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Turkish Journal of Hematology

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Research Articles

Disseminated Intravascular Coagulation in Acute Promyelocytic Leukemia Patients: A Retrospective Analysis of Outcomes and Healthcare Burden in US Hospitals

Rushin Patel, Darshil Patel, Mrunal Patel, Jessica Ohemeng-Dapaah, Afoma Onyechi, Zalak Patel, Chieh Yang, Safia Shaikh; San Bernardino, Chicago, Warren, St. Louis, Riverside, USA

Real-Life Data on the Efficacy and Safety of Letermovir for Primary Prophylaxis of Cytomegalovirus in Allogeneic Hematopoietic Stem Cell Recipients: A Single-Center Analysis

Martyna Włodarczyk, Agata Wieczorkiewicz-Kabut, Krzysztof Białas, Anna Kocłęga, Izabela Noster, Patrycja Zielińska, Grzegorz Helbig; Katowice, Poland

Oncolytic *Myxoma virus* Increases Autophagy in Multiple Myeloma

Alpay Yeşilaltay, Dilek Muz, Berna Erdal; İstanbul, Tekirdağ, Türkiye

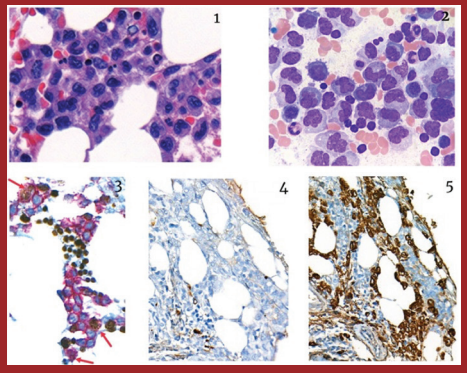
Impact of *CALR* and *JAK2V*617F Mutations on Clinical Course and Disease Outcomes in Essential Thrombocythemia: A Multicenter Retrospective Study in Turkish Patients

Zehra Narlı Özdemir, Yıldız İpek, Püsem Patır, Gözde Ermiş, Rafiye Çiftçiler, Deniz Özmen, Mehmet Baysal, Vildan Gürsoy, Esra Yıldızhan, Serkan Güven, Tarık Ercan, Tayfun Elibol, Sinan Mersin, Eylem Genç, Eren Arslan Davulcu, Volkan Karakuş, Nergiz Erkut, Gürsel Güneş, Reyhan Diz Küçükkaya, Ahmet Emre Eşkazan; İzmir, İstanbul, Antalya, Trabzon, Konya, Bursa, Kayseri, Çanakkale, Muğla, Tekirdağ, Ankara, Türkiye

Perspective in Hematology

Antiphospholipid Syndrome: To Classify or Not to Classify?

Doruk Erkan; New York, USA



Cover Picture:

Asya Tuğçe Bol, Güldane Cengiz Seval, Meral Beksaç, Işınsu Kuzu

The Many Faces of Multiple Myeloma

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Cover Picture

The Many Faces of Multiple Myeloma

1. Neoplastic infiltration by atypical cells with monocytoid morphology on bone marrow trephine biopsy sections (H&E). 2. Neoplastic atypical cells with monocytoid morphology on bone marrow aspirate smears (Giemsa). 3. CD138 (red)-Ki-67 (black) double immunohistochemistry revealing increased proliferative activity (12%) among atypical plasma cells (arrow). 4. Kappa Ig light chain negativity. 5. Lambda Ig light chain restriction.

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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.

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Materials and Methods: Important methods should be written respectively.

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

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1. List all authors

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

2. Organization as author

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

3. Book

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

4. Book Chapter

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

5. Abstract

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

6. Letter to the Editor

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

7. Supplement

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

Brief Reports

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Disseminated Intravascular Coagulation in Acute Promyelocytic Leukemia Patients: A Retrospective Analysis of Outcomes and Healthcare Burden in US Hospitals

Akut Promyelositik Lösemi Hastalarında Yaygın Damariçi Pıhtılaşma: ABD Hastanelerindeki Sonuçların ve Sağlık Hizmeti Yükünün Geriye Dönük Analizi

Rushin Patel[1], Darshil Patel[2], Mrunal Patel[3], Jessica Ohemeng-Dapaah[4], Afoma Onyechi[4], Zalak Patel[5], Chieh Yang[5], Safia Shaikh[6]

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Abstract

Objective: Acute promyelocytic leukemia (APL) is associated with an elevated risk of developing disseminated intravascular coagulation (DIC). The purpose of this study was to assess the outcomes of hospitalizations related to DIC in APL and their impact on healthcare.

Materials and Methods: This study entailed a cross-sectional and retrospective analysis of the US National Inpatient Sample database. We identified adults with APL and categorized them into groups of patients with and without DIC. Our focus areas included in-hospital mortality, length of stay, charges, and complications associated with DIC. Unadjusted odds ratios/coefficients were computed in univariate analysis, followed by adjusted odds ratios (aOR)/coefficients from multivariate analysis that accounted for confounding factors.

Results: Our analysis revealed that APL patients with DIC had a substantially higher aOR for mortality (aOR: 6.68, 95% confidence interval [CI]: 4.76-9.37, p<0.001) and a prolonged length of stay (coefficient: 10.28 days, 95% CI: 8.48-12.09, p<0.001) accompanied by notably elevated total hospital charges (coefficient: $215,512 [95% CI: 177,368-253,656], p<0.001), thereby emphasizing the reality of extended medical care and economic burden. The presence of DIC was associated with increased odds of sepsis, vasopressor support, pneumonia, acute respiratory failure, intubation/mechanical ventilation, and acute kidney injury, reflecting heightened vulnerability to these complications. Patients with DIC demonstrated significantly higher odds ratios for major bleeding, intracranial hemorrhage, gastrointestinal bleeding, red blood cell transfusion, platelet

Öz

Amaç: Akut promyelositik lösemi (APL) yaygın damariçi pıhtılaşma (YDP) gelişim riskinin artışıyla ilişkilidir. Bu çalışmanın amacı APL'de YDP ile ilgili hastane yatışlarının sonuçlarını ve sağlık hizmetleri üzerine etkisini incelemektir.

Gereç ve Yöntemler: Bu çalışma ABD Ulusal Yatan Hasta Örnek veritabanının kesitsel ve geriye dönük bir analizini içermektedir. APL tanılı erişkinleri belirledik ve YDP'si olanlar ve olmayanlar olarak kategorize ettik. Odaklandığımız alanlar hastane mortalitesi, yatış süresi, maliyet ve YDP ile ilişkili komplikasyonlardı. Düzeltilmemiş tahmini rölatif risk oranları (RRO)/katsayılar tek değişkenli analizde hesaplanmış ardından karıştırıcı faktörleri hesaba katan çok değişkenli analizden düzeltilmiş RRO (dRRO)/katsayıları elde edilmiştir.

Bulgular: Analizimiz, YDP'li APL hastalarının mortalite için belirgin şekilde daha yüksek bir dRRO'na sahip olduğunu ortaya koydu (dRRO: 6,68, %95 güven aralığı [GA]: 4,76-9,37, p<0,001). Hastanede kalış süresi belirgin bir şekilde uzamıştı (katsayı: 10,28 gün, %95 GA: 8,48-12,09, p<0,001). Bu durum toplam hastane masraflarını dikkate değer şekilde artırmaktaydı (katsayı: 215,512 $ [95% GA: 177,368-253,656], p<0,001). Bu sonuçlar uzun süreli tıbbi bakımın ve ekonomik yükün gerçekliğini vurgulamaktadır. YDP'li hastalar majör kanama, intrakraniyal kanama, gastrointestinal kanama, kırmızı kan hücresi transfüzyonu, trombosit transfüzyonu, taze donmuş plazma transfüzyonu ve kriyopresipitat transfüzyonu için anlamlı derecede daha yüksek RR oranları göstermiş ve bu da YDP'nin oluşturduğu belirgin hematolojik riskleri işaret etmiştir.

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Abstract

transfusion, fresh frozen plasma transfusion, and cryoprecipitate transfusion, highlighting the pronounced hematological risks posed by DIC.

Conclusion: This study has revealed the significant associations between DIC in APL and various outcomes, underscoring the clinical and economic implications of these conditions. The hematological risks further increase patients' vulnerability to bleeding events and the need for transfusions.

Keywords: Acute promyelocytic leukemia, Disseminated intravascular coagulation, In-hospital mortality, Complications, Length of stay, Charges

Öz

Sonuç: Bu çalışma, APL'de YDP ile çeşitli sonuçlar arasındaki önemli ilişkileri ortaya koymuş ve bu durumun klinik ve ekonomik etkilerinin altını çizmiştir. Hematolojik riskler, hastaların kanamalara karşı hassasiyetini ve transfüzyon ihtiyaçlarını daha da artırmaktadır.

Anahtar Sözcükler: Akut promiyelositik lösemi, Yaygın damar içi pıhtılaşma, Hastane içi mortalite, Komplikasyonlar, Kalış süresi, Ücretlendirme

Introduction

Acute promyelocytic leukemia (APL) is a distinct subtype of acute myeloid leukemia that is physiologically unique from other subtypes. It is distinguished by aberrant proliferation of promyelocytes and has a yearly incidence of 600-800 cases in the United States [1]. It is rare in children under the age of 10, but its prevalence rises during adolescence, peaks in early adulthood, and then remains stable, followed by a decline after 60 years [2]. It presents aggressively and has a complicated coagulopathy that causes bleeding or thrombosis before or during treatment. The clinical symptoms and distinctive morphologic abnormalities of Auer rods on peripheral smears establish APL as a diagnosis.

The most prevalent type of APL is caused by a specific translocation involving the retinoic acid receptors alpha gene (*RARα*) on chromosome 17 and the promyelocytic leukemia (*PML*) gene on chromosome 15 t(15;17)(q22;q12), which results in the formation of the *PML-RARα* fusion gene, thereby leading to clonal promyelocytic expansion [2]. Although it is unclear how this translocation affects leukemogenesis, there is evidence that *PML-RARα* hinders terminal differentiation and subsequently results in the apoptosis of promyelocytes [3].

The most severe clinical manifestation of APL is life-threatening bleeding, primarily caused by increased fibrinolytic-type disseminated intravascular coagulation (DIC). Even though the exact frequency of DIC in APL patients is unknown, a recent epidemiological survey in Taiwan discovered that 90 (77.6%) of 116 APL patients developed overt DIC [4]. The coagulopathy in APL is caused by inappropriate activation of the coagulation cascade by a tissue factor present on the surface of leukemic blasts [5]. This excess release of tissue factor is a common cause of DIC. Another etiology is due to annexin II on the surface of malignant leukocytes, which stimulates the endogenous tissue plasminogen activator and urokinase-type plasminogen activator [5]. This results in hyperfibrinolysis or an excessive breakdown of fibrinogen and occluded fibrin [2]. There is also platelet dysfunction and increased cancer cell cytokine expression (including interleukin [IL]-1β, tumor necrosis factor alpha, and IL-6), which stimulates endogenous tissue factor synthesis, causing the suppression of thrombomodulin and encouraging thrombosis [1,4].

Hemorrhagic episodes due to DIC in cases of APL follow a distinct clinical pattern, and intracranial hemorrhage and pulmonary hemorrhage are the leading cause of death [6,7,8,9]. According to a report from the PETHEMA Group on patients treated in clinical trials with all-trans retinoic acid (ATRA) and idarubicin, 37 of 66 deaths during induction were caused by bleeding, 24 of which were intracranial bleeds and 12 of which were intrapulmonary [7]. According to that study, while most hemorrhagic deaths occurred within the first 10 days following induction, fatal bleeding events persisted until day 23. Excluding patients with subdural hemorrhage, most of the intracranial hemorrhages in the study resulted in death [7].

Induction mortality remains a serious issue and a fundamental cause of treatment failure in managing APL, with hemorrhage contributing to most such cases of early death [1]. The use of ATRA and arsenic trioxide (ATO) in the initial treatment of APL has been one of the most important developments in cancer treatment [8]. This has significantly improved APL outcomes, with over 90% long-term relapse-free survival for individuals who survive the first 30 days after diagnosis. Despite the discovery of highly successful treatment techniques for APL, however, approximately 10% of patients die because of bleeding diathesis in the early course of the disease due to DIC [9]. Our understanding of the prognostic factors related to induction mortality in APL patients has remained extremely limited. Supportive care and other minor diagnostic and therapeutic aspects may play critical roles in patient outcomes, particularly in low socioeconomic settings where early mortality is high and overall survival is poor due to lack of adequate awareness and a multidisciplinary approach to management.

The outcomes of DIC in APL were retrospectively investigated in the present study, thereby offering significant insights into their intricate interaction and their impact on healthcare. This study included a diverse patient population. Hence, the findings may support the development of tailored therapies aimed at reducing mortality and improving the outcomes of APL patients with DIC.

Materials and Methods

This retrospective study employed data extracted from the National Inpatient Sample (NIS) database, which is sponsored by the Agency for Healthcare Research and Quality and constitutes part of the Healthcare Cost and Utilization Project (HCUP) [10]. The NIS database represents an approximate 20% stratified sample of discharges across nearly 1000 US hospitals from all 50 states of the United States. Notably, the NIS database is the largest publicly available all-payer inpatient care database in the United States. This study specifically utilized data from the NIS database spanning the years 2016 to 2019, encompassing hospitalizations from January 1, 2016, to December 31, 2019, and with records of over 24 million hospital stays. Approval from the ethics committee was not needed, as the NIS database that we used contains deidentified data and does not track individuals' information.

Study Population

Using the International Classification of Disease, Tenth Revision, Clinical Modification (ICD-10 CM) codes, we identified hospitalizations involving all adult (≥18 years) patients with APL as their principal or secondary diagnosis. We then categorized the patients into two groups as those with DIC and those without DIC.

Outcomes of Interest

Our areas of focus included in-hospital mortality, length of stay, total hospitalization charges, and complications linked with DIC and other associations. For mortality, we utilized the NIS variable "DIED." Length of stay was determined using the NIS variable "LOS." The length of stay for a patient was defined as the total days spent in the hospital from the day of admission to discharge or death. Total hospitalization charges were assessed using the variable "TOTCHG." Additionally, we referred to the relevant ICD-10 codes provided in Supplemental Table 1 to investigate complications and other associations.

Statistical Analysis

All analyses were conducted using appropriate stratifying, clustering, and weighting samples as provided by the regulations of the HCUP [11]. Statistical analysis was performed using STATA version 17 (Stata Corp LLC, College Station, TX, USA). All ICD-10 CM codes used in our study are listed in Supplemental Table 1.

We calculated the odds ratio for dichotomous variables and the coefficient for continuous variables. To create a multivariate analysis model, we included potential confounding variables, which consisted of age, sex, race, income quartile based on zip code, hospital region, hospital teaching status, hospital division, hospital size by beds, insurance status, and the well-established Charlson Comorbidity Index score. The Charlson Comorbidity Index score encompasses conditions such as myocardial infarction, congestive heart failure, peripheral arterial disease, cerebrovascular disease, dementia, chronic obstructive pulmonary disease, connective tissue disease, peptic ulcer disease, liver disease, diabetes, hemiplegia or paraplegia, chronic kidney disease, diabetes with end-organ damage, solid tumors, leukemia, lymphoma, and AIDS/HIV, all of which are conditions associated with high mortality rates [12].

Initially, we conducted univariate analyses for each of the aforementioned factors to calculate unadjusted odds ratios. Later, in multivariate regression analysis, we used only variables that were associated with the outcomes of interest in univariate regression analysis with a significance level of $p<0.2$. Proportions were compared using the Fisher exact test for categorical variables, and continuous variables were compared using Student's t-test. All p values were two-sided and the significance level was set at $p<0.05$, indicating statistical significance.

Results

Table 1 presents the baseline characteristics of APL hospitalizations in US hospitals. Our study included a total of 2583 hospitalizations of patients with APL, including 2098 hospitalizations without DIC and 485 with DIC. The mean age of the patients without DIC was 53.5 years while the mean age of patients with DIC was 51.4 years, resulting in an overall mean age of 53.1 years. In terms of sex distribution, the sexes were almost equally distributed in both groups and the difference in sex distribution between the groups without and with DIC was not statistically significant. Analyzing the racial distribution, the majority of the patients were white, accounting for 66.0% and 62.0% in the groups with DIC and without DIC, with statistically significant differences observed for both groups. Regarding median household income, the patients were almost equally distributed across all percentile household income categories. An analysis of insurance status revealed that Medicare covered 38.0% of the patients, Medicaid covered 19.1%, private insurance covered 40.0%, and a small percentage of patients

Table 1. Baseline characteristics of acute promyelocytic leukemia hospitalizations.

	Without DIC	With DIC	Total hospitalizations	p
	2098	485	2583	
Mean age (years)	53.5	51.4	53.1	
Sex				0.634
Male	1042 (49.7%)	246 (50.9%)	1289 (49.9%)	
Female	1053 (50.2%)	237 (49.0%)	1291 (50.0%)	
Race				0.037
White	1384 (66.0%)	300 (62.0%)	1686 (65.3%)	
Black	266 (12.7%)	50 (10.4%)	317 (12.3%)	
Hispanic	276 (13.2%)	82 (17.0%)	359 (13.9%)	
Asian or Pacific Islander	63 (3.0%)	18 (3.9%)	82 (3.2%)	
Native American	10 (0.5%)	0 (0%)	10 (0.4%)	
Other	90 (4.3%)	31 (6.5%)	121 (4.7%)	
Median household income				0.025
0^{th} to 25^{th} percentile	618 (29.5%)	110 (22.8%)	728 (28.2%)	
26^{th} to 50^{th} percentile	488 (23.3%)	121 (25.1%)	609 (23.6%)	
51^{st} to 75^{th} percentile	530 (25.3%)	128 (26.5%)	658 (25.5%)	
76^{th} to 100^{th} percentile	455 (21.7%)	123 (25.5%)	578 (22.4%)	
Insurance status				<0.001
Medicare	843 (40.2%)	136 (28.1%)	981 (38.0%)	
Medicaid	394 (18.8%)	98 (20.2%)	493 (19.1%)	
Private insurance	801 (38.2%)	231 (47.7%)	1033 (40.0%)	
No insurance	54 (2.6%)	18 (3.8%)	72 (2.8%)	
Charlson Comorbidity Index score				<0.001
1	0	0	0	
2	767 (36.6%)	229 (47.4%)	997 (38.6%)	
≥3	1328 (63.3%)	254 (52.5%)	1583 (61.3%)	
Admission type				
Non-elective	1795 (85.6%)	450 (92.9%)	2247 (87.0%)	0.151
Elective	302 (14.4%)	33 (7.0%)	335 (13.0%)	
Census division				0.016
New England	90 (4.3%)	24 (5.1%)	116 (4.5%)	
Middle Atlantic	333 (15.9%)	55 (11.5%)	390 (15.1%)	
East North Central	306 (14.6%)	54 (11.1%)	361 (14.0%)	
West North Central	130 (6.2%)	32 (6.6%)	162 (6.3%)	
South Atlantic	434 (20.7%)	102 (21.2%)	537 (20.8%)	
East South Central	153 (7.3%)	30 (6.3%)	183 (7.1%)	
West South Central	211 (10.1%)	55 (11.5%)	268 (10.4%)	
Mountain	113 (5.4%)	24 (5.1%)	136 (5.3%)	
Pacific	312 (14.9%)	102 (21.2%)	415 (16.1%)	
Hospital size by beds				0.041
Small	255 (12.2%)	39 (8.2%)	294 (11.4%)	
Medium	484 (23.1%)	107 (22.2%)	591 (22.9%)	
Large	1355 (64.6%)	336 (69.4%)	1691 (65.5%)	
Hospital location/teaching status				<0.001
Rural	83 (4.0%)	3 (0.6%)	87 (3.4%)	
Urban non-teaching	230 (11.0%)	39 (8.0%)	268 (10.4%)	
Urban teaching	1779 (84.8%)	442 (91.3%)	2223 (86.1%)	

DIC: Disseminated intravascular coagulation.

(2.8%) had no insurance. The majority of patients in both groups were admitted to large hospitals and urban teaching hospitals.

As presented in Table 2, patients who did not have DIC had a lower mortality rate of 4.5% compared to the higher rate of 20.8% among those with DIC. Without DIC, the average length of stay was 13 days, which was shorter than the 25 days observed in the DIC group. Additionally, patients without DIC had lower hospital charges, averaging $166,950, in contrast to the higher mean charge of $396,151 for those with DIC.

Table 3 outlines the risks, outcomes, and interventions linked with DIC in cases of APL.

The adjusted odds ratio (aOR) for mortality was 6.68 (95% confidence interval [CI]: 4.76-9.37, p<0.001), indicating a significant association with DIC and a substantial impact of DIC on patient survival.

The coefficient for length of stay was 10.28 days (95% CI: 8.48-12.09, p<0.001). This implies that patients with DIC experienced significantly more extended hospital stays. The coefficient's magnitude emphasizes this outcome's clinical significance, suggesting that DIC could contribute to prolonged hospitalizations. The coefficient for total hospital charges was $215,512 (95% CI: 177,368-253,656), with a p value of <0.001. The elevated hospital charges highlight the comprehensive impact of this condition on patients and healthcare systems. Moreover, the high aOR values for sepsis and vasopressor support also suggest the high susceptibility of patients with DIC to develop sepsis and require vasopressor support. A statistical difference was observed between patients with and without DIC for respiratory risks including pneumonia, acute respiratory failure, and intubation or mechanical ventilation with respective aOR values of 1.71 (95% CI: 1.25-2.33, p<0.001), 4.27 (95% CI: 3.27-5.59, p<0.001), and 4.46 (95% CI: 3.38-5.89, p<0.001). This demonstrates the high vulnerability of patients with DIC to respiratory complications compared to patients without DIC. The aOR values for major bleeding, intracranial hemorrhage, and gastrointestinal bleeding were 3.86 (95% CI: 2.91-5.10, p<0.001), 5.99 (95% CI: 4.14-8.66, p<0.001), and 1.88

Table 2. Mortality rate, mean length of stay, and mean total hospital charges.

	Without DIC	With DIC
Mortality rate	4.5%	20.8%
Mean length of stay	13 days	25 days
Mean total hospital charges	$166,950	$396,151

DIC: Disseminated intravascular coagulation.

Table 3. Risks, outcomes, and interventions associated with disseminated intravascular coagulation in cases of acute promyelocytic leukemia.

Risks, outcomes, and interventions	Adjusted odds ratio or coefficient	p
Mortality	aOR: 6.68 (95% CI: 4.76-9.37)	**<0.001**
Length of stay (days)	Coefficient: 10.28 (95% CI: 8.48-12.09)	**<0.001**
Total hospital charges ($)	Coefficient: 215,512 (95% CI: 177,368-253,656)	**<0.001**
Sepsis	aOR: 1.84 (95% CI: 1.41-2.39)	**<0.001**
Vasopressor support	aOR: 2.74 (95% CI: 1.44-5.21)	**0.002**
Pneumonia	aOR: 1.71 (95% CI: 1.25-2.33)	**<0.001**
Acute respiratory failure	aOR: 4.27 (95% CI: 3.27-5.59)	**<0.001**
Intubation or mechanical ventilation	aOR: 4.46 (95% CI: 3.38-5.89)	**<0.001**
Clostridioides difficile infection	aOR: 0.63 (95% CI: 0.31-1.28)	0.201
Acute kidney injury	aOR: 2.38 (95% CI: 1.85-3.01)	**<0.001**
Major bleeding	aOR: 3.86 (95% CI: 2.91-5.10)	**<0.001**
Intracranial hemorrhage	aOR: 5.99 (95% CI: 4.14-8.66)	**<0.001**
Gastrointestinal bleeding	aOR: 1.88 (95% CI: 1.27-2.77)	**<0.001**
Pulmonary embolism	aOR: 0.95 (95% CI: 0.50-1.82)	0.870
Red blood cell transfusion need	aOR: 2.80 (95% CI: 2.23-3.52)	**<0.001**
Platelet transfusion need	aOR: 3.78 (95% CI: 2.97-4.82)	**<0.001**
Fresh frozen plasma transfusion need	aOR: 7.32 (95% CI: 4.92-10.87)	**<0.001**
Cryoprecipitate transfusion need	aOR: 10.54 (95% CI: 7.26-15.30)	**<0.001**

aOR: Adjusted odds ratio; CI: confidence interval.

(95% CI: 1.27-2.77, p<0.001), respectively. These results indicate a significant positive association between DIC in APL and the risk of major bleeding. The elevated aOR values underscore the increased susceptibility of these patients to bleeding events. The aOR values for red blood cell transfusion, platelet transfusion, fresh frozen plasma (FFP) transfusion, and cryoprecipitate transfusion were 2.80 (95% CI: 2.23-3.52, p<0.001), 3.78 (95% CI: 2.97-4.82, p<0.001), 7.32 (95% CI: 4.92-10.87, p<0.001), and 10.54 (95% CI: 7.26-15.30, p<0.001), respectively. This suggests significant positive associations between DIC in APL and the need for red blood cell transfusions, platelet transfusions, FFP transfusions, and cryoprecipitate transfusions. The elevated aOR values highlight the heightened likelihood of patients with DIC requiring these transfusions.

Discussion

DIC is life-threatening and causes early morbidity and mortality in patients diagnosed with APL. Our study evaluated the healthcare burdens and outcomes of patients with APL complicated by DIC. Out of the 2583 hospitalizations with APL that we retrospectively assessed, about 19% involved DIC. Although the actual incidence of DIC in APL has not been clearly defined, the incidence of DIC in our study population was much lower compared to previous studies. In Taiwan, an epidemiological study indicated that 90 (77.6%) of 116 APL patients experienced overt DIC [13]. Our investigation did not distinguish between patients who developed DIC before or after chemotherapy induction. The racial distribution in our study population aligned with the patterns previously observed in APL [14]. Patients with DIC have higher odds of developing major life-threatening bleeds, such as intracranial bleeding, as was seen in our study (aOR: 5.99, 95% CI: 4.14-8.66, p<0.001). In a single-center study that evaluated the impact of DIC on induction failure in patients with APL, the leading cause of induction mortality was bleeding, accounting for 66.7% of mortality. The induction mortality rate was 47% in patients with DIC and 11% in patients without DIC [9]. Patients with intracranial hemorrhage tend to have worse outcomes, with higher in-hospital mortality and worse functional outcomes at the time of discharge. In order to reduce secondary brain injury, subsequent therapy focuses on regulating hemostasis, hemodynamics, and intracranial pressure, and most of these cases require observation in an intensive care unit. It is understandable that a substantial number of our cases were managed in metropolitan teaching institutions (84.8% of the DIC-negative and 91.3% of the DIC-positive patients were managed in urban teaching hospitals). In our study population, patients with DIC had more extended hospital stays and higher total hospital charges. Although only a relatively small percentage of our study population (2.8%) lacked insurance, 53.7% of our study population had a household income below the 50th percentile, and the burden of co-pays falls on the patients and society.

The treatment of APL has recently been revolutionized by newer therapies such as ATRA and ATO. Although their exact mechanisms of action remain unclear, ATRA works by various mechanisms, including restoration of autophagy, which serves to degrade the PML-RARa oncoprotein. ATO promotes the autophagy-dependent clearance of the *PML-RARa* gene; synergistically, up to 95% remission may be achieved [15]. Despite these advancements, early death, which is most commonly defined as death within the first 30 days of presentation, remains a major cause of treatment failure. Coagulopathy, leading to intracerebral, gastrointestinal, and pulmonary hemorrhage, is at the forefront of the causes of early death [16,17]. Many clinical trials have failed to include patients with severe coagulopathy/hemorrhage, as they may die even before treatment is initiated. In a Swedish study of 105 APL patients diagnosed between 1997 and 2006, 30 (29%) had early deaths (died within 30 days of diagnosis) and 41% of the early deaths were due to hemorrhage [18]. Our analysis revealed significantly increased odds of mortality from DIC among hospitalized patients with APL (aOR: 6.68, 95% CI: 4.76-9.37, p<0.001). There may be a benefit in further analysis to ascertain whether those deaths were early deaths occurring within 30 days of diagnosis. The management of coagulopathy remains supportive with routine monitoring of platelet count and coagulation parameters and the transfusion of fibrinogen, cryoprecipitate, platelets, and FFP as needed [19]. Our study also revealed statistically higher odds of these patients receiving red blood cell, platelet, FFP, and cryoprecipitate transfusions.

