [7], solid tumors [2], and hemophagocytic lymphohistiocytosis [5]. The cytogenetic abnormalities of t-AML reported in patients with LCH include t(15;17), 11q23, +16, and +21 [8,9,10]. The finding of t(8;21)(q22;q22) in a t-AML patient with LCH has not been reported previously, although it was reported in t-AML patients with other malignant neoplasms, including solid tumors, lymphomas, and other hematologic malignancies [11]. Most of the t-AML cases with t(8;21) reported are t(8;21)(q22;q22); other breakpoints of t(8;21) are rare. However, the current findings indicate a worse outcome for t-AML with t(8;21) compared to de novo AML with t(8;21)(q22;q22) [11]. Throughout the treatment process, the case is more likely to be that of t-AML.

Additionally, previous studies have suggested common neoplastic precursors for LCH and AML [12]. Recent molecular analysis of human LCH samples and mouse models showed that the origin cell may be a myeloid-derived precursor [13]. Furthermore, genomic screening has revealed the presence of BRAF, ARAF, and somatic MAP2KI mutations in the majority of LCH and AML patients' specimens [14,15]. Cases in which LCH occurred concurrently and after AML have also been reported [10,16,17]. Xu et al. [17] reported a case where LCH evolved into AML without chemotherapy including etoposide for LCH. Therefore, researchers have accepted the possibility of genetic predisposition to facilitate the development of pathogenic molecular abnormalities. Yohe et al. [10] reported four patients who presented with acute leukemia of myeloid or ambiguous lineage in association with LCH. One patient had trisomy 21 in both the leukemic blasts and LCH cells, indicative of a clonal relationship. Another patient expressed CD2, CD13, and CD117 on both the LCH cells and the leukemic blasts, suggesting a possible clonal relationship. These reports suggest that LCH and AML might have a common neoplastic stem cell.

In our case, successful allogeneic HSCT not only controlled the patient's AML but also had a long-lasting effect on his relapsed LCH. For these patients, induction chemotherapy combined with allogeneic HSCT is a good choice.

Keywords: Langerhans cell histiocytosis, Acute myeloid leukemia, Allogeneic hematopoietic stem cell transplant

Anahtar Sözcükler: Langerhans hücreli histiositozu, Akut myeloid lösemi, Allojenik hematopoietik kök hücre transplant

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Breast Implant-Associated Anaplastic Large-Cell Lymphoma: A Case Report

Meme İmplantı ile İlişkili Anaplastik Büyük Hücreli Lenfoma: Olgu Sunumu

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To the Editor,

Breast implant-associated anaplastic large-cell lymphoma (BIA-ALCL) is a rare type of peripheral T-cell lymphoma, also recognized as a specific disease in the 2016 revision of the World Health Organization classification of tumors of the hematopoietic and lymphoid tissues [1]. Although BIA-ALCL has an indolent course, infiltrative forms may be life-threatening and 9 deaths have been reported [2]. The annual incidence is estimated as 0.1 to 0.3 per 100,000 women with implants [3]. The median age is 53, with the disease being detected after a median of 8 years following implantation [4]. Herein, we report a rare case of BIA-ALCL, the first from Turkey.

A 40-year-old Caucasian female presented to our clinic with right-sided breast swelling and asymmetry. Five years ago, she was diagnosed with left-sided invasive ductal carcinoma. She received neoadjuvant chemotherapy, followed by mastectomy and axillary lymph node dissection of the left side and nipple-sparing mastectomy of the right side. Macro-textured anatomical silicone gel implants and fat grafting were applied, followed by adjuvant chemotherapy. Five years later, breast ultrasound and MRI revealed effusion in the fibrous capsule surrounding the breast implant (Figures 1A and 1B). Initial evaluation of the effusion was benign and the implant was replaced by another one after partial capsulectomy. However, the seroma recurred. In the third sampling, the immunochemical analysis revealed typically large and pleomorphic CD30-positive so-called hallmark cells (Figures 1C and 1D). She was diagnosed with BIA-ALCL. The Ann Arbor stage was IE and the TNM stage was IA. Complete excision of the breast implant and capsule was performed and no capsule invasion was reported upon pathological evaluation. Neither further surgery nor chemotherapy was applied. She has remained in remission to date, at the 18th month after the surgery.

