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Review

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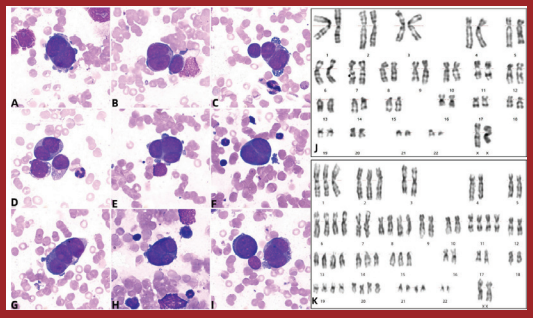

Cover Picture:

Large and Multi-Nuclei Blasts in Acute Myeloid Leukemia with the Hypotriploid Karyotype and *TP53* Mutation with P210 *BCR::ABL1* Transcript

Yihong Huangchuan, Xueyan Chen

2

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Cover Picture

Large and Multi-Nuclei Blasts in Acute Myeloid Leukemia with the Hypotriploid Karyotype and *TP53* Mutation with P210 *BCR::ABL1* Transcript

Bone marrow morphology and chromosome karyotype (A-K): the bone marrow aspirate showed binucleated leukemia cells (A-D) and multinucleated leukemia cells (E-I), with basophilic and vacuolated cytoplasm; Wright-Giemsa staining, 1000x. Eight cells showed translocation between chromosomes 9 and 22, with breakage and rejoining at 9q34 and 22q11.2, 46,XX,t(9;22)(q34;q11.2)[8] (J). The second observed abnormality, hypotriploidy, involves multiple abnormalities in chromosome number. Specifically, there are two groups of t(9;22) present, denoted as 60~65<3n>, XXX,-3,-4,-5,+6,-7,+8,-9, t(9;22)(q34; q11.2)×2,-10,+11,-12,+13,-16,-17,-18,+19,+20,+21,-22[cp5] (K). A change in BCR-ABL1 % was achieved during treatment (L).

International scientific journal published quarterly.

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AIMS AND SCOPE

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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.

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CONTENTS

Turkish Journal of Hematology
The Official Journal of the Turkish Society of Hematology

CONTENTS

Letters to the Editor

REVIEW

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The Advancing Landscape of Paroxysmal Nocturnal Hemoglobinuria Treatment

Paroksismal Noktürnal Hemoglobinüri Tedavisinde Gelişen Manzara

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Abstract

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare chronic bone marrow failure condition characterized by complement-mediated hemolytic anemia and thrombosis. While its initial clinical description occurred in 1882, somatic mutations in *PIGA* were discovered in the 1990s. With an improved understanding of PNH biology, a focused effort on complement inhibitors led to the discovery of eculizumab, a C5 inhibitor initially approved by the US Food and Drug Administration in 2007. Terminal complement pathway inhibition reduced intravascular hemolysis, anemia, and thrombosis. Further advancements in drug development for PNH have included improved pharmacokinetics with ravulizumab in 2018 and the introduction of proximal complement inhibitors such as pegcetacoplan (2021), iptacopan (2023), danicopan (2024), and crovalimab (2024) to enhance patient outcomes. With these new proximal and distal complement inhibitors in the treatment landscape, it is timely for clinicians to review the evolving landscape of PNH treatments and patient selection.

Keywords: Paroxysmal nocturnal hemoglobinuria, Aplastic anemia, Novel complement inhibitors

Öz

Paroksismal nokturnal hemoglobinüri (PNH), kompleman aracılı hemolitik anemi ve tromboz ile karakterize nadir görülen kronik bir kemik iliği yetmezliği durumudur. İlk klinik tanımlaması 1882 yılında yapılmış olsa da, *PIG*'deki somatik mutasyonlar 1990'larda keşfedilmiştir. PNH biyolojisinin daha iyi anlaşılmasıyla, kompleman inhibitörlerine odaklanan çalışmalar, ilk olarak 2007 yılında ABD Gıda ve İlaç Dairesi tarafından onaylanan bir C5 inhibitörü olan ekulizumabın keşfedilmesine yol açmıştır. Terminal kompleman yolu inhibisyonu intravasküler hemolizi, anemiyi ve trombozu azaltmıştır. PNH için ilaç geliştirmedeki diğer ilerlemeler 2018 yılında ravulizumab ile farmakokinetiğin iyileştirilmesi ve hasta sonuçlarını iyileştirmek için pegcetacoplan (2021), iptacopan (2023), danicopan (2024) ve crovalimab (2024) gibi proksimal kompleman inhibitörlerinin piyasaya sürülmesi yer almaktadır. Tedavi sahnesindeki bu yeni proksimal ve distal kompleman inhibitörleri ile klinisyenlerin PNH tedavilerinin ve hasta seçiminin gelişen ortamını gözden geçirmelerinin zamanıdır.

Anahtar Sözcükler: Paroksismal noktürnal hemoglobinüri, Aplastik anemi, Yeni kompleman inhibitörleri

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH), initially described in 1882 by Strübing [1], is a rare clonal hematopoietic stem cell (HSC) disorder. It is characterized by chronic hemolytic anemia due to complement-mediated red cell lysis, thrombosis, smooth muscle dystonia, and bone marrow failure [2,3]. Until 2007, treatment for PNH was largely supportive, with stem cell transplantation being the only definitive therapy. The complement inhibitor eculizumab improved the prognosis for individuals with PNH by inhibiting C5-mediated intravascular hemolysis [4]. Eculizumab therapy greatly reduces mortality, improves quality of life, decreases fatigue and thrombotic risk, and reduces transfusion dependence and other anemia-associated symptoms [4,5,6]. However, a significant proportion of patients with PNH continue to experience breakthrough hemolysis (BTH) and transfusion dependence due to C3-mediated extravascular hemolysis (EVH) despite eculizumab therapy [7,8,9]. The development of novel complement inhibitors has changed the treatment paradigm for patients with PNH suffering from anemia owing to extravascular as well as BTH. These novel agents and the evolving PNH treatment landscape are discussed in this review.

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Pathophysiology

PNH arises from an acquired mutation in the *PIGA* gene, which plays a critical role in the biosynthesis of glycosylphosphatidylinositol (GPI) anchors [10,11,12]. These anchors are essential for attaching various surface proteins, including CD55 (a decay-accelerating factor) and CD59 (the membrane inhibitor of reactive lysis), which regulate complement activation [13,14]. *PIGA*-mutated HSCs produce erythrocytes deficient in CD55 and CD59, leaving them vulnerable to complement-mediated lysis [12]. This loss of complement regulation underlies hemolysis in PNH [11].

PIGA-mutated cells may be present in low and clinically insignificant amounts. Evolution to PNH requires clonal expansion of the *PIGA*-mutated population and frequently occurs in the setting of aplastic anemia (AA). The mechanism of clonal expansion is believed to be due to a survival advantage of *PIGA*-mutated cells compared to the normal HSC population in the setting of AA [15,16]. AA and PNH are closely related disorders due to their shared immune-mediated pathogeneses. While 40% of AA patients have detectable PNH clones, 10%-30% of all AA patients will eventually develop hemolytic PNH [17]. Because PNH is a bone marrow failure disorder linked to T-cell-mediated autoimmunity, it is also associated with an increased risk of secondary myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). Approximately 2% to 6% of PNH patients develop secondary MDS/AML within 10 years, while the prevalence is 9% in PNH patients with AA [17]. Factors associated with progression from PNH to secondary MDS/AML include longer duration of disease, increased telomere attrition, presence of adverse prognostic mutations, and multiple mutations [17,18].

Thrombosis is the most common cause of morbidity and mortality in patients with PNH and is responsible for approximately 40% to 67% of deaths [19]. The mechanism of thrombosis in PNH has not been entirely elucidated but it is attributed to a combination of factors [20,21]. Notably, thrombosis in PNH is more common in patients with large PNH clones (>50% PNH granulocytes) [22]. PNH is associated with both venous and arterial thrombosis, but venous sites are most common [19]. Thrombosis may occur in typical sites such as the limbs and lungs as well as in more unusual sites such as the sagittal and cavernous sinuses, mesenteric veins, and the hepatic vein (Budd-Chiari syndrome). Budd-Chiari syndrome is the most common thrombotic event in patients with PNH [19,20,23].

Management of Paroxysmal Nocturnal Hemoglobinuria

In general, therapy should be initiated for patients exhibiting symptoms of significant hemolysis, such as severe anemia, thrombosis, pain paroxysms, debilitating fatigue, and worsening renal insufficiency [15,16]. The size of the PNH clonal population is another factor to consider in initiating treatment. Patients with small clonal populations (usually <30%) often lack clinical hemolysis and PNH-associated symptoms; therefore, they do not benefit from complement inhibitors. In contrast, those with large PNH clonal populations (usually >50%) do benefit from complement inhibitors. For symptomatic patients with moderate PNH clonal populations (30%-50%), treatment should be based on symptom severity. Providers should assess each case individually, as some patients may still benefit from complement inhibitor therapy [24].

The goals of PNH treatment are to limit complement-mediated hemolysis and subsequent complications and ultimately improve survival patient survival. The efficacy of PNH treatment can be measured by several key endpoints. The degree of hemolysis is estimated using lactate dehydrogenase (LDH) levels, reticulocyte counts, and hemoglobin levels. Other disease- and treatment-related endpoints include thrombotic events, BTH, fatigue as measured by the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) scale, and transfusion dependency. To understand the need for novel therapeutics and their clinical application, we discuss the BTH and EVH seen in PNH in the following subsections, along with the differences from intravascular hemolysis.

Breakthrough Hemolysis

Two humanized monoclonal antibodies (mAbs), eculizumab and ravulizumab, targeting C5 have been approved by the US Food and Drug Administration (FDA) for use in patients with PNH. BTH usually occurs while a patient is on complement inhibitor therapy. BTH can be attributed to suboptimal C5 inhibition and may be pharmacokinetic (i.e., suboptimal C5 inhibition/dosing or missed treatments). Serum drug trough levels may become too low, particularly with eculizumab in the last few days of each biweekly cycle. Pharmacodynamic BTH is likely to be due to conditions that lead to complement activation, such as infections. When the C3b density on the erythrocyte surface is too high, high-affinity C5 convertases can cleave C5 even when bound to a C5 inhibitor or can cause conformational changes to C5, triggering hemolysis [7,8,9].

Extravascular Hemolysis

While C5 inhibitor therapy can effectively halt complement-mediated intravascular hemolysis in patients with PNH, anemia tends to persist in a subset of patients due to EVH [15,25]. The PNH erythrocytes of patients on therapy accumulate C3 fragments, particularly C3d, on their surfaces, which then serve as opsonins. Opsonized erythrocytes are subsequently recognized and phagocytosed by macrophages. Consequently, EVH occurring in the liver and spleen leads to anemia [15,25]. This can be identified based on a persistently high reticulocyte count with concomitant anemia. Notably, C3 fragments are detected

only on PNH erythrocytes after C5 inhibitor treatment. This can be identified using flow cytometry and/or a Coombs test. Normal erythrocytes are not coated by C3 fragments, even after C5 inhibitor treatment. This is likely due to the deficiency of CD55 in PNH cells, as CD55 is a regulator of the C3 convertase. As C5 inhibitors block the complement pathway at the level of C5, the earlier steps in the pathway continue to occur. The activation, deposition, and proteolytic cleavage of C3 to C3b result in the accumulation of C3 fragments on the surfaces of PNH erythrocytes, creating the conditions necessary for significant EVH [25]. Recognition of this limitation in PNH therapy prompted the development of C3 inhibition therapies aimed at preventing both EVH and intravascular hemolysis. The blockade of C3 abolishes intravascular hemolysis by directly inhibiting C3 and prevents EVH by stopping the accumulation of C3d on the surface of PNH erythrocytes [7].

Current Approved Paroxysmal Nocturnal Hemoglobinuria Therapeutics

Eculizumab

The first-line treatment for PNH has historically been eculizumab, an anti-C5 mAb first approved by the FDA in 2007 [26]. Eculizumab compensates for the lack for CD59 by binding C5 and preventing its cleavage by the C5 convertase (Figure 1). Inhibiting C5 cleavage prevents the formation of the membrane attack complex and limits intravascular hemolysis. However, eculizumab does not compensate for the lack of CD55. As a result, the deposition of C3b opsonin will continue and mild to moderate EVH in the liver and spleen may still occur [22].

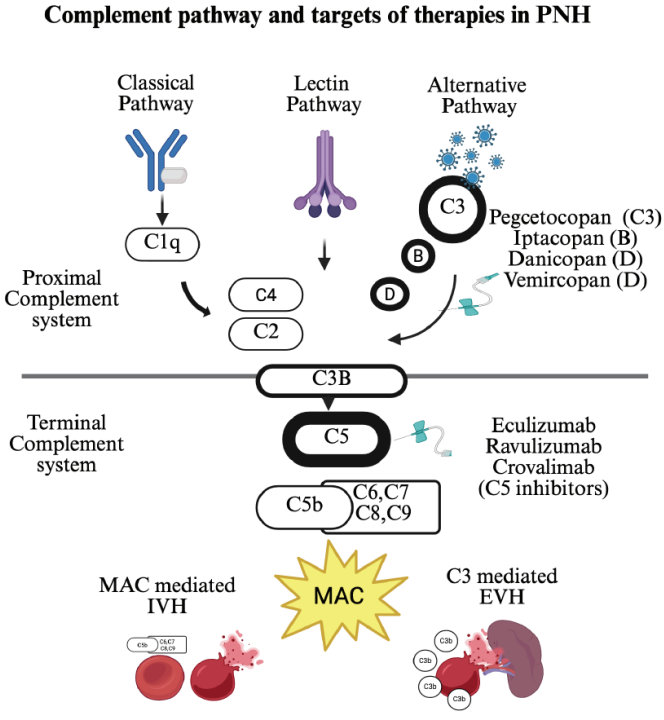

Figure 1. Classical pathway activated by antibodies, lectin pathway activated by ficolins or lectins, and alternative pathway activated by viral or bacterial surfaces.

PNH: Paroxysmal nocturnal hemoglobinuria; MAC: membrane attack complex; IVH: intravascular hemolysis; EVH: extravascular hemolysis.

Eculizumab significantly reduces hemolysis, as demonstrated by marked declines in LDH in all patients to near normal levels after 1 week [5]. Administration of the FACIT-Fatigue scale showed a mean improvement of 12.2 points during the course of a multicenter phase 3 study (scale scores range from 0 to 52, with higher scores indicating improvement) [5]. Additionally, 51% of participants remained transfusion-independent during the course of the study. The average number of red blood cell units required by participants dropped from 12.3 units per patient in the preceding year to 5.9 during the eculizumab trial [5].

During the aforementioned trial, two patients experienced a thrombotic event, including one case of deep vein thrombosis and one of pulmonary embolism. The most common side effect reported was headache (96.4%). Headache is likely associated with the acute increase in nitric oxide levels shortly after beginning eculizumab. Headache is rarely reported after the first few doses [5]. The reduction of thrombotic events results in dramatically improved survival in patients receiving eculizumab therapy. A follow-up retrospective study examined a PNH cohort taking eculizumab between 2002 and 2010. The 5-year survival rate on eculizumab was 95.5%, with no treatment-related deaths, compared to 66.8% in the pre-eculizumab control group. Before initiating eculizumab, there were 34 thrombotic events in 21 of the patients. After eculizumab, there were only 2 thrombotic events. The rate of thrombosis thus decreased from 5.6 to 0.8 thrombotic events per 100 patient years on eculizumab [27]. Furthermore, 21 patients who were previously on prophylactic anticoagulation were able to discontinue therapy without experiencing any thrombotic events after cessation [27].

Ravulizumab

The next advancement in C5 inhibition occurred in 2018 with the approval of ravulizumab, a mAb C5 inhibitor with higher binding affinity for C5 than eculizumab (Figure 1) [28]. Ravulizumab requires dosing every 8 weeks, whereas eculizumab requires biweekly administration (Table 1) [28]. This extended dosing interval is achieved through enhanced endosomal recycling of the mAb. The ravulizumab 301 phase 3 trial compared ravulizumab and eculizumab in complement inhibitor-naïve patients with PNH. Ravulizumab demonstrated non-inferiority for both primary endpoints of the study: transfusion avoidance and hemolysis as measured by LDH. Among patients receiving ravulizumab, 73.6% avoided transfusion compared to 66.1% in the eculizumab group [29]. LDH normalization was observed in 53.6% of patients on ravulizumab compared to 49.4% on eculizumab. BTH occurred at lower rates in the ravulizumab group (4.0%) compared to the eculizumab group (10.7%) [29].

Table 1. FDA-approved treatments for PNH: approval year, mechanism of action, and dosing.

Drug (brand name)	Approval year	Mechanism of action	Loading dose	Maintenance dose	Route	Dosing frequency
Eculizumab (Soliris)	2007	C5 inhibitor	600 mg IV weekly for 4 weeks	900 mg	IV	Q2W
Ravulizumab (Ultomiris)	2018	C5 inhibitor	Weight-based IV dose (2400-3000 mg)	3000-3600 mg	IV	Q8W
Pegcetacoplan (Empaveli)	2021	C3 inhibitor	None	1080 mg	SUBQ	BIW
Iptacopan (Fabhalta)	2023	Factor B inhibitor	None	200 mg	Oral	BID
Danicopan (Voydeya)	2024	Factor D inhibitor	None	150 mg	Oral	TID
Crovalimab (Piasky)	2024	C5 inhibitor	1000 mg IV on day 1, then 4 weekly SUBQ doses of 340 mg	680 mg	SUBQ	Q4W

FDA: US Food and Drug Administration; PNH: paroxysmal nocturnal hemoglobinuria; IV: intravenous; SUBQ: subcutaneous; Q2W: once every 2 weeks; Q8W: once every 8 weeks; BIW: twice every week; BID: twice every day; TID: three times every day; Q4W: once every 4 weeks.

The ravulizumab 302 phase 3 trial evaluated the efficacy of ravulizumab versus eculizumab in patients previously treated with eculizumab. Ravulizumab demonstrated non-inferiority for the study's primary endpoint: change in LDH from baseline. The ravulizumab group experienced a 0.82% reduction in LDH from baseline while the eculizumab group showed an 8.39% increase from baseline [30]. No patients in the ravulizumab group experienced BTH, whereas 5 patients (5.1%) in the eculizumab group did. The mean number of red blood cell units transfused was comparable between the groups, with ravulizumab patients receiving an average of 4.3 units [30]. FACIT-Fatigue scores were also comparable between the groups, with ravulizumab achieving a 2.01-point improvement from baseline and eculizumab showing a 0.54-point improvement [30]. No patients in either group experienced a major adverse vascular event. Headache was the most frequently reported adverse event, occurring in 26.8% of the ravulizumab group and 17.3% of the eculizumab group [29,30].