Study Limitations

The NIS database covers inpatient hospitalizations but lacks pre-admission and post-discharge data, preventing long-term follow-up. Given the cross-sectional nature of our data, our findings demonstrate associations rather than causal relationships with the events studied. Moreover, it is essential to acknowledge that our analyses were conducted using retrospective registry data, introducing the possibility of selection bias due to potential selective reporting and the utilization of ICD codes to form the patient cohort. Since the ICD-10 system provides single codes for APL (C924) and DIC (D65), it is not feasible to stratify patients based on APL risk levels (low/intermediate/high) due to the absence of this information in the dataset and the ICD-10 codes. Additionally, crucial details like coagulation panel results, other laboratory values, treatment strategies, and cause of death analyses are absent from our dataset. These considerations are crucial when interpreting our results and their potential implications for clinical practice.

Despite these limitations, our study provides valuable insights into real-world practices for APL patients with DIC. Our goal is to enhance patient outcomes by informing clinical decision-making.

While coding errors and variations exist, the NIS database is widely used and validated. Our study, based on a substantial sample from this database, represents a diverse population across the United States with data from numerous medical centers.

Conclusion

This study has explored the outcomes of DIC in APL, revealing critical insights into their complex relationship. Our analysis revealed significant connections and important clinical implications. These findings could lead to targeted interventions aimed at lowering mortality, improving hospitalization, and easing the financial burdens of APL patients with DIC. Insights into related complications such as sepsis, respiratory issues, and hematological concerns open possibilities for proactive management approaches. This study also paves the way for refining predictive models and personalized treatment plans, enabling healthcare providers to enhance care quality and patient outcomes.

Ethics

Ethics Committee Approval: Approval from the ethics committee was not needed, as the NIS database that we used contains deidentified data and does not track individuals' information.

Informed Consent: Patient consent was not needed as the NIS dataset is deidentified and does not keep information of individuals.

Authorship Contributions

Concept: R.P., D.P.; Design: R.P., D.P.; Data Collection or Processing: R.P., D.P.; Analysis or Interpretation: R.P., D.P., M.P.; Literature Search: M.P., J.O.D., A.O., C.Y., S.S.; Writing: M.P., J.O.D., A.O., Z.P., C.Y., S.S.

Conflict of Interest: No conflict of interest was declared by the authors.

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Supplemental Table 1. International Classification of Disease, Tenth Revision, Clinical Modification codes used in this study.

Variable	ICD-10 CM/Procedure codes used
Acute promyelocytic leukemia	C924
Disseminated intravascular coagulation	D65
Sepsis	A021, A227, A267, A327, A400, A401, A403, A408, A409, A41, A4101, A4102, A411, A412, A413, A414, A4150, A4151, A4152, A4153, A4159, A4181, A4189, A419, A427, A5486, B377, P360, P3610, P3619, P362, P3630, P3639, P364, P365, P368, P369, R6520, R6521, T8144XA, T8144XD, T8144XS
Vasopressor support	3E030XZ, 3E033XZ, 3E040XZ, 3E043XZ, 3E050XZ, 3E053XZ, 3E060XZ, 3E063XZ
Pneumonia	J180, J181, J182, J188, J189
Acute respiratory failure	J9600, J9601, J9602, J9620, J9621, J9622, J9690, J9691, J9692
Intubation/mechanical ventilation	5A09357, 5A09457, 5A09557, 09HN7BZ, 0CHY7BZ, 0DH57BZ, 0NH17EZ, 5A1935Z, 5A1945Z, 5A1955Z, 0BH07DZ
Clostridioides difficile infection	A047, A0471, A0472
Acute kidney injury	N170, N171, N172, N178, N179
Major bleeding	R58, L7622, K661, I62, I620, I6200, I6201, I6202, I6203, I621, I629, R04, R040, R041, R042, R048, R0481, R0489, R049
Intracranial hemorrhage	I6000, I6001, I6002, I6010, I6011, I6012, I602, I6030, I6031, I6032, I604, I6050, I6051, I6052, I606, I607, I608, I609, I6030, I6030, I610, I611, I612, I613, I614, I615, I616, I618, I619, I6200, I6201, I6202, I6203, I621, I629
Gastrointestinal bleeding	K920, K921, K922, K625, K928, K929, K9281, K9282, K9289, K250, K254, K260, K264, K270, K28, K621
Red blood cell transfusion	30233N0, 30233N1, 30243N0, 30243N1, 30273N1, 30277N1, 30233P0, 30233P1, 30243P0, 30243P1
Platelet transfusion	30233R1, 30243R0, 30243R1, 30273R1, 30277R1
Fresh frozen plasma transfusion	30233L0, 30233L1, 30243L0, 30243L1, 30273L1, 30233K0, 30233K1, 30243K0, 30243K1, 30273K1, 30277L1, 30277K1
Cryoprecipitate transfusion	30233M0, 30233M1, 30243M0, 30243M1, 30273M1, 30277M1, 30233D1, 30243D1
Pulmonary embolism	I26, I260, I2602, I2609, I269, I2692, I2699

ICD-10 CM: International Classification of Disease, Tenth Revision, Clinical Modification.

RESEARCH ARTICLE

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Real-Life Data on the Efficacy and Safety of Letermovir for Primary Prophylaxis of Cytomegalovirus in Allogeneic Hematopoietic Stem Cell Recipients: A Single-Center Analysis

Allojenik Hematopoetik Kök Hücre Alıcılarında Sitomegalovirüsün Primer Profilaksisi için Letermovirin Etkililiği ve Güvenirliğine İlişkin Gerçek Yaşam Verileri: Tek Merkezli Bir Analiz

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Abstract

Objective: Cytomegalovirus (CMV) reactivation is a life-threatening complication after allogeneic hematopoietic stem cell transplantation (HSCT). Introduction of letermovir (LMV) seems to improve post-transplant outcomes, but delayed-onset CMV reactivation still remains a challenge. In this study, we report on our first experience with LMV prophylaxis in 93 CMV-seropositive adult patients receiving HSCT in our center.

Materials and Methods: We retrospectively analyzed the data of 93 adult CMV-seropositive recipients receiving LMV as CMV prophylaxis after HSCT for hematological malignancies between 2019 and 2023. The starting LMV dose was 480 mg daily, reduced to 240 mg daily for those receiving cyclosporin A co-administration. CMV DNA in the blood was measured by real-time polymerase chain reaction weekly for the first 2 months after transplantation, then every other week until the end of immunosuppressive treatment. LMV was continued to day +100 or to CMV reactivation.

Results: The median recipient age at the time of transplant was 51 (range: 20-71) years. All patients received grafts from peripheral blood, mostly for acute myeloid leukemia (60%). The median time from transplantation to LMV initiation was 3 (range: 0-24) days. While 55% of patients were transplanted from matched related donors, 32% had unrelated donors and 13% underwent haploidentical HSCT. Four patients (4%) had CMV "blips" while on LMV, but the drug was continued and repeated assays were negative. Only 2 patients (2%) experienced CMV reactivation while on LMV, on days 48 and 34 after HSCT, respectively. Seven patients (7%) developed late-onset CMV reactivation after a median of 124 days after HSCT (range: 118-152 days) and they were successfully treated with ganciclovir. CMV disease was not observed. Grade III-IV acute graft-versus-host disease occurred in 6 patients (6%) during LMV treatment. LMV treatment was free of side effects.

Öz

Amaç: Sitomegalovirüs (CMV) reaktivasyonu allojenik hematopoetik kök hücre transplantasyonu (HKHT) sonrasında hayatı tehdit eden bir komplikasyondur. Letermovir (LMV) kullanımının nakil sonrası sonuçları iyileştirdiği görülmektedir, ancak gecikmiş başlangıçlı CMV reaktivasyonu hala bir sorun olmaya devam etmektedir. Bu çalışmada, merkezimizde HKHT olan 93 CMV-seropozitif yetişkin hastada LMV profilaksisi ile ilgili ilk deneyimimizi bildiriyoruz.

Gereç ve Yöntemler: 2019-2023 yılları arasında hematolojik maligniteler için HKHT sonrası CMV profilaksisi olarak LMV başlanan 93 yetişkin CMV-seropozitif alıcının verilerini retrospektif olarak analiz ettik. Başlangıç LMV dozu günde 480 mg olup siklosporin A ile birlikte uygulananlar için günde 240 mg'a düşürülmüştür. Kandaki CMV DNA'sı gerçek zamanlı polimeraz zincir reaksiyonu ile transplantasyondan sonraki ilk 2 ay boyunca haftada bir, daha sonra immünosupresif tedavinin sonuna kadar iki haftada bir ölçülmüştür. LMV profilaksisi +100. güne kadar veya CMV reaktivasyonuna kadar devam ettirilmiştir.

Bulgular: Nakil sırasındaki ortanca alıcı yaşı 51 (aralık: 20-71) idi. Tüm hastalar, çoğunlukla miyeloid akut lösemi (%60) nedeniyle periferik kandan nakil yapılmıştır. Transplantasyondan LMV başlangıcına kadar geçen medyan süre 3 (aralık: 0-24) gündü. Hastaların %55'ine doku tipi uyumlu akraba vericilerden nakil yapılırken, %32'sine akraba olmayan vericiler ve %13'üne haploidentik HKHT uygulanmıştır. Dört hastada (%4) LMV kullanırken CMV "*blips*" görüldü, ancak ilaca devam edildi ve tekrarlanan testler negatif çıktı. Sadece 2 hastada (%2) LMV kullanırken, sırasıyla HKHT'den sonraki 34. ve 48. günlerde CMV reaktivasyonu görülmüştür. Yedi hastada (%7) HKHT'den ortanca 124 gün sonra (aralık: 118-152 gün) geç başlangıçlı CMV reaktivasyonu gelişmiş ve bu hastalar gansiklovir ile başarılı bir şekilde tedavi edilmiştir. Bu hastalarda CMV hastalığı gözlenmemiştir. LMV tedavisi sırasında 6 hastada (%6) grade III-IV akut graft-versus-host hastalığı meydana gelmiştir. LMV tedavisi boyunca yan etki görülmemiştir.

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Abstract

Conclusion: LMV prophylaxis was effective in preventing CMV reactivation with a favorable safety profile. CMV reactivation occurred mostly after LMV discontinuation; thus, extending the duration of prophylaxis beyond 100 days could be beneficial.

Keywords: Allogeneic hematopoietic stem cell transplantation, Antiviral prophylaxis, Cytomegalovirus reactivation, Letermovir

Öz

Sonuç: LMV profilaksisi, olumlu bir güvenlik profili ile CMV reaktivasyonunu önlemede etkili olmuştur. CMV reaktivasyonu çoğunlukla LMV kesildikten sonra meydana gelmiştir; bu nedenle profilaksi süresinin 100 günün ötesine uzatılması faydalı olabilir.

Anahtar Sözcükler: Allojenik hematopoietik kök hücre transplantasyonu, Antiviral profilaksi, Sitomegalovirüs reaktivasyonu, Letermovir

Introduction

Cytomegalovirus (CMV) reactivation remains a life-threatening complication after allogeneic hematopoietic stem cell transplantation (HSCT) [1,2]. It affects about 37% of patients and is associated with an increased risk of graft rejection, non-relapse mortality, and decreased overall survival [3,4,5,6]. Without an effective preventive strategy, CMV infection may occur in about 70% of recipients [7,8]. Definitions of CMV infection, reactivation, and disease are presented briefly in Table 1 [9,10,11,12].

Until today, the preferred preventive approach to CMV infection/disease was regular and careful monitoring of CMV blood viremia followed by prompt initiation of preemptive therapy (PET) upon detection of a significant rise in CMV viral load. Although this strategy results in a decline of CMV-related end-organ disease, the issue of frequent CMV reactivations in high-risk patients remains a challenge.

The treatment landscape changed in 2017 with the introduction of letermovir (LMV). The use of LMV as primary CMV prophylaxis has significantly improved clinical outcomes by decreasing the risk of clinically significant CMV (csCMV) infection in allotransplanted patients without causing significant side effects. Moreover, LMV has changed the pattern of CMV management policy in high-risk, CMV-seropositive patients from CMV surveillance and PET to a relatively safer and more effective preventive approach [13]. LMV was granted the recommendation of the European Conference on Infections in Leukemia and was approved in 2017 by the US Food and Drug Administration (FDA) and the European Medicines Agency for prevention of CMV infection/disease in CMV-seropositive HSCT recipients [11,14,15].

Although LMV as post-HSCT prophylaxis is now a well-established strategy, real-world data on delayed-onset CMV reactivations remain scarce. In this study, we report on our first experience with LMV prophylaxis in 93 CMV-seropositive adult patients receiving HSCT in our center.

Materials and Methods

The data of 93 adult patients (57 men) who received LMV prophylaxis between 2019 and 2023 were analyzed. Those who died or were lost to follow-up before the 100th day of observation were excluded from the analysis. Clinical data and transplantation details were obtained from our institutional database of medical records.

Table 1. Definitions of cytomegalovirus infection and disease.

Term	Definition
CMV infection	Isolation of CMV material (proteins or nucleic acid) in body fluid of any type or tissue sample
Primary CMV infection	CMV infection observed for the first time in an individual without known evidence of pre-transplant CMV exposure
Recurrent CMV infection	CMV infection in an individual with known previous evidence of CMV infection when the virus had not been detected during at least 4 consecutive weeks of monitoring, as a result of either reactivation of latent virus or virus reinfection (see below)
CMV reinfection	Detection of a different CMV strain than the one that caused the original CMV infection
CMV reactivation	Detection of two CMV strains (prior and current) that are found to be indistinguishable
Symptomatic CMV infection	Both presence of general symptoms and/or signs (e.g., fever, bone marrow suppression) and detection of CMV genetic material obtained using sensitive methods; no signs of CMV end-organ disease
CMV disease	Detection of CMV material by sensitive tests performed on tissue samples acquired through biopsy or other invasive methods, accompanied by the presence of symptoms and/or signs from the affected organ
"Blip"	An episode of isolated positive PCR assay results where preceding and following tests performed with 7-day intervals remain negative
Late-onset CMV reactivation	CMV reactivation after prophylaxis completion, i.e., beyond the 100th day after allotransplantation

CMV: Cytomegalovirus; PCR: polymerase chain reaction.

All analyzed patients were CMV immunoglobulin (Ig) G-seropositive and CMV IgM-negative before transplantation and received standard antiviral prophylaxis against the herpes simplex virus and varicella zoster virus with acyclovir, trimethoprim-sulfamethoxazole for *Pneumocystis jirovecii* prophylaxis, and fluconazole and/or posaconazole as fungal prophylaxis. The following factors were identified as signifying high risk for CMV reactivation: CMV seropositivity of the recipient (R) prior to transplantation regardless of donor (D) serostatus, cord blood as the stem cell source, unrelated or mismatched donor transplant, haploidentical transplant, use of T-cell depletion, use of corticosteroids at a dose of ≥1 mg/kg, and the occurrence/severity of acute or chronic graft-versus-host disease (GVHD) with treatment [13,16,17,18,19]. Irradiated, leukodepleted, and CMV-negative blood products were transfused after HSCT. GVHD prophylaxis consisted of a calcineurin inhibitor, i.e., either cyclosporin A or tacrolimus, with methotrexate and mycophenolate mofetil as needed. Post-transplant cyclophosphamide was provided in cases of haploidentical HSCT. Anti-thymocyte globulin was administered to every patient at high risk of GVHD (age >50 years, unrelated and/or female donor). The Child-Pugh score was used to rule out severe hepatic impairment. All patients were also screened for the presence of severe kidney failure or any other exclusion criteria. The dose of LMV was adjusted for cyclosporin A co-administration. LMV was continued until day +100 after transplantation or to CMV reactivation.

From the time of neutrophil engraftment, defined as absolute neutrophil count of $\geq 0.5 \times 10^9$/L for 3 consecutive days, patients were screened for CMV reactivation. CMV DNA in the blood was measured using real-time polymerase chain reaction (PCR) weekly for the first 2 months after transplantation, then every other week until the end of immunosuppressive treatment. LMV was continued to day +100 after HSCT or to CMV reactivation. The lower limit of CMV detection was 1 copy/µL.

The detection of measurable CMV DNAemia increasing in 2 consecutive assays was treated as CMV reactivation and PET with (val)ganciclovir [(V)GCV] was then initiated. "Blips" were defined as episodes of isolated positive CMV PCR test results where both the preceding and the succeeding tests performed with 7-day intervals remained negative. When a blip was confirmed, LMV was continued. Late-onset CMV reactivation was defined as virus reactivation after LMV completion. Acute GVHD (aGVHD) was diagnosed and graded according to standard criteria [20,21].

Results

Median recipient age at transplant was 51 (range: 20-71) years. All analyzed patients were CMV IgG-seropositive before transplantation. Thirty-five patients (38%) received an allograft from a seronegative donor (D-/R+) while the remaining patients had seropositive donors (D+/R+). In 76% (n=71) of the cases, myeloid neoplasm was the primary underlying disease, and most patients were transplanted for myeloid acute leukemia (n=60). Peripheral blood was the stem cell source for all transplanted patients. Fifty-one patients (55%) received grafts from human leukocyte antigen (HLA)-matched siblings, 30 patients (32%) were transplanted from unrelated 10/10 HLA-matched (n=16) or 8-9/10 HLA-mismatched (n=14) donors, and 12 patients (13%) underwent haploidentical transplantation. About half of the patients (n=47) received reduced-intensity conditioning, whereas myeloablative conditioning was administered to other patients. The patients' characteristics are summarized in Table 2.

The median time from transplantation to LMV initiation was 3 (range: 0-24) days and the drug was administered orally for all patients. Fifty-two (56%) patients received LMV at 240 mg daily due to concomitant use of cyclosporin A while LMV at 480 mg a day was administered to the remaining 41 patients. One patient's treatment was temporarily interrupted due to severe post-transplant mucositis with dysphagia.

Table 2. Patients' characteristics.

Variable	n=93
Median age, years [range]	51 [20-71]
Male sex, n (%)	57 (61)
Diagnosis, n (%)	
• **Myeloid neoplasms**	71 (76)
AML	56 (60)
MDS	7 (8)
CML	3 (3)
MF	3 (3)
CMML	2 (2)
• **Lymphoid neoplasms**	18 (19)
ALL	11 (12)
DLBCL	2 (2)
BPDCN	2 (2)
MM	1 (1)
BL	1 (1)
ALCL	1 (1)
• **Others**	4 (4)
SAA	4 (4)
Donor, n (%)	
• Sibling	51 (55)
• Unrelated	30 (32)
• Haploidentical	12 (13)
Conditioning, n (%)	
• Myeloablative	46 (49)
• Reduced intensity	47 (51)
CMV serostatus, n (%)	
• D+/R+	58 (62)
• D-/R+	35 (38)

AML: Acute myeloid leukemia; MDS: myelodysplastic syndrome; CML: chronic myeloid leukemia; MF: myelofibrosis; CMML: chronic myelomonocytic leukemia; ALL: acute lymphoblastic leukemia; DLBCL: diffuse large B-cell lymphoma; BPDCN: blastic plasmacytoid dendritic cell neoplasm; MM: multiple myeloma; BL: Burkitt lymphoma; ALCL: anaplastic large cell lymphoma; SAA: severe aplastic anemia; CMV: cytomegalovirus; D: donor; R: recipient.

Four patients (4%) were found to have CMV "blips" at a median of 56 (range: 30-90) days after HSCT with a median number of 31 (range: 16-46) copies/µL, but repeated PCR assays were found to be negative and LMV was continued. Only 2 patients (2.2%) had reactivated CMV during LMV. The first patient had reactivated CMV on day +34 with CMV PCR of 188 copies/µL. The second patient (the one for whom LMV was interrupted for 10 days) had reactivated CMV on day +48 with 232 copies/µL. Both patients had the LMV discontinued and received treatment with GCV with CMV eradication. Seven patients (7%) developed late-onset CMV reactivation at a median of 124 (range: 118-152) days after HSCT with a median CMV load of 81 (range: 11-453) copies/µL. CMV reactivations were treated successfully with (V)GCV.

Six patients (6%) developed grade III-IV aGVHD while on LMV. Despite triple immunosuppressive treatments including a JAK2-inhibitor, ruxolitinib, none of them developed CMV reactivation. LMV was well tolerated and only mild side effects were observed. Nausea, decreased appetite, fatigue, and abdominal pain were among the commonest, but other medications including antibiotics, antifungals, and immunosuppressive agents were being simultaneously administered. No severe or life-threatening adverse events or signs of myelotoxicity or nephrotoxicity were reported.

Three patients qualified for secondary HSCT due to secondary graft failure. Six patients died within the first year after HSCT: 5 due to early relapse and 1 from severe pneumonia. Two of them had previously experienced CMV reactivation. The other patients remain in long-term follow-up and are in good condition overall.

Discussion

The efficacy of primary prophylaxis with LMV in preventing CMV infection in HSCT settings was first demonstrated in a phase II trial [22]. Soon afterwards, Marty et al. [23] performed a pivotal phase III trial including 565 CMV-seropositive allo-recipients. This trial showed that csCMV infection occurred almost 2 times less frequently in the LMV group (37.5% of cases) compared to the placebo group (60.6%) at 24 weeks after HSCT. It also showed that prophylaxis with LMV improved post-transplant survival without causing significant side effects [23]. The success of that phase III trial led to LTV's approval by the FDA and to a shift in anti-CMV policy towards prophylaxis. Post hoc analysis demonstrated that in the LMV group all-cause mortality was lower than in the placebo group not only at week 24 but also at week 48 after HSCT. It has been suggested that the reduction in all-cause mortality achieved by LMV might be related to the delay in the onset of csCMV infection/disease until immune reconstitution is advanced enough to respond to the viral invasion [24,25].

Real-life experiences with the efficacy and safety of LMV as primary CMV prophylaxis reported by transplant centers worldwide were in line with the results of the pivotal study by Marty et al. [20,26,27,28,29,30,31]. According to recent meta-analyses and literature reviews, primary prophylaxis with LMV for adult HSCT recipients reduced the incidence of CMV reactivation, infection, and disease at both day +100 and day +200 compared to controls [24,32,33]. Moreover, no delay in hematological reconstitution and no signs of myelotoxicity or nephrotoxicity were observed in published reports presenting beneficial safety profiles. Our analysis is consistent with those real-life data.

In the aforementioned phase III trial, patients with a high risk of CMV reactivation benefited more from LMV prophylaxis than patients with lower risk. D/R CMV serological status remains the main risk factor influencing the incidence and mortality of CMV reactivation/disease after HSCT [1,16,34,35]. It has been demonstrated that seropositive recipients are more likely to experience CMV reactivation if they received a graft from a seronegative donor than from a seropositive one [36,37,38]. Nevertheless, CMV reactivation occurs in up to 70% of CMV IgG seropositive allo-recipients regardless of donor status according to some recent studies, and LMV prophylaxis is therefore recommended for all CMV seropositive recipients [1,11,39]. In our study, 5 patients out of 7 who had reactivated CMV after 100 days had received grafts from CMV-seronegative donors. This is consistent not only with the results from the pivotal trial by Marty et al. [23] but also with observations from the meta-analysis by Vyas et al. [24], where LMV use was found to be particularly beneficial for high-risk patients [30,40]. Real-world data have shown not only a significant reduction in the risk of any CMV-related complications in all analyzed reports but also a decreased demand for the use of PET, shortened hospitalizations, fewer re-admissions to the hospital, and fewer concurrent complications, particularly fungal or bacterial infections. This, in turn, is associated with a potential economic benefit [41,42].

GVHD increases the risk of CMV reactivation and vice versa. Moreover, GVHD contributes to significant morbidity and mortality, especially when it requires prolonged immunosuppressive treatment that impairs the immune defense of the host [1,11]. It has been suggested that LMV prophylaxis also improves transplantation outcomes in patients with aGVHD. According to recent research, patients with aGVHD had significantly fewer csCMV infections while receiving LMV prophylaxis compared to patients who did not receive LMV [40]. Moreover, improved GVHD-free, relapse-free survival was also demonstrated [43].

Despite the high efficacy of LMV in preventing csCMV infection/disease after HSCT, a higher frequency of delayed-onset CMV

infections has been observed after LMV discontinuation, highlighting the potential role of extended LMV prophylaxis [29,44,45,46,47]. The significance of prolonged LMV prophylaxis for high-risk patients was already addressed by Marty et al. [13]. Discontinuation of LMV on day 100 after HSCT has also been shown to increase CMV-related mortality between days 180 and 364 [46]. A recently published study by Dadwal et al. [48] showed that extending LMV prophylaxis to 200 days after HSCT significantly reduced the incidence of csCMV infections compared to a placebo (2.8% vs. 18.9%) in the high-risk patient group. Despite prolonged administration, LMV was well tolerated and demonstrated a good safety profile with adverse effects similar to those of the placebo. The findings from this trial also suggest that a longer duration of LMV prophylaxis might be particularly beneficial for patients with delayed CMV T-cell reconstitution [41]. In our study, we observed that LMV was effective at reducing csCMV infection/disease during the first 100 days after HSCT, but the incidence of CMV reactivation increased thereafter.

Study Limitations

A potential limitation of this study is its single-center design and short follow-up period. However, the strength of our analysis lies in the fact that we provided real-world data regarding a relatively large population with high CMV seroprevalence compared to other single-arm retrospective cohort studies. More data are needed to confirm our findings in Polish patients.

Conclusion

LMV prophylaxis was effective in preventing CMV reactivation with a favorable safety profile. CMV reactivation occurred most often after LMV discontinuation; thus, extending the duration of prophylaxis beyond 100 days could be beneficial.

Ethics

Ethics Committee Approval: The work described in this article was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans, EU Directive 2010/63/EU for animal experiments, and the uniform requirements for manuscripts submitted to biomedical journals.

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

Authorship Contributions

Surgical and Medical Practices: A.W.K., K.B., A.K., I.N., P.Z.; Concept: M.W., G.H.; Design: M.W.; Data Collection or Processing: M.W., A.W.K., K.B., A.K., I.N., P.Z.; Analysis or Interpretation: M.W., G.H.; Literature Search: M.W.; Writing: M.W., G.H.

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RESEARCH ARTICLE

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Oncolytic *Myxoma virus* Increases Autophagy in Multiple Myeloma

Onkolitik *Miksoma virüsü* Multipl Miyelomda Otofajiyi Artırıyor

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Abstract

Objective: Multiple myeloma, which affects plasma cells, is the second most common hematological malignancy. Despite the development of new drugs and treatment protocols, patient survival has not reached the desired level. In this study, we investigated the effects of *Myxoma virus* (MYXV), an oncolytic virus, on autophagy in myeloma cells.

Materials and Methods: We analyzed protein expressions of ATG-5, p62, Beclin-1, LC3B, and the apoptosis marker Bcl-2 as autophagy markers in human U-266 and mouse MOPC-315 myeloma cell lines subjected to different doses of MYXV. In addition, autophagic images of myeloma cells were investigated using transmission electron microscopy (TEM).

Results: In the first 24 h, which is the early stage of autophagy, ATG-5 and Beclin-1 expression levels were increased in the U-266 and MOPC-315 cell lines in the groups that had received MYXV at a multiplicity of infection of 15. At 48 h, a significant increase was detected in the expression of LC3B, which is a late indicator. Autophagosomes were observed in myeloma cells by TEM.

Conclusion: MYXV shows an antimyeloma effect by increasing autophagy in myeloma cells.

Keywords: *Myxoma virus*, Multiple myeloma, Autophagy, Autophagosome

Öz

Amaç: Plazma hücrelerini etkileyen multipl miyelom, en sık görülen ikinci hematolojik kanserdir. Yeni ilaçların ve tedavi protokollerinin geliştirilmesine rağmen, hasta sağkalımı istenilen düzeye ulaşmamıştır. Bu çalışmada, onkoltik bir virüs olan *Miksoma virüsü*'nün (MYXV), miyelom hücrelerinde otofaji üzerindeki etkilerini araştırdık.