Although it is a very rare entity, detection and diagnosis of BIA-ALCL is an emerging topic. BIA-ALCL is surgically treated and it has an indolent course, with the risk of death being 0.4 micromorts per patient [5]. Most cases are unilateral; however, rare bilateral cases have been reported. Patients mainly present with malignant effusions associated with the fibrous capsule surrounding the implants [6]. Lack of ALK expression and strong membranous expression of CD30 constitute the main immunochemical profile. The largest series published in the literature are summarized in Table 1.

The pathogenesis of BI-ALCL is still unclear. Textured implants are likely to induce a marked local T-cell immune response compared to smooth implants. Textured implants are known

to shed silicone particulate. Macrophages digesting silicone particulate form foamy cells and release cytokines, eliciting T-cell chemotaxis and replication. These findings help us to hypothesize that BI-ALCL originates from aberrant reactive T-cell populations [7]. The main treatment is surgical removal of the implant and total capsulectomy with complete excision of any associated mass until reaching negative margins on final pathologic evaluation, defined as complete surgical excision. Removal of the contralateral breast implant is controversial, as bilateral capsule involvement was reported in the literature [6,8]. Although there is no randomized controlled trial managing patients with incomplete capsulectomy, with residual disease and with stage II-IV disease, the postulated approach is chemotherapy with cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) [6]. CHOP plus etoposide and brentuximab vedotin are alternatives for ALCL treatment [7].

Our patient's diagnosis was based on CD30 positivity and the presence of large pleomorphic cells. Immunohistochemical staining for ALK was not performed and this is a limitation of our report. Immunohistochemical evaluation of the expressions of CD2, CD3, CD4, CD5, CD7, CD8, CD30, and ALK is necessary and constitutes a widely accepted strategy to evaluate seroma samples.