Pegcetacoplan

Pegcetacoplan, a peptide-based inhibitor of C3 designed to control both EVH and intravascular hemolysis, received FDA approval in 2021 for treatment of PNH (Figure 1) [31]. A phase 3 trial evaluated the efficacy and safety of pegcetacoplan monotherapy versus eculizumab in patients with PNH with hemolysis poorly controlled by eculizumab (hemoglobin of 10.5 g/dL). Pegcetacoplan demonstrated superiority to eculizumab in limiting hemolysis. The mean hemoglobin change from baseline in the pegcetacoplan group was an increase of 2.37 g/dL, whereas the eculizumab group experienced a mean decrease of 1.47 g/dL (Table 2). The mean hemoglobin difference between treatments was thus 2.69 g/dL in favor of pegcetacoplan [32]. A total of 85% of patients in the pegcetacoplan group avoided transfusion, compared to only 15% in the eculizumab group [32]. FACIT-Fatigue scores increased by an average of 9.2 points in the pegcetacoplan group and decreased by 2.7 points in the eculizumab group [32]. BTH occurred in 10% of patients receiving pegcetacoplan and 23% of those receiving eculizumab [32]. The most common adverse event in both groups was infusion site reaction. Headache was reported in 7% of those taking pegcetacoplan compared to 23% in the eculizumab group [32].

Iptacopan

Iptacopan was approved in 2023 as the first oral monotherapy for the treatment of PNH in adults (Table 1) [33]. Iptacopan is a selective inhibitor of factor B. Inhibiting factor B prevents the formation of the C3 convertase, thereby limiting complement pathway amplification and significantly reducing hemolysis (Figure 1). Adding iptacopan to treatment led to significant reduction in LDH levels, from 539 IU/L at baseline to 235 IU/L at week 13 [34]. Significant improvements were also observed in other hematological markers and no serious adverse events were reported [34]. A follow-up 12-week phase 2 trial evaluated iptacopan monotherapy in patients with PNH who had not received complement inhibition therapy in the previous 3 months and had a baseline hemoglobin level of 8.58 g/dL [35]. At week 12, LDH levels had decreased by ≥60% from baseline. Hemoglobin levels improved from 8.85 to 11.52 g/dL in cohort 1 and from 7.69 to 10.9 g/dL in cohort 2 (Table 2) [35]. Five patients achieved hemoglobin levels of >12.0 g/dL and an overall upward trend persisted beyond the 12-week study period [35]. All but one patient remained transfusion-independent during the trial. The patient requiring transfusion had preexisting MDS and was enrolled in the trial despite significant reticulocytopenia. No serious adverse events were reported. The most commonly reported adverse event was headache, affecting 4 out of 13 participants [35].

Danicopan

Danicopan is an oral small-molecule inhibitor of factor D approved for PNH treatment in 2024 [36]. Factor D is a serine protease that catalyzes the cleavage of factor B into its active components. Inhibiting factor D prevents the formation of active C3 convertase, thereby blocking amplification of the complement pathway (Figure 1). Factor D is an attractive drug target because it catalyzes a rate-limiting step in complement

Table 2. Summary of clinical trials on PNH treatment.

Drug	Trial name	Cohort	Therapy approach	BTH rate	Thrombotic events	FACIT-Fatigue improvement	Hgb increase (g/dL)
Eculizumab	TRIUMPH	PNH with transfusion dependency	Monotherapy	10.8%	0	6.4	1.4
Ravulizumab	CHAMPION-301	Treatment-naïve	Monotherapy	4.0%	2/125*	7.07	Not reported§
	CHAMPION-302	Eculizumab with persistent anemia	Monotherapy	0.0%	0	2.1	Not reported§
Pegcetacoplan	PEGASUS	C5 inhibitor with persistent anemia	Monotherapy	10.0%	0	9.2	2.37
	PRINCE	Treatment-naïve	Monotherapy	0.0%	0	7.8	2.9
Iptacopan	APPLY	C5 inhibitor with persistent anemia	Monotherapy	3.2%	1/62†	8.0	3.59
	APPOINT	Treatment-naïve	Monotherapy	0.0%	0	Not reported	3.87
Danicopan	ALPHA	C5 inhibitor with persistent anemia	Add-on	8.3%	0	7.9	2.94
Crovalimab	COMMODORE 2	Treatment-naïve	Monotherapy	10.4%	0	7.8	2.2

*: One patient who was using an oral contraceptive developed deep vein thrombosis and one patient discontinued anticoagulation at the initiation of the trial.
†: Transient ischemic attack considered unrelated to iptacopan; iptacopan treatment was ongoing.
§: The ravulizumab trials reported a hemoglobin stabilization rate (avoidance of a ≥2 g/dL decrease in hemoglobin from baseline) and the ravulizumab rate was non-inferior to eculizumab in both trials.
PNH: Paroxysmal nocturnal hemoglobinuria; BTH: breakthrough hemolysis; FACIT-Fatigue: Functional Assessment of Chronic Illness Therapy-Fatigue scale.

amplification and is present at the lowest concentration of any complement protein [37]. In the phase 3 ALPHA clinical trial, danicopan was evaluated as an add-on treatment in patients with clinically significant EVH despite treatment with a C5 inhibitor. A total of 42 participants received danicopan at 150 mg three times daily, while the placebo group continued to receive eculizumab or ravulizumab monotherapy. Danicopan treatment resulted in a clinically significant mean increase in hemoglobin concentration of 2.94 g/dL after 12 weeks (Table 2) [38]. Danicopan in combination with ravulizumab or eculizumab achieved an increase of >2 g/dL in hemoglobin in 60% of patients and transfusion avoidance in 83% of patients [38]. The placebo group experienced a mean increase of 0.5 g/dL in hemoglobin and no members of the placebo group had a hemoglobin increase of >2.0 g/dL [38]. FACIT-Fatigue scores had improved by a mean of 8 points in the danicopan group at 12 weeks compared to 2.6 points in the placebo group [38]. No thromboembolic or other significant adverse events were reported. These findings suggest that danicopan is a promising therapeutic option for patients who continue to have clinically significant EVH despite C5 inhibitor therapy.

Crovalimab

Crovalimab, a humanized anti-complement component C5 (anti-C5) mAb, is the latest development in PNH treatment, having been approved in June 2024 for adult and pediatric patients aged 13 years and older with body weight of at least 40 kg. This approval was based on data from the COMMODORE 2 study (Table 1) [39]. There were 2 primary endpoints of that study: hemolysis control from week 5 to week 25 and transfusion avoidance from baseline to week 25. Key secondary endpoints included BTH from baseline to week 25, stabilized hemoglobin from baseline to week 25, and mean change in fatigue in adult patients from baseline to week 25. Data showed that crovalimab was non-inferior to eculizumab with respect to BTH rates (10.4% vs. 14.5%) and hemoglobin stabilization (63.4% vs. 60.9%) (Table 2) [40]. The advantage of crovalimab is its once-monthly subcutaneous administration [41]. The rates of any-grade treatment-related adverse effects were 33.3% for crovalimab versus 34.8% for eculizumab, while the rates of grade 3-5 adverse effects were 17.8% and 24.6%, respectively [40].

The Role of Allogeneic Hematopoietic Stem Cell Transplantation

While complement inhibitors remain the standard of care for patients with PNH experiencing hemolysis, allogeneic hematopoietic stem cell transplantation (HSCT) may be considered as an alternative for those with refractory disease or severe adverse effects from non-transplant therapies. According to the International Bone Marrow Transplant Registry, the 2-year overall survival (OS) rate was 56% among 48 recipients of human leukocyte antigen (HLA)-identical sibling transplants performed between 1978 and 1995 [42]. A more recent retrospective study evaluated 240 PNH patients who underwent HSCT between 2011 and 2020 across 125 European Society for Blood and Marrow Transplantation transplant centers in the Netherlands and reported a 3-year OS rate of 79%, with infections and graft-versus-host disease (GVHD) identified as

the primary causes of mortality [43]. Survival was associated with both donor type and patient age, and the 3-year OS rates were 86% for HLA-matched sibling donors, 78% for matched unrelated donors, and 62% for mismatched unrelated donors (p=0.003) [43]. Age-stratified OS rates were 83% for patients aged <20 years, 82% for those aged 20-40 years, and 67% for those aged >40 years [43]. Although outcomes following HSCT have improved over the past decade due to advances in donor selection, conditioning regimens, GVHD management, and post-transplant care, HSCT should be reserved for cases where other treatment options are contraindicated or have failed considering the persistent risks of infection and graft failure.

Gene Therapy and Other Biologics

Gene therapy to restore *PIGA* function or block the complement pathway has long been explored as a potential treatment for PNH. In vivo studies have demonstrated that lentiviral vectors can effectively deliver a functional copy of the *PIGA* gene to HSCs, thereby re-establishing the expression of GPI-anchored proteins [44]. HMI-104, an adeno-associated virus-based gene therapy, has shown success in utilizing the hepatic expression of a C5 mAb, resulting in complete inhibition of ex vivo hemolysis [45]. Human studies have yet to be performed, but the one-time application of the therapy and the potential of lifetime cure make such an approach attractive for PNH patients.

Another agent of interest, KP104, is a bifunctional fusion protein that combines an anti-C5 mAB with the regulatory domains of complement factor H, allowing the simultaneous inhibition of the terminal and proximal complement pathways. Results from a phase 2 trial (n=18) demonstrated that KP104 therapy induced rapid and sustained increases in hemoglobin levels, significant reductions in LDH, and complete elimination of transfusion requirements in all treated patients [46].

Patient Selection and Choice of Therapy

Shared decision-making plays a vital role in selecting the most appropriate treatment for PNH. PNH management has advanced significantly as PNH clinicians have widely adopted eculizumab and largely transitioned to ravulizumab due to its more convenient dosing schedule. Now, with subcutaneous injections and oral agents available, PNH management has become increasingly nuanced. While many clinicians await long-term safety data on proximal complement inhibitors, a well-validated quality-of-life tool is needed to determine whether additional proximal inhibition is necessary for patients on C5 inhibitors who do not have severe anemia. At the same time, key considerations when selecting a treatment include the patient's preference for the route of administration, the clinician's assessment of adherence to oral or subcutaneous therapy, and the management strategy for complement activation episodes.

Remaining Challenges in Paroxysmal Nocturnal Hemoglobinuria

With ongoing advances in treatment options, PNH is significantly more manageable today compared to the pre-eculizumab era. However, several challenges remain:

1. Underdiagnosis of PNH remains prevalent due to its rarity.

2. HSCT remains the only curative therapy, and 2% to 6% of patients with PNH develop clonal evolution to MSD or AML [17].

3. Reliable predictive tools to identify clonal evolution do not exist.

4. Assessing residual anemia in patients with PNH is a challenge. The relative contributions of bone marrow failure, clonal evolution, and EVH must be carefully evaluated.

5. BTH remains a concern with novel agents despite its low incidence in trials.

6. Managing hospitalized patients with PNH on oral therapy may be challenging, as most of these medications are non-formulary in hospitals in the United States.

7. The high cost of novel oral agents remains a significant barrier to widespread adoption in the community. Due the expense of proximal complement inhibitors, C5 inhibitors continue to be the first-line therapy in most institutions, with a gradual paradigm shift toward oral agents. Most physicians select a therapy based on extensive discussion with individual patients about their preferences. Long-term safety and real-world evidence will facilitate a shift in the standard of care toward more convenient oral agents.

Conclusion

PNH is a rare clonal HSC disorder characterized by chronic hemolytic anemia, thrombosis, smooth muscle dystonia, and bone marrow failure. HSCT remains the only curative therapy. Over the years, novel complement inhibitors have been developed to improve survival and quality of life, making PNH a more manageable disease. The emergence of multiple novel therapies has made shared decision-making essential, requiring a thorough evaluation of all available treatment options in collaboration with the patient.

Footnotes

Authorship Contributions

Concept: S.T.; Literature Search: C.P., X.V.B.; Writing: C.P., X.V.B., S.T.

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RESEARCH ARTICLE

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The IRF2-INPP4B Pathway Aggravates Acute Myeloid Leukemia

IRF2-INPP4B Yolağı Akut Miyeloid Lösemide Olumsuz Etkilidir

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Abstract

Objective: Interferon-regulatory factor 2 (IRF2) and inositol polyphosphate 4-phosphatase B (INPP4B) are indispensable for differentiating immune T-cells, but the regulatory principle of the IRF2-INPP4B signaling channel in the apoptosis of acute myeloid leukemia (AML) cells remains unclear. This work investigates the function and regulatory principle of IRF2-INPP4B signaling in the progression of AML.

Materials and Methods: $CD4^+$ T-cells were extracted from peripheral blood and characterized via flow cytometry. Flow cytometry was used to estimate apoptosis in the HL60 AML cell line and determine the Th1/Th2 cell ratio. Quantitative real-time polymerase chain reaction was used to measure *IRF2* mRNA. Western blotting was performed to evaluate the protein levels of IRF2, INPP4B, JAK2, p-JAK2, STAT3, p-STAT3, and caspase 3. Interleukin-4 and interferon gamma concentrations were determined using enzyme-linked immunoadsorption assay kits.

Results: We discovered that levels of IRF2 and INPP4B were high in AML-derived $CD4^+$ T-cells. Furthermore, $CD4^+$ T-cells encouraged HL60 cell apoptosis. Downregulation of IRF2 encouraged HL60 cell apoptosis via alterations in the Th1/Th2 ratio while the overexpression of IRF2 stimulated the JAK2-STAT3 signaling channel and downregulated caspase 3.

Conclusion: We revealed that IRF2-INPP4B signaling in $CD4^+$ T-cells stimulated the JAK2-STAT3 signaling channel and downregulated caspase 3, reducing AML cell apoptosis and aggravating AML progression. This work highlights an important regulatory principle concerning AML progression, as the IRF2-INPP4B pathway might impact the JAK2-STAT3 signaling channel. The findings contribute to our knowledge of the complicated interplay of these pathways in AML.

Keywords: Acute myeloid leukemia, Apoptosis, IRF2-INPP4B, JAK2-STAT3 signaling channel

Öz

Amaç: İnterferon düzenleyici faktör 2 (IRF2) ve inositol polifosfat 4-fosfataz B (INPP4B), bağışıklık T-hücrelerinin farklılaşmasında kritik rol oynar. Ancak, IRF2-INPP4B sinyal yolunun akut miyeloid lösemi (AML) hücrelerinin apoptozundaki düzenleyici mekanizması net değildir. Bu çalışma, IRF2-INPP4B sinyalinin AML ilerlemesindeki işlevini ve düzenleyici prensiplerini araştırmaktadır.

Gereç ve Yöntemler: Periferik kandan izole edilen $CD4^+$ T-hücreleri akım sitometri ile tanımlandı. HL60 AML hücre hattında apoptozun hesaplanması ve Th1/Th2 hücre oranı belirlenmesi için akım sitometrisi kullanıldı. *IRF2* mRNA ölçümü kantitatif gerçek zamanlı polimeraz zincir reaksiyonu ile yapıldı. IRF2, INPP4B, JAK2, p-JAK2, STAT3, p-STAT3 ve kaspaz 3 protein düzeyleri, Western blot ile değerlendirildi. İnterlökin-4 ve interferon gama konsantrasyonları ELISA kitleri ile ölçüldü.

Bulgular: AML kaynaklı $CD4^+$ T-hücrelerinde IRF2 ve INPP4B seviyeleri yüksek bulundu. Ayrıca, $CD4^+$ T-hücreleri, HL60 hücre apoptozunu artırdı. IRF2 baskılanması, Th1/Th2 oranını değiştirerek HL60 hücre apoptozunu artırırken IRF2 ifadesindeki artış, JAK2-STAT3 sinyal yolunu aktive etti ve kaspaz 3'ü baskıladı.

Sonuç: IRF2-INPP4B sinyal yolu, CD4+ T-hücrelerinde JAK2-STAT3 yolunu aktive ederek kaspaz 3'ü baskılar ve AML hücre apoptozunu engelleyerek AML ilerlemesini artırır. Bu çalışma, AML ilerlemesinde IRF2-INPP4B yolunun önemini ortaya koymakta ve JAK2-STAT3 sinyalizasyonu ile olan etkileşimini vurgulamaktadır. Bulgular, AML'deki bu karmaşık moleküler mekanizmaların anlaşılmasına katkı sağlamaktadır.

Anahtar Sözcükler: Akut miyeloid lösemi, Apoptoz, IRF2-INPP4B, JAK2-STAT3 sinyal yolu

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Introduction

Acute myeloid leukemia (AML) is a hematological malignancy and the most usual type of acute leukemia in adults [1]. It leads to impaired hematopoietic function and bone marrow decline caused by abnormal proliferation of undifferentiated myeloid precursor cells. The disease progresses rapidly and the rates of complete response and long-term survival are low [2]. Chemotherapy and allogeneic and autologous bone marrow transplantation are currently the three primary therapies for AML [3], but bone marrow transplantation not only requires high costs but also entails the possibility of immune rejection. Furthermore, although 50%-75% of AML patients respond to chemotherapy, there is still a high recurrence rate after the initial cure. The poor prognosis and the high recurrence rate of AML are the primary causes of failed treatments [4]. It is of critical importance to further investigate the principles governing the onset and advance of AML, which would support the designing of innovative and effective therapeutic strategies for its management.

Interferon-regulatory factors (IRFs) belong to the larger category of transcription factors and were originally considered to be transcriptional regulators of the type I interferon system [5]. Research has previously shown that IRFs participate in the differentiating of lymphocytes, cell growth, and apoptosis and play indispensable roles in adaptive immunity and tumorigenesis [6]. Interferon-regulatory factor 2 (IRF2), a member of this family, acts as an antagonist of IRF1 and has been linked to tumor growth [7]. IRF2 can stimulate the malignant proliferation of tumor cells and cause malignant transformation of tumors, and it has been classified as an oncogene in fibroblasts and T lymphocytes [8]. Thus, downregulation of IRF2 could be a potential goal for cancer treatments [9]. Notably, previous works have shown that IRF2 participates in AML progression and may thus be a viable and specific therapeutic factor in treating AML. INPP4B, a newly identified lipid phosphatase, has been ascertained to have indispensable functions in various kinds of cancers [10,11,12]. INPP4B increases proliferation and tumor progression in *PIK3CA*-mutant ER breast cancer cells [11]. Another report suggested that INPP4B-mediated mTORC1 signaling stimulation and cap-dependent translation initiation could facilitate the proliferation of colorectal cancer cells [12]. Additionally, INPP4B, serving as a prognostic and diagnostic marker, regulates the growth of pancreatic cancer cells by triggering AKT [13]. The IRF2-INPP4B signaling channel can promote the progression of AML by encouraging the proliferation and continued survival of AML cells [14], indicating that blocking the IRF2-INPP4B signaling channel might be a vital molecular goal in efforts to treat AML. Researchers have also stated that IRF2 is involved with CD8a dendritic cells via an impact on the differentiation of Th2 cells [15], indicating that IRF2 has a vital function in the differentiating of immune T-cells. Nevertheless, the regulatory principle of the IRF2-INPP4B signaling channel in terms of the apoptosis of AML cells remains unclear.