Gereç ve Yöntemler: İnsan U-266 ve fare MOPC-315 miyelom hücre hatlarına farklı dozlarda MYXV uygulanarak ve otofaji belirteçleri olarak ATG-5, p62, Beclin-1, LC3B ve apoptoz belirteci Bcl-2 protein ekspresyonları analiz edilmiştir. Ayrıca, miyelom hücrelerinin otofajik görüntüleri, transmisyon elektron mikroskobu (TEM) kullanılarak incelenmiştir.

Bulgular: İlk 24 saatte, yani otofajinin erken aşamasında, U-266 ve MOPC-315 hücre hatlarında MYXV'nin moi 15 enfektif dozda alındığı gruplarda ATG-5 ve Beclin-1 ekspresyon düzeyleri artmıştır. Kırk sekiz saatte, geç bir belirteç olan LC3B'nin ekspresyonunda önemli bir artış tespit edilmiştir. Miyelom hücrelerinde otofagozomlar TEM ile gözlemlenmiştir.

Sonuç: MYXV, miyelom hücrelerinde otofajiyi artırarak antimiyelom etkisi göstermektedir.

Anahtar Sözcükler: *Miksoma virüsü*, Multipl miyelom, Otofaji, Otofagozom

Introduction

Multiple myeloma (MM), which is most commonly diagnosed between the ages of 60 and 70 years, is the second most common blood cancer, accounting for 10% of all hematological malignancies [1]. MM, caused by clonal plasma cell (PC) proliferation, progresses with bone destruction, anemia, frequent infections, and decreased kidney function. The treatment of MM involves a combination of various therapies including corticosteroids, anthracyclines, alkylating agents, immunomodulatory drugs (IMiDs), proteasome inhibitors (PIs), histone deacetylase inhibitors, monoclonal antibodies, and autologous stem cell transplantation [2,3]. In recent years, median overall survival has increased from 2-3 years to 8-10 years due to a better understanding of the pathophysiology and heterogeneity of MM and with the help of new therapeutic approaches [4,5]. However, due to the development of resistance to both IMiDs and PIs, the desired patient survival could not be

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achieved in the treatment of MM patients [6]. Therefore, patients with MM continue to exhibit recurrent patterns of remission and relapse. Moreover, as the disease progresses, tumor cells become more aggressive, the remission period becomes shorter, and patients die from refractory disease [7].

As treatment-free periods are reduced in patients with MM and treatment-resistant disease persists, therapeutics with new mechanisms of action are needed to completely control the disease. Researchers have focused on effective new strategies for cancer treatment in recent years. Since the 1900s, when natural viral infection was observed to cause tumor regression, the idea that viruses could be used for cancer treatment has become widespread [8]. However, due to toxicity and viral pathogenicity, studies on this subject could not be conducted for many years. Thanks to advances in genetic engineering, a new generation of viruses called oncolytic viruses (OVs) that can be used effectively in therapy have been developed. OVs can selectively kill tumor cells without affecting healthy cells [9]. OVs used therapeutically can induce inflammatory responses [10,11], serve as in situ vaccine agents [12], act as adjuvant therapeutics [13,14], and cause DNA damage in cancer cells [15]. Furthermore, OVs can trigger various forms of programmed cell death, including apoptosis, pyroptosis, necroptosis, and autophagy [16,17].

OVs such as reoviruses, the measles virus, *Vaccinia virus*, and the vesicular stomatitis virus have been shown to have therapeutic potential for the treatment of MM [18,19,20,21,22]. *Myxoma virus* (MYXV), which is classified in the genus *Leporipoxvirus* of the family *Poxviridae*, has a double-stranded DNA genome. Having a strict tropism for rabbits and hares, MYXV causes no obvious pathology in either humans or mice [23,24]. The therapeutic effect of MYXV, an OV whose therapeutic potential has recently been recognized, has been investigated in pancreatic cancer, melanoma, glioma, and rhabdoid tumors [25,26,27,28,29,30,31,32]. MYXV has been reported to target leukemia cells in AML tumor xenografts without harming normal hematopoietic stem cells [33]. MYXV has also been shown to induce oncolysis by increasing apoptosis in myeloma cells [34]. Moreover, it has been observed that intravenous injection of MYXV causes reductions of 70%-90% in tumor volume [35].

Autophagy is a natural cleaning and recycling program that breaks down damaged or unnecessary structures within the cell and, if necessary, puts them back into use. There are three subtypes of autophagy in eukaryotic cells: macroautophagy, microautophagy, and chaperone-mediated autophagy [36]. In an unstressed cell, the basal level of autophagy is usually low, removing damaged proteins and organelles to maintain homeostasis. Under stressful conditions, autophagy is induced to restore homeostasis, produce essential amino acids, and maintain cellular life [37]. However, autophagic genomic damage or autophagic imbalance can occur in a wide variety of diseases and disorders, including aging, neurodegeneration, autoimmune diseases, and cancer [38].

Autophagy has a complex relationship with cancer, as it can have both positive and negative effects on tumor growth [39]. In cases of certain types of cancer, such as hepatocellular, pancreatic, and colorectal tumors, as well as lymphoma, leukemia, and myeloma, autophagy is impaired and can lead to resistance to chemotherapy treatments [40]. However, the absence of genes required for autophagy in tumors suggests that it may also act as a tumor suppressor. In breast cancer, for instance, there is a decrease in the expression of the autophagy protein Beclin-1 [41].

After the demonstration of the effectiveness of autophagy in cancer cells, autophagy became the new target of treatment in cancer cells. The oncolytic mammalian reovirus has been proven to trigger autophagy in colorectal cancer [42], while an oncolytic adenovirus equipped with Beclin-1, a key player in autophagy, causes autophagic cell death [43]. The Beclin-1-armed oncolytic *Vaccinia virus* (OVV) was determined to increase the efficacy of chemotherapeutics against lymphoma in vivo and in vitro [44]. Similarly, OVV armed with Beclin-1 has increased therapeutic efficacy in myeloma and leukemia [45].

It has been suggested that PCs have high levels of autophagic activity and autophagy has an important role in PC oncogenesis [46]. Furthermore, autophagy is a survival mechanism in long-lived human PCs [47]. MM cells, like PCs, have both an enlarged endoplasmic reticulum (ER) network and secrete immunoglobulin (Ig). For these reasons, there are misfolded or unfolded proteins that can be toxic in the cytoplasm of MM cells. Thus, MM cells use molecular pathways such as the ubiquitin-proteasome system (UPS), proteasomal degradation, and autophagy to protect against damage [36]. Autophagy in myeloma cells both removes UPS-ubiquitinated proteins [48] and plays a role in determining sensitivity to PIs, which are among the important drugs in MM therapy [49]. MM cells treated with emodin/carfilzomib overexpressed the autophagic protein p62 and LC3B compared to control cells [50]. Patients with high immunoreactivity of autophagic markers such as Beclin-1 and LC3 have been found to have longer survival [51]. In addition, increased Beclin-1 and LC3 expression in MM cells led to median overall survival results of 1171 and 934 days, respectively. Recently, it has been reported that inhibiting the late autophagy phase with elaiophylin inhibits autophagic flux, activates ER stress-mediated apoptosis, and consequently leads to anti-MM cell activity [52].

To our knowledge, there has been no research to date investigating the effect of MYXV on autophagy in MM cells. In this study, we aimed to investigate the expression levels of

autophagic proteins and damage to cells by applying different concentrations of MYXV to myeloma cells.

Materials and Methods

Cells, Reagents, and Virus

MYXV was kindly provided by the University of South Carolina School of Medicine (USA). The U-266 (*Homo sapiens* B lymphocyte cells), MOPC-315 (*Mus musculus* B lymphocyte cells), and Vero (African green monkey kidney-CCL81) cell lines were purchased from ATCC (USA). Fetal bovine serum (FBS), trypsin-EDTA, Dulbecco's modified eagle medium (DMEM), phosphate-buffered saline (PBS), L-glutamine, amino acids, and vitamins were purchased from Pan Biotech (Germany). RPMI 1640 medium and streptomycin were purchased from Sigma-Aldrich (USA), rapamycin was purchased from Biosynth (Switzerland), and WST-1 solution was purchased from Roche (Germany).

MOPC-315 and U-266 cells were used to investigate the apoptotic and autophagic effects of the virus in MM cells. RPMI 1640 supplemented with 10% FBS, 1X penicillin/streptomycin, 2 mM L-glutamine, 1X amino acids, and 1X vitamins was used for culturing U-266 and MOPC-315 cells in suspension in tissue culture flasks at 37 °C with 5% CO_2. To prepare the subculture, cells were centrifuged at 1000 rpm for 10 min. The supernatant was then removed, viability was checked with trypan blue staining, and cells were counted on Thomas slides and suspended in tissue culture flasks.

Virus Titration and Purification

Vero cells were used to produce and titrate MYXV. The Vero cells were grown in DMEM with 10% FBS and 1X penicillin/streptomycin at 37 °C with 5% CO_2. Subculturing was carried out using trypsin-EDTA and PBS solutions. MYXV was purified and titrated as previously described [53]. Vero cells prepared at 1-1.5x10^5/mL were infected with green fluorescent protein-labeled MYXV and evaluated under a fluorescence microscope (see Figure 1). To determine the titer of MYXV, a microtitration test using a focal assay was conducted as previously reported [53]. The process involved preparing logarithmic 10-fold dilutions of MYXV and applying quadruple repetitions for each dilution. After a 48-h incubation period, the tissue culture infective dose ratio was calculated by counting the growth foci of MYXV. Virus purification was performed in an ultracentrifuge (Optima XPN-100 and Max-XP, Beckman Coulter, USA) with 24%-40% sucrose solutions with double and triple gradients created. The virus layer and two consecutive centrifugation protocols were collected at 50,000 x g for 40 min and at 33,000 x g for 40 min. Finally, the pellet obtained by centrifugation at 18,000 rpm was resuspended in PBS.

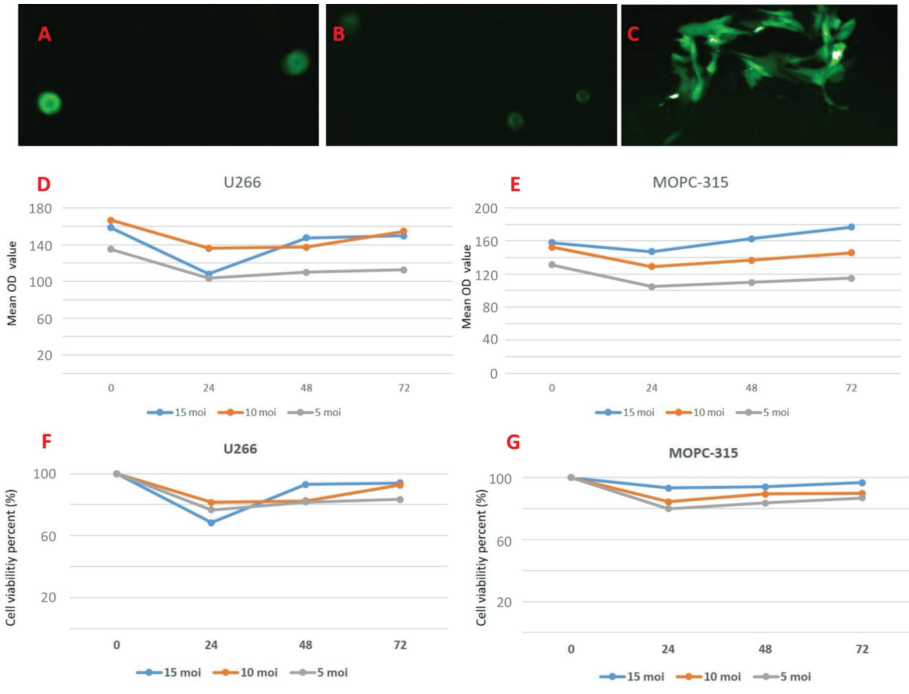

Figure 1. *Myxoma virus* (MYXV) infection in different cells and WST-1 results. MYXV expresses green fluorescent protein, and virus-infected cells were visualized as green under a fluorescent microscope. A) Virus-infected MOPC-315 cells, 10^x. B) Virus-infected U-266 cells, 10^x. C) Virus-infected Vero cells, 10^x. D) WST-1 results of MYXV-infected U-266 cells. E) WST-1 results of MYXV-infected MOPC-315 cells. F) MYXV-infected U-266 cell viability percentage for each incubation period. G) MYXV-infected MOPC-315 cell viability percentage for each incubation period. WST-1 results are shown as 15 multiplicity of infection (moi), 10 moi, and 5 moi virus-infected U-266 and MOPC-315 cells for 0, 24, 48, and 72 h of incubation.

WST-1 Assay

In this study, a set of cell groups was prepared to investigate the cytotoxicity, apoptosis, and autophagy caused by MYXV infection. The U-266 and MOPC-315 cells were prepared in 96-well microplates at a density of 3-4x10^4 cells per well. The cells were infected by diluting MYXV to a multiplicity of infection (moi) of 0.1, 1, 5, 10, or 15. All applications were performed in four repetitions. PBS was added to the wells for the control group. A rapamycin trial group was prepared in separate wells with MYXV-uninfected cells to induce autophagy. Cell viability rates after infection were tested at 24, 48, 72, 96, and 120 h. Cell viability was measured with 420-nm, 450-nm, 480-nm, and 630-nm filters using a spectrophotometer. The ideal incubation period for the cells was determined as 48 h. At the end of the 24, 48, and 72 h of incubation, cell supernatants were collected and analyzed.

Enzyme-Linked Immunosorbent Assay (ELISA)

For autophagy and apoptosis tests, cells were prepared as described above, and 500 nM rapamycin (Biosynth) was added to wells as a positive control. All experimental cell groups were incubated at 37 °C with 5% CO_2. Cell supernatants were collected after 24, 48, and 72 h of incubation and ELISA tests were performed.

In MM cell lines, the levels of apoptotic protein Bcl-2 (catalog no: E1832Hu, BT LAB, China) and the autophagic proteins Beclin-1 (catalog no: E2011Hu, BT LAB), human sequestosome-1 (p62) (catalog no: E6779 Hu, BT LAB), human LC3B (catalog no: MBS1603826, MyBioSource, USA), and human ATG-5 (catalog no: EH1729, FineTest, USA) were measured by ELISA according to the manufacturer's instructions.

ELISA tests were performed in triplicate, with each marker being tested twice. Thus, six measurements were made in each group for comparisons. The utilized kit contains 96-well microplates that are coated with capture antibodies, and biotinylated antibodies are used as detection antibodies. The standards in the kit are diluted in a twofold logarithmic manner. Standards, samples, and biotin antibodies are added to the wells, followed by the addition of HRP-streptavidin conjugate after washing. After another washing step, a substrate solution is added, and the target protein concentration is calculated by reading the optical density absorbance at 450-nm on a microplate reader (Epoch 2, BioTek, USA) and then adding the acidic stopping solution.

Transmission Electron Microscopy (TEM) Analysis

Cells were fixed with Karnovsky fixative (2.5% glutaraldehyde, 2% paraformaldehyde in cacodylate buffer) for 24 h. After fixation, the cells were pelleted by centrifugation at 300x g for 5 min. Cells embedded in 1% agar were then washed with distilled water and postfixed with 2% osmium tetroxide for 30 min. After washing with distilled water, cells were kept in 1% uranyl acetate at 4 °C overnight. The next day, after washing with distilled water, cells were kept in a lead aspartate solution at 65 °C for 1 h, then rinsed with distilled water and passed through an increasing alcohol series (30%, 50%, 70%, 80%, 90%, 96%, and 100%) for 1 h. Cells were dehydrated by putting them into pure acetone after alcohol and were kept in 1:1 acetone-Epon and 1:2 acetone-Epon mixtures for 2 h. After being kept in pure Epon for 2 h, cells were placed into an embedding mold and kept at 60 °C for 24 h to form a block. Thin sections of 60-100 nm in thickness were taken from the obtained cell blocks using an ultramicrotome (UC7, Leica, Germany) and a diamond blade, and the sections were dried on formvar-coated gold grids. The prepared sections were visualized at 30 kV using a GeminiSEM 500 electron microscope and a STEM detector (ZEISS, Germany). At least fifty cells were evaluated in each group.

Statistical Analysis

IBM SPSS Statistics (IBM Corp., USA) was used for statistical analysis. In group comparisons, the Kruskal-Wallis analysis of variance test was used for three or more groups. The Bonferroni-corrected Mann-Whitney U test was used for subgroup comparisons. For all statistics, $p<0.05$ was accepted as significant.

Results

WST-1 Assay Results

The WST-1 test results revealed the cell viability/toxicity in all experiments with viral infections of 5 moi, 10 moi, and 15 moi at 24, 48, and 72 h of incubation. It was determined for both cell types that cell viability decreased in the first 24 h following viral infection. There was no statistical difference in cell viability according to virus amounts ($p>0.05$). No significant increase in cell toxicity was detected in other incubation periods ($p>0.05$) (Figure 1).

ELISA Results

The results of autophagic proteins in MYXV-infected cell lines are given in Table 1 and Figures 2-5. The highest LC3B expression in the U-266 cell line was observed in the rapamycin group at 24 and 72 h, and in the group that received 5 moi MYXV at 48 h. At 24 h, the LC3B expression of the 15 moi MYXV group was significantly lower than the LC3B expression of the control group ($p=0.009$). The LC3B expression of the 5 moi MYXV group at 48 h was significantly higher than the LC3B expression of the control group at 48 h ($p=0.009$). The LC3B expression of the rapamycin group was significantly higher at 72 h compared to the LC3B expression of the control group ($p=0.009$). The highest expression of LC3B in the MOPC-315 cell line was observed in the control group at 24 and 72 h, and in the group that received 10 moi

Table 1. ELISA results.

			Control	Rapamycin	5 moi MYXV	10 moi MYXV	15 moi MYXV	Post-hoc	p
			$\bar{X} \pm SD$	$\bar{X} \pm SD$	$\bar{X} \pm SD$	$\bar{X} \pm SD$	$\bar{X} \pm SD$		
U-266	LC3B (ng/L)	24 h	2377±0.577	3427±0.577	1487±0.577	1417±0.577	987±0.577	[7]*	**0.009**
		48 h	1677±0.577	2247±0.577	2502±0.577	2232±0.577	1902±0.577	[2]*	**0.009**
		72 h	1537±0.577	2587±0.577	1542±0.577	1872±0.577	1577±0.577	[1]*	**0.009**
	p62 (ng/mL)	24 h	2.033±0.057	2.033±0.057	3.033±0.057	3.033±0.057	3.033±0.057	ns	0.055
		48 h	3.033±0.057	3.033±0.057	3.033±0.057	2.033±0.057	1.533±0.057	ns	0.089
		72 h	3.533±0.057	3.033±0.057	3.033±0.057	3.033±0.057	3.033±0.057	ns	0.087
	Beclin-1 (ng/mL)	24 h	6.033±0.057	6.033±0.057	8.033±0.057	9.033±0.057	14.033±0.057	[4]*, [7]*	**0.011**
		48 h	6.033±0.057	6.033±0.057	6.033±0.057	8.033±0.057	7.033±0.057	ns	0.065
		72 h	5.033±0.057	8.033±0.057	8.033±0.057	8.033±0.057	8.033±0.057	ns	0.078
	ATG-5 (ng/mL)	24 h	0.650±0.043	0.650±0.043	8.033±0.057	16.033±0.057	20.033±0.057	[4]*, [7]*	**0.011**
		48 h	0.650±0.043	0.650±0.043	14.033±0.057	20.033±0.057	20.033±0.057	ns	0.067
		72 h	0.650±0.043	0.650±0.043	14.033±0.057	19.033±0.057	20.033±0.057	[4]*, [7]*	**0.011**
	Bcl-2 (U/mL)	24 h	5.033±0.057	5.033±0.057	6.033±0.057	5.033±0.057	5.033±0.057	ns	0.087
		48 h	4.033±0.577	4.033±0.577	4.033±0.577	5.033±0.577	5.033±0.577	ns	0.075
		72 h	5.033±0.057	6.033±0.057	5.033±0.057	4.033±0.057	5.033±0.057	[6]*	**0.020**
MOPC-315	LC3B (ng/L)	24 h	2632±0.577	2162±0.577	2562±0.577	1047±0.577	1687±0.577	[3]*	**0.009**
		48 h	1607±0.577	1732±0.577	1947±0.577	1987±0.577	1812±0.577	[3]*	**0.009**
		72 h	2547±0.577	1657±0.577	1297±0.577	1627±0.577	1492±0.577	[2]*	**0.009**
	p62 (ng/mL)	24 h	5592±0.577	4863±0.577	4706±0.577	4784±0.577	4916±0.577	[2]*	**0.009**
		48 h	9870±0.577	6333±0.577	6121±0.577	6280±0.577	6439±0.577	[2]*	**0.009**
		72 h	5658±0.577	5022±0.577	5101±0.577	3645±0.577	6227±0.577	[10]*	**0.009**
	Beclin-1 (ng/mL)	24 h	8.067±0.115	7.033±0.057	6.033±0.057	6.100±0.173	6.067±0.115	ns	0.101
		48 h	5.033±0.057	8.067±0.115	7.100±0.173	5.100±0.173	7.067±0.115	[1]*, [6]*	**0.013**
		72 h	5.100±0.173	7.067±0.115	8.067±0.115	8.100±0.173	6.033±0.057	[2]*, [3]*	**0.011**
	ATG-5 (ng/mL)	24 h	0.650±0.043	0.650±0.043	7.067±0.115	17.100±0.173	18.067±0.115	[4]*, [7]*	**0.011**
		48 h	0.650±0.043	0.650±0.043	14.033±0.057	18.100±0.173	19.067±0.115	[4]*, [7]*	**0.040**
		72 h	0.650±0.043	0.683±0.101	15.067±0.115	18.100±0.173	20.067±0.115	[4]*, [7]*	**0.011**
	Bcl-2 (U/mL)	24 h	5.033±0.057	5.067±0.115	8.033±0.057	6.033±0.057	5.067±0.115	ns	0.058
		48 h	4.100±0.173	5.100±0.173	4.067±0.115	6.100±0.173	4.033±0.057	ns	0.084
		72 h	3.033±0.057	5.033±0.057	5.067±0.115	6.033±0.057	5.100±0.173	[3]*	**0.021**

[1]: Control versus rapamycin; [2]: control versus 5 moi; [3]: control versus 10 moi; [4]: control versus 15 moi; [5]: rapamycin versus 5 moi; [6]: rapamycin versus 10 moi; [7]: rapamycin versus 15 moi; [8]: 5 moi versus 10 moi; [9]: 5 moi versus 15 moi; [10]: 10 moi versus 15 moi; *: $\alpha<0.05$, ns: nonsignificant; ELISA: enzyme-linked immunosorbent assay; moi: multiplicity of infection; MYXV: *Myxoma virus*; SD: standard deviation.

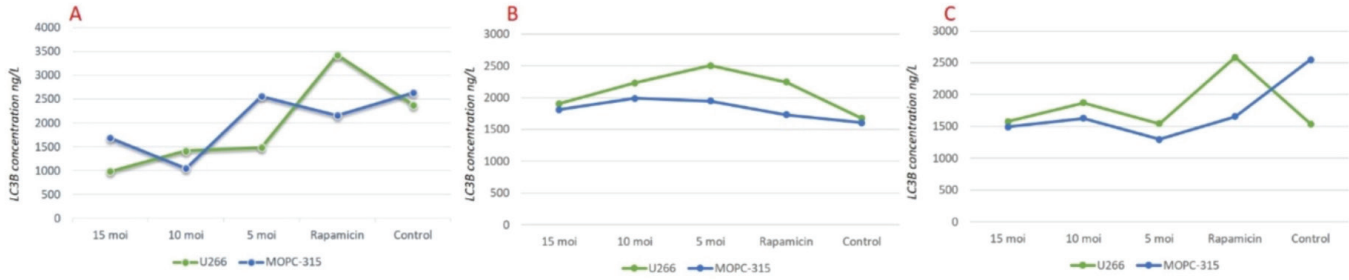

Figure 2. ELISA results of LC3B protein concentrations in MYXV-infected U-266 and MOPC-315 cells according to hours of incubation: A) 24 h of incubation period, B) 48 h of incubation, C) 72 h of incubation.

ELISA: Enzyme-linked immunosorbent assay; MYXV: *Myxoma virus.*

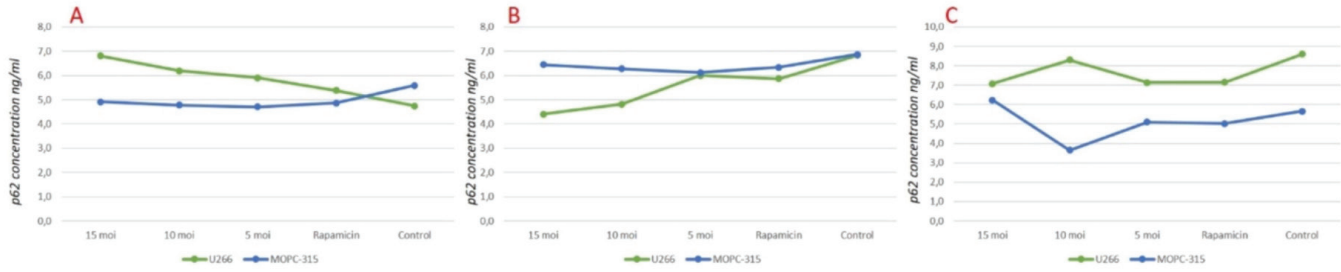

Figure 3. ELISA results of p62 protein concentrations in MYXV-infected U-266 and MOPC-315 cells according to hours of incubation: A) 24 h of incubation period, B) 48 h of incubation, C) 72 h of incubation.

ELISA: Enzyme-linked immunosorbent assay; MYXV: *Myxoma virus.*

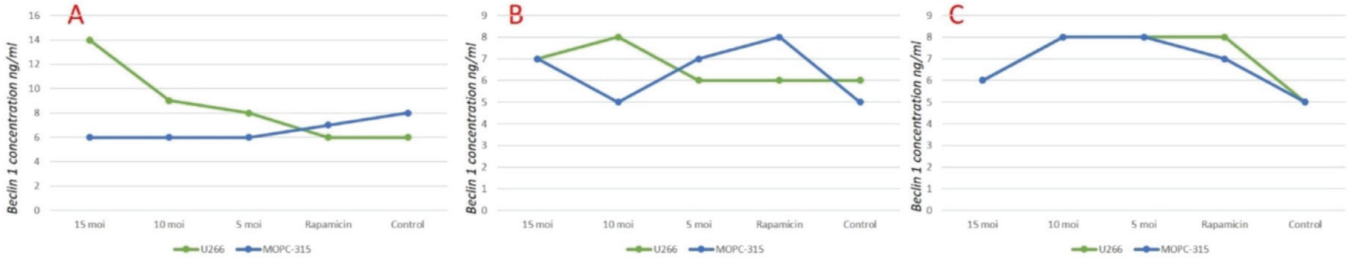

Figure 4. ELISA results of Beclin-1 protein concentrations in MYXV-infected U-266 and MOPC-315 cells according to hours of incubation: A) 24 h of incubation period, B) 48 h of incubation, C) 72 h of incubation.

ELISA: Enzyme-linked immunosorbent assay; MYXV: *Myxoma virus.*

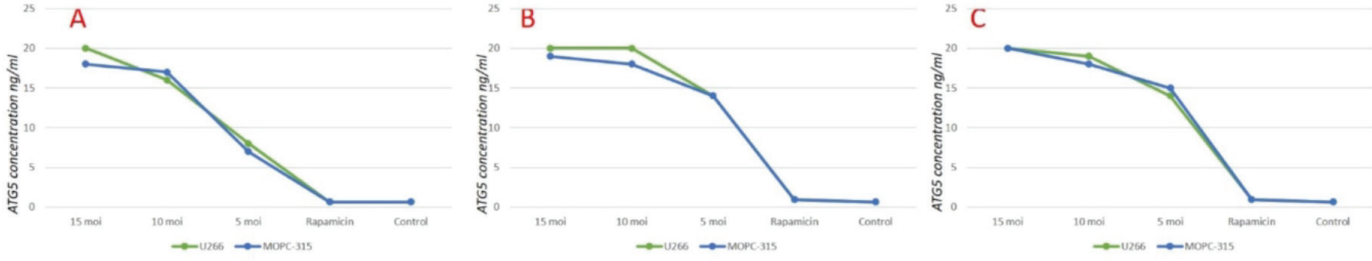

Figure 5. ELISA results of ATG-5 protein concentrations in MYXV-infected U-266 and MOPC-315 cells according to hours of incubation: A) 24 h of incubation period, B) 48 h of incubation, C) 72 h of incubation.