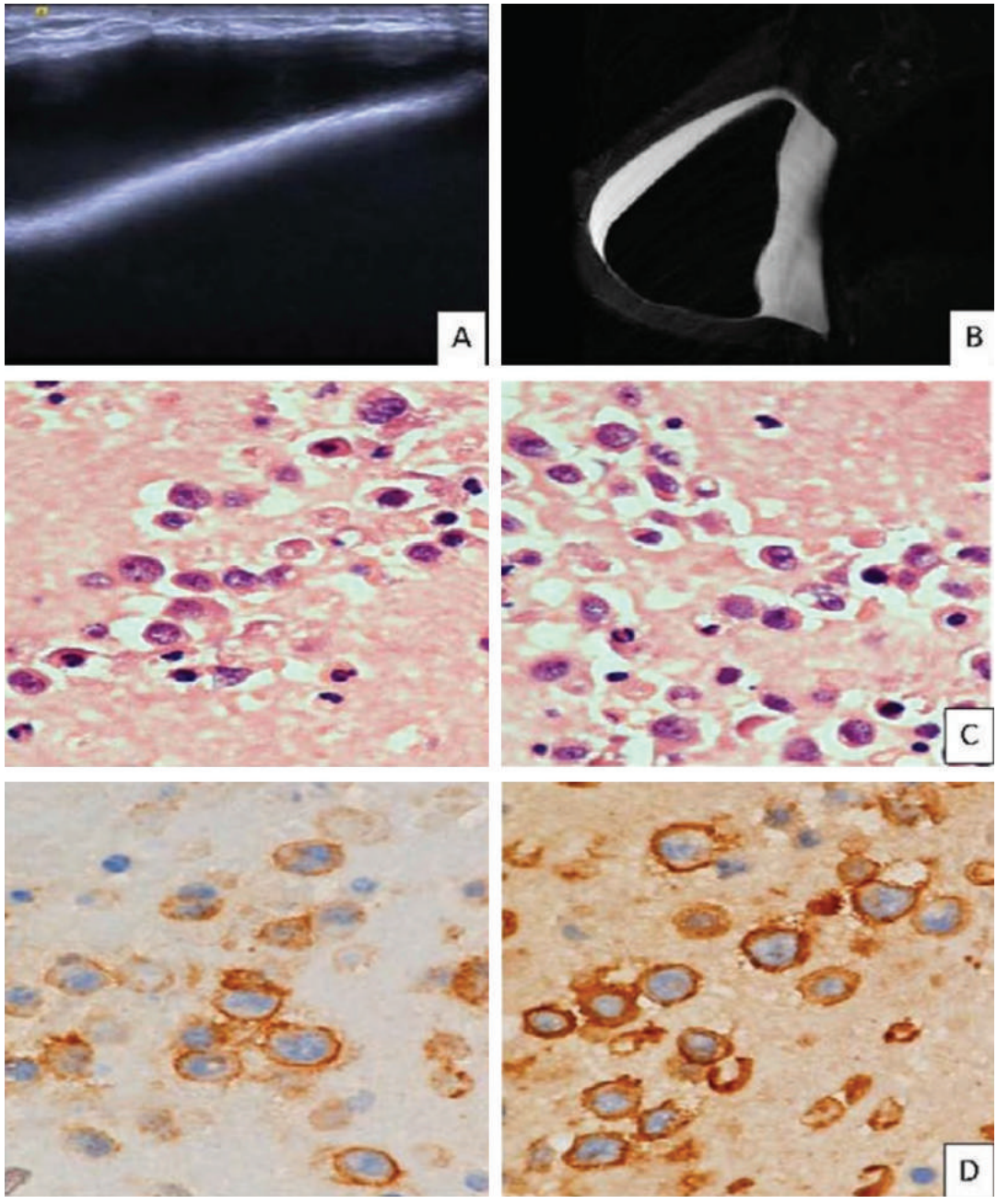

Figure 1. A&B: Ultrasound (A) and magnetic resonance imaging (B) of the capsule of the implant and the seroma at breast. C: Hematoxylin eosin staining, large cells, pleomorphic cells with abundant cytoplasm. D: CD30 (+) lymphocytes.

Table 1. Summary of large series of breast implant-associated anaplastic large-cell lymphoma cases.

Study	n	Age	Interval from implant to diagnosis	Implant characteristics	Treatment and outcome
Doren et al. [9]	100	Mean 53.2±12.3 years	Mean 10.7±4.6 years	Textured (n=51) Smooth (n=0) Unknown (n=49)	No data available.
Loch-Wilkinson et al. [10]	55	Median 47.1 years	Median 7.46 years	Biocell (n=44) Polyurethane (n=15) Salt loss (n=5) Siltex (n=5) Poly Implant Prothèse (n=2) Smooth (n=4)	All patients underwent total capsulectomy and removal of implants on both the diseased and non-diseased sides. Twelve patients had evidence of tumor infiltrating the capsule. Nine patients had adjuvant chemoradiotherapy, 1 patient had adjuvant chemotherapy; 5 of them had local recurrence. Two had positive tumor margins in histopathology. Three patients survived and remained disease-free. One patient received neoadjuvant chemotherapy. One patient had autologous bone marrow transplant. Overall, 1 patient who presented with seroma and 3 patients who presented with mass and/or metastatic disease died.
de Boer at al. [11]	32	Median 59 years	Median 13 years	Macro-textured (n=23) Micro-textured (n=5) Unknown (n=4)	Surgery only (n=11). Neoadjuvant chemotherapy (n=2). Neoadjuvant chemoradiotherapy (n=1). Adjuvant radiotherapy (n=2). Adjuvant chemotherapy (n=9). Adjuvant chemoradiotherapy (n=6). Chemotherapy only (n=1). Autologous stem cell transplantation performed for 5 patients. Twenty-nine patients were in complete remission after first-line (n=23) or second-line (n=6) treatment. Two patients died of disseminated disease after second-line treatment.
Campanale et al. [12]	22	Mean 49.6 years	Mean 7.8 years	Textured surface and silicone filler device (n=21) Textured surface and a double lumen saline/silicone filler device (n=1)	Implant removal with total capsulectomy only (n=14). Adjuvant chemotherapy and autologous stem cell transplantation (n=2). Adjuvant chemotherapy + anti-CD30 (n=1). Adjuvant chemotherapy (n=3). Adjuvant chemoradiotherapy (n=1). Mastectomy and chemotherapy (n=1). Nineteen patients are apparently free of disease, 1 patient died, and 2 are still undergoing chemotherapy.