JAK2, belonging to the protein-tyrosine kinase family, acts as a key regulator in diverse physiological and pathological processes such as cell proliferation and differentiation [16]. Other signaling molecules such as STAT1, STAT3, and STAT5 are also regulated by JAK2. Specifically, the phosphorylation of STAT3 can be stimulated by JAK2, and the JAK2-STAT3 pathway has a key function in human tumorigenesis by modulating cell proliferation, survival, immune response, and differentiation [17]. Researchers have also suggested that the JAK2-STAT3 pathway affects AML progression [18]. It was recently revealed that JARID2, a histone demethylase, has a crucial function in breast cancer growth and advancement by modulating the activities of INPP4B and the JAK2-STAT3 pathway [19]. The question of whether IRF2 and INPP4B take part in processes of AML by modulating the JAK2-STAT3 pathway remains unanswered, however. The present work accordingly aimed to investigate the role of IRF2 and INPP4B in the progression of AML.

Materials and Methods

Reagents

JAK2 inhibitor AG-490 was bought from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). STAT3 inhibitor Stattic was bought from MedChemExpress (Monmouth Junction, NJ, USA).

Clinical Sample Collection and $CD4^+$ T-Cell Isolation

We collected a total of 20 peripheral blood samples at our hospital in 2021 from 10 AML patients and 10 healthy individuals (control group). The diagnostic criteria for AML were based on the guidelines from the 2017 edition of the European LeukemiaNet recommendations for AML in adults [20]. $CD4^+$ T-cells were extracted from peripheral blood via a commercial $CD4^+$ T-cell separation kit (HS-SJ078, Crondabio, Shanghai, China). Briefly, fresh blood samples were collected in collection tubes and peripheral monocytes were subjected to a preparation process based on density gradient centrifugation. Subsequently, magnetic live cell classification and a $CD4^+$ T-cell separation kit were used to separate $CD4^+$ T-cells from peripheral monocytes. The obtained $CD4^+$ T-cells were then maintained in RPMI-1640 medium supplied by Gibco (Rockville, MD, USA), supplemented with 10% fetal bovine serum (FBS), 1 mM L-glutamine, and 55 μM β-mercaptoethanol. Ethical permission for this work was granted by the First Affiliated Hospital of Bengbu Medical College on June 25, 2021, with permit number [2021] No. 205. All experiments were conducted following the institutional ethical standards and all participants granted their written informed consent.

CD4+ T-Cell Identification

Flow cytometry was used to determine the purity of the CD4+ T-cells. Briefly, the CD4+ T-cells ($1x10^6$ cells) were blocked with 2% FBS for 30 min in phosphate-buffered saline (PBS) and then underwent incubation with fluorochrome-conjugated anti-CD4 (ab133616, 1:100, Abcam, Cambridge, UK) for 30 min. The cells were then evaluated with the BD FACSVerse device (BD Biosciences, Franklin Lakes, NJ, USA) and findings were evaluated in more detail using FlowJo software (BD Biosciences).

Cell Culture

HL60 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were maintained in RPMI-1640 medium (Gibco) supplemented with 10% FBS under 5% CO_2 and 95% air at 37 °C.

Treatment and Transfection of Cells

Cells were seeded at $2x10^5$ cells per 6-well plate and permitted to grow to 70%-90% confluency before transfection. The cells were then transfected with 20 μM pcDNA3.1-IRF2 overexpression vectors (Geneseed Biotech, Guangzhou, China) or 20 μM IRF2 siRNA (Genechem, Shanghai, China) using Lipofectamine 2000 Reagent (11668-027, Invitrogen, Carlsbad, CA, USA) following the manufacturer's directions. For transfection, four units (15 μL) of Lipofectamine were diluted in 150 μL of Opti-MEM medium (Gibco). Meanwhile, the specified DNA amount (14 μg) was mixed with 70 μL of Opti-MEM medium. The diluted DNA and Lipofectamine were combined at a 1:1 ratio and incubated at room temperature for 5 min, and the resulting DNA-lipid mixture was gently added to the cells. Culturing continued for 48 h. A negative control (NC) siRNA sequence or pcDNA3.1 plasmid served as the control group. For the CD4+ T-cell co-culture, HL60 cells were maintained in CD4+ T-cell medium with different co-culture conditions. The siRNA sequences targeting IRF2 were: si-IRF2-1, sense: 5′-AGUUAAGCACAUCAAGCAAGA-3′, antisense: 5′-UUGCUUGAUGUGCUUAACUUU-3′; si-IRF2-2, sense: 5′-GGUGAACAUCAUAGUUGUAGG-3′, antisense: 5′-UACAACUAUGAUGUUCACCGU-3′; si-IRF2-3, sense: 5′-GGUCCUGACUUCAACUAUAAA-3′, antisense: 5′-UAUAGUUGAAGUCAGGACCGC-3′.

Flow Cytometry

For HL60 cell apoptosis analysis, the HL60 cells were cultured with CD4+ T-cell medium and then the apoptosis of the HL60 cells was determined by flow cytometry [21]. HL60 cells cultured in RPMI-1640 medium served as the control. Briefly, the HL60 cells were collected and washed three times with PBS and then stained with the FITC Annexin V Apoptosis Detection Kit (HS-SJ069, Crondabio) at 4 °C in darkness according to the manufacturer's directions. Apoptosis was detected using the FACSVerse (BD Biosciences) and the findings were evaluated in detail with FlowJo software (BD Biosciences). The ratio of Th1 cells positive for IFN-γ to Th2 cells positive for IL-4 among the cells was determined by staining with the INF-γ-PerCP-Cy5.5 antibody (BioLegend, San Diego, CA, USA) and PE-IL-4 antibody (BioLegend), respectively.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from the cells using TRIzol reagent (HS-SJ012, Crondabio) following the manufacturer's directions. The cDNA was then obtained via reverse transcription using the PrimeScript RT Master Mix Kit (R223-01, Vazyme, Nanjing, China). Subsequently, quantitative real-time polymerase chain reaction (qRT-PCR) was conducted with the LightCycler 480II instrument (Roche, Basel, Switzerland) using ChamQ Universal SYBR qPCR Master Mix (Q711-02, Vazyme). The PCR protocol consisted of 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 20 s. Gene expression levels were determined using the $2^{-\triangle\triangle Ct}$ method [22], with β-actin mRNA serving as the internal control. The primer sequences utilized in the experiments were as follows: IRF2, forward: 5′-GTTGTAGGACAGTCCCATCT-3′, reverse: 5′-CTATCAGTCGTTTCGCTTT-3′; INPP4B, forward: 5′-GTGTCTGATGCTGACGCTAA-3′, reverse: 5′-AAATCGGAAATGCCAACG-3′; actin, forward: 5′-TGTGACGTGGACATCCGCAAAG-3′, reverse: 5′-TGGAAGGTGGACAGCGAGGC-3′.

Western Blotting

Proteins were extracted from the cells using RIPA buffer (HS-SJ011, Crondabio) and then separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Beyotime, Shanghai, China), followed by transfer to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were then blocked with Tris-buffered saline with Tween (TBST; 1.5 mM Tris, 5 mM NaCl, 0.1% Tween-20) containing 5% skim milk at room temperature for 1 h and subjected to incubation with specific primary antibodies anti-IRF2 (ab124744, 1:1000, Abcam), anti-INPP4B (ab81269, 1:2000, Abcam), anti-JAK2 (ab108596, 1:5000, Abcam), anti-STAT3 (ab68153, 1:1000, Abcam), anti-p-JAK2 (ab32101, 1:5000, Abcam), anti-p-STAT3 (ab267373, 1:2000, Abcam), anti-caspase 3 (ab32351, 1:2000, Abcam), and anti-actin (GB12001, 1:2000, Servicebio, Wuhan, China) at 4 °C overnight. The membranes were subsequently incubated with a horseradish peroxidase-conjugated secondary antibody (BL003A, 1:4000, Biosharp, Hefei, China) for 1 h at room temperature. Finally, membrane bands were visualized using an enhanced chemiluminescence reagent (WBKLS0100, Millipore, Billerica, MA, USA). Actin served as a loading control and band grayscale values were analyzed using ImageJ software (Bio-Rad). Changes in protein levels were quantified by analyzing the grayscale values of the protein of interest and actin bands.

Cytokine Analysis by ELISA

Supernatants were harvested from CD4+ T-cell cultures and analyzed using IL-4 (E-EL-H0101c, Elabscience, Wuhan, China) and IFN-γ (E-EL-H0108c, Elabscience) ELISA kits following the manufacturer's directions.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Inc., San Diego, CA, USA) with a minimum of three repetitions. Data were expressed as mean ± standard deviation values. Statistical significance was estimated by utilizing Student's t-test for two groups or one-way analysis of variance for multiple groups. Values of $p<0.05$ were deemed statistically significant.

Results

IRF2 and INPP4B Levels Were High in CD4+ T-Cells from Acute Myeloid Leukemia Patients

To investigate the function of IRF2 and INPP4B in CD4+ T-cells, CD4+ T-cells were obtained from the peripheral blood of healthy individuals and patients with AML. As displayed in Figure 1A, the CD4+ T-cells accounted for 94.6% of all obtained cells. The levels of IRF2 and INPP4B in CD4+ T-cells obtained from healthy individuals and patients with AML were subsequently comparatively evaluated and qRT-PCR revealed a marked increase in the expression of IRF2 and INPP4B mRNA in CD4+ T-cells from AML patients compared to those from the healthy control group (Figure 1B). Furthermore, western blotting indicated significant increases in IRF2 and INPP4B in CD4+ T-cells obtained from AML patients compared to the healthy control group. Normalized to the control group, the IRF2 and INPP4B protein levels in the CD4+ T-cells of the patient group were respectively 2.06±0.1 and 2.11±0.13 (Figure 1C). These findings revealed that IRF2 and INPP4B were expressed in CD4+ T-cells isolated from AML patients at significantly high levels, indicating that IRF2 and INPP4B may be involved in the progression of AML.

CD4+ T-Cells Encouraged the Apoptosis of HL60 Cells

To clarify the function of CD4+ T-cells in the apoptosis of AML cells, isolated CD4+ T-cells were co-cultured with HL60 cells. Flow cytometry analysis showed that CD4+ T-cells markedly induced HL60 cell apoptosis compared to the control group, with apoptosis rates of 3.95±0.74% and 20.49±1.13% in the control and CD4+ T-cell groups, respectively (Figure 2A). After confirming the elevated IRF2 and INPP4B expression in CD4+ T-cells isolated from AML patients, we investigated whether CD4+ T-cell-induced apoptosis correlated with the regulation of

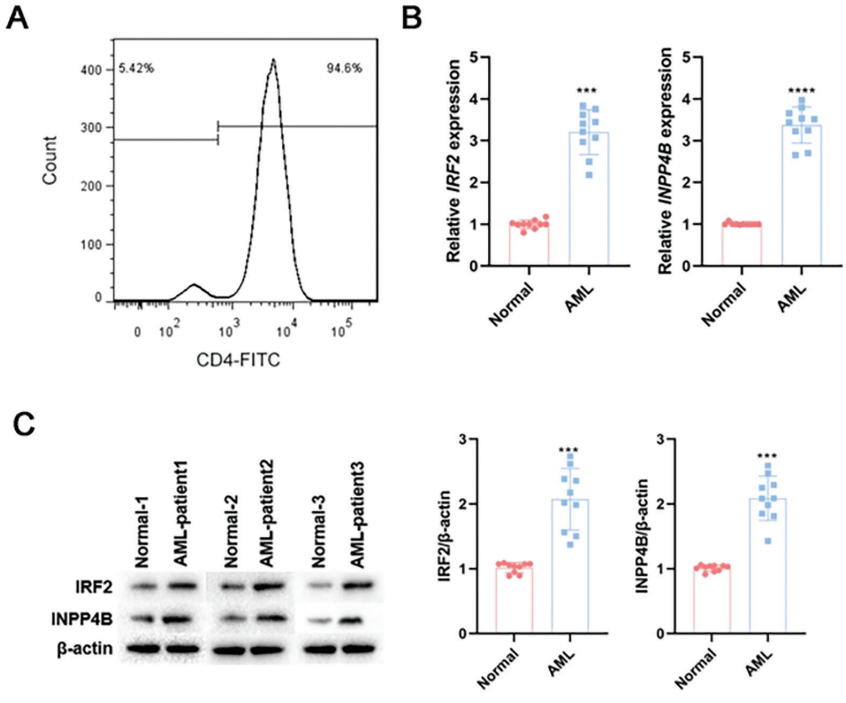

Figure 1. The expression levels of IRF2 and INPP4B were significantly increased in CD4+ T-cells isolated from patients with acute myeloid leukemia (AML). A) CD4+ T-cells were isolated from the peripheral blood of healthy donors and AML patients, followed by flow cytometry analysis. B) Quantitative real-time polymerase chain reaction was used to measure the expression of *IRF2* and *INPP4B* in CD4+ T-cells from healthy individuals and AML patients. C) Western blotting was used to estimate the protein expression levels of IRF2 and INPP4B in CD4+ T-cells from healthy donors and AML patients.

***: $p<0.001$; ****: $p<0.0001$. IRF2: Interferon-regulatory factor 2; INPP4B: inositol polyphosphate 4-phosphatase B.

IRF2 and INPP4B expression. The expression of IRF2 and INPP4B in HL60 cells was evaluated, and both qRT-PCR and western blotting revealed markedly reduced IRF2 and INPP4B expression in the CD4⁺ T-cell group versus the control group at mRNA and protein levels, respectively. When normalized to the control group, the protein expression levels of IRF2 and INPP4B in the patient-derived CD4⁺ T-cell group were respectively 0.57±0.04 and 0.51±0.22 (Figures 2B and 2C). These findings suggest that the CD4⁺ T-cells encouraged HL60 cell apoptosis through the downregulation of IRF2 and INPP4B.

Downregulation of IRF2 Encouraged Apoptosis of HL60 Cells by Influencing the Th1/Th2 Ratio

We further investigated whether IRF2 regulated the differentiation of Th1/Th2 cells in CD4⁺ T-cells. The loss and gain functions of IRF2 for the differentiating of Th1/Th2 cells in CD4⁺ T-cells were investigated by transfection with overexpressing IRF2 plasmids and the siRNA of *IRF2*. As displayed in Figure 3A, the siRNA-2 of *IRF2* exerted the best knockdown effect against *IRF2* in CD4⁺ T-cells. Accordingly, the siRNA-2 of *IRF2* was used for further work. Further analysis demonstrated that IFN-γ was markedly increased in the Si-IRF2 group and decreased in the Over-IRF2 group compared to the corresponding NC group. The opposite result was obtained for IL-4 in the different groups (Figure 3B). We also discovered that the knockdown of IRF2 markedly elevated the ratio of Th1/Th2 while increased expression of IRF2 reduced the ratio of Th1/Th2. The Th1/Th2 ratios in the control, Si-NC, Si-IRF2, Over-NC, and Over-IRF2 groups were 1.07±0.04, 1.08±0.04, 17.14±1.41, 1.07±0.03, and 0.28±0.03, respectively (Figure 3C). To explore the functional effect of the IRF2-regulated Th1/Th2 ratio in CD4⁺ T-cells on HL60 cells, HL60 cells were co-cultured in medium containing CD4⁺ T-cells transfected with either an IRF2 overexpression plasmid or IRF2-targeting siRNA. Subsequently, the CD4⁺ T-cells transfected with the IRF2 overexpression plasmid or IRF2 siRNA were co-cultured with HL60 cells. Flow cytometry analysis revealed that silencing IRF2 further enhanced HL60 cell apoptosis compared to the CD4⁺ T-cell group. The apoptosis rates in the control, CD4⁺ T, Si-NC-CD4⁺ T, Si-IRF2-CD4⁺ T, Over-NC-CD4⁺ T, and Over-IRF2-CD4⁺ T-cell groups were 4.21±0.46%, 20.13±0.48%, 20.68±0.81%, 41.17±1.14%, 20.59±0.65%, and 12.42±1.11%, respectively (Figure 3D). Additionally, qRT-PCR and western blotting revealed that INPP4B and IRF2 mRNA expression and protein levels were significantly decreased in the CD4⁺ T-cell group. Compared to the CD4⁺ T-cell group, the obtained values were further reduced in the Si-IRF2 CD4⁺ T-cell group, whereas they were elevated in the Over-IRF2 CD4⁺ T-cell group compared to the NC-CD4⁺ group. Normalized to the control group, the protein expression levels of IRF2 in the CD4⁺ T, Si-NC-CD4⁺ T, Si-IRF2-CD4⁺ T, Over-NC-CD4⁺ T, and Over-IRF2-CD4⁺ T-cell groups were 0.56±0.03, 0.56±0.05, 0.3±0.03, 0.55±0.02, and 0.83±0.07 while the values for INPP4B were 0.51±0.04, 0.52±0.05, 0.22±0.02, 0.52±0.04, and 0.88±0.04 (Figures 3E and 3F). These findings suggested

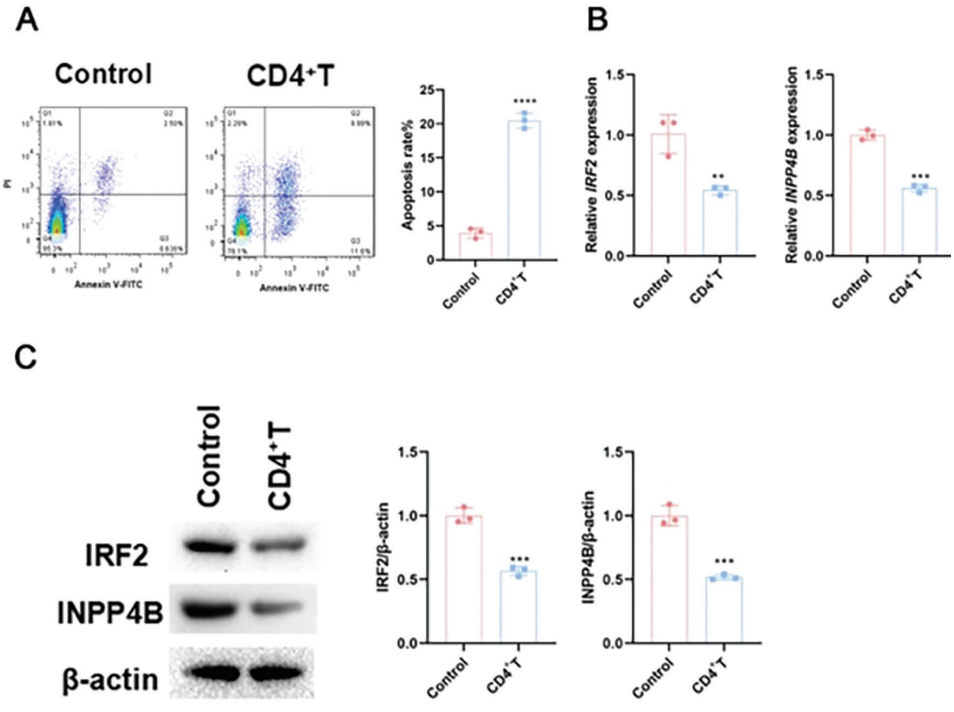

Figure 2. CD4⁺ T-cells induced apoptosis in HL60 cells. A) Flow cytometry was used to identify apoptosis in HL60 cells co-cultured with CD4⁺ T-cells. B, C) Quantitative real-time polymerase chain reaction and western blot analysis were used to measure the expression of IRF2 and INPP4B in HL60 cells, respectively.

**: p<0.01; ***: p<0.001, ****: p<0.0001. IRF2: Interferon-regulatory factor 2; INPP4B: inositol polyphosphate 4-phosphatase B.