ELISA: Enzyme-linked immunosorbent assay; MYXV: *Myxoma virus.*

MYXV at 48 h. The LC3B expression of the 10 moi MYXV-treated group was significantly lower at 24 h and significantly higher at 48 h compared to the LC3B expression of the control group (p=0.009). The LC3B expression of the 5 moi MYXV group was significantly lower than the LC3B expression of the control group at 72 h (p=0.009).

There was no significant difference observed in the levels of p62 expressed in the U-266 cells (p>0.05). However, in the MOPC-315 cell line, the control group had the highest levels of p62 expression at 24 and 48 h. At 72 h, the group administered 15 moi MYXV had the highest p62 expression. The 5 moi MYXV group had significantly lower p62 expression than the control group at both 24 and 48 h (p=0.009). Additionally, at 72 h, the group administered 15 moi MYXV had significantly higher p62 expression than the group administered 10 moi MYXV (p=0.009).

The highest expression of Beclin-1 in the U-266 cells was observed in the group administered 15 moi MYXV at 24 h, and this value was significantly higher than the expression of Beclin-1 in the control and rapamycin groups. There was no significant difference in Beclin-1 expression between the groups at 48 and 72 h (p>0.05). In the MOPC-315 cells, the highest Beclin-1 expression was observed in the control group at 24 h, in the rapamycin group at 48 h, and in the group administered 10 moi MYXV at 72 h. While no difference was observed between the groups at 24 h (p>0.05), Beclin-1 expression in the rapamycin group at 48 h was significantly higher than in the control and the

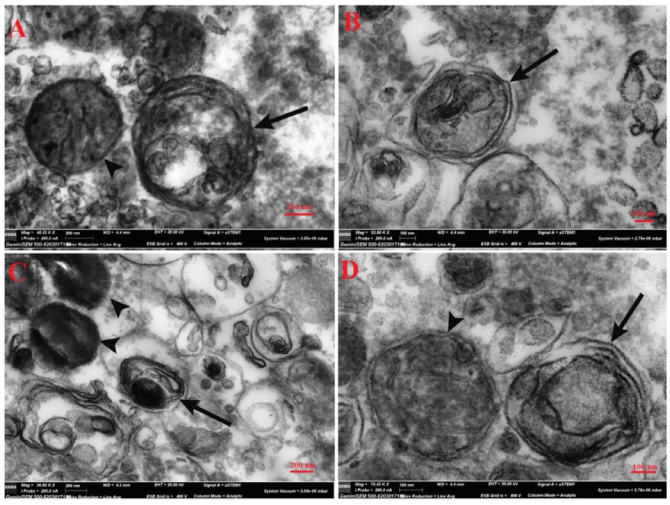

Figure 6. A) Electron microscopic image of cells from the MOPC-315 cell line. An autophagosome (arrow) characterized by a double-layered membrane structure containing various vesicles and degenerate structures and a mitochondrion (arrowhead) that maintains its normal structure are seen. B) Electron microscopic image of cells from the MOPC-315 cell line. An autophagosome (arrow) is seen, which is characterized by a double-layered membrane structure containing vesicles and membranes. C) Electron microscopic image of cells obtained from cell line U-266. An autolysosome (arrow) and larger lysosomes (arrowhead) adjacent to it are in the process of fusion of autophagosome membranes and lysosome. D) Electron microscopic image of cells obtained from cell line U-266. An autophagosome (arrow) and adjacent mitochondrion (arrowhead) are seen, characterized by a double-layered membrane structure containing membranes.

10 moi MYXV group (p=0.009). At 72 h, the Beclin-1 expression levels of the groups that received 5 moi MYXV and 10 moi MYXV were significantly higher than the Beclin-1 expression of the control group (p=0.009).

The highest expression levels of ATG-5 in the U-266 cells at 24, 48, and 72 h were determined in the 15 moi MYXV group (Figure 5). At 24 and 72 h, ATG-5 expression was significantly higher in the 15 moi MYXV group than in the control and rapamycin groups (p=0.011). There was no difference between the groups at 48 h (p>0.05). The highest expression levels of ATG-5 in the MOPC-315 cells at 24, 48, and 72 h were observed in the 15 moi MYXV group, being significantly higher than the ATG-5 expression in the control and rapamycin groups (p=0.011).

There was no significant variation in the expression of Bcl-2 in the U-266 cells after 24 or 48 h (p>0.05). However, after 72 h, the group that was administered rapamycin exhibited significantly higher expression of Bcl-2 compared to the 10 moi MYXV group (p=0.020). Similarly, no significant difference was observed in Bcl-2 expression in the MOPC-315 cell line after 24 or 48 h (p>0.05). Nevertheless, after 72 h, the group that was administered 10 moi MYXV exhibited significantly higher Bcl-2 expression compared to the control group (p=0.021).

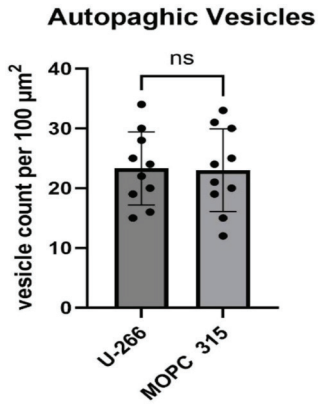

Figure 7. Autophagic vesicle counts for MYXV-infected U-266 and MOPC-315 cells.

MYXV: *Myxoma virus;* ns: not significant.

Electron Microscopic Screening

Several cells in all examined groups showed deterioration in their cell membrane integrity and ultrastructural structure, while a small number of cells maintained their integrity. Electron microscopic images were obtained from the structurally intact cells, which revealed autophagic vesicles and autophagosome structures at different stages (early and late) in all groups, as shown in Figure 6.

Autophagosomes were counted in a unit area of cells (100 µm^2) in the autophagic particle count groups (as seen in Figure 7). There was no statistical difference between the cell groups based on unpaired t-tests and Mann-Whitney U tests (p>0.05).

Discussion

The targeted survival in MM has not been achieved although different treatment modalities have been applied for many years. The heterogeneity of MM cells and the emergence of drug-resistant clones preclude a complete cure. Recently, both experimental and clinical studies have suggested that OVs could be a potential therapeutic alternative to treat hematological malignancies [45,54]. OVs can be used for therapeutic purposes alone and/or in combination with standard chemotherapeutic agents [55].

Autophagy exerts an oncosuppressive effect in the early phase of tumorigenesis by preventing genome instability, inflammation, and chronic tissue damage [56]. Conversely, once the tumor has grown, autophagy promotes tumor growth, progression, and drug resistance [57]. Therefore, targeting autophagy in tumor treatments would be a good approach. Indeed, a link between OVs and autophagic cell death has also been demonstrated [58]. Various OVs such as the herpes simplex virus [59], adenovirus [60], paramyxovirus [61], and Newcastle disease virus [62] have been reported to induce autophagic cell death. In addition, OV-induced autophagy has been found to induce immunogenic

cell death in cancer cells, thereby inducing stronger antitumor immunity [63].

Survival has improved significantly with the use of new therapeutic agents such as PIs in the treatment of MM, but resistance to these drugs develops over time [64]. When PIs are administered to MM patients, PCs activate autophagy for protein degradation for survival [57]. Therefore, the co-targeting of proteasome and autophagy in the treatment of MM will have an enhanced antimyeloma effect. Vogl et al. [65] found high antitumor efficacy in MM with the concomitant use of bortezomib, a PI, and hydroxychloroquine, an autophagy inhibitor. Lei et al. [45] reported that OVV armed with Beclin-1 induced enhanced cell death by inducing autophagic cell death in leukemia and MM cell lines in vitro and in vivo. In the same study, they determined that OVV armed with Beclin-1 increased necrosis in tumor tissue and exhibited increased Beclin-1 expression and irregular p62 expression; autophagosomes and autolysosomes were observed by TEM study [45].

In our study, the expression of proteins involved in autophagy was evaluated. The levels of ATG-5 and Beclin-1, which are involved in the formation of phagophores, constituting the initial stage of autophagy, were found to be significantly higher in MM cell lines at 24 h in our study ($p<0.05$). The highest expression levels of both ATG-5 and Beclin-1 were observed in the group administered 15 moi MYXV. LC3B, which helps phagophore elongation by joining the late autophagosome ring of autophagy, was found to be significantly higher at 48 h in our study. The highest expression of LC3B was seen in the group treated with 5 moi MYXV in the U-266 cell line and in the group treated with 10 moi MYXV in the MOPC-315 cell line. In terms of p62 expression, no significant difference was observed in the U-266 cell line ($p>0.05$), while there was an irregular expression pattern in the MOPC-315 cell line. TEM analysis was performed on MYXV-treated cell lines to confirm the ELISA results and visualize autophagy. As a result of TEM images, autophagosomes were determined in both early and late autophagy processes. These findings show that MYXV causes the death of malignant PCs by autophagy in MM cell lines. Both the ELISA and TEM results of our study reaffirm the results of previous studies showing increased expression of autophagy proteins in MM cell lines [45].

Study Limitations

There are some limitations of our study. First, the results of the autophagy proteins whose expressions we examined in this study could also be confirmed by methods such as western blotting, immunohistochemistry, and/or flow cytometry. Experimental animal studies with MM cell lines or MM cell lines from clinical patients would also strengthen the results. It is suggested that autophagy is responsible for drug resistance, especially in MM patients. Therefore, adding a PI to the study would have been helpful to elucidate the cause of drug resistance.

Conclusion

The development of resistance to the drugs used in MM is the most important obstacle to improving the survival of these patients. Autophagy is one of the mechanisms that may be responsible for the development of drug resistance in MM. OVs with successful results in treating hematological malignancies can also induce autophagy. Our study determined that the expression of autophagy proteins ATG-5, Beclin-1, and LC3B increased in MM cell lines with the application of MYXV, an OV. Targeting autophagy with OVs is promising in the treatment of MM to prevent drug resistance and increase the effectiveness of MM treatment. More research is needed to understand the role of autophagy in MM fully and to develop targeted therapies.

Ethics

Ethics Committee Approval: The sample was not derived from patients. Commercial cell lines were utilized.

Informed Consent: The sample was not derived from patients. Commercial cell lines were utilized.

Authorship Contributions

Concept: A.Y., D.M., B.E.; Design: A.Y., D.M., B.E.; Data Collection or Processing: A.Y.; Analysis or Interpretation: A.Y.; Literature Search: A.Y., D.M., B.E.; Writing: A.Y., D.M., B.E.

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RESEARCH ARTICLE

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Impact of *CALR* and *JAK2V*617F Mutations on Clinical Course and Disease Outcomes in Essential Thrombocythemia: A Multicenter Retrospective Study in Turkish Patients

Esansiyel Trombositemide *CALR* ve *JAK2V*617F Mutasyonlarının Klinik Seyir ve Hastalık Sonuçlarına Etkisi: Türk Hastalarda Geriye Dönük Çok Merkezli Çalışma

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Abstract

Objective: In this study, we investigated the effects of calreticulin (*CALR*) and *JAK2*V617F mutational status on clinical course and disease outcomes in Turkish patients with essential thrombocythemia (ET).

Materials and Methods: Seventeen centers from Türkiye participated in the study and *CALR*- and *JAK2*V617F-mutated ET patients were evaluated retrospectively.

Results: A total of 302 patients were included, of whom 203 (67.2%) and 99 (32.8%) were *JAK2*V617F- and *CALR*-positive, respectively. *CALR*-mutated patients were significantly younger (51 years vs. 57.5 years, p=0.03), with higher median platelet counts ($987x10^9$/L vs. $709x10^9$/L, p<0.001) and lower median hemoglobin levels (13.1 g/dL vs. 14.1 g/dL, p<0.001) compared to *JAK2*V617F-mutated patients. Thromboembolic events (TEEs) occurred in 54 patients (17.9%), 77.8%

Öz

Amaç: Bu çalışmada Türk esansiyel trombositemi (ET) hastalarında *CALR* ve *JAK2*V617F mutasyon durumunun klinik seyir ve hastalık sonuçlarına etkilerini araştırdık.

Gereç ve Yöntemler: Çalışmaya Türkiye'den 17 merkez katılmış olup, *CALR* ve *JAK2*V617F mutasyonu pozitif olan ET hastaları geriye dönük olarak değerlendirilmiştir.

Bulgular: Çalışmaya toplam 302 hasta dahil edildi. Bunların 203'ü (%67,2) *JAK2*V617F ve 99'u (%32,8) *CALR* pozitifti. *CALR* mutasyonlu hastalar *JAK2*V617F pozitif olanlara göre daha gençti (sırasıyla; 51 yaş, 57,5 yaş, p=0,03), daha yüksek ortanca trombosit sayısına (sırasıyla; $987x10^9$/L, $709x10^9$/L, p<0,001) ve daha düşük ortanca hemoglobin düzeylerine (sırasıyla; 13,1 g/dL, 14,1 g/dL, p<0,001) sahipti. Tromboembolik olaylar (TEO) 54 hastada (%17,9) meydana geldi ve bunların %77,8'i arteriyeldi. *CALR* mutasyonu ile karşılaştırıldığında

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Abstract

of which were arterial. Compared to *CALR* mutation, *JAK2*V617F was associated with a higher risk of thrombosis (8.1% vs. 22.7%, p=0.002). Rates of transformation to myelofibrosis (MF) and leukemia were 4% and 0.7%, respectively, and these rates were comparable between *JAK2*V617F- and *CALR*-mutated cases. The estimated overall survival (OS) and MF-free survival of the entire cohort were 265.1 months and 235.7 months, respectively. OS and MF-free survival durations were similar between *JAK2*V617F- and *CALR*-mutated patients. Thrombosis-free survival (TFS) was superior in *CALR*-mutated patients compared to *JAK2*V617F-positive patients (5-year TFS: 90% vs. 71%, respectively; p=0.001). Age at diagnosis was an independent factor affecting the incidence of TEEs.

Conclusion: In our ET cohort, *CALR* mutations resulted in higher platelet counts and lower hemoglobin levels than *JAK2*V617F and were associated with younger age at diagnosis. *JAK2*V617F was strongly associated with thrombosis and worse TFS. Hydroxyurea was the most preferred cytoreductive agent for patients with high thrombosis risk.

Keywords: *CALR* mutation, Essential thrombocythemia, *JAK2*V617F mutation, Myeloproliferative neoplasm

Öz

JAK2 V617F daha yüksek tromboz riski ile ilişkiliydi (%8,1'e karşı %22,7, p=0,002). Miyelofibroz (MF) ve lösemiye dönüşüm oranları sırasıyla %4 ve %0,7 idi ve bu oranlar *JAK2*V617F ve *CALR* mutasyonlu olgular arasında benzerdi. Tüm kohortta tahmini toplam sağkalım (OS) ve MF'siz sağkalım sırasıyla 265,1 ay ve 235,7 aydı. *JAK2*V617F ve *CALR* mutasyonlu hastalar arasında OS ve MF'siz sağkalım benzerdi. *CALR* mutasyonlu vakalarda trombozsuz sağkalım (TFS), *JAK2*V617F pozitif hastalara göre daha üstündü (5 yıllık TFS sırasıyla; %90, %71 [p=0,001]). Tanı yaşı TEO insidansını etkileyen bağımsız bir faktördü.

Sonuç: ET kohortumuzda *CALR* mutasyonları, *JAK2*V617F'ye göre daha yüksek trombosit sayısı, daha düşük hemoglobin düzeyi ve tanı anında daha genç yaşla ilişki bulundu. *JAK2*V617F, tromboz ve daha kötü TFS ile güçlü bir şekilde ilişkiliydi. Hidroksiüre yüksek tromboz riski olan hastalarda en çok tercih edilen sitoredüktif ilaçtı.

Anahtar Sözcükler: *CALR* mutasyonu, Esansiyel trombositemi, *JAK2*V617F mutasyonu, Miyeloproliferatif neoplazm

Introduction

Essential thrombocythemia (ET) is a Philadelphia-negative myeloproliferative neoplasm (MPN) characterized by high platelet counts due to the clonal proliferation of the megakaryocytic lineage within the bone marrow. Patients with ET may display molecular markers such as Janus kinase 2 (*JAK2*; 9p24), calreticulin (*CALR*; 19p13.2), or myeloproliferative leukemia virus (*MPL* oncogene; 1p34) in a mutually exclusive manner [1,2]. The valine-to-phenylalanine (V617F) alteration constitutively activates *JAK2*, resulting in overproduction of myeloid cells. The *JAK2*V617F mutation is present in approximately 55% of patients with ET, 25% of patients with *CALR*, and 3% of patients with *MPL* [1,3]. The statuses of these driver mutations are relevant not only for their diagnostic contribution but also for their prognostic significance [4].

Frameshift mutations in the *CALR* gene encoding molecular chaperones in the endoplasmic reticulum are the second most common somatic mutation in ET. Two mutations of the *CALR* gene, type 1 (c.1092_1143del; L367fs*46) and type 2 (c.1154_1155insTTGTC; K385fs*47), represent more than 80% of *CALR* mutations [1,2,5]. The mutant CALR protein interacts with the thrombopoietin receptor, MPL, via its extracellular domain, activating the downstream JAK-STAT pathway and resulting in cytokine-independent growth [6,7].

In patients with ET, the presence of *JAK2*V617F is associated with an increased risk of thrombosis and a lower risk of post-ET myelofibrosis (MF) [8]. Compared to *JAK2*V617F, mutant *CALR* is associated with younger age, male sex, higher platelet count, lower hemoglobin level, lower leukocyte count, and lower incidence of thrombotic events. In addition, patients with the type 2 *CALR* mutation tend to have higher platelet counts than patients with type 1 [9,10].

Previous studies have shown that in patients with ET, median survival is approximately 20 years [11,12], although life expectancy in ET is inferior to that of the general population, regardless of mutational status [12]. Young ET patients have clearly longer survival compared to their older counterparts, which requires appropriate action during patient management [13]. Risk factors for survival in ET are advanced age, leukocytosis, and thrombosis history. Mutational status does not seem to affect survival in patients with ET [14].

The prevalence of *JAK2*V617F and *CALR* mutations in Turkish patients with ET and the relationships of these driver mutations with clinical outcomes remain undetermined. In this study, we aim to investigate the effects of *CALR* and *JAK2*V617F mutation status on the clinical course and disease outcomes of Turkish ET patients.

Materials and Methods

*JAK2*V617F- and *CALR*-mutated ET patients aged ≥18 years were included in the study. *MPL*-mutated and triple-negative ET patients were excluded. Demographic data, clinical and laboratory characteristics, treatment modalities, and disease outcomes were evaluated retrospectively. Bone marrow

aspiration and biopsy were performed for all patients to exclude those with pre-fibrotic MF according to the 2016 revision of the World Health Organization's classification of myeloid neoplasms and acute leukemia [15].

*JAK2*V617F mutations were detected by quantitative polymerase chain reaction and *CALR* mutations were detected by next-generation sequencing of *CALR* exon 9. The 52-bp deletion (p.L367fs*46) was defined as *CALR* type 1 and the 5-bp TTGTC insertion (p.K385fs*47) as *CALR* type 2, while the others were grouped as "other." *CALR* subgroup analyses were performed, excluding patients with unknown *CALR* type.

At diagnosis, the International Prognostic Score of Thrombosis in Essential Thrombocythemia (IPSET-thrombosis) and the revised IPSET-thrombosis were used to define the risk of thromboembolic events (TEEs). The revised IPSET-thrombosis defines four risk categories according to three adverse variables (thrombosis history, age >60 years, and *JAK2*V617F): very low (no adverse features), low (presence of *JAK2*V617F), intermediate (age >60 years), and high (presence of thrombosis history or presence of both advanced age and *JAK2*V617F) [3,16]. Deep vein thrombosis (DVT), pulmonary thromboembolism (PTE), pulmonary arterial hypertension (PAH) due to chronic PTE, and TEEs occurring in any venous vessel were considered as venous-type TEEs. Myocardial infarction (MI), angina pectoris (AP), transient ischemic attack, ischemic stroke or cerebrovascular accident (CVA), thrombosis in the carotid artery, peripheral arterial occlusive disease, and TEEs in any arterial vessel were classified as the arterial type.

All procedures were performed in accordance with the ethical standards of the relevant institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Ethical approval was obtained from the Local Ethics Committee of İzmir Bozyaka Training and Research Hospital with approval number 2022/127 on August 10, 2022.

Statistical Analysis

IBM SPSS Statistics 22 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The conformity of the variables to normal distribution was examined using visual (histogram and probability graphs) and analytical (Kolmogorov-Smirnov/Shapiro-Wilk test) methods. Descriptive statistics were presented as mean ± standard deviation, median (minimum-maximum), frequency distribution, and percentage. Comparisons between groups were made by chi-square, Fisher exact, and Mann-Whitney U tests as appropriate. Two major outcomes were assessed accordingly: overall survival (OS) and MF-free survival, calculated from the time of development of fibrosis in the bone marrow. Survival estimations were assessed by Kaplan-Meier method and the log-rank test was used for comparisons of survival distributions among groups. Thrombosis-free survival (TFS) was calculated from the date of diagnosis to date of thrombosis (uncensored) or last contact (censored). A Cox proportional hazard regression model was used for multivariable analysis. Values of $p<0.05$ were considered statistically significant.

In a one-way analysis of variance (ANOVA) study, sample sizes of 53, 33, and 9 were obtained from the 3 *CALR*-positive groups whose means were to be compared. According to the one-way ANOVA test, the total sample of 95 subjects achieved 100% power to detect differences among the means versus the alternative of equal means using an F-test with a 0.05000-significance level.

Results

Entire Patient Cohort

Patients with ET from 17 centers in Türkiye, diagnosed between January 1999 and July 2022, were included in the study. A total of 302 patients, 203 of whom had *JAK2*V617F-mutated ET and 99 of whom had *CALR*-mutated ET, were analyzed. While 174 (57.6%) of the patients were female, 128 (42.4%) were male. The median age was 55 years (range: 20-88 years). Median follow-up was 41.7 months (range: 0.17-278.3 months). Two hundred and three (67.2%) and 99 (32.8%) patients were *JAK2*V617F- and *CALR*-positive, respectively. At the time of diagnosis, 24.8% (n=74) of the patients had splenomegaly and 4 patients (1.3%) had splenectomy secondary to trauma. Median platelet count was $784x10^9$/L (range: $452-2635x10^9$/L), median hemoglobin level was 13.8 g/dL (range: 8.2-17.8 g/dL), and median leukocyte count was $9.6x10^9$/L (range: $3.4-33.4x10^9$/L) (Table 1).

According to the IPSET-thrombosis risk score, 84 (27.8%) patients were in the low-risk group, 81 (26.8%) patients were in the intermediate-risk group, and 137 (45.4%) patients were in the high-risk group. When we evaluated the revised IPSET-thrombosis risk score, 70 (23.2%) patients were in the very low-risk group, 99 (32.8%) were in the low-risk group, 25 (8.3%) were in the intermediate-risk group, and 108 (35.7%) were in the high-risk group.

Including the period before diagnosis, the time of diagnosis, and follow-up, TEEs occurred in 17.9% (n=54) of the patients, 77.8% of which were in arterial sites, while 14.8% were in venous sites and 7.4% were in both venous and arterial sites. Thirty-two patients (59.3%) had MI or AP, 2 patients (3.7%) experienced both MI and PTE, and thrombosis in the carotid artery occurred in 2 cases (3.7%). While 2 patients (3.7%) had both CVA and PAH due to chronic PTE, 7 (13%) had CVA. DVT occurred in 4 cases (7.4%), cerebral venous sinus thrombosis in 3 cases (5.6%), PTE in 1 case (1.9%), and portal vein thrombosis in 1 case (1.9%).

During the follow-up of 258 patients with no history of TEEs prior to or at the time of diagnosis, the TEE rate was 3.9%

Table 1. Demographic and biological features of patients with essential thrombocythemia.

	All patients, n=302 (100%)	*JAK2*V617F-positive, n=203 (67.2%)	*CALR*-positive, n= 99 (32.8%)	p
Median age (years)	55 (20-88)	57.5 (20-87)	51 (20-88)	0.03
Median follow-up time (months)	41.7 (0.17-278.3)	40.9 (0.47-240.3)	48.6 (0.17-278.3)	0.07
Sex				
Female	174 (57.6%)	120 (59.1%)	54 (54.5%)	0.4
Male	128 (42.4%)	83 (40.9%)	45 (45.5%)	
Splenomegaly				
Yes	75 (24.8%)	52 (25.6%)	23 (23.2%)	0.7
No	223 (73.8%)	149 (73.4%)	74 (74.7%)	
Splenectomy	4 (1.3%)	2 (1.0%)	2 (2.0%)	
At diagnosis (median)				
PLT (10^9/L)	784 (304-2635)	709 (304-2635)	987 (343-2449)	**<0.001**
Hb (g/dL)	13.8 (8.2-17.8)	14.1 (8.3-17.8)	13.1 (8.2-17.2)	**<0.001**
WBC (10^9/L)	9.6 (3.4-33.4)	9.8 (3.9-33.3)	8.9 (3.4-20.6)	0.06
Thromboembolic events				
Yes	54 (17.9%)	46 (22.7%)	8 (8.1%)	**0.002**
No	248 (82.1%)	157 (77.3%)	91 (91.9%)	
Type of thrombosis				
Arterial	42 (77.8%)	35 (76.0%)	7 (87.5%)	0.7
Venous	8 (14.8%)	7 (15.2%)	1 (12.5%)	
Venous and arterial	4 (7.4%)	4 (8.8%)	0 (0%)	
Comorbidities				
None	136 (45.0%)	84 (41.4%)	52 (52.5%)	0.2
CVD and/or metabolic	132 (43.7%)	97 (47.8%)	35 (35.4%)	
Neuropsychiatric	13 (4.3%)	9 (4.4%)	4 (4.0%)	
Others	21 (7.0%)	13 (6.4%)	8 (8.1%)	
IPSET-thrombosis				
Low	84 (27.8%)	0 (0%)	84 (84.8%)	**<0.001**
Intermediate	81 (26.8%)	70 (34.5%)	11 (11.1%)	
High	137 (45.4%)	133 (65.5%)	4 (4.0%)	
Revised IPSET-thrombosis				
Very low	70 (23.2%)	0 (0%)	70 (70.7%)	**<0.001**
Low	99 (32.8%)	99 (48.8%)	0 (0%)	
Intermediate	25 (8.3%)	0 (0%)	25 (25.3%)	
High	108 (35.7%)	104 (51.2%)	4 (4.0%)	
Treatments				
Only ASA	67 (22.2%)	51 (25.1%)	16 (16.2%)	**<0.001**
HU	178 (58.9%)	130 (64.0%)	48 (48.5%)	
Anagrelide	24 (7.9%)	8 (3.9%)	16 (16.2%)	
HU + anagrelide	17 (5.6%)	7 (3.4%)	10 (10.1%)	
IFN-α	16 (5.3%)	7 (3.4%)	9 (9.1%)	
MF transformation				
Yes	12 (4.0%)	8 (3.9%)	4 (4.0%)	0.9
No	290 (96.0%)	195 (96.1%)	95 (96.0%)	
Leukemic transformation				
Yes	2 (0.7%)	1 (0.5%)	1 (1.0%)	0.6
No	300 (99.3%)	202 (99.5%)	98 (99.0%)	

JAK2: Janus kinase 2; *CALR:* calreticulin; PLT: platelet count; Hb: hemoglobin; WBC: white blood cell count; CVD: cardiovascular disease; IPSET-thrombosis: International Prognostic Score of Thrombosis in Essential Thrombocythemia; ASA: acetylsalicylic acid; HU: hydroxyurea; IFN-α: interferon alpha; MF: myelofibrosis.