As the number of breast implant surgeries is rising continuously, the diagnosis of BIA-ALCL is increasing. Patients undergoing breast implantation should be informed of the risk of lymphoma development. Recurring effusions around the capsule may reveal the suspicion of BIA-ALCL. Patients should be treated with surgery-based treatments. Randomized controlled studies are needed to determine standard chemotherapy protocols.

Keywords: Breast implants, Lymphoma, Large-cell, Anaplastic, Seroma

Anahtar Sözcükler: Meme implantı, Lenfoma, Büyük hücreli, Anaplastik, Seroma

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A Rare Cause of Cyanosis Since Birth: Hb M-Iwate

Doğumdan İtibaren Mevcut Olan Siyanozun Nadir Bir Nedeni: Hb M-Iwate

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To the Editor,

Cyanosis in an apparently healthy newborn baby may be caused by hemoglobin (Hb) variants associated with the formation of methemoglobin. Such Hb variants are collectively known as M Hbs [1]. Hb M-Iwate [alpha2 87(F8) His>Tyr, HBA2:c.262C>T] is one of the Hb variants associated with methemoglobinemia [2].

Many Hb variants have been reported so far from Turkey [3,4,5]. We report herein a newborn baby from Bursa, Turkey, with methemoglobinemia and (pseudo) cyanosis having Hb M-Iwate as the underlying cause. To our knowledge, this is only the second report of Hb M-Iwate from Turkey, and more than four decades have passed since its first observation in Turkey in a 21-year-old male by Ozsoylu [6]. In addition, our case represents the first case of Hb M-Iwate from Turkey identified through genetic analysis of the α-globin chain gene (*HBA*).

The boy, born at term to a 32-year-old mother, was noted to be cyanotic immediately after birth. He had findings of dyspnea and he received oxygen by hood.

In the family history, the mother had history of cyanosis, particularly in the peroral area, and was otherwise healthy. In addition, the maternal grandfather and his mother, who had migrated from Thessaloniki (Greece), also had a history of cyanosis.

The oxygen saturation (SpO_2) of the baby, measured by pulse oximeter, was between 50% and 60%. Administration of oxygen did not result in an increase of the measured SpO_2. In venous blood gas analysis, pH was 7.43, pCO_2 was 34.6 mmHg, pO_2 was 45.3 mmHg, and the p_{50} value was 39.2 mmHg (normal range: 22.6-29.4 mmHg). Methemoglobin relative concentration was 13.5% (normal: <1.5%). Complete blood

count testing (Table 1) and echocardiographic examination were both normal.

In the follow-up of the case, findings of dyspnea resolved by the 3rd postnatal day, although cyanosis persisted. The baby was discharged on the 4th day in good condition.

Genetic analysis by Sanger sequencing of the *HBA* genes identified a pathogenic variant, HBA2:c.262C>T, corresponding to the already described Hb M-Iwate [alpha2 87(F8) His>Tyr] in the propositus and in his similarly affected mother (Figure 1A). This Hb variant could be detected by high-performance liquid chromatography (HPLC) (Beta-Thalassemia Program, Bio-Rad) (Figures 1B and 1C).

Table 1. Complete blood count parameters of the proband and his mother on the first postnatal day.

	Proband	Mother
Hb (g/dL)	19.1	11.2
Hct (%)	50.2	32.3
RBC (10^6/µL)	5.06	3.74
MCV (fL)	99	87
MCH (pg)	37.7	29.9
MCHC (g/dL)	38.0	34.2
RDW (%)	13.8	10.6
WBC (10^3/µL)	23.2	9.0
Plt (10^3/µL)	246	258

Hb: Hemoglobin, Hct: hematocrit, RBC: red blood cell count, MCV: mean corpuscular volume, MCH: mean corpuscular Hb, MCHC: mean corpuscular Hb concentration, RDW: red cell distribution width, WBC: white blood cell count, Plt: platelet count.