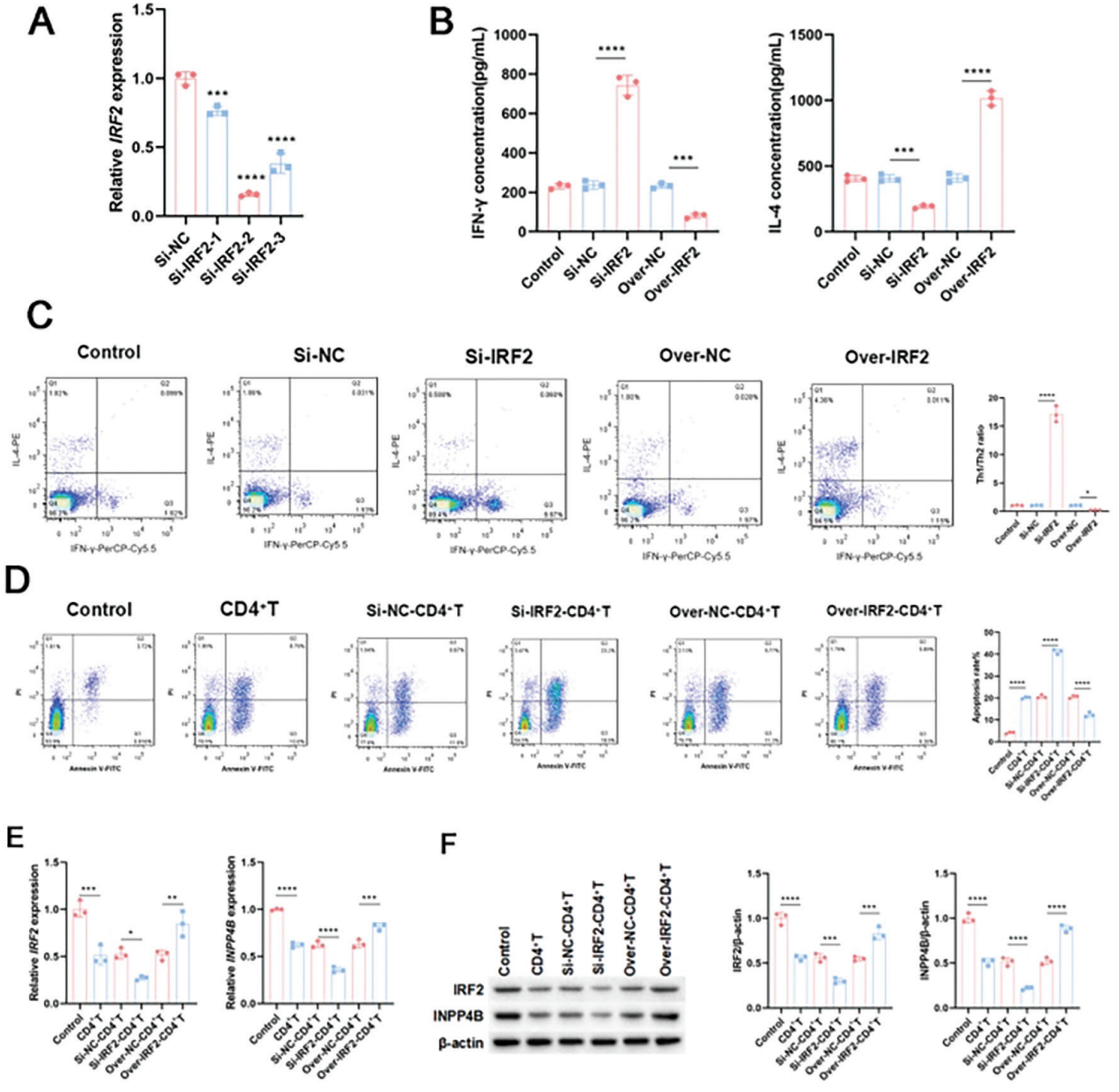

Figure 3. Downregulation of IRF2 induced HL60 cell apoptosis by modulating the Th1/Th2 ratio. A) *IRF2* expression levels in CD4+ T-cells transfected with *IRF2* siRNA. B) Interferon gamma and interleukin-4 levels measured by ELISA in the context of IRF2 manipulation. C) Th1/Th2 ratios analyzed by flow cytometry. D) Flow cytometric assessment of apoptosis in HL60 cells co-cultured with CD4+ T-cells overexpressing or knocking down IRF2, respectively. E, F) INPP4B expression measured by quantitative real-time polymerase chain reaction and western blotting in relation to IRF2, respectively.

*: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001. IRF2: Interferon-regulatory factor 2; INPP4B: inositol polyphosphate 4-phosphatase B.

that decreasing the expression of IRF2 encouraged HL60 cell apoptosis by influencing the Th1/Th2 ratio.

Overexpression of IRF2 Stimulated the JAK2-STAT3 Signaling Channel and Downregulated Caspase 3

To explore the impact of IRF2 on the JAK2-STAT3 signaling channel and caspase 3 expression, we examined key proteins of the JAK2-STAT3 pathway, including JAK2, p-JAK2, STAT3, p-STAT3, and caspase 3. Total JAK2 and STAT3 expression remained unchanged by IRF2. In contrast, p-JAK2 and p-STAT3 expression showed significant downregulation in the Si-IRF2 $CD4^+$ T-cell group and upregulation in the Over-IRF2-$CD4^+$ T-cell group compared to the NC-$CD4^+$ T-cell group. Normalized to the control group, the values obtained for p-JAK2 protein expression in the $CD4^+$ T, Si-NC-$CD4^+$ T, Si-IRF2-$CD4^+$ T, Over-NC-$CD4^+$ T, and Over-IRF2-$CD4^+$ T-cell groups were 0.51±0.03, 0.51±0.05, 0.23±0.02, 0.51±0.04, and 0.88±0.04, while the respective values for p-STAT3 were 0.54±0.04, 0.54±0.04, 0.23±0.02, 0.53±0.04, and 0.8±0.03 (Figure 4A). The opposite result was obtained for caspase 3. Normalized to the control group, the values obtained for caspase 3 protein expression in the $CD4^+$ T, Si-NC-$CD4^+$ T, Si-IRF2-$CD4^+$ T, Over-NC-$CD4^+$ T, and Over-IRF2-$CD4^+$ T-cell groups were 1.86±0.17, 1.86±0.16, 2.71±0.15, 1.87±0.12, and 1.28±0.08, respectively. To confirm the regulatory effect of the JAK2-STAT3 signaling channel on caspase 3, the HL60 cells were exposed to JAK2 inhibitor AG-490 and STAT3 inhibitor Stattic. This showed that caspase 3 was markedly elevated with both AG-490 and Stattic compared to the $CD4^+$ T-cell group. Normalized to the control group, the protein expression levels of p-JAK2, p-STAT3, and caspase 3 in the $CD4^+$ T, $CD4^+$ T + AG-490, and $CD4^+$ T + Stattic groups were respectively 0.52±0.03, 0.23±0.01, and 0.5±0.04; 0.61±0.03, 0.33±0.03, and 0.19±0.02; and 1.78±0.16, 2.49±0.15, and 2.47±0.15 (Figure 4B). These findings indicated that heightened expression of IRF2 might repress apoptosis in HL60 cells by stimulating the JAK2-STAT3 signaling channel to inhibit caspase 3.

Discussion

AML is an extremely heterogeneous disease both biologically and clinically. The incidence of AML in China ranks among the top in the world and the mortality rate of young AML patients is high [4]. The pathogenesis of AML is still not fully understood, which hinders the identification of new avenues for treatment [3]. Thus, it is vital to explore the key regulators of AML progression and clarify their mechanisms. In this study, we found that IRF2 and INPP4B were expressed in $CD4^+$ T-cells isolated from AML patients at significantly high levels. Mechanistically, we showed that IRF2 overexpression in $CD4^+$ T-cells stimulated the JAK2-STAT3 signaling channel and downregulated caspase 3 expression to promote AML cell survival, thereby facilitating AML progression.

With the advancement of cytogenetic techniques and molecular biology, the pathogenesis of AML has been further explored and the roles of additional leukemia-related genes, such as *TP53*, *TRIM62*, and *EBF3*, have been elucidated. This has yielded new opportunities for early diagnosis, prognostic estimation, and targeted therapies for AML [23]. IRF2, a multifunctional transcription factor, has crucial functions in influencing apoptosis and the cell cycles of cancer cells. For instance, IRF2 suppresses cancer cell proliferation by promoting AMER-1 transcription in human gastric cancer cells [24]. Guo et al. [25] reported that the IRF2-β-catenin axis drives the proliferation of hepatocellular carcinoma (HCC) cells, enhances their resistance to lenvatinib, and blocks HCC cell apoptosis. Additionally, evidence indicates that IRF2 is vital for the differentiation of immune T-cells. Our prior studies similarly showed that the IRF2-INPP4B signaling channel promotes AML progression by facilitating leukemic cell proliferation and survival while inhibiting apoptosis [14,26], indicating that restricting the IRF2-INPP4B signaling channel might be a potential molecular goal for new AML therapies. Further analysis demonstrated that the IRF2-INPP4B axis had a role in the differentiating of Th1/Th2 cells that allowed it to inhibit the apoptosis of AML cells [27]. Nevertheless, the precise regulatory actions by which the IRF2-INPP4B axis impacts that process of differentiation are still not fully elucidated. In the present work, we showed that expression values of IRF2 and INPP4B were significantly higher in $CD4^+$ T-cells obtained from patients with AML. Further analysis revealed that the $CD4^+$ T-cells encouraged the apoptosis of HL60 cells. These outcomes were consistent with those of a previous work reporting that although regulatory $CD4^+$ T-cells were increased in a subset of cultures, in vitro human CD80/IL2 lentivirus-transduced AML cells showed enhanced cytolytic activity [27]. Additionally, we found that reducing the expression of IRF2 encouraged the apoptosis of HL60 cells via an impact on the Th1/Th2 differentiation process, indicating that the IRF2-INPP4B axis inhibited AML cell apoptosis by modulating the differentiating of Th1/Th2, which could lead to AML progression. The JAK/STAT signaling channel is stimulated by cytokines [28,29]. An increasing body of literature confirms that the JAK/STAT signaling channel has vital roles in tumor growth via its influences on various biological processes, such as proliferation, migration, invasion, and drug resistance [28]. Zhao et al. [30] concluded that the viability of myelodysplastic syndrome and AML cells is inhibited by chidamide due to its ability to inhibit the JAK2-STAT3 signaling channel. Mesbahi et al. [18] reported that the antitumor efficacy of arsenic trioxide in AML cells was improved by the induction of reactive oxygen species, which blocked JAK2-STAT3 signaling. Wang et al. [31] showed that miR-146a encouraged AML progression by stimulating JAK2-STAT3 signaling and decreasing the expression of CNFR.

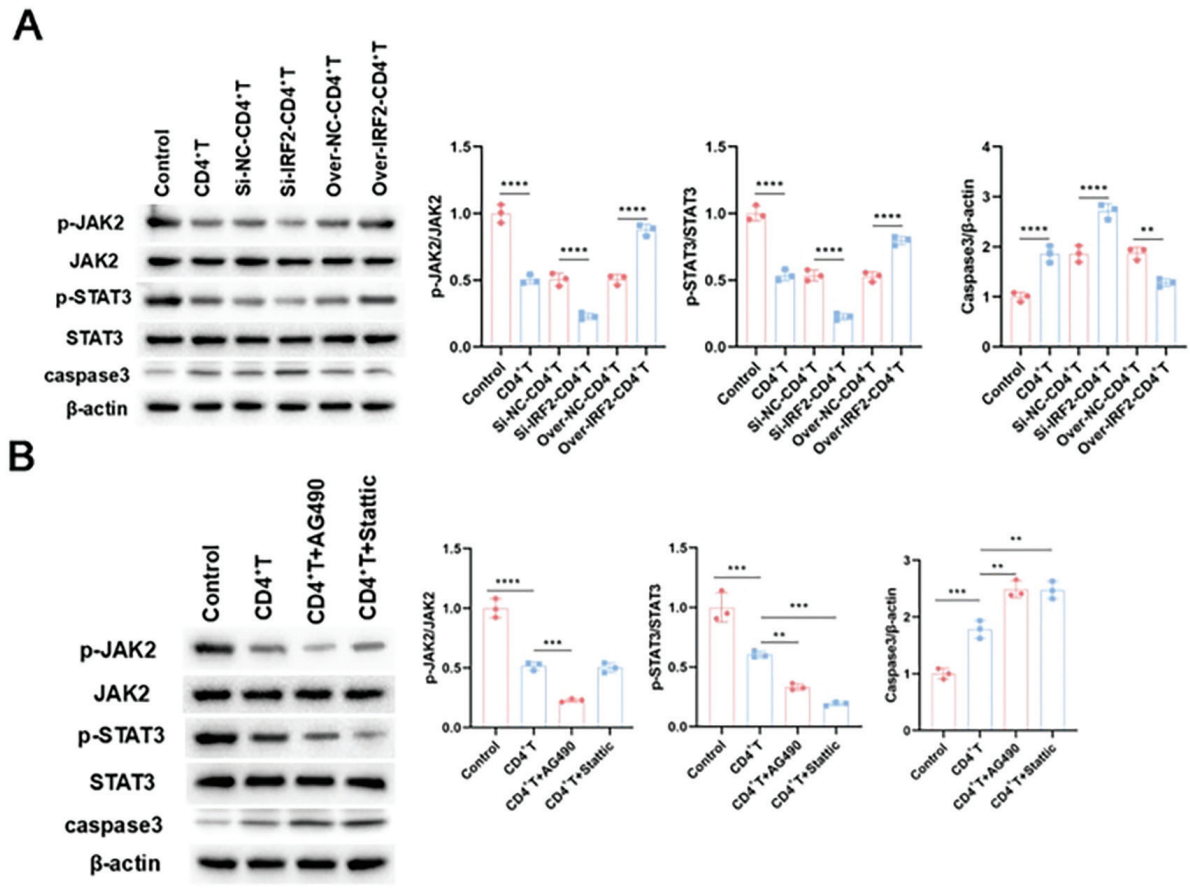

Figure 4. Overexpression of IRF2 stimulated the JAK2-STAT3 signaling channel and downregulated caspase 3 expression. A) Western blotting was used to measure the expression levels of JAK2-STAT3 signaling channel-related proteins and caspase 3 as impacted by IRF2, with the proteins including JAK2, STAT3, p-JAK2, and p-STAT3. B) Effects on the expression of caspase 3 via JAK2 inhibitor AG-490 and STAT3 inhibitor Stattic were detected with western blotting.

**: p<0.01; ***: p<0.001; ****: p<0.0001. IRF2: Interferon-regulatory factor 2.

Nevertheless, it is not yet clear whether the IRF2-INPP4B axis participates in the modulation of JAK2-STAT3 signaling. In the present work, we have offered evidence demonstrating that increasing the expression of IRF2 stimulated the JAK2-STAT3 signaling channel and downregulated caspase 3. Further analysis showed that inhibiting the JAK2-STAT3 signaling channel could elevate caspase 3, implying that JAK2-STAT3 might inhibit AML apoptosis. Thus, this work highlights a previously uncharacterized link between the IRF2-INPP4B and JAK2-STAT3 pathways, offering data on a new potential regulatory mechanism in AML progression. This finding could pave the way for new therapeutic strategies that focus on this interplay.

Study Limitations

Our work has several limitations. First, the sample size of the patient group was relatively small and we could not analyze patient subgroups; thus, the ensuing heterogeneity may have introduced some bias in the interpretation of the obtained data. Furthermore, this work lacked animal experiments to further verify the findings. This work constituted a pilot study and we plan to carry out further large-scale research including animal experiments in the future to further validate our findings.

Conclusion

This work has revealed that IRF2 and INPP4B have significantly high expression levels in the $CD4^+$ T-cells of patients with AML. We also showed that reducing the expression of IRF2 encouraged apoptosis in HL60 cells via an impact on the Th1/Th2 ratio. In contrast, the overexpression of IRF2 stimulated the JAK2-STAT3 signaling channel and downregulated caspase 3. Our work has thus revealed that the activation of the IRF2-INPP4B axis in $CD4^+$ T-cells exacerbated AML progression by stimulating the JAK2-STAT3 signaling channel and inhibiting the apoptosis of AML cells. This might help clarify the factors involved in AML progression while offering innovative guidance for the design of new AML therapies.

Ethics

Ethics Committee Approval: Ethical permission for this work was granted by the First Affiliated Hospital of Bengbu Medical College on June 25, 2021, with permit number [2021] No. 205. All experiments were conducted following the institutional ethical standards.

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

Acknowledgment

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Footnotes

Authorship Contributions

Surgical and Medical Practices: X.X., M.Z., F.Z.; Concept: Y.Y., M.W.; Design: X.X., M.Z., F.Z.; Data Collection or Processing: X.X., M.Z., S.T., F.S.; Analysis or Interpretation: S.T., J.Z., J.L.; Literature Search: J.L., P.Z.; Writing: X.X., M.Z., F.Z.

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

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miR-379-5p Inhibited the Proliferation of Acute Myeloid Leukemia Cells Through Negative Regulation of *YBX1*

miR-379-5p *YBX1* Negatif Düzenlemesi Aracılığı ile Akut Miyeloid Lösemi Hücrelerinde Proliferasyonu İnhibe Etti

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Abstract

Objective: Acute myeloid leukemia (AML) highly lethal hematological malignancy that is difficult to treat. This study aimed to clarify the molecular mechanisms of miR-379-5p in AML progression.

Materials and Methods: Quantitative real-time polymerase chain reaction was utilized to evaluate miR-379-5p expression levels in AML patients and a control group. A receiver operating characteristic curve was created to assess the clinical predictive value of miR-379-5p in AML, while cell experiments used the CCK-8 assay, flow cytometry, and transwell chambers. Potential target genes of miR-379-5p were predicted by employing online bioinformatics tools, followed by validation using a dual luciferase reporter assay.

Results: miR-379-5p expression was significantly decreased in AML patients and had clinical predictive value for the disease. In AML cell lines, miR-379-5p was downregulated; conversely, the upregulation of miR-379-5p inhibited proliferation, migration, and invasion while promoting apoptosis. Notably, *YBX1* was a potential target gene of miR-379-5p and its upregulation reduced the effects of miR-379-5p on AML cell behavior.

Conclusion: miR-379-5p has potential as a biomarker for AML by regulating cell proliferation and apoptosis through the targeting of *YBX1*.

Keywords: Acute myeloid leukemia, miR-379-5p, *YBX1*, Cell proliferation, Apoptosis, Diagnostic significance

Öz

Amaç: Akut miyeloid lösemi (AML) tedavisi zor olan ve oldukça ölümcül bir hematolojik malignitedir. Bu çalışmada AML progresyonunda miR-379-5p nin moleküler mekanizmalarını netleştirmeyi hedefledik.