(n=10). Six of those patients were *JAK2*V617F-mutated and in the high IPSET-thrombosis risk group at the time of diagnosis, while 4 of them were *CALR*-mutated and in the low-risk group. Multivariate analysis was performed for analyzing the effects of hemoglobin levels, platelet counts, and age at diagnosis on TEE occurrence. Only age was found to be a factor independently affecting the cumulative TEE incidence (p=0.008) (Table 2).

Sixty-five (21.5%) female patients were of reproductive age and a total of 3 pregnancies occurred in 2 of those cases. While 2 of them resulted in delivery with the use of interferon alpha (IFN-α), 1 pregnancy resulted in intrauterine fetal death in the 6th gestational week. Demographic and biological features of patients according to mutational status are presented in Table 1.

Table 2. Multivariate analysis for factors that may influence the incidence of thromboembolic events.

	Thrombosis-free survival	
Variables	**HR (95% CI)**	**p**
Age at diagnosis (≤60 years and >60 years)	0.48 (0.28-0.83)	**0.008**
Hemoglobin level (g/dL)	0.31 (0.02-3.43)	0.34
PLT count (x10^9/L)	1.1 (1.0-2.81)	0.99
WBC count (x10^9/L)	1.0 (0.07-2.12)	0.95

PLT: Platelet; WBC: white blood cell; HR: hazard ratio; CI: confidence interval.

Characteristics and Treatments of the *JAK2*V617F- and *CALR*-Mutated Subgroups

CALR-mutated patients were significantly younger (median age at diagnosis: 51 years [range: 20-88 years] vs. 57.5 years [range: 20-87 years], p=0.03) than patients harboring the *JAK2*V617F mutation. Compared to *JAK2*V617F-mutated cases, patients with *CALR* mutations had higher median platelet counts (987x10^9/L [range: 458-2449x10^9/L] vs. 709x10^9/L [range: 452-2635x10^9/L], p<0.001) and significantly lower hemoglobin levels (13.1 g/dL [range: 8.2-17.2 g/dL] vs. 14.1 g/dL [range: 8.3-17.8 g/dL], p<0.001). The leukocyte counts of patients with *CALR* mutations were lower than those of patients with *JAK2*V617F mutations, but the difference did not reach statistical significance (8.9x10^9/L [range: 3.4-20.6x10^9/L] vs. 9.8x10^9/L [range: 3.9-33.3x10^9/L], p=0.06).

Regarding the IPSET-thrombosis risk stratification, 34.5% of *JAK2*V617F-mutated patients were in the intermediate-risk group and 65.5% in the high-risk group, while 84.8% of the *CALR*-mutated patients were in the low-risk group, 11.1% in the intermediate-risk group, and 4% in the high-risk group (p<0.001). According to the revised IPSET-thrombosis risk score, 48.8% of *JAK2*V617F-mutated patients were in the low-risk group and 51.2% in the high-risk group, while 70.7% of the *CALR*-mutated patients were in the very low-risk group, 25.3% in the intermediate-risk group, and 4% in the high-risk group (p<0.001). Compared to *CALR* mutations, *JAK2*V617F mutations were associated with a higher incidence of TEEs (8.1% vs. 22.7%, p=0.002). The incidence of thrombosis in arterial sites was higher than the incidence of thrombosis in venous sites in both groups, although the difference was not statistically significant (Table 1).

All patients received acetylsalicylic acid (ASA) for thromboprophylaxis. The percentage of patients treated with ASA alone was significantly higher in the *JAK2*V617F-mutated group compared to patients with *CALR* mutations (25.1% vs. 16.2%, p<0.001). Similarly, hydroxyurea (HU) therapy was found to be more commonly used for patients with *JAK2*V617F than *CALR*-mutated patients (64% vs. 48.5%, p<0.001). Other treatment modalities including anagrelide monotherapy, IFN-α, and the combination of HU and anagrelide were more common among *CALR*-mutated patients than patients harboring *JAK2*V617F (p<0.001) (Table 1).

There was no difference between the two groups in terms of median follow-up duration, sex distribution, splenomegaly rate, median leukocyte count, comorbidities, or rates of MF progression and leukemia (Table 1).

Patients with *CALR* Mutations

CALR-mutated patients were further analyzed according to subgroups as type 1, type 2, and others. There was no difference between cases of type 1, type 2, and other *CALR* mutations in terms of sex, splenomegaly, hemoglobin, platelet and leukocyte counts, comorbidities, IPSET-thrombosis score, revised IPSET-thrombosis score, treatment modalities, incidence of thrombotic events, or leukemic transformation (Table 3).

The median ages of patients with type 1, type 2, and other *CALR* mutations were 54.5, 50, and 37 years, respectively (p=0.005). The rate of progression to MF was higher in the others group than in cases of type 1 and type 2 *CALR* mutations (22.2%, 1.9%, and 0%, respectively; p=0.02). Similarly, the median follow-up of the others group was significantly longer than the median follow-up durations of patients with type 1 and type 2 mutations (68.5, 49.1, and 45 months, respectively; p=0.04) (Table 3).

Survival

Progression to MF was observed in 4% (n=12) of the entire cohort and leukemic transformation occurred in 0.7% (n=2) of the patients. One patient with leukemic transformation harbored a *TP53* mutation with a complex karyotype.

The estimated median OS for the entire cohort was 265.1 months (range: 255.8-274.3 months). There was no difference in OS between *CALR*- and *JAK2*V617F-mutated ET cases (254.9 months [range: 235.1-274.7 months] vs. 234.6 months [range: 228.1-241.2 months], p=0.1) (Figure 1). Estimated MF-free survival was 235.7 months (range: 205.7-265.8 months). Like OS, the MF-free survival duration was also similar between the *CALR*- and *JAK2*V617F-mutated groups (247.5 months [range: 213.9-281.2 months] vs. 199.7 months [range: 165.7-233.7 months], p=0.4) (Figure 1). In *CALR*-mutated patients, OS and MF-free survival were similar between the subgroups established according to *CALR* type (p=0.4 and p=0.2, respectively) (Figure 2). OS and MF-free survival were also similar between men and women (p=0.4 and p=0.06, respectively).

The 2-year and 5-year TFS rates were respectively 93% and 90% in *CALR*-mutated patients, while TFS was 77% and 71% at 2 years and 5 years in *JAK2*V617F-mutated patients, respectively. *CALR*-mutated patients thus had significantly longer TFS

Table 3. Clinical and biological features of patients with *CALR*-mutated essential thrombocythemia.

	All patients, n=95 (100%)	Type 1, n=53 (55.8%)	Type 2, n=33 (34.7%)	Others, n=9 (9.5%)	p
Median age (years)	52 (20-88)	54.5 (24-88)	50 (20-86)	37 (21-51)	**0.005**
Median follow-up time (months)	50.6 (0.2-278.3)	49.1 (0.2-278.3)	45.0 (3.7-161.3)	68.5 (35.4-236.2)	**0.04**
Sex					
Female	52 (54.7%)	28 (52.8%)	21 (63.6%)	3 (33.3%)	0.2
Male	43 (45.3%)	25 (47.2%)	12 (36.4%)	6 (66.7%)	
Splenomegaly					
Yes	22 (23.2%)	11 (20.8%)	6 (18.2%)	4 (44.4%)	
No	71 (74.7%)	40 (75.5%)	27 (81.8%)	5 (55.6%)	0.1
Splenectomy	2 (2.1%)	2 (3.7%)	0 (0%)	0 (0%)	
At diagnosis (median)					
PLT (x10^9/L)	994 (394-2449)	936 (394-2412)	1030 (343-2449)	865 (671-2442)	0.8
Hb (g/dL)	13.0 (8.2-17.2)	12.9 (8.2-16.4)	13.6 (10.2-17.2)	12.4 (10.2-15.0)	0.4
WBC (x10^9/L)	9.0 (3.4-20.6)	9.7 (3.4-18.4)	9 (3.7-15.9)	8.6 (4.2-20.6)	0.5
Thromboembolic events					
Yes	8 (8.4%)	4 (7.5%)	3 (9.1%)	1 (11.2%)	0.8
No	87 (91.6%)	49 (92.5%)	30 (90.9%)	8 (88.9%)	
Type of thrombosis					
Arterial	7 (87.5%)	3 (75.0%)	3 (100%)		
Venous	1 (12.5%)	1 (25.0%)	0 (0%)	-	0.6
Venous and arterial	0 (0%)	0 (0%)	0 (0%)		
Comorbidities					
None	50 (52.6%)	26 (49.1%)	17 (51.5%)	7 (77.8%)	
CVD and/or metabolic	34 (35.8%)	21 (39.6%)	12 (36.4%)	1 (11.1%)	0.7
Neuropsychiatric	5 (5.3%)	2 (3.8%)	2 (6.1%)	1 (11.1%)	
Others	6 (6.3%)	4 (7.5%)	2 (6.1%)	0 (0%)	
IPSET-thrombosis					
Low	80 (84.2%)	44 (83.0%)	27 (81.8%)	9 (100%)	
Intermediate	11 (11.6%)	7 (13.2%)	4 (12.1%)	0 (0%)	0.7
High	4 (4.2%)	2 (3.8%)	2 (6.1%)	0 (0%)	
Revised IPSET-thrombosis					
Very low	67 (70.2%)	34 (63.5%)	24 (72.7%)	9 (100%)	
Low	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0.2
Intermediate	24 (25.5%)	17 (32.7%)	7 (21.2%)	0 (0%)	
High	4 (4.3%)	2 (3.8%)	2 (6.1%)	0 (0%)	
Treatments					
Only ASA	18 (18.9%)	10 (18.9%)	1 (11.1%)	0 (0%)	
HU	45 (47.4%)	29 (54.7%)	2 (22.2%)	2 (28.6%)	0.5
Anagrelide	14 (14.7%)	5 (9.4%)	3 (33.3%)	3 (42.9%)	
HU + anagrelide	9 (9.5%)	4 (7.5%)	1 (11.1%)	1 (14.3%)	
IFN-α	9 (9.5%)	5 (9.4%)	2 (22.2%)	1 (14.3%)	
MF transformation					
Yes	3 (3.2%)	1 (1.9%)	0 (0%)	2 (22.2%)	**0.002**
No	92 (96.8%)	52 (98.1%)	33 (100%)	7 (77.8%)	
Leukemic transformation					
Yes	1 (1.1%)	1 (1.9%)	0 (0%)	0 (0%)	0.5
No	94 (98.9%)	52 (98.1%)	33 (100%)	9 (100%)	

CALR: Calreticulin; PLT: platelet count; Hb: hemoglobin; WBC: white blood cell count; CVD: cardiovascular disease; IPSET-thrombosis: International Prognostic Score of Thrombosis in Essential Thrombocythemia; ASA: acetylsalicylic acid; HU: hydroxyurea; IFN-α: interferon alpha; MF: myelofibrosis.

compared to *JAK2*V617F-mutated patients (p=0.001) (Figure 3).

Discussion

ET is a type of MPN characterized by an increased rate of TEEs, a varying burden of symptoms, and an intrinsic risk of progression to MF and acute leukemia; however, survival is only modestly reduced in most cases. In this study, we have presented the retrospective analysis of 302 patients with ET according to their *JAK2*V617F and *CALR* mutational statuses. Furthermore, we compared the clinical courses and disease outcomes of the *CALR*-mutated patients according to the *CALR* subtypes of type 1, type 2, and others.

There is growing evidence that *CALR*-mutated ET cases are phenotypically different from other molecular types of ET,

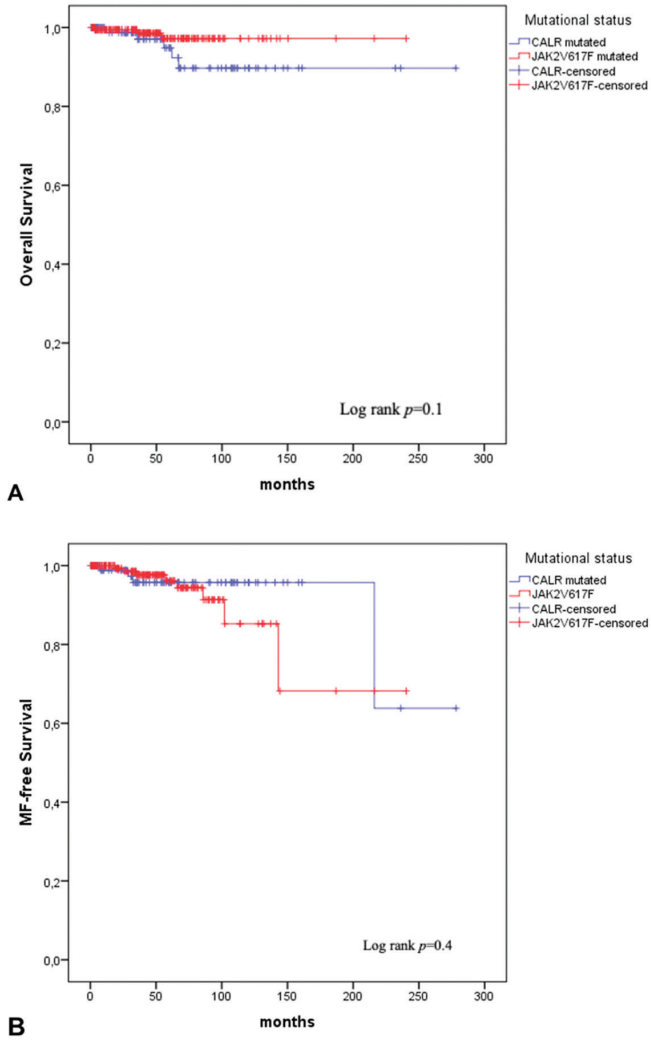

Figure 1. Overall (A) and myelofibrosis-free (B) survival according to the mutational statuses of the patients.

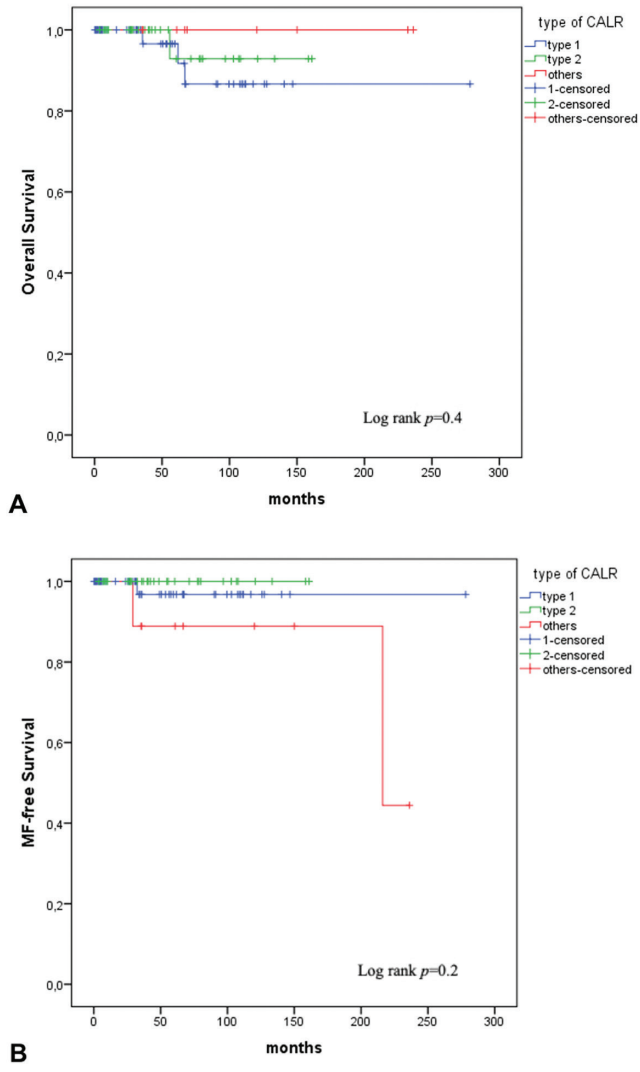

Figure 2. Overall (A) and myelofibrosis (MF)-free (B) survival of *CALR*-mutated patients according to *CALR* subtypes.

including *JAK2*V617F-mutated, *MPL*-mutated, and triple-negative cases, in terms of both clinical and hematological presentations and survival outcomes [17,18,19,20]. Moreover, Alvarez-Larrán et al. [17] underlined the need for new treatment modalities for *CALR*-mutated ET, arguing that conventional cytoreductive agents are less effective in these patients compared to other ET cases. However, in this study, we compared the outcomes of *CALR*-mutated patients with only those who were *JAK2*V617F-positive. Triple-negative ET is a heterogeneous group of diseases and may harbor additional non-driver mutations that affect disease outcome. For this reason, we excluded triple-negative patients from our study due to the heterogeneous nature of the disease and we also excluded *MPL* cases due to their low frequency.

Nangalia et al. [2] reported that patients with *CALR*-mutated MPNs presented with higher platelet counts and lower hemoglobin levels than patients carrying the *JAK2*V617F mutation. Among Han Chinese patients, *CALR* mutations

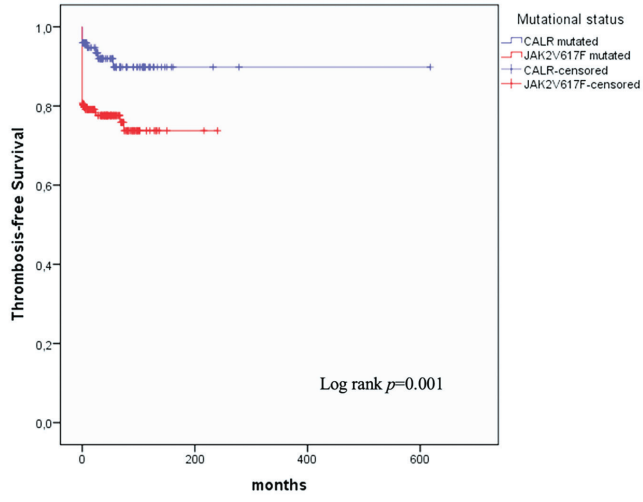

Figure 3. Thrombosis-free survival according to mutational status.

were associated with younger age and higher platelet counts compared to *JAK2*V617F mutations [21]. In addition, patients with *CALR* mutations in Tunisian and Belgian cohorts had higher platelet counts and lower hemoglobin levels and leukocyte counts than patients with *JAK2*V617F mutations [18,22]. Ceylan et al. [23] showed that patients with *CALR* mutations presented with higher platelet counts and lactate dehydrogenase levels compared to *JAK2*- and *MPL*-mutated patients. Similarly, in our patient cohort, *CALR*-mutated patients had significantly higher platelet counts and lower hemoglobin levels (p<0.001). In addition, these patients tended to have lower leukocyte counts (p=0.06).

In patients with ET, the risk for thrombotic complications may exceed 20% [7,24], and previous studies have shown that the risk of thrombosis is higher in *JAK2*V617F-mutated cases [25]. Among the 300 patients with ET reported by Gangat et al. [26], 106 (35%) experienced arterial (n=75) or venous (n=43) events. In univariate analysis, compared to *JAK2*V617F-mutated cases, *CALR*-mutated patients had better TFS (hazard ratio [HR]: 0.53, 95% confidence interval [CI]: 0.30-0.92). However, the authors concluded that the favorable effect of *CALR* mutations might be confined to younger patients [26]. Guglielmelli et al. [27] reported thrombosis (arterial or venous) in 152 (30%) of 502 ET patients, and of those cases, 96 (19%) were arterial and 82 (16%) were venous. In multivariate analysis, venous thrombosis risk at any time was significantly higher in *JAK2*V617F-mutated patients than that observed in *CALR*-mutated cases. This study confirmed the prothrombotic influence of *JAK2*V617F [28] as opposed to *CALR* mutations [27]. In a study that included 168 ET patients, 51 (30.35%) experienced thrombotic events, 60% of which were arterial [29], and *JAK2*V617F-mutated cases exhibited a 1.5-fold higher risk of developing thrombotic events. In our study, the total incidence of thrombotic complications was 17.9%, and most of them (77.8%) were arterial. In the literature, similar to our study, TEEs in ET patients are more common in arterial sites than venous sites, but the incidence of TEEs is generally reported to be higher than that of our patient cohort. Although many factors such as age, comorbidities, and smoking might have an impact on the incidence of TEEs in ET patients, the ASA use in all cases of our cohort might have contributed positively to the low incidence of thrombosis in our study. We also showed in multivariate analysis that age was the only factor independently affecting the incidence of TEEs. Since patients with *CALR* mutations were younger, age may have contributed positively to the lower incidence of TEEs in these patients. Like previous studies, in our cohort the incidence of all TEEs was significantly higher in *JAK2*V617F-mutated cases than *CALR*-mutated cases. Furthermore, *JAK2*V617F-mutated patients had high risk scores for both IPSET-thrombosis and the revised IPSET thrombosis scoring system compared to *CALR*-positive patients. The 2-year and 5-year TFS rates were superior in *CALR*-mutated patients compared to *JAK2*V617F-positive patients (p=0.001, HR: 0.71, 95% CI: 0.68-0.74). In a Japanese population, *JAK2*V617F mutations were related to more thrombotic events and more splenomegaly than *CALR* mutations [30]. In our population, the incidence of splenomegaly was similar between *JAK2*V617F- and *CALR*-mutated cases.

In ET, the leukemic transformation rates at 10 years are estimated to be <1% while those at 20 years average 5%, and the rates of developing MF are slightly higher [25]. Historical data about the probability of MF transformation in ET patients revealed rates of 2.7% (95% CI: 2.4-2.9) at 5 years, 8.3% (95% CI: 7.8-8.9) at 10 years, and 15.3% (95% CI: 6.1-24.5) at 15 years [31]. Barbui et al. [8] revealed that leukemic transformation rates at 10 and 15 years were 0.7% and 2.1%, respectively, and progression rates to overt MF at 10 and 15 years were 0.8% and 9.3%, respectively. They claimed that the absence of *JAK2*V617F was a risk factor for overt MF progression. The rate of leukemic transformation in our cohort (0.7%) is comparable to those mentioned in the literature, while the rate of progression to MF (4%) is slightly higher than the previously published values. The presence of *JAK2*V617F and *CALR* mutations had no impact on progression to MF and leukemic transformation in our study.

Among our patient cohort, OS and MF-free survival rates were similar between *JAK2*V617F- and *CALR*-mutated patients. In *CALR* subgroup analysis, the rates of MF occurrence were significantly higher in patients with other (non-type 1 and non-type 2) *CALR* mutations, but OS and MF-free survival were similar among all *CALR*-mutated patients. Our results support previous data suggesting that driver mutations do not have an impact on survival in ET [25]. In a study of 1494 ET patients, the independent adverse effect of male sex on survival was confirmed with multivariable analysis (HR: 1.6, 95% CI: 1.1-2.5, p=0.02), and in the context of the IPSET, the HRs (95% CI) were 1.6 (1.1-2.5) for male sex, 7.5 (3.1-18.3) for high-risk IPSET scores, and 4.1 (1.8-9.5) for intermediate-risk IPSET scores. Tefferi et al. [32] suggested that women with ET live longer than their male counterparts and that sex might supersede thrombosis history as a risk factor for OS. However, in our study, sex had no effect on survival outcomes, similar to previous findings in a Romanian cohort [29].

Recent studies revealed the adverse impact on survival of non-driver mutations such as *ASXL1*, *SF3B1*, *SRSF2*, and *TP53* [25,33]. *TP53* mutations were highly predictive for leukemic transformation in previous studies [33,34]. In our population, one of the two patients who experienced acute myeloid leukemia progression had *TP53* mutation with a complex karyotype.

The management of ET mainly focuses on reducing the risk of thrombosis, controlling myeloproliferation, and managing

disease-related symptoms and complications [35]. HU is recommended as a front-line cytoreductive therapy for patients with high-risk ET [25]. In the UK-PT1 study, HU was superior to anagrelide in reducing arterial thrombosis, major bleeding, and fibrotic progression [36]. However, in the ANAHYDRET study, HU and anagrelide were found to be similar in the prevention of thrombotic end points [37]. The majority of our patients (77.8%) were treated with cytoreductive agents. In our study, the overall cytoreduction rate was comparable to that of a Romanian cohort (77.8% vs. 76.8%, respectively), although approximately half of the Romanian patients received cytoreductive therapy without ASA/antiplatelets or anticoagulants [23]. HU was the most preferred cytoreductive treatment in both patient cohorts.

In our study, the percentage of patients receiving HU was higher among *JAK2*V617F-mutated patients than those with *CALR* mutations (64% vs. 48.5%), but the overall cytoreduction rate was higher among *CALR*-mutated patients (74.9% vs. 83.8%). The higher rate of HU use among patients with *JAK2*V617F was probably due to the higher incidence of thrombosis observed in this patient population. In addition, *CALR* mutations causing higher platelet counts seem to have prompted more cytoreductive therapy initiation. Although most of these patients had a low IPSET-thrombosis score, their exposure to cytoreductive treatment may be considered over-treatment in ET [38]. In contrast, extreme thrombocytosis has previously been associated with *CALR* mutations [10] and a lower risk of arterial thrombosis [39].

Guglielmelli et al. [27] confirmed the prothrombotic influence of *JAK2* as opposed to *CALR* mutations and suggested that extreme thrombocytosis might also play a part in contributing to the observed decreased risk of arterial thrombosis in *CALR*-mutated ET.

Study Limitations

Since our study was retrospective in nature, covering approximately 20 years, it has some limitations. The major limitations are the lack of comprehensive data regarding non-driver mutations and different median follow-up periods for different *CALR* mutation types. While acknowledging the limitations, we anticipate that our study may contribute to the literature as it includes a large number of Turkish patients and provides real-life data.

Conclusion

This is the first multicenter study investigating the disease characteristics and clinical courses of *CALR*-mutated ET patients in a Turkish population. Our results proved that *JAK2*V617F was strongly associated with thromboembolic complications and HU was the most preferred cytoreductive agent for patients with high thrombosis risk. Mutant *CALR* resulted in higher platelet counts and lower hemoglobin levels than mutant *JAK2*V617F and was related to younger ages at the time of diagnosis. We suggest that ET cases should be managed meticulously considering the disease characteristics caused by driver and non-driver mutations.

Ethics

Ethics Committee Approval: Ethical approval was obtained from the Local Ethics Committee of İzmir Bozyaka Training and Research Hospital with approval number 2022/127 on August 10, 2022.

Informed Consent: Retrospective study.

Authorship Contributions

Surgical and Medical Practices: P.P., D.Ö., S.G., R.Ç., S.M., T.E., V.G., T.El., N.E., G.G., R.D.K., E.A.D., E.E.G., Y.İ., M.B., E.Y.; Concept: Z.N.Ö., P.P., D.Ö., S.G., R.Ç., S.M., T.E., V.G., T.El., G.E., G.G., R.D.K., E.A.D., E.E.G., Y.İ., M.B., E.Y., A.E.E.; Design: Z.N.Ö., P.P., D.Ö., S.G., R.Ç., S.M., T.E., V.G., T.El., G.E., G.G., R.D.K., E.A.D., E.E.G., Y.İ., M.B., E.Y., A.E.E.; Data Collection or Processing: Z.N.Ö., P.P., D.Ö., S.G., R.Ç., S.M., T.E., V.G., T.El., G.E., G.G., R.D.K., E.A.D., E.E.G., Y.İ., M.B., E.Y., A.E.E., V.K.; Analysis or Interpretation: Z.N.Ö, A.E.E.; Literature Search: Z.N.Ö, A.E.E.; Writing: Z.N.Ö, A.E.E.

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Antiphospholipid Syndrome: To Classify or Not to Classify?

Antifosfolipid Sendromu: Sınıflandırmalı mı Yoksa Sınıflandırmamalı mı?

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Abstract

Antiphospholipid syndrome (APS) is a systemic autoimmune disorder resulting in thrombosis, microvascular disease, morbidity in pregnancy, and/or non-thrombotic manifestations. The recently introduced 2023 American College of Rheumatology (ACR) and European Alliance of Associations for Rheumatology (EULAR) APS classification criteria, with significantly higher specificity compared to the revised Sapporo criteria, now reflect the current thinking about APS and provide a new foundation for future APS research. The purpose of this short commentary is to discuss the appropriate circumstances under which the 2023 ACR/EULAR classification criteria could be used and to demonstrate how the new criteria can be applied to simple case scenarios.