The M Hbs are transmitted in an autosomal dominant fashion and the existence of familial cyanosis with this pattern of inheritance was first recognized in Japan more than 200 years ago. In the 1950s, Shibata et al. [7] discovered the cyanosis to be due to an abnormal Hb in a large family with about 70 affected individuals. This abnormal Hb was later given the name Hb M-Iwate. In the vivid description of the clinical picture by Shibata et al. [8], "The patients with this disease are cyanotic from childhood, looking like a man who has been swimming in a cold water pool for a long time".

In conclusion, M Hbs should be considered in the differential diagnosis of cyanosis in the newborn period. HPLC can identify the presence of an Hb variant but gene sequencing is necessary for the identification of abnormal variants. Except for cosmetic consequences, the clinical course of patients with Hb M-Iwate is unremarkable.

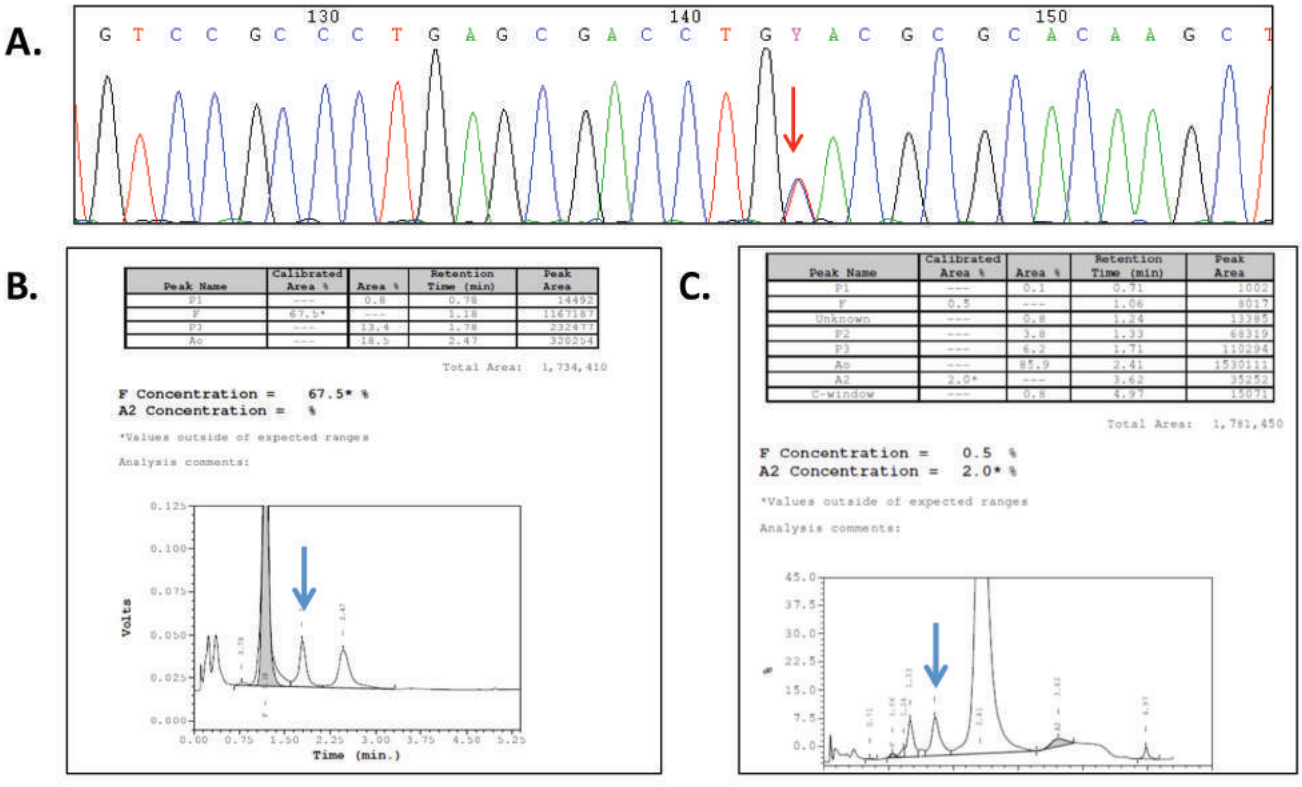

Figure 1. A) DNA sequence of a segment of exon 2 of the *HBA2* gene showing the c.262C>T mutation. B) HPLC of the propositus (newborn). Peaks corresponding to Hb F (67.5%), Hb M-Iwate (identifield as P3) (13.4%; arrow), and Hb A (18.5%) are observed. C) HPCL of the mother. Peaks corresponding to Hb F (2.5%), Hb M-Iwate (identified as P3) (6.2%; arrow), Hb A2 (2.0%), and a small fraction (C-window), corresponding to HbA2var ($a^{Iwate}{}_2\delta_2$), are observed.

Keywords: Hb M-Iwate, Cyanosis, Methemoglobinemia

Anahtar Sözcükler: Hb M-Iwate, Siyanoz, Methemoglobinemi

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Hodgkin Lymphoma, Tuberculosis, and Atypical Radiologic Image

Hodgkin Lenfoma, Tüberküloz ve Atipik Radyolojik Görüntü

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To the Editor,

We read the report by Büyükşimşek et al., [1] "Atypical Radiologic Image Characterized by Cavitary Lung Lesions in a Case of Hodgkin Lymphoma" (HL), with great interest. Büyükşimşek et al. [1] reported on a case of HL presenting with abnormal lung radiologic imaging and mentioned that "Disseminated cavitary lesions mimicking tuberculosis or other opportunistic infections in a case of HL is interesting and differential diagnosis is very important". We would like to share our ideas regarding this observation. Indeed, lung involvement due to lymphoma is possible. Nevertheless, the concurrence between HL and tuberculosis is detectable. In endemic areas of tuberculosis, such as Southeast Asia, tuberculosis