Gereç ve Yöntemler: AML hastaları ve kontrol grup örneklerinde kantitatif gerçek zamanlı polimeraz zincir reaksiyonu kullanılarak miR-379-5p ekspresyon düzeyleri değerlendirildi. AML'de klinik prediktif miR-379-5p değerini ortaya çıkarabilmek için alıcı taraflı karakteristik bir eğri oluşturuldu. Hücre deneyleri ise CCK-8, akım sitometri ve transwell migrasyon tetkikini kullandı. miR-379-5p'nin potansiyel hedefleri online biyoinformatik tahmin araçları ile öngörülmüştür, peşinden dual lusiferaz raportör yöntemi ile doğrulanmıştır.

Bulgular: AML hastalarında miR-379-5p ekspresyonu belirgin şekilde azalmıştır ve hastalık için klinik prediktif değeri olduğu görülmüştür. AML hücre dizelerinde miR-379-5p azalmıştır, oysa ki artmış düzeyleri proliferasyonu, migrasyonu ve invazyonu inhibe ederek apopitozu tetikler. Özellikle *YBX1* miR-379-5p geni potansiyel hedefidir ve artmış seviyeleri miR-379-5p nin AML hücre davranışındaki etkilerini azaltmaktadır.

Sonuç: miR-379-5p *YBX1*'i hedef alarak hücre proliferasyonu ve apopitozisin düzenlenmesi üzerinden AML için potansiyel bir biyobelirteç olabilir.

Anahtar Sözcükler: Akut miyeloid lösemi, miR-379-5p, *YBX1*, Hücre proliferasyonu, Apopitozis, Tanısal önem

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Introduction

Acute myeloid leukemia (AML) interferes with the differentiation of myeloid cells, resulting in unchecked proliferation of leukemia progenitor cells and compromised development of normal blood cells [1]. AML has a high mortality rate and accounts for about two-thirds of adult leukemia cases [2]. Hematopoietic stem cell transplantation and chemotherapy are the most important means for treating AML. However, for patients with relapsed or metastatic advanced AML, these methods are still associated with poor prognosis [3]. AML is a malignant proliferative disease and abnormal gene mutations and gene expression might be linked to the abnormal proliferation of AML-associated cancer cells [4]. Therefore, identifying AML-related biomarkers is essential for effective clinical diagnosis.

Studies have confirmed that microRNA (miRNA) significantly regulates cell proliferation, angiogenesis, and the bone marrow microenvironment, thereby promoting the progression of leukemia [5]. Notable examples include miR-203 [6] and miR-217 [7]. As a tumor suppressor, miR-379-5p plays a crucial role in various cancers, including breast cancer [8], hepatocellular carcinoma [9], and oral squamous cell carcinoma [10]. Research has shown that the expression levels of miR-379-5p are reduced in $CD34^+$ cells from patients with primary myelofibrosis, suggesting its potential involvement in the pathogenesis of chronic myeloid neoplasms [11]. However, the precise mechanisms underlying the role of miR-379-5p in AML remain poorly understood.

YBX1 is a multifunctional cancer-related protein. Research indicates that *YBX1* is elevated in bladder [12], pancreatic [13], and nasopharyngeal cancer [14]. Furthermore, *YBX1* is significantly upregulated in small extracellular vesicles released from the cells of most pediatric AML patients [15]. However, the mechanisms of miR-379-5p and *YBX1* in AML remain poorly understood.

We hypothesized that miR-379-5p was downregulated in AML and involved in the regulation of AML cells based on previous findings. In this study, we enrolled 70 healthy individuals and 75 patients with AML. miR-379-5p was detected and compared between the two groups. We also explored the molecular mechanisms of miR-379-5p in AML and its clinical diagnostic value, providing guidance for the diagnosis and treatment of AML.

Materials and Methods

Patients and Specimens

This study enrolled 75 AML patients from Changchun University of Chinese Medicine, while the control group included 70 healthy individuals. Among the AML patients, 46 were younger than 60 years old, whereas 29 were 60 years old or older. The mean age of the AML group was 51.73±9.30 years. The patient cohort comprised 43 men and 32 women. In the control group, the mean age was 49.56±9.89 years, with 52 participants under 60 years old and 18 participants aged 60 years or older. This group consisted of 39 men and 31 women. According to the French-American-British (FAB) classification criteria, the subtypes of AML were distributed as follows: 6 patients had M0 type, 43 had M1/M2 type, and 26 had M4/M5 type. From a cytogenetic perspective, patients were further categorized based on prognosis: 30 patients had a favorable prognosis, 35 had an intermediate prognosis, and 10 had a poor prognosis. In this study, the age threshold for distinguishing adult from pediatric patients was set at 18 years. All 75 AML patients were adults as determined by their ages. All enrolled AML patients were newly diagnosed and had not yet received treatment. Ethical approval was obtained from the Ethics Committee of the Changchun University of Chinese Medicine (approval number: 2018005, date: August 15, 2018). Written informed consent was obtained from all participants and all procedures were conducted in strict accordance with the Declaration of Helsinki. After confirming the participants' informed consent, peripheral blood samples were collected. The collected samples were left standing at room temperature for 2 h and then centrifuged for 10 min, and upper serum samples were collected. The serum samples were stored in a refrigerator at -20 °C for subsequent experiments.

Cell Culture and Cell Transfection

Human bone marrow stromal cells of the HS-5 line and AML cells of lines K562, THP-1, KG-1, and HL-60 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in RPMI-1640 medium (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum in a humidified incubator (SANYO, Osaka, Japan) at 37 °C with 5% CO_2.

Lipofectamine 3000 (Invitrogen, Waltham, MA, USA) was utilized for the transfection of synthetic sequences, including miR-379-5p-mimic (miR-mimic) (GenePharma, Shanghai, China) and its negative control (mimic-NC), pcDNA-3.1 empty vector (p-control), and YBX1 recombinant pcDNA-3.1 plasmid (p-YBX1). The concentration of both miR-379-5p-mimic and the miR-379-5p inhibitor was 50 nM.

Quantitative Real-Time Polymerase Chain Reaction

The total RNA of miR-379-5p and *YBX1* was respectively extracted. Subsequently, the obtained cDNA was amplified using reverse transcription reagents on the LightCycler 96 device (Roche, Basel, Switzerland). Real-time polymerase chain reaction (RT-qPCR) was used to detect the expression levels of miR-379-5p and *YBX1* using cDNA as the template. The RT-qPCR reaction protocol was as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles consisting of denaturation at

95 °C for 5 s, annealing at 55 °C for 10 s, and extension at 72 °C for 15 s. Gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method with *U6* and *GAPDH* serving as internal controls.

CCK-8 Assay

Approximately $2x10^3$ cells were carefully plated in a 96-well plate. Experimental groups were established based on the experimental objectives and measurements were taken at designated time points of 0, 24, 48, and 72 h. Subsequently, CCK-8 solution was added to each well, followed by an additional incubation period of 4 h. The absorbance at 450 nm was then measured using a spectrophotometer. Cell viability, as an indicator of cell proliferation, was evaluated according to the manufacturer's instructions provided with the CCK-8 kit (Hamby Biotechnology, Beijing, China). Each experiment was performed in triplicate for each cell group.

Measurement of Cell Apoptosis

The necessary samples were collected, washed repeatedly with phosphate-buffered saline, resuspended, and counted. Annexin V-FITC and propidium iodide reagents (Sigma-Aldrich, St. Louis, MO, USA) were added. Cell samples were incubated on ice for 15-20 min before being analyzed by flow cytometry. The apoptosis rate was calculated based on the intensity of the fluorescence signal.

Cell Migration and Invasion Experiments

Transwell chambers (BD Biosciences, San Jose, CA, USA) with 8-µm pores were coated with Matrigel (BD Biosciences) for invasion assays, while uncoated chambers were used for migration assays. In brief, $1x10^4$ cells were suspended in serum-free medium and seeded into the upper chamber. Following 24 h of incubation at 37 °C, non-migrated cells in the upper chamber were gently removed. Migrated or invaded cells were then fixed with methanol and stained with crystal violet. All assays were performed in triplicate.

Bioinformatics Analysis

miR-379-5p targets were predicted using the miRDB, miRTarBase, and TargetScan databases, and a Venn diagram was constructed to identify the overlapping targets.

Dual Luciferase Reporter Assay

The primers for the 3'-UTR of the *YBX1* gene were designed using Primer Premier 5.0 (PREMIER Biosoft, San Francisco, CA, USA) and subsequently amplified by PCR. The resulting PCR product was cloned into the pGLO plasmid (Promega, Madison, WI, USA) to generate the wild-type *YBX1* vector (WT-*YBX1*). Additionally, site-directed mutagenesis was performed on the 3'-UTR sequence of *YBX1* to construct the mutant vector (MUT-*YBX1*). These vectors were co-transfected with miR-379 mimics or appropriate controls using Lipofectamine 3000 (Invitrogen). Following 48 h of incubation, luciferase activity was measured using a luciferase reporter assay kit.

Statistical Analysis

Data analysis was conducted using SPSS (SPSS Inc., Chicago, IL, USA) and GraphPad Prism (GraphPad Inc., San Diego, CA, USA) software. For inter-group data comparisons, Student t-tests, chi-square tests, and one-way analysis of variance were employed. The diagnostic efficacy of miR-379-5p in AML was assessed via receiver operating characteristic (ROC) curve analysis. Each experimental condition was replicated three times to ensure reliability. Statistical significance was set at $p<0.05$.

Results

miR-379-5p was Expressed at Lower Levels in Acute Myeloid Leukemia Cells

We investigated the correlation between miR-379-5p expression levels and various clinicopathological parameters in patients with AML. No significant differences were observed in age or sex distribution between the low-expression and high-expression groups of miR-379-5p ($p>0.05$). While there were some variations in white blood cell count and bone marrow blast percentage, these differences did not reach statistical significance ($p>0.05$). However, significant differences in miR-379-5p expression were noted for platelet counts, FAB subtypes, and cytogenetic characteristics ($p<0.05$) (Table 1). RT-qPCR results indicated that the miR-379-5p expression of AML patients was significantly lower than that observed in the control group (Figure 1A). Serum miR-379-5p had clinical diagnostic value for AML with diagnostic specificity of 85.71%, sensitivity of 86.67%, and ROC curve area of 0.929 (Figure 1B). Moreover, miR-379-5p expression was significantly lower in HL-60, KG-1, K562, and THP-1 cells compared to HS-5, with the lowest levels obtained in HL-60 and THP-1 (Figure 1C), leading to further experiments using those two cell lines.

miR-379-5p Regulated Cellular Behaviors

RT-qPCR results showed that miR-mimic transfection significantly increased miR-379-5p levels in the HL-60 and THP-1 cell lines compared to the control group (Figures 2A and 2B). CCK-8 results showed a significant decrease in proliferation capacity in the miR-379-5p overexpression group (Figures 2C and 2D). Transwell assay results indicated that the migration levels of HL-60 and THP-1 cells transfected with miR-mimic were significantly lower (Figures 2E and 2F) and cell invasion ability was significantly inhibited (Figures 2G and 2H). Cell apoptosis experiments revealed a significant increase in the apoptosis rate in the miR-mimic group (Figures 2I and 2J).

Table 1. Correlation of miR-379-5p levels with clinicopathological parameters of acute myeloid leukemia patients.

Characteristics	Number	Serum miR-379-5p		p
		Low expression (n=39)	High expression (n=36)	
Age, years				
<60	46	24 (52.17%)	22 (47.83%)	0.970
≥60	29	15 (51.72%)	14 (48.28%)	
Sex				
Male	43	21 (48.83%)	22 (51.16%)	0.525
Female	32	18 (56.26%)	14 (43.75%)	
WBC count (x10^9/L)				
<10	33	13 (39.39%)	20 (60.61%)	0.053
≥10	42	26 (61.9%)	16 (38.10%)	
Platelet count (x10^9/L)				
<50	39	16 (41.03%)	23 (58.97%)	0.048
≥50	36	23 (63.89%)	13 (36.11%)	
BM blasts (%)				
<50	44	19 (43.18%)	25 (56.82%)	0.069
≥50	31	20 (64.51%)	11 (35.48%)	
FAB subtype				
M0	6	1 (16.67%)	5 (83.33%)	0.013
M1/M2	43	19 (44.19%)	24 (55.81%)	
M4/M5	26	19 (73.08%)	7 (26.92%)	
Cytogenetics				
Favorable	30	9 (30.00%)	21 (70.00%)	0.005
Intermediate	35	22 (62.86%)	13 (37.14%)	
Poor	10	8 (80.00%)	2 (20.00%)	

WBC: White blood cells; BM: bone marrow; FAB: French-American-British classification.

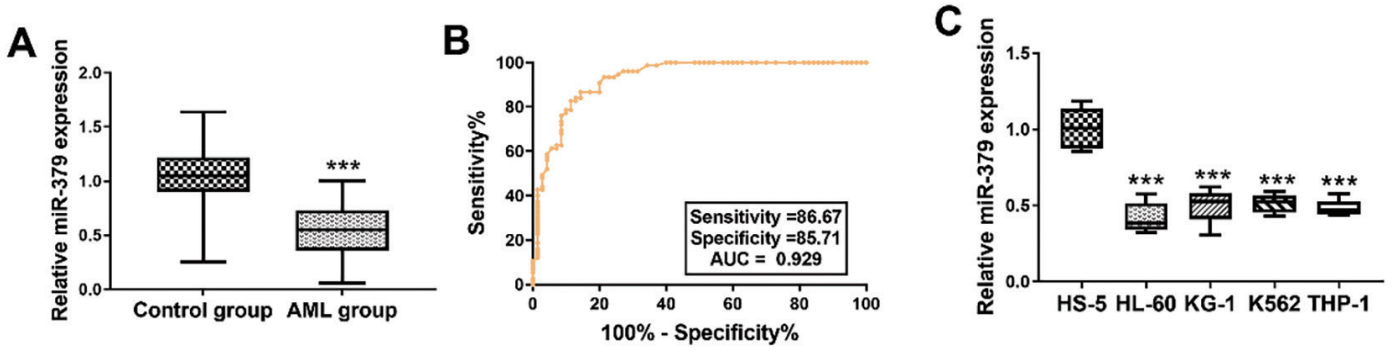

Figure 1. Expression level and diagnostic value of miR-379-5p. A) miR-379-5p was downregulated in acute myeloid leukemia. B) miR-379-5p possesses diagnostic value. C) miR-379-5p was downregulated in acute myeloid leukemia cell lines.

***: p<0.001; AML: acute myeloid leukemia; AUC: area under the curve.

The Targeting Relationship of miR-379-5p and *YBX1*

Venn diagram analysis identified 10 overlapping target genes: *EIF4G2*, *ELMOD2*, *NHLRC3*, *HSPA5*, *YBX1*, *EDN1*, *EDEM3*, *PCGF3*, *SLC20A1*, and *LIN28B* (Figure 3A). *YBX1* was identified as a target gene of miR-379-5p, with its targeting sequence predicted (Figure 3B). The luciferase reporter assay confirmed that in the WT-*YBX1* group, miR-mimic co-transfection significantly reduced luciferase activity, while no substantial alteration was witnessed in the MUT-*YBX1* group (Figure 3C).

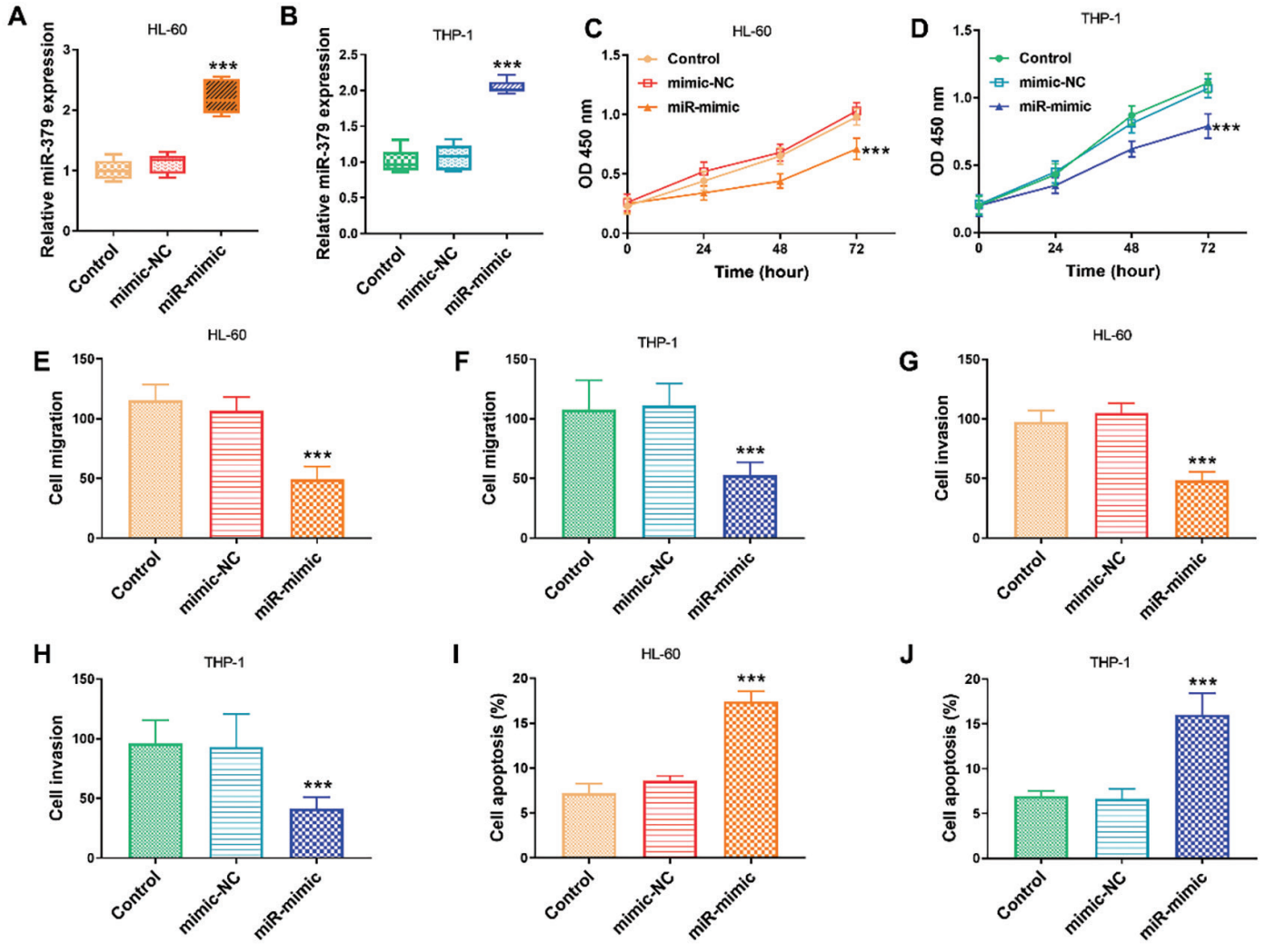

Figure 2. Role of miR-379-5p in HL-60 and THP-1 cells. A, B) After transfection with miR-379-5p mimics, the expression level of miR-379-5p was elevated. C, D) miR-379-5p suppressed proliferation. E, F) miR-379-5p suppressed migration. G, H) miR-379-5p inhibited invasion. I, J) miR-379-5p promoted apoptosis.