Keywords: Antiphospholipid syndrome, Classification criteria, Antiphospholipid antibodies

Öz

Antifosfolipid sendromu (AFS) tromboz, mikrovasküler hastalık, gebelik morbiditesi ve/veya trombotik olmayan belirtilerle sonuçlanan sistemik otoimmün bir bozukluktur. 2023'te tanıtılan Amerikan Romatoloji Koleji (ACR) ve Avrupa Romatoloji Birlikleri İttifakı (EULAR) AFS sınıflandırma kriterleri, revize edilmiş Sapporo kriterlerine kıyasla önemli ölçüde daha yüksek özgüllüğe sahip olduğundan güncel AFS yorumunu yansıtmakta ve gelecekte AFS araştırmaları için yeni bir temel sağlamaktadır. Bu kısa yorumun amacı, 2023 ACR/EULAR sınıflandırma kriterlerinin kullanılabileceği uygun durumları tartışmak ve yeni kriterlerin nasıl basit vaka senaryolarına uygulanabileceğini göstermektir.

Anahtar Sözcükler: Antifosfolipid sendromu, Sınıflandırma kriterleri, Antifosfolipid antikorları

Introduction

Antiphospholipid syndrome (APS) is a systemic autoimmune disorder resulting in thrombosis, microvascular disease, morbidity in pregnancy, and/or non-thrombotic manifestations such as cardiac valve disease or thrombocytopenia [1]. The three commonly used tests to detect the antibodies responsible for APS, namely antiphospholipid antibodies (aPLs), are the anticardiolipin antibody (aCL) enzyme-linked immunosorbent assay (ELISA), anti-β_2-glycoprotein-I antibody (aβ_2GPI) ELISA, and lupus anticoagulant (LA) functional coagulation assay.

Disease classification criteria are used to capture well-defined homogeneous cohorts for research. Given the strict and standardized definitions included in classification criteria, the goal is not to identify the "entire universe" of all possible patients, but rather to capture a majority of patients who share the key features of the condition of interest [2]. Thus, classification criteria are not "diagnostic criteria" and they should not be used for diagnostic and therapeutic decisions in clinical settings.

The APS classification for research was established based on the Sapporo criteria, published in 1999 [3] and revised in 2006 [4]. Given the limitations of the Sapporo criteria [5], including a lack of strict definitions, an international multidisciplinary effort was initiated, supported by the American College of Rheumatology (ACR) and European Alliance of Associations for Rheumatology (EULAR), to develop new APS classification criteria. These recently introduced 2023 ACR/EULAR APS classification criteria, with significantly higher specificity (99%) compared to the revised Sapporo criteria (86%), now reflect the current thinking about APS and provide a new foundation for future APS research. The new criteria have hierarchically clustered and weighted independent clinical and laboratory domains; APS classification based on the new criteria requires a threshold to be achieved (Table 1).

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The purpose of this short commentary is to discuss the appropriate circumstances under which the 2023 ACR/EULAR classification criteria could be used and to demonstrate how the new criteria can be applied to simple case scenarios. Detailed discussion about the diagnosis and management of APS can be found elsewhere [6,7].

When to Classify and When Not to Classify?

The new 2023 ACR/EULAR APS classification criteria aim to identify homogeneous APS cohorts for research purposes; thus, both researchers and clinicians should fully understand the implications of the criteria. In fact, for both research and clinical practice settings, it will be helpful to clarify two potential misunderstandings:

"This patient does not meet APS classification criteria; thus she/he cannot participate in any research study": this is an incorrect statement because if a patient does not meet the APS classification criteria, i.e., falls below the threshold at which a significant number of experienced physicians would feel comfortable calling the case "APS" for research purposes, the case may still be uncertain, equivocal, or controversial rather than a case of "no APS." As discussed above, classification criteria do not necessarily capture patients with rare and unusual manifestations of a disease. Thus, rather than performing no research with these patients, they should be studied or analyzed separately, i.e., not combined with cases meeting the 2023 ACR/EULAR APS classification criteria. Ideally, those patients who do not fulfill the classification criteria should meet either clinical OR laboratory criteria. In fact, the results of such studies may

Table 1. Summary of 2023 ACR/EULAR antiphospholipid syndrome (APS) classification criteria (please refer to the original publication [1] or the online calculator [8] for details and definitions; patients accumulating at least three points each from the laboratory and clinical domains are classified as having APS).

Entry criteria	
At least one clinical criterion listed below (domains 1-6) plus positive antiphospholipid antibody (aPL) test (lupus anticoagulant test, or moderate-to-high titers of anticardiolipin or anti-β_2-glycoprotein-I antibodies [IgG or IgM]) within 3 years of the clinical criterion	
Clinical domains and criteria:	**Weight**
Domain 1. Macrovascular (venous thromboembolism [VTE])	
• VTE with a high VTE risk profile	1
• VTE without a high VTE risk profile	3
Domain 2. Macrovascular (arterial thrombosis [AT])	
• AT with a high cardiovascular disease (CVD) risk profile	2
• AT without a high CVD risk profile	4
Domain 3. Microvascular*	
• Suspected	2
• Established	3
Domain 4. Obstetric	
• Three or more consecutive pre-fetal (<10w) and/or early fetal (10w 0d to 15w 6d) deaths	**1**
• Fetal death (16w 0d to 33w 6d) in the absence of preeclampsia (PEC) with severe features or placental insufficiency (PI) with severe features	**1**
• PEC with severe features (<34w 0d) or PI with severe features (<34w 0d) with/without fetal death	**3**
• PEC with severe features (<34w 0d) and PI with severe features (<34w 0d) with/without fetal death	**4**
Domain 5. Cardiac valve	
Thickening	**2**
Vegetation	**4**
Domain 6. Hematology	
Thrombocytopenia (lowest 20-130x10^9/L)	**2**
Laboratory (aPL) domains and criteria:	**Weight**
Domain 7. aPL test by coagulation-based functional assay (lupus anticoagulant test [LA])	
Positive LA (single - one time)	**1**
Positive LA (persistent)	**5**
Domain 8. aPL test by solid-phase assay (anti-cardiolipin antibody [aCL] ELISA and/or anti-β_2-glycoprotein-I antibody [aβ_2GPI] ELISA [persistent])**	
Moderate-high positive (IgM) (aCL and/or aβ_2GPI)	**1**
Moderate positive (IgG) (aCL and/or aβ_2GPI)	**4**
High positive (IgG) (aCL or aβ_2GPI)	**5**
High positive (IgG) (aCL and aβ_2GPI)	**7**

***Suspected:** Livedo racemosa, livedoid vasculopathy lesions by exam, or acute/chronic aPL nephropathy by physical examination and/or laboratory, or pulmonary hemorrhage by symptoms and imaging; **Established:** Livedoid vasculopathy by pathology; acute/chronic aPL nephropathy by pathology; pulmonary hemorrhage by bronchoalveolar lavage or pathology; myocardial disease by imaging or pathology; or adrenal hemorrhage by imaging or pathology.
****Moderate** (40-79 U) and **high** (>80 U) levels of aCL/aβ_2GPI are based on ELISA.
ELISA: Enzyme-linked immunosorbent assay; ACR: American College of Rheumatology; EULAR: European Alliance of Associations for Rheumatology.

guide future updates of the 2023 ACR/EULAR APS classification criteria.

"If this patient fulfills the classification criteria, then we can confirm the diagnosis and start treatment": this is also an incorrect statement given, as discussed above, the fact that classification criteria should serve research, not clinical decision-making. Meanwhile, the diagnosis of APS is a complex equation performed by physicians, which should be based on the aPL profile, the strength of the association between aPLs and the event, and the potential other causes of the event. For instance, in an aPL-positive patient with deep vein thrombosis and multiple additional venous thromboembolism (VTE) risk factors, the diagnosis of APS can be easily questioned. Thus, the treatment recommendations may deviate from standard APS recommendations. Similarly, some aPL-positive patients, e.g., those with obstetric morbidity, may be managed as having APS even if they do not fulfill the classification criteria. Future research based on the new criteria is expected to provide better management guidance to clinicians.

How to Classify?

The 2023 ACR/EULAR APS classification criteria include entry criteria (at least one positive aPL test within 3 years of an aPL-associated clinical criterion) followed by weighted criteria clustered into six clinical (macrovascular VTE, macrovascular arterial thrombosis, microvascular, obstetric, cardiac valve, and hematological) and two laboratory (LA functional coagulation assay and aCL and/or $a\beta_2$GPI IgG/M ELISA) domains. For different aPL-related items included in these domains: a) strict definitions, based on a literature review and steering committee consensus, are also provided [1]; b) when "equally or more likely" causes exist (except the consideration of VTE and cardiovascular disease risk factors), then the item in question should not be scored; and c) the highest weighted item in each domain should be counted toward the total score. Patients accumulating at least three points each from the clinical and laboratory domains are classified as having APS. For the details of the classification criteria and item definitions, please refer to the original publication [1] or the online criteria calculator [8]. Some of the novel features of the new criteria, with the guidance of simple case scenarios to demonstrate the criteria in action, are summarized in Table 2.

Conclusion

The highly specific 2023 ACR/EULAR APS classification criteria will increase the quality of APS research and hopefully trigger further interest in developing and conducting well-designed, risk-stratified, and controlled clinical trials of aPL-positive patients. Thus, the long-term goal would be to provide clinicians with high-quality evidence-based study results and guidelines for improved management decisions and patient outcomes. In the short run, the new classification criteria should not be used for APS diagnosis and management;

Table 2. Novel features of the 2023 ACR/EULAR antiphospholipid syndrome (APS) classification criteria summarized with the guidance of the simple case scenarios (please refer to the original publication [1] or the online calculator [8] for details and definitions; patients accumulating at least three points each from the laboratory and clinical domains are classified as having APS).

Laboratory (aPL) results (item weight in parentheses)	Clinical presentation (item weight in parentheses)	Classçification met[a]?
Persistent LA positivity[b] (5)	VTE with active malignancy[c] (1)	No
	VTE with active malignancy (1) + history of thrombocytopenia[d] (2)	Yes
	Unprovoked VTE (3)	Yes
Persistent triple aPL positivity with high positive IgG aCL and IgG $a\beta_2$GPI[e] (12)	Pulmonary hemorrhage (suspected)[d, f] (2)	No
	Pulmonary hemorrhage (suspected)[d, f] (2) + cardiac valve thickening[d] (2)	Yes
	Pulmonary hemorrhage (established)[d, f] (5)	Yes
Persistent moderate positive IgG aCL and IgG $a\beta_2$GPI[e] (5)	Fetal death (28w) without placental insufficiency (PI) (severe)[d] (1)	No
	Fetal death (28w) without PI (severe)[d] (1) + livedo racemosa (2)	Yes
	Fetal death (28w)[d] with PI (severe) (3)	Yes
Persistent high positive IgM aCL and IgM $a\beta_2$GPI[e, g] (1)	Stroke without high-risk CVD profile[c] (4)	No

[a]Patients accumulating at least three points each from the laboratory and clinical domains are classified as having APS; [b]Performed according to International Society for Thrombosis and Hemostasis guidelines [9]; [c]Risk stratification of thrombotic events is required for macrovascular domains by traditional VTE and CVD risk factors; [d]Otherwise unexplained; [e]Two levels of aCL/$a\beta_2$GPI positivity are defined, moderate (40-79 U) and high (>80 U), based on enzyme-linked immunosorbent assay, not based on new automated systems; [f]Suspected pulmonary hemorrhage is based on symptoms and imaging, whereas established pulmonary hemorrhage is based on symptoms, imaging, and bronchoalveolar lavage or biopsy; [g]Isolated persistent IgM aCL/$a\beta_2$GPI positivity is not sufficient for APS classification, even when clinical criteria are met.

aCL: Anticardiolipin antibody; aPL: antiphospholipid antibody; $a\beta_2$GPI: anti-β_2-glycoprotein-I antibody; CVD: cardiovascular disease; LA: lupus anticoagulant test; VTE: venous thromboembolism; ACR: American College of Rheumatology; EULAR: European Alliance of Associations for Rheumatology.

however, they can partially serve as a guide while evaluating aPL-positive patients.

Ethics

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The Many Faces of Multiple Myeloma

Multipl Myelomun Farklı Yüzleri

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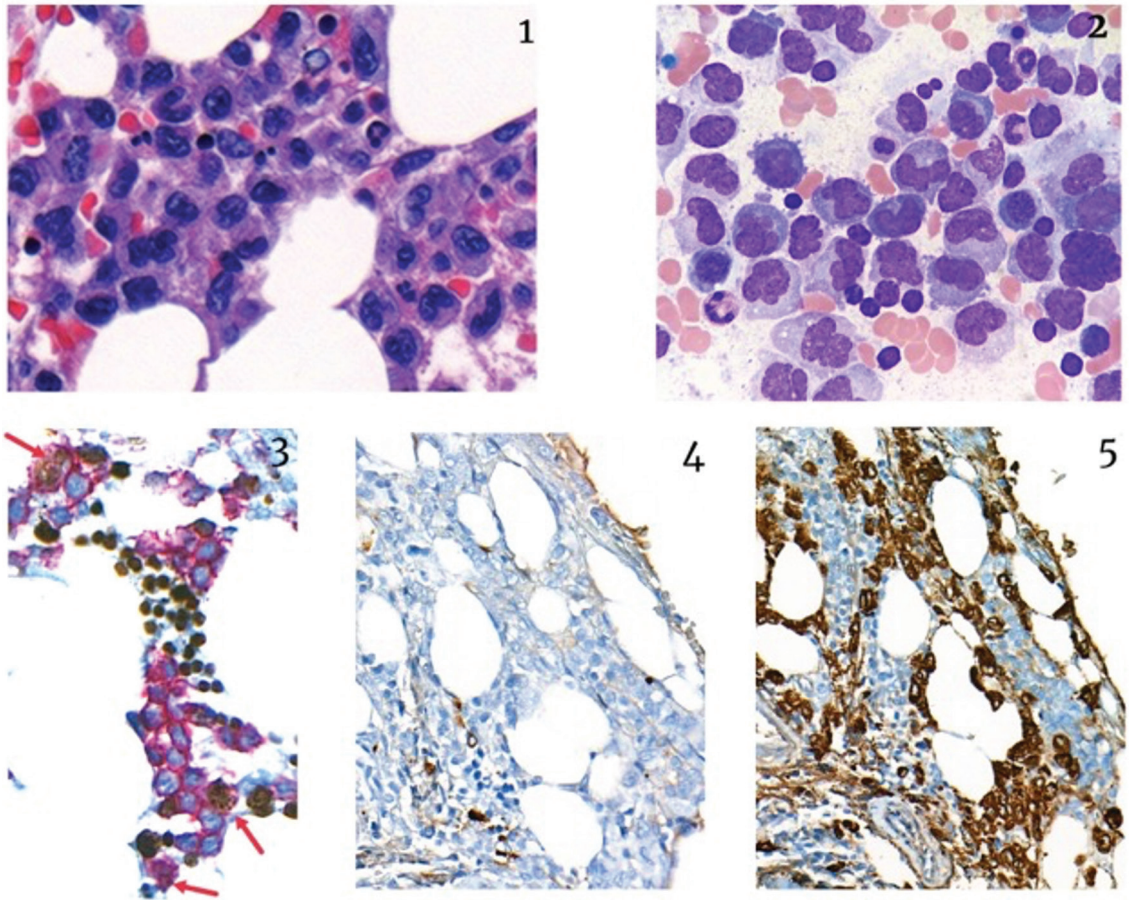

Figures 1-5. 1. Neoplastic infiltration by atypical cells with monocytoid morphology on bone marrow trephine biopsy sections (H&E). **2.** Neoplastic atypical cells with monocytoid morphology on bone marrow aspirate smears (Giemsa). **3.** CD138 (red)-Ki-67 (black) double immunohistochemistry revealing increased proliferative activity (12%) among atypical plasma cells (arrow). **4.** Kappa Ig light chain negativity. **5.** Lambda Ig light chain restriction.

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A 66-year-old man with a 2-year history of multiple myeloma (MM) with t(4;14) (bone marrow analysis) was admitted to our hospital with severe pancytopenia. His hemoglobin level was 5.9 g/dL, white blood cell count was $1.47x10^9$/L, and platelet count was $44x10^9$/L. A free light chain assay confirmed the progression of MM with an increase of 2152.5 mg/dL in serum lambda light chain levels. The patient was treated initially with bortezomib-cyclophosphamide-dexamethasone followed by autologous stem cell transplantation. Due to an early relapse under lenalidomide maintenance, bortezomib-lenalidomide-dexamethasone treatment was initiated, to which he remained refractory.

Bone marrow biopsy was consistent with interstitial infiltration by large pleomorphic, atypical cells with prominent large irregular and multilobate nuclei, visible nucleoli, thin granular chromatin, and some vacuolated cytoplasm, suggesting high-grade lymphoma or acute leukemia with monoblastic differentiation (Figures 1 and 2).

Flow cytometry revealed CD38, CD138, and CD56 expression, suggesting plasma cell origin, which was confirmed by strong CD138 and lambda expression by immunohistochemistry (Figures 3-5).

Anaplastic morphology and resistance to conventional therapy are compatible with the unfavorable genetic abnormality observed in this case [1]. Anaplastic MM is characterized by pleomorphic and markedly enlarged plasma cells and poor clinical outcome [2,3,4,5]. The abnormal morphology can be confused with acute leukemia or aggressive lymphoma at the time of the initial diagnosis. The clinical history, flow cytometry, molecular findings, and immunohistochemistry are helpful in confirming the diagnosis.

Keywords: Myeloma and other plasma cell dyscrasias, Neoplasia, Immunology, Marrow

Anahtar Sözcükler: Myelom ve diğer plazma hücre diskrazileri, Neoplazi, İmmunoloji, Kemik iliği

Ethics

Informed Consent: Informed consent was obtained from the patient for the anonymous use of materials taken from him in all kinds of research following the necessary procedures.

Authorship Contributions

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

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Advanced Cutaneous Peripheral T-cell Lymphoma-Not Otherwise Specified with Extensive Ulceronecrotic Dyschromic Plaques and Poor Outcome

İlerlemiş Kutanöz Periferik T-hücre Lenfoma-Başka Türlü Belirtilmeyen, Yaygın Ülseronekrotik Diskromik Plaklar ve Kötü Sonuçlar

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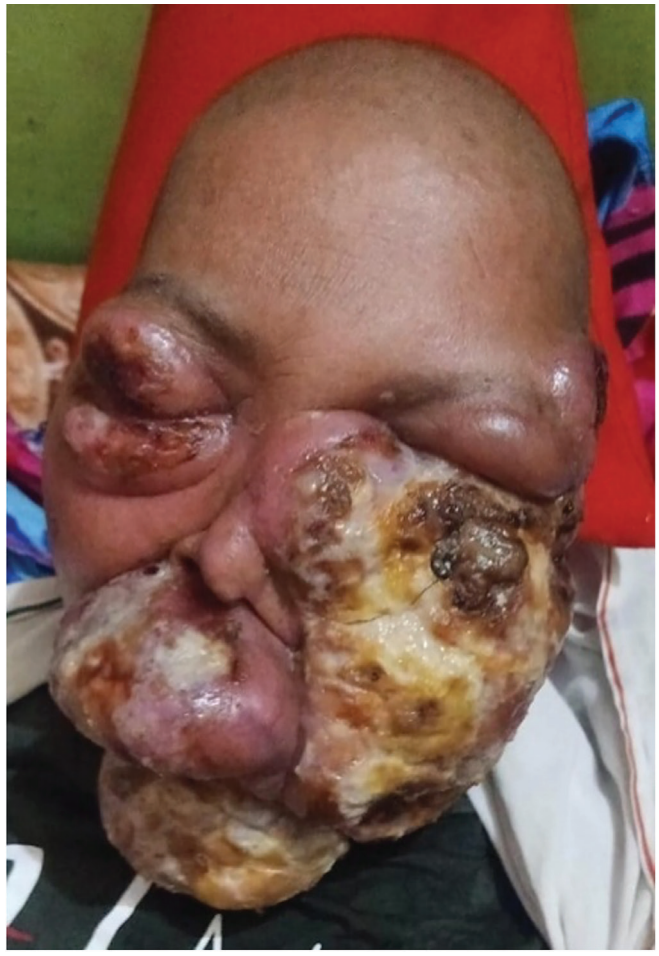

Figure 1. Extensiveulceronecrotic dyschromic plaques in a patient with cutaneous peripheral T-cell lymphoma-not otherwise specified.

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We present the case of a 42-year-old male patient with a known history of cutaneous peripheral T-cell lymphoma-not otherwise specified. The patient had received two cycles of cyclophosphamide, doxorubicin, vincristine, etoposide, and prednisone (CHOEP regimen) but discontinued treatment abruptly [1]. After a 6-month interval, the patient presented to our tertiary care center with a significantly deteriorated general condition, marked weight loss, and persistent fever. A large ulceronecrotic dyschromic plaque measuring 12x6 cm was observed, involving the entire left cheek, nose, and lower eyelid. The surface of the lesion exhibited oozing and crusted areas, with additional ulcerative lesions present on the left upper eyelid and adjacent temple. Similar ulceronecrotic dyschromic plaques were also identified on the chin, right upper lip, and right lower eyelid (Figure 1). A biopsy of the lesion confirmed the earlier diagnosis of cutaneous peripheral T-cell lymphoma-not otherwise specified without bone marrow and peripheral blood involvement.

The patient's chemotherapy treatment was resumed considering the recurrence of the lymphoma; however, due to the extensive nature of the lesion, debridement was not deemed appropriate. Unfortunately, the patient presented to us in an extremely debilitated condition with advanced disease progression and succumbed to neutropenic sepsis within 2 months of restarting chemotherapy.

Keywords: Lymphoma, T-cell neoplasms, Ulceronecrotic dyschromic plaques, Acquired neutropenia

Anahtar Sözcükler: Lenfoma, T-hücreli neoplazmlar, Ülseronekrotik diskromik plaklar, Kazanılmış nötropeni

Ethics

Informed Consent: Informed consent was received from the patient.

Authorship Contributions

Surgical and Medical Practices: V.S.; Concept: V.S.; Design: V.B.; Data Collection or Processing: V.B.; Analysis or Interpretation: V.S.; Literature Search: V.B.; Writing: V.S., V.B.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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Unusual Clasmatosis Morphology

Anormal Klazmatoz Morfolojisi

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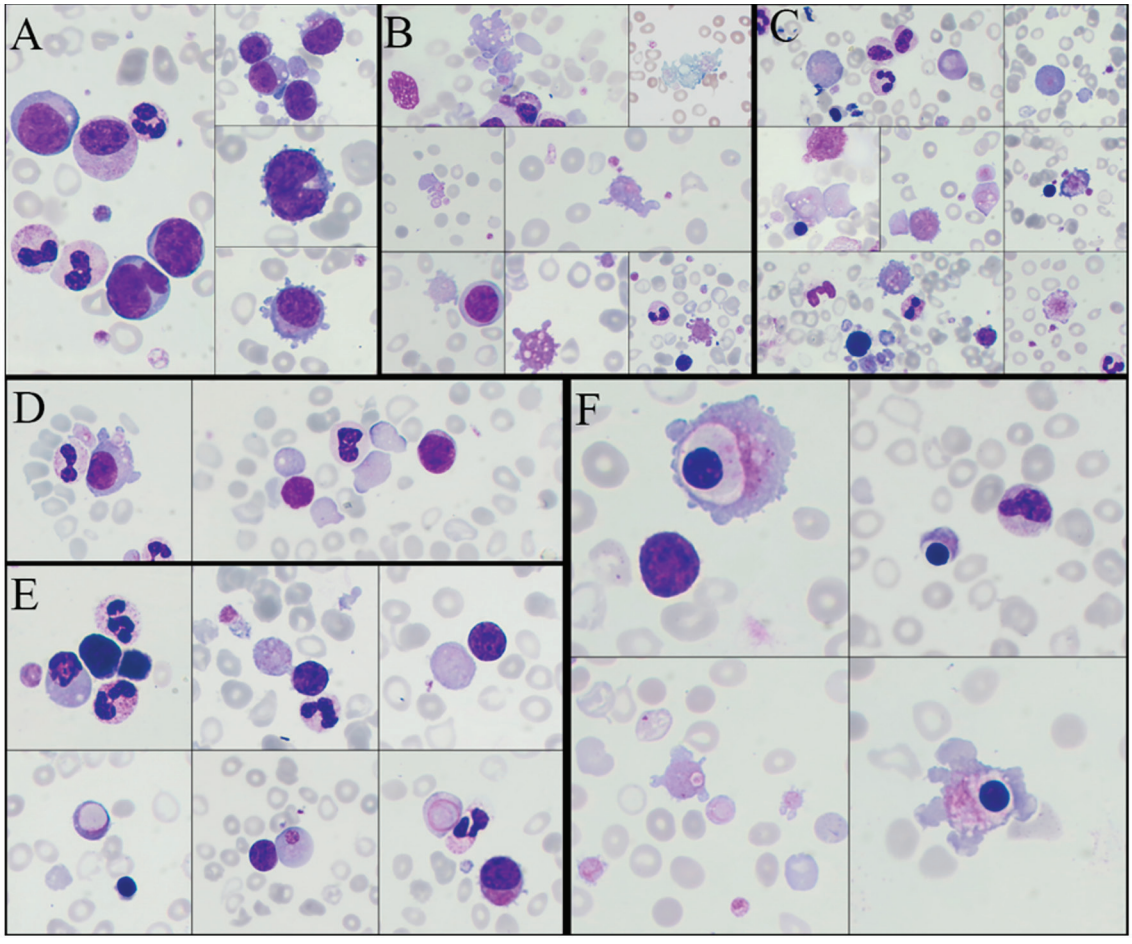

Figure 1. Clasmatosis in peripheral blood. May-Grünwald Giemsa staining, 1000^{x} magnification. (A) Circulating blast cells and megakaryocytes/megakaryoblasts. (B, C) Different morphologies of fragments or whole empty cytoplasm. (D, E) Different modes of clasmatosis formation: cytoplasmic fragmentation (D) and nuclear expulsion (E). (F) Cytoplasmic residues of phagocytosis.

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Clasmatosis refers to cytoplasmic fragments in the peripheral blood (PB) or bone marrow [1,2]. We describe clasmatosis of unusual morphology in the PB of an 83-year-old man with primary myelofibrosis diagnosed 2 years earlier according to the following criteria of the World Health Organization (WHO): megakaryocytic atypical hyperproliferation, JAK2-V617F positivity, absence of WHO criteria for other myeloid neoplasms, anemia, and splenomegaly [3].

PB review showed red blood cells with anisopoikilocytosis, dysplastic neutrophilia ($23.0x10^9$/L), and immature granulocytes ($2.4x10^9$/L). Bone marrow aspiration yielded a dry tap. Blast cells, nucleated red blood cells, and micromegakaryocytes/megakaryoblasts were also observed (Figure 1A).

The clasmatosis morphology displayed both regular and irregular contours (Figure 1B) and giant forms without or with internal granules (Figure 1C), sometimes indistinguishable from giant platelets (Figures 1B and 1C).

Small-to-medium clasmatosis originates from cytoplasmic budding and fragmentation (Figure 1D). Clasmatosis also occurred in this case after nuclear extrusion, as seen in the sequence portrayed in Figure 1E: i) the beginning of nuclear exit from the cellular boundary, ii) detachment from the last cytoplasmic ribbon, and iii) complete exit. Afterwards, various cellular remnants could be observed, including naked nuclei and denucleated cytoplasm, sometimes containing the negative mold of the pre-existing nucleus, as well as Cabot-like rings and chromatin fragments.

Whole cytoplasm or fragments were observed after nuclear detachment retaining the contents of the previous phagocytosis (Figure 1E).

Keywords: Clasmatosis, Primary myelofibrosis, Peripheral blood review

Anahtar Sözcükler: Klazmatoz, Primer miyelofibrozis, Çevresel kan değerlendirmesi

Ethics

Informed Consent: According to the Local Ethics Committee, the retrospective nature of this study allowed for a waiver of consent.