screening is routinely done for any cancerous patients, including those with HL. Pathophysiologically, a common pathway that can result in increased risk for tuberculosis among patients with HL is the alteration of the antioxidative system. The depletion of glutathione (GSH) due to HL [2] can increase the risk for tuberculosis since GSH plays an important role in defending against mycobacterial pathogens [3]. Considering the present report by Büyükşimşek et al., [1] there is an interesting question of whether the present case of HL had a concurrent tuberculosis infection or not. Büyükşimşek et al. [1] used the QuantiFERON test for exclusion of tuberculosis. In a recent report, the sensitivity and specificity of the QuantiFERON test were found to be poor [4]. In cases with underlying vitamin B12 deficiency, false negative results by QuantiFERON are possible [5]. In a recent report, vitamin B12 deficiency was observable in 0.54% of patients with HL and anemia [6].

Keywords: Hodgkin Lymphoma, Tuberculosis, Radiology

Anahtar Sözcükler: Hodgkin Lenfoma, Tüberküloz, Radyoloji

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Reply from the Authors

To the Editor,

We thank Drs. Yasri and Wiwanitkit for their interest and for sharing their thoughts on our case report. We agree with them about the co-occurrence of tuberculosis and lymphoma, especially in endemic areas. Additionally, it is very well known that infections with *Mycobacterium tuberculosis* and other intracellular microorganisms are common in cases of Hodgkin lymphoma (HL) due to underlying T-cell defects [1,2]. On the other hand, clinical symptoms and signs including fever, night sweats, and weight loss are very common in tuberculosis and in HL, and sometimes it may be very difficult to differentiate HL and/or accompanying tuberculosis in a case of HL. For this reason, as we discussed before, tuberculosis was the first diagnosis in our case when the patient presented with fever and night sweats. To differentiate and to exclude tuberculosis, we tried different technologies, including culture for tuberculosis and follow-up radiologic imaging, and also clinical signs and symptoms. Of course QuantiFERON was not the only applied test in our case, but due to the journal's space limitations we could not mention the other tests: culture for tuberculosis was reported as negative and the patient responded very well to anti-lymphoma therapy only.

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