***: p<0.001.

YBX1 Reversed the Cellular Behavior of miR-379-5p

To further explore the working principles of the miR-379-5p/*YBX1* axis, a rescue experiment was performed. RT-qPCR results revealed that *YBX1* expression declined in the miR-mimic group. However, in the co-transfection group, the miRNA expression level of *YBX1* recovered to a certain extent. This indicated that miR-379 suppressed *YBX1* expression, while co-transfection of miR-mimic and *YBX1* reversed this trend (Figures 4A and 4B).

Cell proliferation assays showed decreased proliferation in the miR-mimic group, which was reversed in the miR-mimic + p-*YBX1* group (Figures 4C and 4D). Transwell assay results indicated that migration and invasion were reduced in the miR-mimic group, while *YBX1* upregulation mitigated these effects (Figures 4E-4H). Apoptosis assay results showed increased apoptotic capacity in the miR-mimic group, which was counteracted by *YBX1* upregulation (Figures 4I and 4J).

Discussion

AML is characterized by aberrant proliferation of primitive myeloid cells in the bone marrow and blood, but its pathogenesis is still unclear [16]. The incidence and mortality rates of AML are continuously rising [17]. Our study demonstrated that miR-379-5p has significant diagnostic value for AML.

miRNAs have a propensity for loss and transfer, influencing the proliferation and migration of malignant tumor cells [18]. miR-379-5p inhibits hepatocellular carcinoma invasion [19] and is also found to play significant roles in studies on endometrial cancer [20] and lung cancer [21]. Our study revealed that miR-

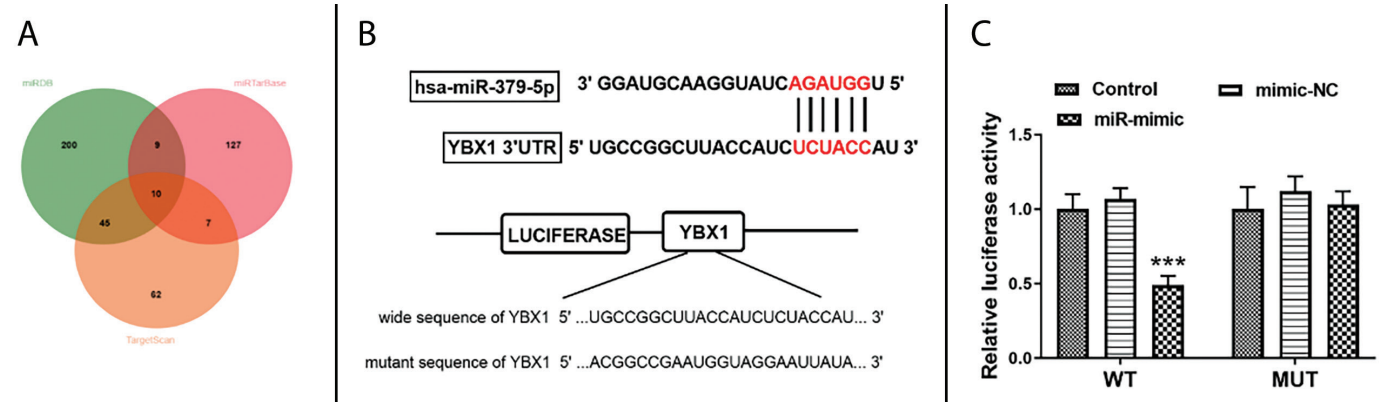

Figure 3. The targeting relationship of *YBX1* and miR-379-5p. A) The predicted results of target genes of miR-379-5p. B) Targeted binding sites. C) Validation of the targeted relationship.

***: p<0.001.

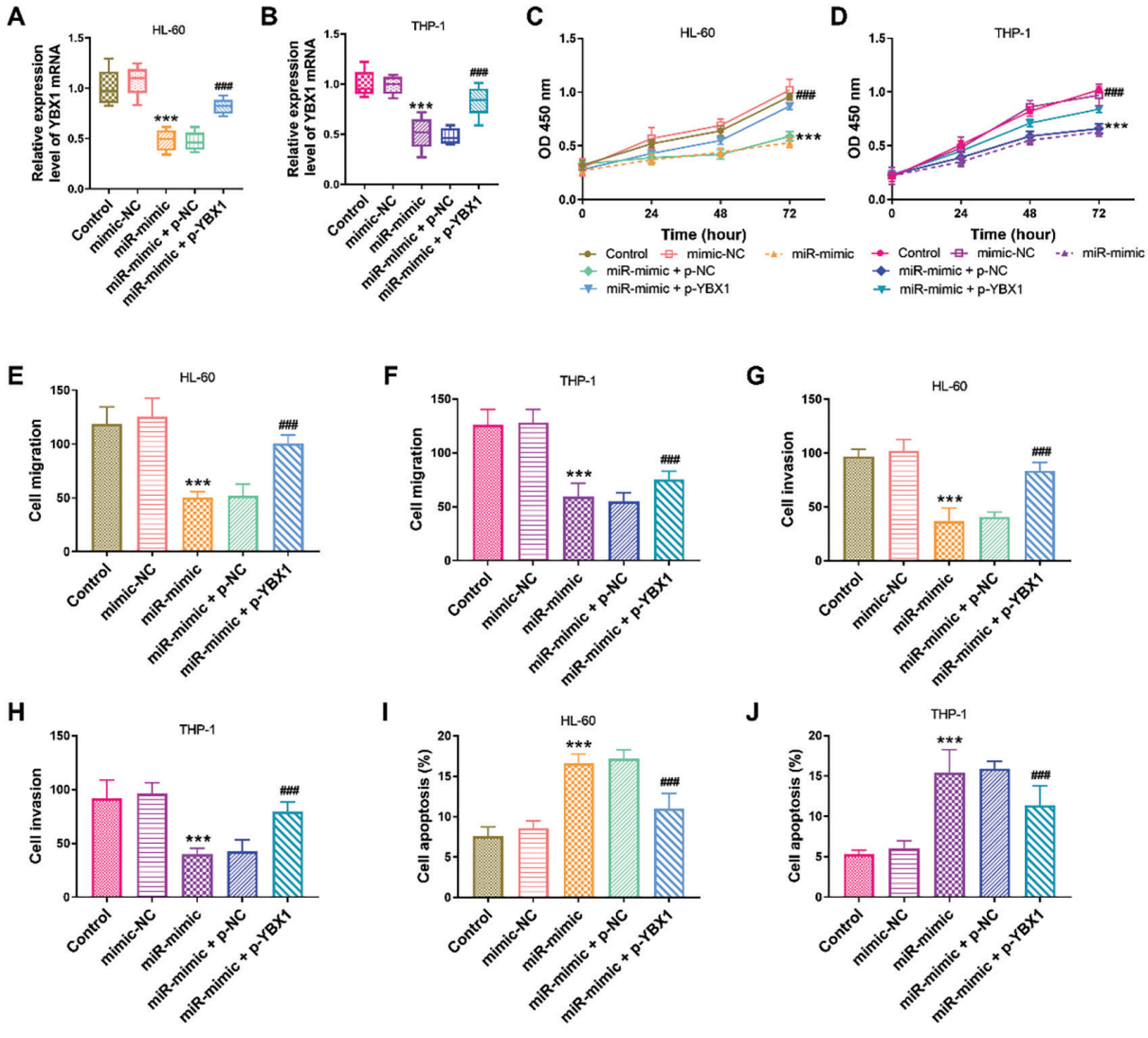

Figure 4. Regulation of HL-60 and THP-1 cells by the miR-379-5p/*YBX1* axis. A, B) Regulation of *YBX1* expression. C, D) Effect of miR-379-5p/*YBX1* on proliferation ability. E, F) Influence of miR-379-5p/*YBX1* on migration. G, H) Influence of miR-379-5p/*YBX1* on invasion. I, J) Impact of miR-379-5p/*YBX1* on apoptosis.

***: p<0.001.

379-5p suppressed the proliferation, migration, and invasion of AML cell lines and promoted apoptosis. Collectively, these findings provided robust evidence that miR-379-5p functions as a potent tumor suppressor in AML cells. Specifically, miR-379-5p might inhibit cell proliferation by targeting key genes involved in cell cycle progression [22]. In the context of apoptosis signaling pathways, miR-379-5p probably activates the expression of both intrinsic and extrinsic apoptosis-related genes [23], thereby promoting apoptosis in AML cells. For cell migration and invasion, miRNA may exert an inhibitory effect, likely by modulating the expression of cytoskeletal proteins or the function of intercellular adhesion molecules [23]. miR-379-5p may downregulate the expression of matrix metalloproteinases, which play critical roles in cell migration and invasion [24], thereby reducing the degradation of the extracellular matrix and impeding the ability of AML cells to migrate and invade surrounding tissues. In addition, research has determined that miR-379-5p also exhibited inhibitory effects against the cell proliferation of non-small-cell lung cancer [25] and glioma cells [26], promoting cancer cell apoptosis. These findings collectively suggested that miR-379-5p may act as a tumor suppressor of AML cells.

YBX1 was recognized as the target gene of miR-379-5p. Our study has revealed that miR-379-5p exhibited significantly reduced expression in AML patients, which was frequently correlated with unfavorable prognosis. Specifically, elevated *YBX1* expression in AML cells promotes cell proliferation, whereas the knockout of *YBX1* inhibits this process. High *YBX1* levels in adult AML patients were often associated with poor prognosis. miR-379-5p exerts its effects by negatively regulating *YBX1*; thus, decreased miR-379-5p expression has a weaker suppressive effect on *YBX1*, thereby enhancing the proliferative and other oncogenic effects of *YBX1*. Moreover, *YBX1* can counteract the inhibitory effects of miR-379-5p on AML cell proliferation, apoptosis, migration, and invasion. These findings are in alignment with the results of prior research studies. Previous studies have shown that miR-379-5p expression was decreased in osteoarthritis and it stimulated cell activity by negatively regulating *YBX1* [27]; furthermore, miR-379-5p and *YBX1* were shown to collaborate to regulate the biological activities of nasopharyngeal carcinoma cells [28]. In addition, research indicated that *YBX1* was upregulated in AML cells and enhanced their proliferation [29], while the knockout of this gene inhibited cell proliferation [30]. In adult AML patients, high expression of *YBX1* was often accompanied by an adverse prognosis [31]. These findings are in agreement with our research outcomes, demonstrating that *YBX1* counteracted the effects of miR-379-5p on AML cell proliferation, apoptosis, migration, and invasion.

Conclusion

This study has demonstrated that miR-379-5p inhibited the proliferation of AML cells by downregulating *YBX1*. The relative expression level of miR-379-5p was significantly reduced in both AML patients and AML cell lines, suggesting its potential as a clinical biomarker. Elevated levels of miR-379-5p were associated with inhibited cell proliferation, enhanced apoptosis, and suppressed cell migration and invasion. *YBX1*, identified as a target gene of miR-379-5p, promotes cell proliferation, migration, and invasion, thereby inhibiting apoptosis and exerting an opposing effect on miR-379-5p. We chose to isolate miR-379-5p from serum. Because serum samples are relatively easy to obtain through non-invasive or minimally invasive methods, they impose a significantly smaller burden on patients with AML compared to tissue sampling. Furthermore, the ability to collect multiple serum samples at different treatment stages is an important consideration in sample selection. Additionally, miRNA in serum can reflect the overall physiological and pathological state of the body. For systemic diseases such as AML, detecting miR-379-5p expression in serum may provide a more comprehensive reflection of the disease's impact on the entire body and its potential role in disease progression, rather than being confined to changes in specific local tissues. This approach can complement tissue-based studies to better elucidate disease mechanisms. Therefore, we chose to isolate miR-379-5p from serum. Our findings may provide novel insights into AML treatment. However, our study possessed certain limitations, such as the absence of tissue samples. Further investigation is warranted to explore miR-379-5p as a potential biomarker.

Ethics

Ethics Committee Approval: Ethical approval was obtained from the Ethics Committee of Changchun University of Chinese Medicine (approval number: 2018005, date: August 15, 2018).

Informed Consent: Written informed consent was obtained from all participants.

Footnotes

Authorship Contributions

Surgical and Medical Practices: H.W., Y.X., X.T.; Concept: L.Z., H.G., Y.X., J.H.; Design: H.W., H.G., Y.X.; Data Collection or Processing: H.W., J.H., X.T.; Analysis or Interpretation: L.Z., J.H., X.T.; Literature Search: H.G., X.T.; Writing: H.W., L.Z., H.G., Y.X., J.H., X.T.

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

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RESEARCH ARTICLE

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Exploration of Leucine-Rich Alpha-2 Glycoprotein 1 (LRG1) and Its Association with Proangiogenic Mediators in Sickle Cell Disease: A Potential Player in the Pathogenesis of the Disease

Orak Hücre Hastalığında Lösinden Zengin Alfa-2 Glikoprotein 1 (LRG1) ve Proanjiyogenik Mediyatörlerle İlişkisinin Araştırılması: Hastalığın Patogenezinde Potansiyel Bir Oyuncu

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Abstract

Objective: Leucine-rich alpha-2-glycoprotein 1 (LRG1) is a novel mediator involved in abnormal angiogenesis. We aimed to investigate circulating LRG1 levels and their relationship with proangiogenic mediators in sickle cell disease (SCD).

Materials and Methods: A total of 50 patients with SCD, with 25 in steady-state condition (SCD-SS) and 25 in periods of painful vaso-occlusive crisis (SCD-VOC), and 25 healthy controls were included in the study. Demographical and clinical data were collected from hospital records. Serum LRG1, vascular endothelial growth factor A (VEGFA), and hypoxia-inducible factor 1-alpha (HIF1A) levels were measured by enzyme-linked immunosorbent assay (ELISA), and C-reactive protein (CRP) was measured by the nephelometric method. Routine biochemical parameters were assessed using an autoanalyzer. Multinomial logistic regression was used to analyze ELISA parameters, and receiver operating characteristic (ROC) curves were constructed to determine the optimal cut-off point for HIF1A to predict VOCs in SCD patients.

Results: LRG1 and VEGFA levels were significantly higher in SCD patients than controls (p<0.001), with no difference between the SCD-SS and SCD-VOC groups. HIF1A, CRP, and lactate dehydrogenase levels differed significantly across all groups, being highest in the SCD-VOC group (p<0.001). After adjusting for age and sex, LRG1, HIF1A, and VEGFA remained elevated in the SCD groups. HIF1A correlated with CRP (r=0.351, p=0.024), but LRG1 showed no correlation with proangiogenic mediators in the SCD-VOC group. The area under the ROC curve was calculated as 0.694 (95% confidence interval: 0.542-0.845, p=0.021) and the optimal cut-off point was 494.5 pg/mL for HIF1A in predicting vaso-occlusive crises in patients with SCD.

Öz

Amaç: Lösinden zengin alfa-2-glikoprotein 1 (LRG1), anormal anjiyogenezde rol oynayan yeni bir mediyatördür. Bu çalışmada, dolaşımdaki LRG1 düzeylerini ve bu düzeylerin orak hücre hastalığında (OHH) proanjiyogenik mediyatörlerle ilişkisini araştırmayı amaçladık.

Gereç ve Yöntemler: Çalışmaya, 25'i stabil durumda (OHH-SS) ve 25'i ağrılı vazooklüzif kriz (OHH-VOK) döneminde olmak üzere toplam 50 OHH hastası ile 25 sağlıklı kontrol birey dahil edildi. Demografik ve klinik veriler hastane kayıtlarından elde edildi. Serum LRG1, vasküler endotelyal büyüme faktörü A (VEGFA) ve hipoksi ile indüklenebilir faktör 1-alfa (HIF1A) düzeyleri ELISA yöntemiyle, C-reaktif protein (CRP) düzeyleri ise nefelometrik yöntemle ölçüldü. Rutin biyokimyasal parametreler otoanalizör ile değerlendirildi. ELISA parametreleri çoklu lojistik regresyon analizi ile incelendi ve OHH hastalarında VOK'leri öngörmede HIF1A için optimal eşik değeri belirlemek amacıyla alıcı işletim karakteristik eğrisi (ROC) analizi yapıldı.

Bulgular: LRG1 ve VEGFA düzeyleri OHH hastalarında kontrol grubuna kıyasla anlamlı şekilde yüksek bulundu (p<0,001); ancak OHH-SS ve OHH-VOK grupları arasında anlamlı fark gözlenmedi. HIF1A, CRP ve laktat dehidrogenaz düzeyleri tüm gruplar arasında anlamlı farklılık gösterdi ve en yüksek düzeyler OHH-VOK grubunda saptandı (p<0,001). Yaş ve cinsiyete göre düzeltme yapıldıktan sonra LRG1, HIF1A ve VEGFA düzeylerinin OHH gruplarında yüksek seyretmeye devam ettiği görüldü. HIF1A düzeyi CRP ile pozitif korelasyon gösterdi (r=0,351, p=0,024); ancak OHH-VOK grubunda LRG1 düzeyi ile proanjiyogenik mediyatörler arasında bir korelasyon saptanmadı. ROC eğrisi altında kalan alan 0.694 olarak hesaplandı (güven aralığı, %95: 0,542-0,845, p=0,021) ve HIF1A için OHH hastalarında VOK'leri öngörmede optimal kesim noktası 494,5 pg/mL olarak belirlendi.

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Abstract

Conclusion: Circulating LRG1 levels may reflect neutrophil activation and contribute to the cross-talk between proangiogenic mediators released in SCD.

Keywords: Sickle cell disease, LRG1, Angiogenesis, VEGF, HIF1A

Öz

Sonuç: Dolaşımdaki artmış LRG1 düzeyleri nötrofil aktivasyonunu yansıtabilir ve OHH'de salınan proanjiyogenik mediyatörler arasındaki etkileşime katkı sağlayabilir.

Anahtar Sözcükler: Orak hücre hastalığı, LRG1, Anjiyogenez, VEGFA, HIF1A

Introduction

Sickle cell disease (SCD), constituting a group of autosomal recessive hemoglobinopathies, are prevalent in the Çukurova region of Türkiye, and particularly in Hatay province [1]. It is a monogenetic disorder caused by a single base-pair mutation in the *β-globin* gene. This mutation results in the substitution of valine for glutamic acid at the 6th position of the β chain, forming abnormal hemoglobin (HbS) [2,3]. The polymerization of deoxygenated HbS results in the transformation of red blood cells from their biconcave shape into a sickle cell shape under low-oxygen conditions, leading to vaso-occlusion, especially in small vessels. Continuous and intermittent microvascular blockages initiate complex pathophysiological mechanisms, encompassing ischemia/reperfusion injury, hypercoagulability, heightened leukocyte adhesion to the endothelium, and ultimately tissue damage [4].