Authorship Contributions

Concept: A.L.G., F.F.; Design: A.L.G.; Data Collection or Processing: M.M., F.F.; Analysis or Interpretation: M.M.; Literature Search: M.M.; Writing: A.L.G., F.F.

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LETTER TO THE EDITOR

Turk J Hematol 2024;41:47-48

Epithelial Cells or Vascular Smooth Muscle Cells in a Peripheral Blood Smear?

Periferik Kan Yaymasında Epitel Hücresi mi Vasküler Düz Kas Hücresi mi?

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To the Editor,

We read the paper submitted to this journal by Lee et al. [1] with great interest. They reported rare epithelial cells in the peripheral blood smear of a 56-year-old male patient. In this blood smear, a few clusters of medium-to-large cells containing elongated oval-grooved nuclei with pale blue frayed cytoplasm at both ends were found at the tail-end of the blood smear. The authors reasoned that these cells were likely epithelial cells, reported as non-hematopoietic cells. The authors then stated that the presence of these abnormal cells could be due to improper mixing before aspiration, to a blunted-tip needle being used, or to repeated unsuccessful venipuncture attempts, and these abnormal cells can also be rarely seen from finger or heel pricks due to transference of skin into the blood tube.

However, confirmation of epithelial cells should be validated by immunohistochemistry, and Lee et al. [1] did not mention the venipuncture status of this patient. As we know, alcohol disinfection is mandatory before venipuncture. Thus, the transference of skin into the blood tube is rare. For this reason, we propose other possible causes of the abnormal cells reported by Lee et al. [1], including vascular smooth muscle cells, subcutaneous fibroblasts, or even the synoviocytes around the elbow joint, from venipuncture of the synovium of the elbow joint. However, whether these cells had the cluster feature is unclear, and other immunohistochemistry methods are needed for corroboration.

Additionally, these abnormal cells reported by Lee et al. [1] also resemble vascular endothelial cells. Vascular endothelial cells have a highly irregular cell morphology, mostly in the shape of long tails or spindles with intact cell membranes and irregular nuclei, often lacking nucleoli. However, vascular endothelial cells could be excluded in this case from our perspective because vascular endothelial cells are often arranged in a single layer and are sparse, regardless of whether they are brought out by vein or bone marrow puncture. Single or several endothelial cells have a certain trend of arrangement, which is inconsistent with the cell cluster feature in the study discussed here.

In conclusion, we appreciate the report offered by Lee et al. [1] for giving us an excellent opportunity to discuss these rare abnormal cells that are seldom seen in hematological examinations.

Keywords: Epithelial cells, Vascular smooth muscle cells, Peripheral blood smear

Anahtar Sözcükler: Epitelial hücreler, Vasküler düz kas hücreleri, Periferik kan yayması

Ethics

Informed Consent: Not applicable.

Authorship Contributions

Concept: W.Y., Y.W., W.P.; Data Collection or Processing: W.Y., Y.W., W.P.; Analysis or Interpretation: W.Y., Y.W., W.P.; Literature Search: W.Y., Y.W., W.P.; Writing: W.Y., Y.W., W.P.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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Reply from the Authors:

To the Editor,

We would like to thank Yang et al. [1] for their insights and comments on our article and we would like to offer some additional points for consideration.

First, Yang et al. [1] proposed that the abnormal cells observed in our study could be vascular smooth muscle cells, subcutaneous fibroblasts, or synoviocytes from around the elbow joint, suggesting that these cells might have been the source of the observed anomalies. While these suggestions are valid and should be considered, the presence of these cell types in a peripheral blood smear is extremely rare. To confirm their identity, as Yang et al. [1] rightly pointed out, further immunohistochemistry methods should be employed to corroborate their origin. However, it is crucial to acknowledge that in our paper, we did not definitively identify these cells as epithelial cells but rather suggested that as a possibility. The suggestions made by Yang et al. [1] offer alternative avenues for investigation but do not definitively rule out the possibility of epithelial cells.

Furthermore, it is noteworthy that Yang et al. [1] raised the issue of venipuncture status and alcohol disinfection as important factors that can influence the presence of foreign cells in a blood smear. Proper venipuncture technique and disinfection are essential aspects of the procedure that significantly reduce the risk of contamination. Nevertheless, it is not entirely inconceivable that, despite following correct procedures, rare instances of contamination might occur. While the chances of transference of skin into the blood tube are indeed minimal, it is not entirely impossible, and we acknowledge this possibility.

In conclusion, the comments made by Yang et al. [1] offer highly valid alternative hypotheses and facilitate further discussion on the presence of abnormal cells in blood smears.

Sincerely,

Phebe En Ni Lee, Gloria Yuquan Chen, Eng Soo Yap

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LETTER TO THE EDITOR

Turk J Hematol 2024;41:49-50

Importance of Rare Gene Alterations in the Prognosis of B-Cell Acute Lymphoblastic Leukemia

B-Hücre Akut Lenfoblastik Lösemi Prognozunda Nadir Gen Değişikliklerinin Önemi

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[#]*These authors contributed equally to this work.*

To the Editor,

We read the recently published study by Ray et al. [1] with great interest. They reported amplification of the *BCR::ABL1* fusion gene as a rare phenomenon in B-cell acute lymphoblastic leukemia (ALL). In their study, *BCR::ABL1* and *ETV6::RUNX1* translocations, *TCF3* and *KMT2A* rearrangements, and the t(9,22)(q34;q11) *BCR::ABL1* translocation were revealed by fluorescent in situ hybridization (FISH) along with multiple copies of the fusion gene appearing separately in a patient with B-cell ALL as a rare case. This patient died within a month of the diagnosis.

However, the two major isoforms of the oncogenic *BCR-ABL1* tyrosine kinase, p210 and p190, were not mentioned in the context of this patient's case. p210 is the hallmark of chronic myelogenous leukemia, whereas p190 occurs in the majority of B-cell ALL cases [2]. The resulting fusion oncogene is a tyrosine kinase, which in turn results in the uncontrolled proliferation of cells. Importantly, the combination of chemotherapy with second- or third-generation tyrosine kinase inhibitors further improved the outcomes of *BCR-ABL1*-positive B-cell ALL patients [3]. However, in the case discussed here, the *BCR-ABL1* tyrosine kinase p210 and p190 status of the patient and the possible use of tyrosine kinase inhibitors such as imatinib or dasatinib remains unclear since the patient died within a month of diagnosis.

Besides the *BCR::ABL1* translocation, the *ETV6::RUNX1* translocation and *TCF3* and *KMT2A* rearrangements were also revealed in this patient. Among these genes, *ETV6-RUNX1* is the most frequent genetic fusion in pediatric B-ALL. The distinct *KMT2A* rearrangements are independent dismal prognostic factors, and *TCF3* gene rearrangements were also described as being associated with significant differences in ALL prognosis [4,5]. As we know, the molecular hallmark of ALL entails recurrent prognostic genetic alterations, many of which are cryptic by conventional cytogenetics [5,6]. Thus, Ray et al. [1] highlighted the need for FISH or other conventional cytogenetic approaches over reverse-transcriptase polymerase chain reaction studies to confirm disease progression. However, FISH only uses several commercial probes, resulting in limited results for rare genes. Therefore, besides FISH, we want to emphasize that whole-genome sequencing could provide standalone, reliable genetic testing to detect all subtype-defining genetic abnormalities in B-ALL, accurately classifying patients for risk-directed treatment stratification [7]. Moreover, RNA sequencing is also a powerful next-generation sequencing technology that can simultaneously identify cryptic gene rearrangements, sequence mutations, and gene expression profiles in a single assay, including genetic alterations not detected by conventional methods that confer potential prognostic and therapeutic impact [6]. Thus, in addition to FISH, we suggest that whole-genome sequencing or RNA sequencing could be better tools to more accurately classify ALL patients for risk-directed treatment stratification.

Keywords: Rare gene, Prognosis, B-cell acute lymphoblastic leukemia

Anahtar Sözcükler: Nadir gen, Prognoz, B-hücre akut lenfoblastik lösemi

Ethics

Informed Consent: Not applicable.

Authorship Contributions

Concept: L.X., Y.W., W.P.; Data Collection or Processing: L.X., Y.W., W.P.; Analysis or Interpretation: L.X., Y.W., W.P.; Literature Search: L.X., Y.W., W.P.; Writing: L.X., Y.W., W.P.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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Reply from the Authors:

To the Editor,

We thank Xiang et al. [1] for their interest in our article [2]. We would like to clarify that in our patient, only the *BCR::ABL1* translocation was detected along with the amplification of this fusion gene. The *ETV6::RUNX1*, *TCF3*, and *KMT2A* translocations were not detectable in our patient. It is very unlikely to have translocations of multiple types in the same patient as they are mutually exclusive, although we and a few other hematologists have seen the *CRLF2* rearrangement in patients with the *BCR::ABL1* translocation. However, we acknowledge their insights into the roles of different *BCR::ABL1* isoforms and their prognostic significance. Testing for p190 and p210 was planned on fresh samples, due to technical reasons, but could not be performed as the patient died soon after the initial diagnosis, even before tyrosine kinase inhibitors could be initiated. We acknowledge that whole-genome sequencing and RNA sequencing provide more comprehensive genetic assessment and may help unravel cryptic and novel aberrations not picked up by conventional approaches. However, the exorbitant cost and limited availability of these advanced techniques is a significant hindrance preventing their routine application for all patients in resource-constrained settings. The objective of our paper was simply to highlight the rarity of *BCR::ABL1* amplification and the utility of FISH testing in its diagnosis in the era of advanced molecular diagnostics.

Sincerely,

Debadrita Ray, Praveen Sharma, Arihant Jain, Sreejesh Sreedharanunni

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LETTER TO THE EDITOR

Turk J Hematol 2024;41:51-52

Immunoglobulin Replacement Therapy for Hypogammaglobulinemia in Multiple Myeloma Should Not Be Ignored

Multipl Myelomda Hipogamaglobulinemi için İmmünoglobulin Replasman Tedavisi İhmal Edilmemelidir

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[#]*These authors contributed equally to this work.*

To the Editor,

The letter entitled "Invasive Aspergillosis and Candidiasis in a Patient with Plasma Cell Myeloma," written by Khadwal et al. [1], as well as Yavaşoğlu's [2] comment on it, published in recent issues of your journal, were quite interesting. Here we would like to highlight another critical issue that may be overlooked, which is the role of immunoglobulin replacement therapy (IgRT) for hypogammaglobulinemia in multiple myeloma (MM) in the prevention of invasive fungal infection. Khadwal et al. [1] reported that a 65-year-old woman diagnosed with immunoglobulin G (IgG) kappa MM 4 years previously had died of her illness within 5 days of admission to the hospital and that invasive fungal infections were found in this patient. We noticed that this patient had severe hypogammaglobulinemia at the time of her final admission, including IgG of 364 mg/dL (normal: 658-1837 mg/dL), IgM of <21.0 mg/dL (normal: 40-263 mg/dL), and IgA of 41.0 mg/dL (normal: 71-263 mg/dL). However, from our perspective, severe hypogammaglobulinemia was not temporarily induced, and there was a long period since the diagnosis or first chemotherapy of this MM patient.

As we know, the overall survival of MM patients has significantly improved since the introduction of bortezomib and lenalidomide. However, chemotherapy is significantly associated with myelosuppression or DNA synthesis inhibition, further impairing humoral or cellular immunity. These chemotherapies also significantly reduce Ig production and result in secondary immunodeficiency (SID) in MM patients. Therefore, MM patients who have received these chemotherapies are at a high risk of infection, and infections are still the main cause of morbidity and mortality in MM patients.

IgRT and prophylactic antibiotics are two main strategies for the care of patients with SID, especially secondary to hematological diseases [3]. Recent data demonstrated that MM patients receiving IgRT had a lower reduction in the use of antibiotics, fewer days of hospitalization, and fewer infections compared to patients not receiving IgRT [4]. Moreover, in our country, hematological SID specialists are still lacking, and IgRT for SID patients is not covered by health insurance, which results in a high infection rate among MM patients.

Thus, in this letter, we want to highlight another critical issue that may overlooked, which is the role of IgRT for hypogammaglobulinemia in MM in the prevention of invasive fungal infection. Moreover, monitoring Ig levels at diagnosis or after chemotherapy for MM could further enhance the surveillance of fungal infection risk and reduce the mortality rate in MM patients.

Keywords: Immunoglobulin replacement therapy, Hypogammaglobulinemia, Multiple myeloma

Anahtar Sözcükler: İmmünoglobulin replasman tedavisi, Hipogamaglobulinemi, Multipl myelom

Ethics

Informed Consent: Not applicable.

Authorship Contributions

Concept: Q.Z., Y.W., W.P.; Design: Q.Z., Y.W., W.P.; Data Collection or Processing: Q.Z., Y.W., W.P.; Analysis or Interpretation: Q.Z., Y.W., W.P.; Literature Search: Q.Z., Y.W., W.P.; Writing: Q.Z., Y.W., W.P.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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Reply from the Authors:

To the Editor,

We thank Zhang et al. [1] for their interest in our report titled "Invasive Aspergillosis and Candidiasis in a Patient with Plasma Cell Myeloma" [2]. We appreciate their comments and valid queries regarding that publication. Our patient was a non-smoker but had comorbidities including type 2 diabetes mellitus, hypertension, chronic renal disease, and hypogammaglobulinemia with serum immunoglobulin G (IgG) of 364 mg/dL (normal: 658-1837 mg/dL), IgM of <21.0 mg/dL (normal: 40-263 mg/dL), and IgA of 41.0 mg/dL (normal: 71-263 mg/dL). Since her diagnosis 4 years previously with IgG kappa multiple myeloma (MM) of stage III, the best response to therapy had been very good partial response until she developed the final relapse. She was scheduled to receive daratumumab in addition to VCD (bortezomib, cyclophosphamide, and dexamethasone) to manage that relapse, but it could not be administered due to active infection and poor general condition. It is evident that she had most risk factors predisposing to invasive fungal infections, including steroids, diabetic state, broad-spectrum antibiotics, hypogammaglobulinemia, two prior lines of chemotherapy and pre-terminal neutropenia prior to her death during her week-long hospitalization. She was not receiving antifungal prophylaxis. Bone marrow examination performed 3 weeks prior to her final admission had shown 50% plasma cells but no microbial agents were identified at that time. In the autopsy, the bone marrow revealed small clusters of plasma cells (<5%) with relative depletion of normal hemopoietic elements. No fungal hyphae were identified in the sections.

We agree with Zhang et al. [1] regarding the use of fungal prophylaxis for patients receiving high-dose chemotherapy, commonly given during acute leukemia therapy and hematopoietic stem cell transplantation. While there was previously no definite consensus for fungal prophylaxis, the International Myeloma Working Group recently published guidelines and recommendations on risk-adapted prophylaxis for infections in cases of MM [3]. They suggest bacterial, fungal, and antiviral prophylaxis for intermediate-risk and high-risk MM patients.

In view of the above guidelines, which became available 1 year after the death of the patient that we described, antifungal prophylaxis is indicated and should be given to all relapsed/refractory MM patients with underlying risk factors such as diabetes mellitus, renal failure, or hypogammaglobulinemia and those receiving high cumulative doses of steroids during induction as well as the maintenance phase, resulting in a net state of immunosuppression.

Sincerely,

Alka Khadwal, Kirti Gupta, Nabhajit Mallik, Madhurima Sharma, Pankaj Malhotra

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Turk J Hematol 2024;41:53-54

Circulating Monocytes Phagocytosing Lymphocytes in the Small-Cell Variant of T-Cell Prolymphocytic Leukemia

T-Hücreli Prolenfositik Löseminin Küçük Hücreli Varyantında Lenfositleri Fagosite Eden Monositler

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To the Editor,

A 66-year-old man was admitted because of wheezing after physical activity for 1 month. Physical examination showed multiple enlarged lymph nodes in the neck, axilla, and groin, and the largest one was about 4x1.5 cm. The liver was palpable 7 cm below the right subcostal margin and the spleen 10 cm below the left. The patient developed abdominal distention and decreased breath sounds in the right lung without tenderness or rebound pain. A complete blood count showed remarkable leukocytosis (white blood count: $317.96x10^9$/L) with 98% abnormal mature lymphocytes, hemoglobin concentration of 111 g/L, and severe thrombocytopenia (platelets: $23x10^9$/L). Other laboratory results indicated elevated levels of lactic dehydrogenase (LDH; 1232 U/L) and β2-microglobulin (6.36 mg/L). Chest computed tomography revealed hepatosplenomegaly and right-sided pleural effusion with atelectasis of the right lung, and the pleural effusion had high levels of LDH (4877 U/L) and adenosine deaminase (24.2 U/L).

A peripheral blood smear revealed small mature lymphocytes with clumped chromatin, regular nuclei, invisible nucleoli, and scant basophilic cytoplasm (Figure 1a-1d). Strikingly, monocytes engaging in phagocytosis of lymphocytes were observed, showing nuclear condensation and scant cytoplasm (Figure 1a-1c), along with occasional cocci with both intracellular and extracellular monocytes (Figures 1c and 1d). Phagocytosis was not seen in the bone marrow. Flow cytometric analysis of the bone marrow aspiration demonstrated lymphocytes positive for CD3, CD4, CD2, CD7 (bright), CD5, CD45RA, and TRBC1 and negative for CD8, CD10, CD25, CD30, CD45RO, CD56, CD57, CD279, and TCRγδ. The morphological features and immunophenotyping of abnormal cells in the pleural fluid were identical to those of the marrow samples. Cytogenetic analysis showed 46,XY,inv(9)(p12q13)[18]. Fluorescence in situ hybridization revealed TRA/D rearrangement in 83% of the cells (Figures 1e and 1f). A diagnosis of T-cell prolymphocytic leukemia (T-PLL) of the small-cell variant type was made. The patient underwent chemotherapy with bendamustine, but there was no significant improvement. For economic reasons, he was discharged and blood culture was not performed.

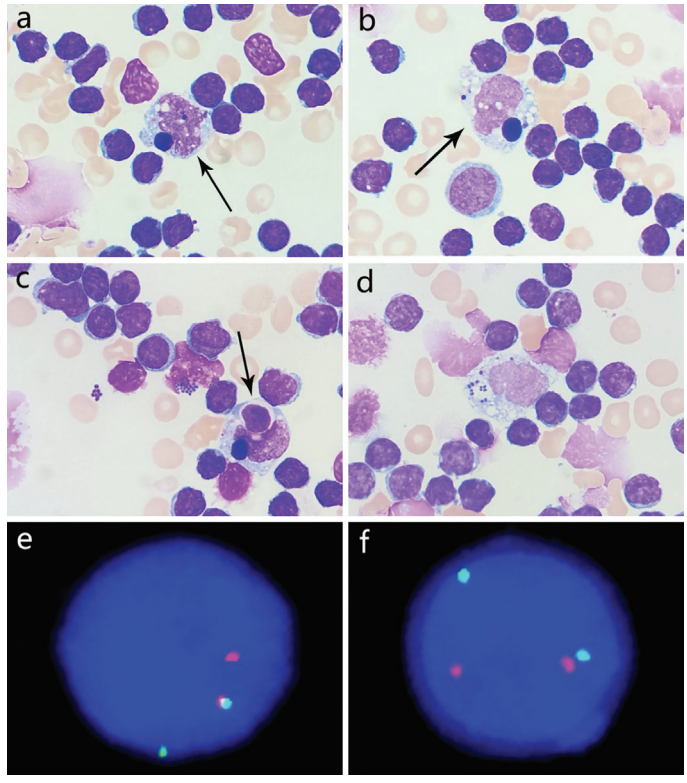

Figure 1. Peripheral blood smear revealed mature lymphocytes with clumped chromatin, regular nuclei, invisible nucleoli, and scant cytoplasm (a, b, c, d, Wright-Giemsa staining, 1000× magnification). Strikingly, monocytes engaged in the phagocytosis of lymphocytes were observed, showing nuclear condensation and scant cytoplasm (a, b, c, black arrows), along with occasional cocci with both intracellular and extracellular monocytes (c, d). Fluorescence in situ hybridization detected TRA/D rearrangement in 83% of all cells (e, f).

Very few cases of phagocytosis of lymphocytes by circulating monocytes have been reported in the literature. To our knowledge, circulating monocytes are generally considered as committed precursors for phagocytes, such as macrophages and dendritic cells [1]. Moreover, Kovács et al. [2] found that phagocytic activities of monocytes occur in patients with ovarian cancer. Phagocytic activity of circulating monocytes may be present in cases of hematological disorders or various infections. Coincidentally, Li et al. [3] documented an unusual anaerobic infection in a 46-year-old man showing the presence of phagocytosis of lymphocytes by circulating monocytes on Wright-stained blood smears. In our case, the occurrence of small lymphocytes in a small-cell variant of T-PLL together with pleural involvement and the phagocytosis of lymphocytes by circulating monocytes was extremely uncommon. In brief, we have described a rare case of the small-cell variant of T-PLL, presenting with pleural effusion and circulating monocytes phagocytosing lymphocytes on blood smears.

Keywords: Circulating monocytes, Phagocytosis, Small-cell variant, T-cell prolymphocytic leukemia, Flow cytometric analysis

Anahtar Sözcükler: Dolaşımdaki monositler, Fagositoz, Küçük hücreli varyant, T-hücreli prolenfositik lösemi, Akım sitometri analizi

Ethics

Informed Consent: Informed consent was obtained for the publication.

Authorship Contributions

Surgical and Medical Practices: S.Z., M.M., Y.L., Y.B., Z.Z., Y.Z.; Concept: S.Z., M.M., Y.L.; Design: S.Z., M.M., Y.L.; Data Collection or Processing: Y.B., Z.Z., Y.Z.; Analysis or Interpretation: Y.B., Z.Z., Y.Z.; Literature Search: Y.B., Z.Z., Y.Z.; Writing: Y.B., Z.Z., Y.Z.

Conflict of Interest: No conflict of interest was declared by the authors.

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LETTER TO THE EDITOR

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Successful Management of Ibrutinib-Induced Thrombocytopenia in a Patient with Chronic Lymphocytic Leukemia: No Interruption, Only Reduction

Kronik Lenfositik Lösemili Bir Hastada İbrutinibe Bağlı Trombositopeninin Başarılı Yönetimi: İlacı Kesme, Doz Azalt

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To The Editor,

Ibrutinib, an irreversible inhibitor of Bruton's tyrosine kinase, was approved for treating chronic lymphocytic leukemia (CLL). Its utilization is associated with an increased risk of transient thrombocytopenia [1]. Studies have reported that grade 3 to 4 thrombocytopenia induced by ibrutinib occurs in 2% to 17% of patients undergoing treatment [2].

Lipsky et al. [3] found that a significant number of patients exhibited a slight decline in platelet (PLT) counts by day 2 and notable elevation in PLT counts several days later with ibrutinib. In real-world clinical settings, the administration of ibrutinib has demonstrated an improvement in PLT counts among CLL patients with pre-existing thrombocytopenia [2,4].

Herein we present a patient with CLL who experienced grade 4 thrombocytopenia with ibrutinib treatment and our management of the case by decreasing the dose of ibrutinib.

An 85-year-old man showed lymphocytosis compatible with CLL in flow cytometric analysis ($CD5^+$, $CD19^+$, $CD20^+$, $CD23^+$) in 2015. He was reassessed in October 2021 when B symptoms appeared while being followed without treatment. There was diffuse lymphadenomegaly; however, the liver and spleen sizes were normal. In laboratory tests, the hemoglobin level was 10.4 g/dL; leukocyte, lymphocyte, and PLT counts were $102.8x10^9$/L, $87x10^9$/L, and $72x10^9$/L, respectively. Lactate dehydrogenase, C-reactive protein, and hematinic parameters were found to be within normal reference ranges. Peripheral blood fluorescence in situ hybridization examination was negative for del 17p and trisomy 12, 44% positive for del 11q, and 24% positive for del13q. On October 13, 2021, ibrutinib (140 mg/day) treatment was started. To manage the side effects, it was planned to increase the dose to a daily dose of 420 mg over time.

On the 9^{th} and 19^{th} days of treatment, the PLT count had decreased to $48x10^9$/L and $16x10^9$/L, respectively, as confirmed by peripheral blood smear. On the 19^{th} day, ibrutinib was not interrupted and the dose was revised to 140 mg every other day. Supportive therapy was not given because of the lack of symptoms. One week after the dose revision, the PLT count increased to over $50x10^9$/L and remained within the range of $50\text{-}100x10^9$/L for 10 weeks. By January 2022, it surpassed $100x10^9$/L, leading to an elevation of the ibrutinib dose to 140 mg/day. After 4 months, with the PLT count exceeding $150x10^9$/L, the dose was further escalated to 280 mg/day.

Figure 1 illustrates how the patient's lymphocyte and PLT counts changed throughout the course of treatment. At the time of writing, in August 2023, the PLT count was $146x10^9$/L and the lymphocyte count was $3.9x10^9$/L under ibrutinib administration of 280 mg/day.

Typically, ibrutinib-related hematotoxicity manifests within the initial months of therapy, but its impact tends to diminish over time [4,5]. Although dose reduction has been implemented in response to hematological toxicities, there is currently no conclusive evidence regarding the effectiveness of this strategy [6].

The reason behind the temporary decrease in PLT counts observed in patients undergoing ibrutinib treatment is still not fully understood. It appears to primarily result from the inhibition of early-stage megakaryopoiesis. Further research is required to investigate the factors contributing to the PLT recovery observed in response to ibrutinib [7].

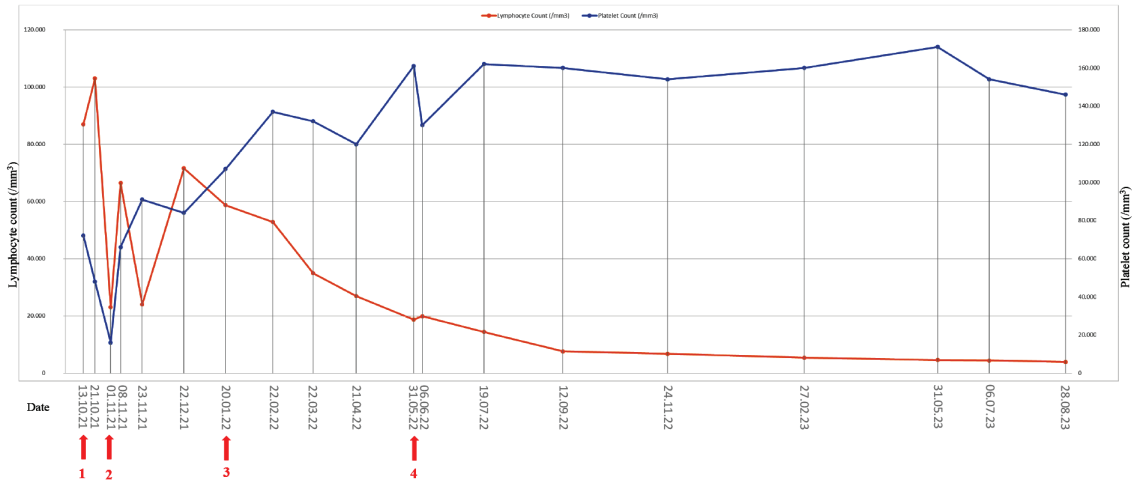

Figure 1. Changes in platelet and lymphocyte counts with ibrutinib therapy. Point 1: Ibrutinib therapy was initiated at 140 mg/day. Point 2: The dose was reduced to 140 mg every other day. Point 3: The dose was increased again to 140 mg/day. Point 4: The dose was increased to 280 mg/day.

Keywords: B-cell neoplasms, Chronic lymphocytic leukemia, Ibrutinib, Megakaryocytes, Thrombocytopenia

Anahtar Sözcükler: B-hücreli neoplaziler, Kronik lenfositik lösemi, İbrutinib, Megakaryositler, Trombositopeni

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

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LETTER TO THE EDITOR

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Ibrutinib-Associated Leukocytoclastic Vasculitis in a Patient with Chronic Lymphocytic Leukemia

Kronik Lenfositik Lösemi Tanılı Hastada Gelişen İbrutinib ile İlişkili Lökositoklastik Vaskülit

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To the Editor,

Ibrutinib is a Bruton's tyrosine kinase inhibitor approved for treatment of chronic lymphocytic leukemia (CLL). Leukocytoclastic vasculitis is one of the cutaneous adverse events seen with ibrutinib. Here we report a patient with CLL who developed multiple skin lesions 13 days after the initiation of ibrutinib.

A 63-year-old man, who was diagnosed with CLL in 2012 and treated with fludarabine, cyclophosphamide, and rituximab in 2014, presented with rapid doubling time of absolute lymphocyte count and fatigue after 8 years of observation. He was also diagnosed with prostatic adenocarcinoma and goserelin was administered 1 month ago. He was started on combination therapy with ibrutinib at 420 mg/day and allopurinol at 150 mg/day. On the 13th day after initiation of ibrutinib and allopurinol therapy, the patient developed a painless, non-pruritic, violaceous rash on his extremities and entire trunk. Physical examination revealed multiple violaceous lesions that did not fade with pressing. Treatment with allopurinol and ibrutinib was paused and a skin biopsy performed. An oral corticosteroid was started and then tapered off. The skin biopsy revealed fibrinoid necrosis of the vessel walls in the dermis and leukocytoclasis around those vessels, and the patient was diagnosed with leukocytoclastic vasculitis (Figure 1).

After his rash, which was thought to be due to the allopurinol, was completely resolved, ibrutinib re-challenge was attempted with a dose of 140 mg once daily. However, the rash developed again and resolved after ibrutinib administration was discontinued.

Leukocytoclastic vasculitis is a very rare side effect of ibrutinib treatment. We initially blamed allopurinol for the patient's rash. However, when treatment with ibrutinib alone was re-started, the rash appeared again. Thus, we concluded that the rash was not associated with allopurinol treatment and ibrutinib treatment was stopped.

The most common cutaneous adverse events with ibrutinib treatment are bruising (12%-51%), rash (12%-29%), petechiae (11%-16%), and skin infections (14%-16%). The possible mechanism of ibrutinib-associated skin toxicity is thought to be related to epidermal growth factor inhibition [1,2,3]. In a previous study, two types of skin reactions were described: palpable purpuric, pruritic rashes and non-palpable, petechial eruptions [2]. In our case, the skin rash was painless, non-pruritic, and purpuric with centripetal spread, which differs from the two aforementioned distinct types. Similarly to cases

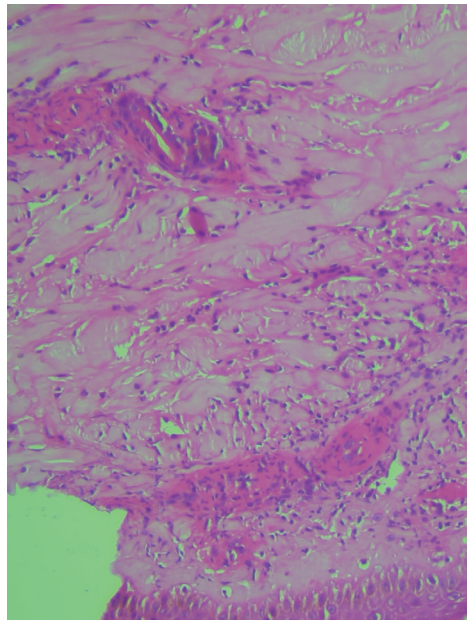

Figure 1. Skin biopsy revealed fibrinoid necrosis of the vessel walls in the dermis and leukocytoclasis around those vessels.

described in the literature, our patient was diagnosed with leukocytoclastic vasculitis by skin biopsy.

In a previous study, leukocytoclastic vasculitis was seen in three of 25 patients. One of those three cases was followed as CLL and the time of onset of the rash was 260 days [4]. In two other cases of patients with CLL treated with ibrutinib, leukocytoclastic vasculitis developed [4,5].

In conclusion, skin side effects are common with ibrutinib treatment, but leukocytoclastic vasculitis is a serious complication of treatment and may require ibrutinib discontinuation. In our case and some of the cases in the literature, ibrutinib treatment had to be discontinued. We wanted to draw attention to leukocytoclastic vasculitis, a serious complication of ibrutinib.

Keywords: Ibrutinib, Leukocytoclastic vasculitis, Chronic lymphocytic leukemia, Ibrutinib-associated vasculitis

Anahtar Sözcükler: İbrutinib, Lökositoklastik vaskülit, Kronik lenfositik lösemi, İbrutinib ilişkili vaskülit

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LETTER TO THE EDITOR

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Nilotinib-Associated Multiple Silent Arterial Stenoses in a Patient with Chronic Myeloid Leukemia

Kronik Miyeloid Lösemi Tanılı Hastada Nilotinib ile İlişkili Çoklu Sessiz Arteriyel Darlık

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To the Editor,

Nilotinib, a second-generation tyrosine kinase inhibitor (TKI), is employed in the treatment of chronic myeloid leukemia (CML) [1]. However, previous studies have indicated an association between nilotinib and vascular adverse events, including peripheral arterial occlusive disease, cerebrovascular disease, and coronary artery disease [2,3]. We report a case of significant bilateral carotid artery stenoses without any neurologic symptoms and, at the same time, left subclavian and celiac artery stenoses without any atherosclerotic or cardiovascular risk factors except age in a patient treated with nilotinib.

A 70-year-old non-smoking, non-diabetic Caucasian female with no history of vascular disease was diagnosed with CML in 2004. The patient was initially treated with imatinib at 400 mg/day, but due to loss of cytogenetic response, it was switched to dasatinib in the 6th year. The patient then experienced complications with recurrent pleural effusion despite appropriate management (dose reduction and diuretics along with steroid interventions) and therapy was changed to nilotinib at 2x400 mg/day in 2016. The nilotinib dose was reduced to 2x300 mg due to bicytopenia a few weeks after initiation. Although major molecular response was not achieved, a cytogenetic remission was obtained at that dose and the patient tolerated it well. Serum hemoglobin A1c (5.3%) and low-density lipoprotein cholesterol (92 mg/dL) were within the normal ranges during follow-up. In the 3rd year of nilotinib therapy, an inter-arm blood pressure difference (150/90 mmHg for the right arm and 110/70 mmHg for the left arm) was detected in a routine visit. A computed tomography angiogram (CTA) showed significant stenosis in the left subclavian artery, total occlusion in the celiac artery, and severe stenosis in the right internal carotid artery (ICA) (North American Symptomatic Carotid Endarterectomy Trial [NASCET] criterion: more than 50% stenosis), stenosis in the left vertebral artery orifice (NASCET criterion: more than 50% stenosis) with fibrofatty plaque in February 2019. Endovascular stent placements were performed in the celiac and left subclavian arteries. Dual antiplatelet therapy (aspirin at 100 mg/day and clopidogrel at 75 mg/day) was initiated. Diffusion magnetic resonance imaging of the brain showed no ischemic pathology. The administration of nilotinib was continued to control the CML. One year later, a subsequent CTA showed progressive stenosis of the right ICA (NASCET criterion: 70%) and new stenosis of the left proximal ICA (NASCET criterion: less than 50%) within the fibrofatty plaque (Figure 1). Although she still had no neurologic symptoms, nilotinib was replaced with bosutinib to reduce the risk of vascular disease progression. Bosutinib was started at a dose of 200 mg and increased to 500 mg/day within weeks for better tolerability. After a few months, the patient left our follow-up and has not been admitted to our center again. Therefore, further information about the course of arterial stenosis after the drug change cannot be provided.

According to long-term evidence, arterial occlusive diseases are more strongly associated with nilotinib than other TKIs [2]. In most reported cases, the patients have baseline vascular risk factors such as hypertension, coronary artery disease, smoking, diabetes mellitus, or dyslipidemia. They also present with stroke, transient ischemic attack, or myocardial infarction at dramatically higher rates [4,5,6]. In our case, significant bilateral ICA stenoses developed asymptomatically without major vascular risk factors other than age. To the best of our knowledge, only one previous report described bilateral severe ICA stenoses without neurological complications in a patient with 10 years of nilotinib usage [7]. Our case also indicates that multiple and radiologically severe but asymptomatic arterial stenoses may occur in nilotinib-using CML patients. A study of nilotinib-induced vasculopathy showed that nilotinib has pro-atherogenic and anti-angiogenic effects on endothelial cells by suppressing normal endothelial cell proliferation and migration [8].

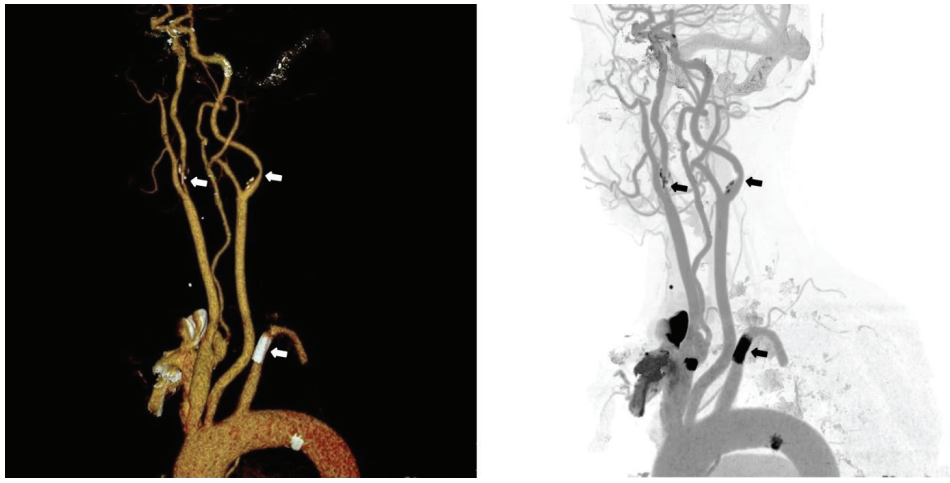

Figure 1. Computed tomography angiography results. Arrows show right and left internal carotid artery stenosis (>50%) and endovascular stent in the left subclavian artery.

These previous reports and our present case show that future studies are needed to investigate the causality between nilotinib and arterial stenotic disease beyond atherogenic pathways. We suggest that patients using nilotinib remain under active clinical surveillance to detect possible arterial stenoses, even if they are asymptomatic. Routine cardiovascular examination and awareness of these complications are of vital importance in detecting nilotinib-associated vascular stenoses.

Keywords: Nilotinib, Chronic myeloid leukemia, Arterial stenosis

Anahtar Sözcükler: Nilotinib, Kronik miyeloid lösemi, Arteriyel darlık

Ethics

Informed Consent: Written informed consent for publication of these details was obtained from the patient.

Authorship Contributions

Surgical and Medical Practices: M.T., R.I., O.E.Ç., İ.C.H.; Concept: M.T., R.I., O.E.Ç., İ.C.H.; Design: M.T., R.I., O.E.Ç., İ.C.H.; Data Collection or Processing: M.T., R.I.; Analysis or Interpretation: R.I., O.E.Ç.; Literature Search: M.T., R.I.; Writing: M.T., R.I.

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LETTER TO THE EDITOR

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Ruxolitinib for the Treatment of Refractory Idiopathic Multicentric Castleman Disease: A Case Report

Refrakter İdiyopatik Multisentrik Castleman Hastalığının Tedavisinde Ruxolitinib: Bir Olgu Sunumu

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To the Editor,

A 46-year-old woman with a 4-month history of severe fatigue was admitted to our hospital in November 2018. Positron emission tomography/computed tomography revealed hepatosplenomegaly and multiple enlarged lymph nodes with slightly elevated ^{18}F-fluorodeoxyglucose uptake. Biopsy of the right axillary lymph node was consistent with the plasma cell subtype of Castleman disease (CD), with immunohistochemistry results revealing positivity for CD20, CD138, CD3, immunoglobulin (Ig) G, kappa, lambda, and Ki-67 (index: 10%). Laboratory tests showed mild anemia (hemoglobin: 113 g/L), elevated platelet count (406x10^9 cells/L), impaired renal function with elevated serum creatinine level (141 µmol/L), elevated C-reactive protein (CRP) (69.20 mg/L), and hypergammaglobulinemia (IgG, 30.48 g/L) without monoclonal gammopathy. The serum albumin level was 36 g/L. Human herpes virus-8 (HHV-8)/HIV test results were negative. According to the Castleman Disease Collaborative Network (CDCN) diagnostic criteria [1], she was diagnosed with non-severe idiopathic multicentric CD-not otherwise specified subtype (iMCD-NOS).

Siltuximab, the only US Food and Drug Administration-approved therapy, was not available in China at the time, and other recommended treatment options for non-severe iMCD, such as tocilizumab and rituximab, were off-label regimens in China and required additional intravenous administration. Therefore, the TCD regimen (thalidomide, cyclophosphamide, and dexamethasone) was initiated as the first-line therapy for this patient [2]. Six cycles were given, but her condition did not improve and was evaluated as stable disease according to the CDCN response criteria [3]. A subsequent BCD regimen (bortezomib, cyclophosphamide, and dexamethasone) [4] was started and partial remission (PR) was achieved after 3 months [3]. However, disease progression occurred soon, and ruxolitinib (10 mg/day) was started as a third-line treatment with a rapid response (Table 1). After 12 months, the patient was free of constitutional symptoms. Physical examination showed no abnormalities in superficial lymph nodes. Hemoglobin, albumin, and CRP values normalized and renal function was improved. PR was achieved again [3]. She has had no flares or adverse events for more than 2 years and is still receiving ruxolitinib treatment.

iMCD represents a group of poorly understood lymphoproliferative disorders [1]. Interleukin-6 (IL-6) is the most important established cytokine in iMCD and the overactivation of IL-6 signaling, probably through Janus kinase (JAK)-signal transduction and transcriptional activator 3 (STAT3), has been considered to be the main pathogenic pathway in at least a portion of iMCD cases [5,6]. IL-6 blocking, although ineffective in more than 50% of patients, is the recommended frontline treatment for iMCD regardless of severity classification [3,7]. Recent serum proteomics found that IL-6-JAK-STAT3 signaling was significantly enriched even in IL-6 blocking non-responders [5], and that result was further supported by lymph node tissue-based immunohistochemistry with significantly increased phosphorylated-STAT3 expression in both non-responders and responders. These results suggest that the JAK-STAT3 pathway may be generally involved in the pathogenesis of iMCD. In addition, links between type I interferon stimulation and mammalian target of rapamycin (mTOR) activation in patients with iMCD and furthermore between IL-6 and mTOR were described, both of which could be eliminated by JAK1/2 inhibition [6].

Therefore, targeting JAK1/2 may be useful in iMCD treatment. Ruxolitinib is a potent and selective JAK1/2 inhibitor approved for the treatment of myelofibrosis [8]. Successful treatment of iMCD-thrombocytopenia, anasarca, fever, reticulin fibrosis, and organomegaly (TAFRO) with ruxolitinib has been reported in some cases [9,10]. However, it is not evident whether these

Table 1. Changes in key features with ruxolitinib treatment.

	Months since initiation of ruxolitinib treatment										
Variables	-3	0	3	6	9	12	15	18	21	24	27
Treatment											
Ruxolitinib, mg/day	-	10	15	15	10	10	10	10	10	10	5
Dexamethasone, mg/week	20	20	10	5	5	-	-	-	-	-	-
Key features											
CRP, mg/L	56.26	59.06	21.34	23.15	14.17	7.9	16.06	10.2	-	7.18	9.08
Hemoglobin, g/L	133	127	124	121	130	142	140	134	139	134	132
Platelet count, $x10^9$/L	268	325	416	476	352	375	269	349	295	349	337
Albumin, g/L	42	41	42	43	43	46	45	46	46	45	44
Creatinine, µmol/L	123	113	120	103	96	108	107	108	106	104	111
IgG, g/L	10.16	11.38	9.83	12.14	11.7	12.95	12.2	13.18	-	13.68	-
IL-6, pg/mL	10.3	10.4	7.9	7.9	4.9	4.3	3.2	2.9	-	3.3	5
TNF-α, pg/mL	15.7	10.9	8.8	9.1	7.9	7.8	11.7	7.1	-	9.5	-

CRP: C-reactive protein; IgG: immunoglobulin G; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha.

findings can be generalized to a larger cohort of patients with iMCD, as patients with iMCD-NOS have different profiles from iMCD-TAFRO [1]. Our case provides original clinical data for JAK1/2 inhibition in iMCD-NOS. Future prospective studies are needed to determine the effectiveness of JAK1/2 inhibitors in the treatment of iMCD.

Keywords: Idiopathic multicentric Castleman disease, Janus kinase inhibitor, Ruxolitinib, Treatment

Anahtar Sözcükler: İdiyopatik multisentrik Castleman hastalığı, Janus kinaz inhibitörü, Ruxolitinib, Tedavi

Ethics

Informed Consent: Written informed consent was obtained from the patient for publication.

Authorship Contributions

Data Collection or Processing: M.H.D., L.Z.; Analysis or Interpretation: J.L., L.Z.; Writing: Y.H.G.

Conflict of Interest: No conflict of interest was declared by the authors.

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LETTER TO THE EDITOR

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Rifampicin-Induced Toxic Hepatitis in a Patient with Hemophilia After Chemical Synovectomy

Bir Hemofili Hastasında Kimyasal Sinovektomi Sonrası Rifampisin ile İlişkili Toksik Hepatit

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To the Editor,

Chemical synovectomy with rifampicin has the potential to be an important option because the radioactive agents required for radioisotope synovectomy are not available in Türkiye [1,2]. For countries where radioisotope synovectomy cannot be performed, chemical synovectomy with intraarticular rifampicin may be an easy, inexpensive, and successful treatment option. It seems to be more effective when used in small joints such as the elbow and ankle rather than the knee joint, which requires large volumes [3].

We planned to administer 250 mg of rifampicin (with 3-5 mL of lidocaine) for the ankle and elbow and 500 mg of rifampicin (with 7-10 mL of lidocaine) for the knee joint intraarticularly at 2-week intervals until treatment response [4]. Treatment response was determined as reduction in joint pain, improvement in joint function, and regression in synovitis. It was evaluated 1 month after the first injection or after at least 2 injections. We included patients whose synovitis was stage I-III and refractory to secondary prophylaxis for at least 6 months and who signed the treatment consent form.

Case: A 41-year-old patient with inhibitor-negative severe hemophilia A presented with recurrent bleeding in the right elbow. The patient had been receiving tertiary prophylaxis for the last 2 years. In the last 1 year, bleeding at the right elbow occurred three times a month (30-35 per year). The patient had a history of radioisotope synovectomy of the right elbow once in 2001. Physical examination of the right elbow revealed 20 degrees of flexion, 30 degrees of extension loss, swelling, and pain. The Hemophilia Joint Health Score (HJHS) 2.1 for a single joint was 9. A score of 60 was determined on a visual analog scale (VAS) for quality of life.

A total of 4 doses of 250 mg of rifampicin were administered. Two days after the last injection, the patient presented with the complaint of jaundice of the eyes. Total bilirubin was 4.9 mg/dL, direct bilirubin was 3.3 mg/dL, aspartate aminotransferase was 158 U/L, alanine transaminase 317 U/L, and alkaline phosphatase was 232 U/L. Abdominal ultrasonography revealed normal results. The patient did not receive any concurrent treatment that could cause hyperbilirubinemia. Although we did not have the opportunity to examine the serum rifampicin level, the case was evaluated as rifampicin-associated toxic hepatitis because we could not detect any cause. Follow-up was planned with a gastroenterological opinion. Liver function test results were followed closely. On the 12th day, the biochemistry values decreased to normal ranges.

In the 3rd month follow-up of this patient, joint movement limitation continued but the pain completely disappeared. There was no bleeding after the last injection. The HJHS 2.1 score for the right elbow was calculated as 7. The VAS score had increased to 90 points. After treatment, decreases in the number of bleedings and the HJHS 2.1 score and an increase in the quality-of-life score were observed.

Despite the regression of the patient's pain and improvement of the target joint with treatment, we thought that the development of toxic hepatitis despite the local use of rifampicin was related to systemic absorption from the intraarticular area. However, the symptoms resolved spontaneously without any other systemic side effects. No similar case report has been found in the literature.

Keywords: Hemophilia, Chemical synovectomy, Rifampicin, Toxic hepatitis

Anahtar Sözcükler: Hemofili, Kimyasal sinovektomi, Rifampisin, Toksik hepatit

Ethics

Informed Consent: The patient signed the treatment consent form.

Authorship Contributions

Surgical and Medical Practices: S.A., E.K.B.; Concept: M.C.U., C.B., K.K.; Design: S.A., K.K.; Data Collection or Processing: M.C.U., E.K.B.; Analysis or Interpretation: M.C.U., C.B., K.K.; Literature Search: M.C.U.; Writing: M.C.U.

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LETTER TO THE EDITOR

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Analysis of Hereditary FXII Deficiency Caused by Three Mutations Including a Novel Mutation

Biri Yeni Olmak Üzere Üç Mutasyon İlişkili Kalıtsal FXII Eksikliğinin Analizi

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To the Editor,

Congenital coagulation factor XII (FXII) deficiency is an autosomal disorder that primarily affects blood clotting. Patients exhibit prolonged activated partial thromboplastin time (aPTT) in vitro, but there is no significant predisposition to bleeding as expected. Most cases are typically identified incidentally during routine health checks or preoperative coagulation screening tests [1,2]. This report describes two Chinese patients with FXII deficiency, neither of whom had significant abnormal bleeding symptoms as expected. Both patients had prolonged aPTT, with FXII:C and FXII:Ag levels reduced to approximately 3% and 3.1 U/dL of normal, respectively. Relatives of the two affected patients had slightly prolonged aPTT, with FXII:Ag levels decreasing to approximately 50 U/dL of the normal range (Table 1). After conducting DNA analysis, it was discovered that Proband A carried homozygous deletion mutation c.303_304delCA (His82Glnfs*36) in exon 5 of the *F12* gene. The frequency of homozygous types ranges from 1 in 500,000 to 1 in 2 billion [3]. Consanguineous unions lead to the expression of traits associated with recessive genes in a homozygous state within a family [4]. The homozygous His82Glnfs*36 mutation in Proband A is likely to have originated from the parents due to their consanguineous marriage. Proband B was found to carry a compound heterozygous mutation consisting of the c.712_713insT (Cys219Leufs*73) mutation in exon 8 and the c.1792_1796delGTCTA (Val579Hisfs*32) deletion mutation in exon 14 (Figure 1).

Biological studies demonstrated that Cys219Leufs*73 and Val579Hisfs*32 were completely conserved in the homologous sequence, and His82Glnfs*36 was found to be highly conserved (Figure 1). MutationTaster predicted all three mutations to be pathogenic.

The His82Glnfs*36 and Cys219Leufs*73 mutations result in a partial truncation of the FXII protein. The Val579Hisfs*32 mutation is situated in the catalytic reaction site. These mutations alter the polarity of the amino acids and hydrogen bonding in this area. Previous in vitro expression studies have demonstrated that mutations at this site result in

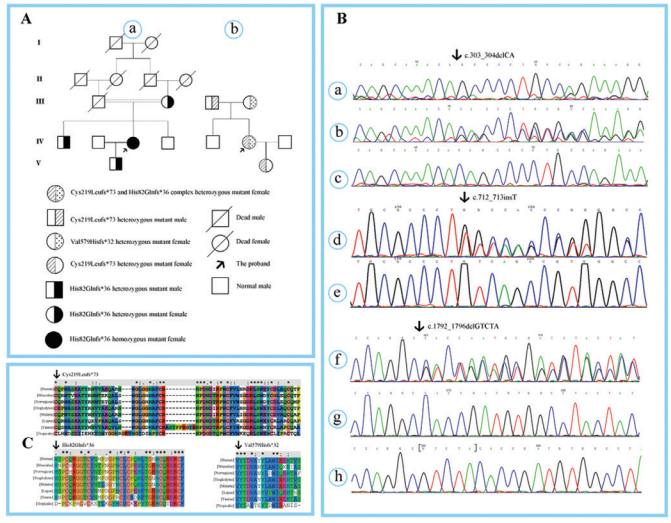

Figure 1. Genetic sequencing and conservation analysis of three FXII mutations. A) Pedigree investigation for two families. B) Chromatogram of DNA sequencing. (a) is a homozygous His82Glnfs*36 sequencing map, (b) is a heterozygous His82Glnfs*36 sequencing map, (c) is a wild-type forward sequencing of His82Glnfs*36, (d) is a heterozygous Cys219Leufs*73 sequencing map, (e) is a Cys219Leufs*73 wild-type forward sequencing, (f) is a heterozygous sequencing map of Val579Hisfs*32, (g) is a clonal sequencing map of Val579Hisfs*32, and (h) is a wild-type forward sequencing of Val579Hisfs*32. The position of the mutational base is indicated with an arrow. C) Conservation analysis of the three mutations. The target amino acids are indicated by arrows.

FXII: Congenital coagulation factor XII.

Table 1. Phenotypes and genotypes of two families with hereditary FXII deficiency.

Family members	PT (s)	aPTT (s)	FXII:C (%)	FXII:Ag(U/dL)	AA substitution	Genotype
Family A						
Mother (III_2)	12.4	46.9	45	45.7	His82Glnfs*36	Heterozygous
Brother (IV_1)	12.8	48.7	42	46.1	His82Glnfs*36	Heterozygous
Husband (IV_2)	13.0	36.5	98	103.2	-	Wild type
Proband (IV_3)	14.1	145	3	3.1	His82Glnfs*36	Homozygous
Brother (IV_4)	13.9	34.7	102	110.1	-	Wild type
Son (V_1)	12.9	49.8	46	49.7	His82Glnfs*36	Heterozygous
Family B						
Father (III_3)	13.4	49.7	45	46.7	Cys219Leufs*73	Heterozygous
Mother (III_4)	13.7	46.5	43	50.7	Val579Hisfs*32	Heterozygous
Brother (IV_5)	12.8	37.6	95	102.1	-	-
Proband (IV_6)	14.1	126.5	3	3.2	Cys219Leufs*73 Val579Hisfs*32	Compound heterozygous
Husband (IV_7)	14.0	40.1	110	108.9	-	-
Daughter (V_2)	13.6	44.9	44	45.8	Cys219Leufs*73	Heterozygous
Normal range	12.6-14.4	29.0-43.0	72-113	72-113	-	-

PT: Prothrombin time; aPTT: activated partial thromboplastin time; AA: amino acid; FXII: congenital coagulation factor XII.

the production and secretion of defective FXII proteins [5]. We hypothesized that the Val579Hisfs*32 mutation would also have deleterious effects. In addition, studies have shown that if the mutation is more than 50-55 nucleotides upstream of the last exon-exon junction after splicing, it can induce nonsense-mediated mRNA degradation [6]. It was hypothesized that the RNA surveillance systems of these two patients would eliminate some of the FXII mRNA from the alleles encoding the mutation.

By 2023, a total of 69 *F12* gene variants were registered in the Human Gene Variation Database (https://www.hgmd.cf.ac.uk/ac/all.php). These variants have fewer than 20 small insertions or deletions. It is important to note that the three mutations presented in this study are deletion/insertion mutations. Furthermore, the Cys219Leufs*73 mutation has never been reported before in the world. These mutations may have caused the FXII defect in the two pedigrees. However, the specific mechanism needs to be confirmed by further in vitro expression experiments.

Acknowledgment

We are grateful to the patients and their family members for their cooperation.

Keywords: Factor XII deficiency, Novel mutation, Genetic mutation

Anahtar Sözcükler: Faktör XII eksikliği, Yeni mutasyon, Genetik mutasyon

Ethics

Informed Consent: Participants carefully read and fully understood the informed consent form for the research. This ensured that participants had a clear understanding of the purpose, procedures, and potential risks and benefits of the study and how personal information would be handled. The rights and privacy of participants were strictly respected.

Authorship Contributions

Surgical and Medical Practices: L.Y.; Concept: Y.X.; Design: L.Y.; Data Collection or Processing: L.Y., M.W.; Analysis or Interpretation: L.Y., M.L.; Literature Search: Li.Y., L.Y.; Writing: L.Y.

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