While the ischemic events of SCD have been extensively investigated, the role of hypoxia-induced angiogenic responses has not been fully elucidated in patients with SCD. Hypoxia-inducible factor 1-alpha (HIF1A) plays a pivotal role in maintaining oxygen homeostasis and is upregulated during hypoxia [5]. Under hypoxic or ischemic conditions, HIF1A initiates the upregulation of gene transcription and associated mediators responsible for promoting angiogenesis, such as vascular endothelial growth factor A (VEGFA). There are limited studies showing increased HIF1A expression levels in SCD [6,7]. Elevated levels of VEGFA have also been observed in patients with SCD, attributed to factors including hypoxia, ischemia, and vascular damage [7,8,9]. However, some studies have reported inconsistent findings [10]. While these observations suggest a correlation between hypoxia and angiogenesis, the presence of proangiogenic mechanisms in SCD remains unclear. Among the emerging factors, leucine-rich alpha-2-glycoprotein 1 (LRG1) is a secreted glycoprotein belonging to the leucine-rich repeat protein family [11]. LRG1 is primarily secreted from neutrophils, but it is also released from other cell types, including endothelial cells, epithelial cells, and fibroblasts [12]. Its primary function involves modulating angiogenesis, specifically pathological angiogenesis, by promoting vascular remodeling through the transforming growth factor beta (TGF-β) signaling pathway [13]. Elevated levels of LRG1 have been documented in diseases characterized by abnormal angiogenesis, such as diabetic kidney disease [14], multiple myeloma [15], and osteoarthritis [16].

However, there have been no studies investigating LRG1 levels in SCD. Considering the unbalanced angiogenesis resulting from hypoxia in patients with SCD, investigating the role of LRG1 may contribute to the elucidation of the cross-talk between angiogenetic and inflammatory mediators, including VEGF and HIF1A.

This is the first study evaluating serum LRG1 levels in patients with SCD. The aim of this study is to examine serum LRG1 levels and their relationships with VEGFA, HIF1A, and acute-phase reactants in patients with SCD during steady-state (SS) conditions and painful vaso-occlusive crisis (VOC) episodes.

Materials and Methods

Study Design

This was a prospective case-control study. A total of 50 patients with SCD as proven by Hb electrophoresis (homozygous sickle cell disease [HbSS] or sickle cell beta thalassemia [HbS/β-thal]) presenting to the Hematology Department of Hatay Mustafa Kemal University Hospital (Hatay, Türkiye) between December 2021 and January 2023 were included in the study. The control group consisted of 25 age- and sex-matched healthy subjects. Data on all relevant demographic and clinical features were obtained from the hospital's information system and recorded. Of the 50 patients with SCD, 25 were in SS condition, constituting the SCD-SS group, and 25 were in a period of painful VOC, constituting the SCD-VOC group. An acute VOC phase was clinically defined as a painful episode lasting more than 2 hours with the patient feeling that the pain was specific to vaso-occlusion. Doctors could not identify another etiology for the pain in these cases and the patients sought treatment for pain in the emergency department. The patients in the SS period were clinically defined as patients who had not been in VOC for at least 1 month before study inclusion [17].

Informed written consent was obtained from all patients and healthy control participants. This study was performed according to the Declaration of Helsinki's ethical principles for human medical research and the study protocol was approved by the Hatay Mustafa Kemal University Ethics Committee (protocol date and number: 06.05.2021 and 2021/47, decision no: 11).

Exclusion Criteria

Individuals with any hematological disorders other than SCD and patients with chronic pain unrelated to SCD were excluded.

Patients who had acute chest syndrome or other SCD-related complications within the past year as well as those with chronic kidney disease, inflammatory or connective tissue disease, acute or chronic infections, diabetes, obesity, liver failure, or coronary heart disease were excluded, as were smokers, alcohol consumers, those who had received transfusions in the last 3 months, individuals under 18 years of age, pregnant women, and breastfeeding women.

Sample Collection and Measurement of Biochemical Parameters

Whole blood (8 mL) for clot-activator gel tubes (BD Vacutainer Serum Separation Tubes, Becton Dickinson, Franklin Lakes, NJ, USA) and samples of blood with EDTA (4 mL) (BD Vacutainer K_2EDTA tubes, Becton Dickinson) were collected from SCD patients and the members of the healthy control group. In the SCD-VOC group, blood samples were collected within the first hour of presentation before administering any medication to the patient. After centrifugation at 1500 x g for 10 min (NF 1200 centrifuge, Nüve, Akyurt, Türkiye), samples were aliquoted for the measurement of routine biochemical data and enzyme-linked immunosorbent assay (ELISA) analyses and stored at -80 °C. Serum albumin, blood urea nitrogen, and creatinine levels and alanine aminotransferase, aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) activities were measured by the spectrophotometric method using an autoanalyzer (Advia 2400, Siemens, Tokyo, Japan), and serum high-sensitivity C-reactive protein (CRP) levels were measured by the nephelometric method (Advia 1200, Siemens). Hematological parameters were assayed using a whole blood count analyzer (Mindray BC-6800, Mindray, Shenzhen, China) within 2 hours of collection.

The serum LRG1, HIF1A, and VEGFA levels of all samples were measured by the ELISA method using commercially available ELISA kits (Elabscience, Wuhan, China) according to the manufacturer's protocol for the ELISA device (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA). Concentrations were calculated using a 4P-logic calibration curve, and the performance characteristics of the kits were as follows:

LRG1 ELSIA Kit (LOT: E-EL-H1287): Analysis range of 7.81-500 ng/mL, with sensitivity of 4.69 ng/mL; intra-assay coefficient of variability (CV) of <8% and inter-assay CV of <10%; final concentrations calculated by multiplying the dilution factor and given as µg/mL.

HIF1A ELISA Lit (LOT: E-EL-H6066): Analysis range of 62.5-4000 pg/mL, with sensitivity of 37.5 pg/mL; intra-assay CV of <8% and inter-assay CV of <10%.

VEGFA ELISA Kit (LOT: E-EL-H0111): Analysis range of 31.25-2000 pg/mL, with sensitivity of 18.75 pg/mL; intra-assay CV of <8% and inter-assay CV of <10%.

Statistical Analysis

IBM SPSS Statistics for Windows 21.0 (IBM Corp., Armonk, NY, USA) was used to analyze the data. Power analysis was performed using an effect size (Cohen's f) of 0.44, with alpha level of 0.05 and power of 0.80. The effect size was determined based on similar studies in the literature and pilot data previously obtained. The required sample size for each group was calculated to be at least 18 individuals. The normality of the data was evaluated using the Shapiro-Wilk test. Normally distributed variables were presented as mean ± standard deviation, whereas non-normally distributed variables were characterized by median (25^{th}-75^{th} interquartile range) values. Categorical data were analyzed using the chi-square test. Continuous variables with normal distribution were analyzed by one-way analysis of variance (ANOVA), followed by post-hoc Tukey or Tamhane T2 tests as appropriate. For non-normally distributed continuous variables, the Kruskal-Wallis test with post-hoc Bonferroni multiple comparison testing was utilized. The relationships between variables were assessed using the Pearson correlation test for normally distributed data and the Spearman correlation test for non-normally distributed data. Multinomial logistic regression analysis was conducted to evaluate the relationship between SCD groups and ELISA parameters. In the regression model, the ELISA parameters were defined as independent variables, while the disease groups (SCD-SS and SCD-VOC) were defined as dependent variables, with the control group serving as the reference category. Adjustments were made for age and sex, and the resulting odds ratios and 95% confidence intervals (CIs) were reported. We also constructed receiver operating characteristic (ROC) curves and calculated the sensitivities and specificities of HIF1A. The optimal cut-off point for HIF1A was detected for the prediction of VOC in patients with SCD. Values of $p<0.05$ were considered statistically significant.

Results

The demographic distribution and laboratory results of the participants are presented in Table 1. Of the 50 patients with SCD, 25 (50%) were male and 25 (50%) were female, with a mean age of 34±9 years. There were no statistically significant differences in age or sex between any of the three groups. The majority of the patients (n=47, 94%) had HbSS, while only 3 (6%) had sickle/β-thalassemia (HbS/β-thal). Fifteen patients (60%) in the SCD-VOC group and 13 patients (52%) in the SCD-SS group were using hydroxyurea.

Serum CRP levels and LDH activities were significantly different between all three groups, being highest in the SCD-VOC group ($p<0.001$). White blood cell (WBC) and neutrophil counts and AST activities were found to be higher in SCD patients compared to the healthy controls ($p<0.001$), but there was no significant difference between the SCD-VOC and SCD-SS groups. Hb

levels were significantly different between all three groups, being lowest in the SCD-VOC group, but albumin levels were significantly lower only in the SCD-VOC group (p<0.001) (Table 1). Serum LRG1 levels were significantly higher in both the SCD-SS and SCD-VOC groups compared to the control group (p=0.003 and p=0.001, respectively). There was no significant difference observed in serum LRG1 levels between the SCD-SS and SCD-VOC groups (Figure 1A). Serum HIF1A levels were significantly higher in both the SCD-SS and SCD-VOC groups compared to the controls (p<0.001) (Figure 1B), and the highest levels were observed in the SCD-VOC group (p=0.006). VEGFA levels were significantly higher in both the SCD-SS and SCD-VOC groups compared to the control group (p=0.004 and p<0.001, respectively), with no significant difference observed between the SCD-SS and SCD-VOC groups (Figure 1C). In the VOC-SCD group, serum HIF1A levels positively correlated with CRP levels (r=0.351, p=0.024). There were no significant correlations observed between LRG1 and VEGFA (r=0.047, p=0.832) or LRG1 and HIF1A (r=0.085, p=0.699) levels in the SCD-VOC and SCD-SS groups. According to the multinomial logistic regression analysis, after adjusting for age and sex, the SCD-SS and SCD-VOC groups had significantly higher levels of LRG1, HIF1A, and VEGFA compared to the control group (Table 2). The area under the curve (AUC) was calculated as 0.694 (95% CI: 0.542-0.845, p=0.021) and the optimal cut-off point was 494.5 pg/mL for HIF1A in predicting VOC in patients with SCD (Figure 2). The HbS levels of the SCD-SS and SCD-VOC groups were 76.5% (58.6%-88.4%) and 79.5% (76.5%-84.4%), respectively, with no significant difference between the groups (p=0.572). HbS levels were not correlated with LRG1, HIF1A, or VEGFA levels in SCD patients (r=0.099 and p=0.748, r=0.421 and p=0.152, and r=-0.022 and p=0.940, respectively).

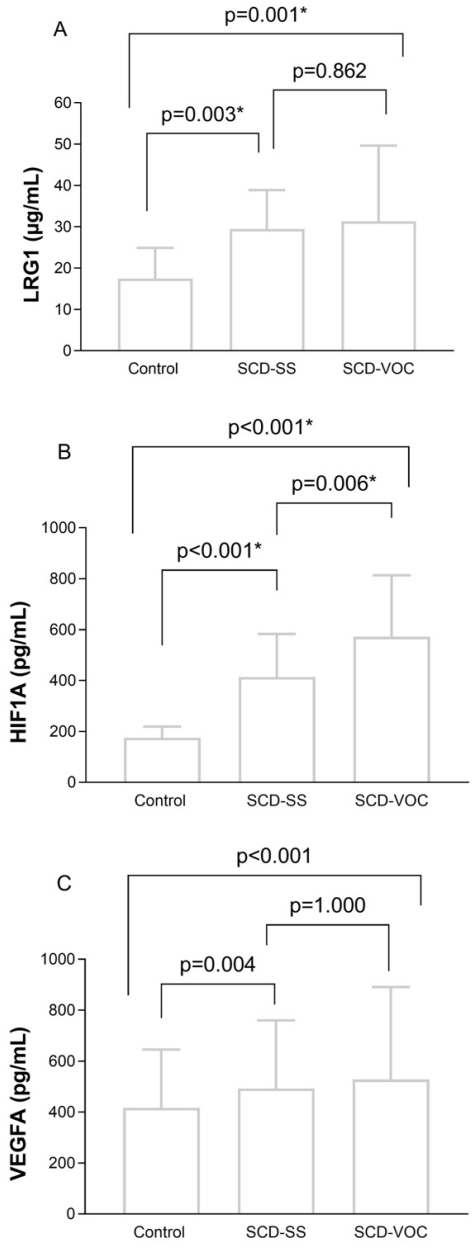

Figure 1. Comparisons of serum LRG1 (A), HIF1A (B), and VEGFA (C) among the groups.

*: ANOVA testing was used for normally distributed parameters. Kruskal-Wallis tests were conducted for other parameters. SCD-SS: Patients with sickle cell disease in steady-state condition; SCD-VOC: patients with sickle cell disease in painful periods of vaso-occlusive crisis; LRG1: leucine-rich alpha-2-glycoprotein 1; HIF1A: hypoxia-inducible factor 1-alpha; VEGFA: vascular endothelial growth factor A.

Discussion

In this study, serum levels of LRG1 and proangiogenic mediators HIF1A and VEGFA were measured at different stages of SCD.

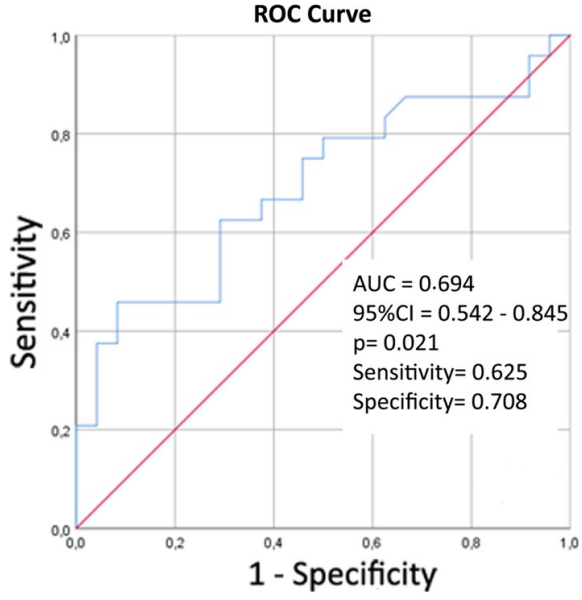

Figure 2. Receiver-operating characteristic (ROC) curve to determine the optimal threshold value for HIF1A in patients with SCD. The optimal cut-off point was 494.5 pg/mL for HIF1A parameter to estimate VOC in patients with SCD.

HIF1A: Hypoxia-inducible factor 1-alpha; SCD: sickle cell disease; VOC: vaso-occlusive crisis; AUC: area under the curve; CI: confidence interval.

Both LRG1 and VEGFA levels were significantly elevated in SCD patients compared to the healthy control group, with no significant differences observed between SS and VOC periods, which displayed similar patterns. Serum HIF1A levels were also significantly higher in SCD patients, with the highest levels observed in the VOC group, in contrast to LRG1 and VEGFA. Additionally, a significant but low correlation was observed between serum HIF1A and CRP levels, while no correlation was found between LRG1 and the proangiogenic mediators. HIF1A demonstrated better ability to distinguish VOC and SS conditions in SCD patients.

LRG1 levels were significantly elevated in both the SCD-SS and SCD-VOC groups compared to the healthy control group in this study (p=0.003 and p=0.001, respectively) (Figure 1A; Table 1).

Table 1. Comparisons of age, sex, and levels of biochemical parameters among the study groups.

Variables	Control (n=25) Mean ± SD Median (25^{th}-75^{th})	SCD-SS (n=25) Mean ± SD Median (25^{th}-75^{th})	SCD-VOC (n=25) Mean ± SD Median (25^{th}-75^{th})	p
Female, n (%)	17 (68)	11 (44)	14 (56)	0.232*
Age, years	33±7	35±9	33±9	0.715**
WBC, 10^3/µL	7.2 (5.9-8.3)	11.4 (6.9-13.7)	13.4 (10.7-17.6)	0.014[a] <0.001[b] 0.164[c]
Neutrophil counts, 10^3/µL	4.1 (3.4-5.2)	6.1 (4.1-8.9)	9.3 (6.3-13.1)	0.024[a] <0.00[b] 0.145[c]
Hemoglobin, g/dL**	14±1.6	9.3±2.1	7.7±1.1	<0.001[a] <0.001[b] 0.002[c]
Platelets, 10^3/µL	243 (211-288)	361 (219-444)	317 (184-415)	0.065
hsCRP, mg/L	0.92 (0.64-1.71)	4.39 (3.13-7.73)	34.7 (10.5-61.5)	<0.001[a] <0.001[b] 0.003[c]
Albumin, g/dL	4.6 (4.4-4.7)	4.3 (4.2-4.7)	3.7 (3.6-3.9)	0.398[a] <0.001[b] <0.001[c]
ALT, U/L	17 (14-26)	18 (14-29)	25 (14-33)	0.416
AST, U/L	15 (13-20)	40 (27-62)	50 (36-60)	<0.001[a] <0.001[b] 0.791[c]
LDH, U/L	145 (89-201)	339 (238-542)	638 (491-680)	<0.001[a] <0.001[b] 0.016[c]
BUN, mg/dL	11 (10-14)	9 (8-13)	8 (6-11)	0.280[a] 0.252[b] 0.002[c]
Creatinine, mg/dL	0.69 (0.61-0.79)	0.49 (0.42-0.61)	0.42 (0.34-0.55)	<0.001[a] <0.001[b] 0.813[c]
LRG1 (µg/mL)**	17.5±7.5	29.5±9.4	31.3±18.3	0.003[a] 0.001[b] 0.862[c]
HIF1A (pg/mL)**	174.8±44.7	413.5±169.3	572.1±241.5	<0.001[a] <0.001[b] 0.006[c]
VEGFA (pg/mL)	416 (374-484)	492 (417.5-630.5)	528 (446.5-589)	0.004[a] <0.001[b] 1.000[c]

*: Chi-square test; **: analysis of variance (ANOVA). Kruskal-Wallis tests were conducted for other parameters. [a]: Control group vs. SCD-SS group; [b]: Control group vs. SCD-VOC group; [c]: SCD-SS group vs. SCD-VOC group.

SCD-SS: Patients with sickle cell disease in steady-state condition; SCD-VOC: patients with sickle cell disease in painful periods of vaso-occlusive crisis. WBC: White blood cells; hsCRP: high-sensitivity C-reactive protein; ALT: alanine aminotransferase; AST: aspartate aminotransferase; LDH: lactate dehydrogenase; BUN: blood urea nitrogen; LRG1: leucine-rich alpha-2-glycoprotein 1; HIF1A: hypoxia-inducible factor 1-alpha; VEGFA: vascular endothelial growth factor A; SD: standard deviation.

Table 2. Multinomial logistic regression for the association between study groups and ELISA parameters.

		LRG1	HIF1A	VEGFA
Control		1 (reference)	1 (reference)	1 (reference)
SCD-SS	OR (95% CI)*	1.15 (1.053-1.256)	1.025 (1.010-1.042)	1.014 (1.005-1.022)
	p	0.002	0.002	0.002
SCD-VOC	OR (95% CI)*	1.2 (1.076-1.339)	1.029 (1.013-1.046)	1.015 (1.007-1.024)
	p	0.001	<0.001	<0.001

*: Adjusted for age and sex. The dependent variables were the control, SCD-SS, and SCD-VOC groups as categorical groups, with the control group serving as the reference. The presence of multicollinearity among independent variables was assessed using variance inflation factors (VIFs), and no variable with VIF of >5 was detected. Odds ratios (ORs) and their corresponding 95% confidence intervals (CIs) are presented.
SCD-SS: Patients with sickle cell disease in steady-state condition; SCD-VOC: patients with sickle cell disease in painful periods of vaso-occlusive crisis; LRG1: leucine-rich alpha-2-glycoprotein 1; HIF1A: hypoxia-inducible factor 1-alpha; VEGFA: vascular endothelial growth factor A.

Additionally, serum LRG1 levels remained significantly higher in the SCD-SS and SCD-VOC groups in multivariable models after adjusting for age and sex (Table 2). However, there was no significant difference between the VOC and SS periods. The elevated levels of LRG1 in SCD patients could possibly be associated with its secretion kinetics because LRG1 is packaged into the granule compartment of human neutrophils and secreted upon neutrophil activation to modulate the microenvironment [18]. Considering the increased granulopoiesis in SCD patients [19,20,21], we can say that LRG1 may be upregulated by the chronic systemic inflammation observed in SCD patients and contribute to microcirculatory irregularities. The significantly elevated WBC and neutrophil counts in both the SCD-VOC and SCD-SS groups compared to the control group in this study further support this idea (Table 1).

We also found higher serum CRP levels in patients compared to the control group, but its levels were highest in the SCD-VOC group (Table 1). CRP is a well-known acute-phase reactant and a laboratory indicator of the severity of inflammation in SCD. However, in the VOC group, there was no significant correlation between LRG1 levels and CRP, WBC, or neutrophil counts. Levels of LRG1 did not vary with disease severity. We can say that LRG1 is not an effective biomarker for distinguishing between crisis and remission periods in cases of SCD. Therefore, further studies are needed to understand the role of LRG1 as an acute-phase protein in patients with SCD. However, the significantly elevated levels of circulating LRG1 in patient groups suggest its potential involvement in the angiogenic imbalance associated with SCD. LRG1 exerts these effects by activating the proangiogenic pathway, promoting endothelial cell proliferation, migration, and tubulogenesis [11]. It has also been reported that LRG1 enhances TGF-β signaling in the endothelium, contributing to abnormal vascular growth in inflammatory and ischemic conditions [13]. This mechanism may also play a role in the pathogenesis of SCD. In a study conducted on a cardiomyocyte cell line, it was demonstrated that LRG1 significantly enhanced the expression of HIF1A [22]. Another study reported that LRG1 induces HIF1A and regulates epithelial-mesenchymal transition and angiogenesis in colorectal cancer [23]. Moreover, LRG1 has been demonstrated to guide glomerular endothelial cells toward a proangiogenic pathway [24] and to enhance ocular neovascularization in diabetic retinopathy [13]. In the present study, serum levels of VEGFA and HIF1A as proangiogenic mediators were also measured. Serum HIF1A levels were significantly increased in both the SCD-SS and SCD-VOC groups compared to healthy controls (p<0.001) (Figure 1B). This difference was significant after adjustment for age and sex (Table 2). HIF1A is a widely recognized key transcription factor that becomes activated in response to hypoxia and plays a vital role in restoring oxygen homeostasis [25,26]. Increased expression of HIF1A in SCD patients has been demonstrated in a limited number of studies [7,27]. Our findings are consistent with those of previous studies and support the idea of elevated HIF1A levels in SCD patients. Unlike LRG1, its levels were significantly higher in the SCD-VOC group compared to the SCD-SS group (p=0.006) (Figure 1B) and correlated positively with CRP levels (r=0.351, p=0.024). This was an anticipated result, as elevated levels of HIF1A in the SCD-VOC group may be associated with the severe hypoxia experienced by these patients. We also performed ROC analysis to determine the optimal threshold value for HIF1A in patients with SCD. The AUC was calculated as 0.694 (95% CI: 0.542-0.845, p=0.021) (Figure 2). Thus, we can say that a serum HIF1A cut-off value of 494.5 pg/mL is feasible for predicting VOC in SCD patients.

The elevated HIF1A levels observed in patients during the SS period were likely a result of ongoing chronic inflammation, as suggested by a previous study [28]. During hypoxia, it has been demonstrated that HIF1A rapidly binds to the regulatory region of the VEGFA-expressing gene, thereby initiating its transcription and translation [29,30]. In the present study, VEGFA levels were also found to be elevated in patients during the SS and VOC periods compared to the controls (p=0.004 and p<0.001, respectively), similar to LRG1. However, no significant difference was observed between the two periods (Figure 1C). VEGFA has been previously investigated in patients with SCD; however, conflicting results have been reported. While some

studies have found no differences in VEGFA levels between patients with SCD during VOC and SS periods [10,31], others have reported higher VEGFA levels during VOC episodes [32]. The differences between studies could be related to the antiangiogenic effects of hydroxyurea, a drug used in SCD treatment. One study suggested that hydroxyurea reduces VEGFA and HIF1A expression under both in vivo and in vitro conditions [33]. Although the underlying mechanism has not yet been elucidated, hydroxyurea treatment may affect serum LRG1 and VEGF levels through novel vascular mechanisms in the patients at different stages of SCD. The use of hydroxyurea was comparable between the two patient groups during the SS (52%) and VOC (60%) periods in the present study. This may explain the lack of a significant difference between the two groups. Serum VEGFA levels demonstrated a pattern similar to that of serum LRG1 in patients with SCD in this study. A similar relationship was reported in another study conducted on tumor cells, wherein LRG1 was shown to directly induce VEGFA expression and promote angiogenesis in colorectal cancer cells [23]. Another study showed that knockdown of LRG1 dramatically reduced VEGFA expression in the mouse retina [13]. In the present study, the elevated LRG1 and VEGFA levels of SCD patients may have caused the formation of non-functional vessels, contributing to the unbalanced angiogenesis triggered by ischemic processes.

Study Limitations

The first limitation of this study is its small sample size due to its single-center design. Another limitation is drug use. More than half of the patients were receiving hydroxyurea treatment, which has previously been reported to affect the serum levels of some markers. Therefore, further studies with larger case groups considering drug usage are needed.

Conclusion

In this study, we observed elevated levels of LRG1 in SCD patients together with VEGF and HIF1A as other proangiogenic mediators. HIF1A may prove to be more useful in distinguishing between VOC and SS periods of SCD. Circulating LRG1 levels may reflect neutrophil activation in SCD and contribute to the cross-talk between proangiogenic mediators released during hypoxia. However, the small cohort of this study does not allow the definitive confirmation of LRG1 as a biomarker for VOC. Larger studies with more extensive sample sizes are needed to validate its potential as a biomarker in patients with SCD during the VOC period.

Ethics

Ethics Committee Approval: This study was performed according to the Declaration of Helsinki's ethical principles for human medical research and the study protocol was approved by the Hatay Mustafa Kemal University Ethics Committee (protocol date and number: 06.05.2021 and 2021/47, decision no: 11).

Informed Consent: Informed written consent was obtained from all patients and healthy control participants.

Footnotes

Authorship Contributions

Surgial and Medical Practices: M.K., L.S.D.B.; Concept: O.Ö., M.K.; Design: O.Ö., M.K.; Data Collection or Processing: F.H.E., H.O., L.S.D.B.; Analysis or Interpretation: O.Ö., F.H.E., H.O.; Literature Search: O.Ö. F.H.E., L.S.D.B.; Writing: O.Ö., M.K., H.O. H.K., A.A.

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RESEARCH ARTICLE

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hsa_circRNA_092488 Exacerbates the Progression of Deep Vein Thrombosis Through the NLRP3/NF-κB Signaling Pathway

hsa_circRNA_092488, NLRP3/NF-κB Sinyal Yolu Aracılığı ile Derin Ven Trombozunun İlerlemesini Şiddetlendirir

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Abstract

Objective: Deep vein thrombosis (DVT) is a vascular disorder with an incidence rate of about 0.1%. Endothelial progenitor cells (EPCs) are precursor cells of endothelial cells and contribute to vascular repair and regeneration. Circular RNA (circRNA) has become a new focus of research as circRNAs are involved in various biological processes including the progression of DVT. This study explored the upregulation of hsa_circRNA_092488 in DVT patients.

Materials and Methods: The expression of hsa_circRNA_092488 was evaluated in venous blood samples obtained from DVT patients (n=42) and healthy controls (n=42). Gain- and loss-of-function studies of hsa_circRNA_092488 were carried out. The expression levels of related RNAs and proteins were examined by quantitative real-time reverse-transcription polymerase chain reaction, western blotting and immunofluorescence assays. The proliferation, migration, cell cycle progression, and apoptosis of transfected cells were measured by CCK-8 assay, transwell assay, and flow cytometry. The association of hsa_circRNA_092488 and NOD-like receptor protein 3 (NLRP3) in EPCs was revealed using RNA pull-down analysis. Furthermore, the stability of NLRP3 mRNA was examined in transfected EPCs.

Results: Upregulation of hsa_circRNA_092488 was detected in blood samples from DVT patients and it had the ability to suppress the proliferation and migration of EPCs, induce cell cycle arrest from the S to the G0/G1 phase, and trigger cellular apoptosis. Furthermore, NLRP3 was identified as the potential downstream target molecule of hsa_circRNA_092488 and it could exert its regulatory functions by activating the NLRP3/nuclear factor (NF)-κB signaling pathway. Overexpression of hsa_circRNA_092488 in cells notably elevated the protein expression of caspase-1, interleukin-1β, P-NF-κB-p65/NF-κB-p65, and P-IκBα/IκBα, while knockdown of hsa_circRNA_092488 significantly reduced the levels of those proteins in EPCs.

Conclusion: hsa_circRNA_092488/NLRP3/NF-κB signaling could be a novel therapeutic candidate for the treatment of DVT.

Keywords: hsa_circRNA_092488, NLRP3, NF-κB, Deep vein thrombosis

Öz

Amaç: Derin ven trombozu (DVT), yaklaşık %0,1'lik bir insidans oranına sahip bir vasküler hastalıktır. Endoteliyal progenitor hücreler (EPC), endoteliyal hücrelerin öncü hücreleridir ve vasküler onarım ve rejenerasyona katkıda bulunurlar. Dairesel RNA (circRNA), DVT'nin ilerlemesi de dahil olmak üzere çeşitli biyolojik süreçlerde yer alması nedeniyle yeni bir araştırma odağı haline gelmiştir. Bu çalışmada, DVT hastalarında hsa_circRNA_092488'in yukarı düzenlenmesi araştırıldı.

Gereç ve Yöntemler: hsa_circRNA_092488 ekspresyonu DVT hastalarından (n=42) ve sağlıklı kontrollerden (n=42) alınan venöz kan örneklerinde değerlendirildi. hsa_circRNA_092488'in fonksiyon kazanımı ve kaybı çalışmaları yapıldı. İlgili RNA ve proteinlerin ekspresyon düzeyleri kantitatif gerçek zamanlı ters transkripsiyon polimeraz zincir reaksiyonu, western blotting ve immünofloresan analizleri ile incelendi. Transfekte hücrelerin proliferasyonu, göçü, hücre döngüsü ilerlemesi ve apoptozu CCK-8 analizi, transwell analizi ve akış sitometrisi ile ölçüldü. hsa_circRNA_092488 ve NOD benzeri reseptör protein 3'ün (NLRP3) EPC'lerdeki ilişkisi RNA çekim analizi kullanılarak ortaya kondu. Ayrıca, NLRP3 mRNA'nın stabilitesi transfekte EPC'lerde incelendi.

Bulgular: DVT hastalarının kan örneklerinde hsa_circRNA_092488'in yukarı yönlü düzenlenmesi tespit edildi ve bu proteinin EPC'lerin çoğalmasını ve göçünü baskılama, hücre döngüsünün S'den G0/G1 fazına kadar durmasını sağlama ve hücresel apoptozu tetikleme yeteneği olduğu görüldü. Ayrıca, NLRP3, hsa_circRNA_092488'in potansiyel alt akış hedef molekülü olduğu ve düzenleyici işlevlerini NLRP3/nükleer faktör (NF)-κB sinyal yolunu aktive ederek uygulayabileceği tanımlandı. Hücrelerde hsa_circRNA_092488'in aşırı ekspresyonu, kaspaz-1, interlökin-1β, P-NF-κB-p65/NF-κB-p65 ve P-IκBα/IκBα'nın protein ekspresyonunu belirgin şekilde yükseltirken, hsa_circRNA_092488'in baskılanması, bu proteinlerin EPC'lerdeki seviyelerini önemli ölçüde azalttı.

Sonuç: hsa_circRNA_092488/NLRP3/NF-κB sinyal iletimi DVT tedavisinde yeni bir tedavi adayı olabilir.

Anahtar Sözcükler: hsa_circRNA_092488, NLRP3, NF-κB, Derin ven trombozu

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Introduction

Deep vein thrombosis (DVT) is a multifactorial disorder that may be triggered by numerous risk factors including pregnancy, immobility, and thrombophilia [1]. The risk factors for DVT particularly include elements that favor the formation of thrombi, including venous stasis, vascular injury, and hypercoagulability [2]. However, the detailed mechanisms underlying the onset and progression of DVT are not fully understood. It has been reported that alterations in venous blood flow, endothelial activation, adhesion of platelets and leukocytes, and activation of coagulation play essential roles in the pathogenesis of DVT [1,2]. The diagnosis is based on a high degree of suspicion considering the risk factor history, D-dimer measurements, and ultrasound scans showing the presence of deep vein clots [1]. Other relevant tests include pulmonary artery computed tomography scans and ventilation-perfusion scans when pulmonary embolism is suspected [1,2].

Endothelial progenitor cells (EPCs) are precursor cells of endothelial cells and contribute to vascular repair and regeneration [3]. Under physiological or pathological stimulation, they can be mobilized from the bone marrow into the peripheral blood to participate in the repair of damaged blood vessels [4]. Studies have shown that EPCs play important roles in cardiovascular and cerebrovascular diseases, peripheral vascular diseases, tumor angiogenesis, and wound healing [5].

Circular RNAs (circRNAs) are associated with vascular dysfunction in several vascular disorders and could be used as disease biomarkers [6]. They constitute a group of essential post-transcriptional regulators and they are formed by the head-to-tail splicing of exons, reflecting the unrecognized regulatory potential of coding sequences [6]. For instance, circ_0020123 and hsa_circ_0001020 may affect EPC migration, invasion, and tube formation during the development of DVT [7,8]. The knockdown of circ_0020123 enhanced the proliferation and angiogenesis of human umbilical vein endothelial cells in vitro and it was considered as a putative biomarker for the onset of DVT in pregnant women [7]. In addition, the upregulation of hsa_circ_0001020 was observed in both DVT patients and a mouse model, and it suppressed the migration and invasion of EPCs as well as tube formation by regulating the miR-29c-3p/MDM2 signaling pathway [8]. The knockdown of hsa_circ_0001020 in mice with DVT inhibited thrombosis and enhanced the homing ability of EPCs into thrombi [8], while the upregulation of hsa_circRNA_092488 was observed in DVT and its involvement in inflammation was also reported [9,10].

In this study, we explore the upregulation of hsa_circRNA_092488 in patients with DVT and its ability to inhibit the proliferation and migration of EPCs, cause a shift in the cell cycle from the S to the G0/G1 stage, and induce apoptosis. In addition, the ability of hsa_circRNA_092488 to exert its regulatory functions via the NOD-like receptor protein 3 (NLRP3)/nuclear factor (NF)-κB pathway was evaluated.

Materials and Methods

Characterization of CircRNAs

For reverse transcription, oligo-dT and random primers were mixed with isolated RNA and then the levels of circular and linear RNAs were evaluated. The incubation of total RNA (~2 μg) and RNase R (3 U/μg; Epicentre Technologies, Madison, WI, USA) was carried out at 37 °C for 30 min. After treatment with RNase R, RNA expression was measured using quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR).

Patient Specimens and Cell Cultures

The expression of hsa_circRNA_092488 was evaluated in venous blood samples obtained from patients with DVT (n=42) and healthy individuals (n=42) who had presented to the First Affiliated Hospital of Jinzhou Medical University between June 2018 and May 2020. Venous blood samples were collected from the median cubital vein after 6 h of fasting immediately after diagnosis. The median value of hsa_circRNA_092488 expression was used as the cutoff. Samples with values below the median were classified as "low" and those above the median were classified as "high." Demographic factors including sex, age, history of smoking, and obesity were assessed and no significant differences in these factors were observed between the DVT patients with low-level and high-level hsa_circRNA_092488. The clinical features of the analyzed patients are summarized in Table 1. This study included patients who were diagnosed with DVT using color Doppler ultrasound and lower extremity angiography. Individuals with a history of diabetes mellitus, hypertension, other chronic diseases, and recent immobilization or surgery were excluded, as were those using anticoagulants or platelet inhibitors, undergoing hormone therapy, or having concurrent tumors. All procedures were performed in line with the Declaration of Helsinki and the protocols were reviewed by the Ethics Committee of the First Affiliated Hospital of Jinzhou Medical University (decision no: 202047). Informed consent was obtained from all analyzed patients and controls.

To isolate mononuclear cells (MNCs), circulating blood (~100 mL) was obtained from the patients with DVT and the healthy controls using Vacutainer EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and samples were kept in the dark before being processed. The isolation of MNCs was performed at 5003 x g for 30 min using a density-gradient centrifuge (Biocoll, Biochrom, Berlin, Germany). Samples were then rinsed with phosphate-buffered saline (PBS) three times. Isolated cells were inoculated on cell culture dishes precoated with commercial fibronectin (Sigma, Darmstadt, Germany) and cultured using EGM growth medium for endothelial cells (GE Healthcare Life

Table 1. Clinical features of analyzed patients.

Parameter	Number of patients	Low hsa_circRNA_092488 expression (n=21)	High hsa_circRNA_092488 expression (n=21)	p
Age, years				
≤40	22	12	10	0.54
>40	20	9	11	
Sex				
Female	21	11	10	0.76
Male	21	10	11	
Smoking				
Yes	19	8	11	0.35
No	23	13	10	
BMI (kg/m^2)		24.62±6.17	25.24±6.35	0.75

BMI: Body mass index.

Sciences, Chicago, IL, USA), which contains human epidermal growth factor (10 ng/mL), bovine brain extract (12 mg/mL), hydrocortisone (1 mg/mL), human insulin-like growth factor-1 (50 ng/mL), and penicillin/streptomycin (all from Gibco, Grand Island, NY, USA). In addition, heparin (10 U/mL) was added to prevent platelet coagulation, and cells were incubated at 37 °C with 5% CO_2. EPC colonies were visible after about 2 weeks.

Transfection

The annealing of small hairpin RNA segments was carried out and then the plasmids were cloned into the pU6-Luc-Puro lentivirus vector (Genepharm Co. Ltd., Pallini, Greece). To produce a cell model with the overexpression of hsa_circRNA_092488, WT (oe- hsa_circRNA_092488) and MUT (oe-NC) fragments were amplified by PCR and then the segments were inserted into the PLCDH-cir expression vector (Invitrogen, Waltham, MA, USA). To verify the transfection efficiencies, down- or upregulation of hsa_circRNA_092488 was confirmed using qRT-PCR. Briefly, cells were seeded onto a 24-well plate and 1 μg of plasmids were used per well for each transfection.

Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction

The extraction of total RNA was performed using TRIzol reagent (Sobao Biotechnology, Shanghai, China). Subsequently, reverse transcription was carried out using the PrimeScript RT Reagent Kit (Invitrogen, Shanghai, China), and the SYBR Green PCR reagent was used for qRT-PCR on the 7500 RT-PCR System (Applied Biosystems, Foster City, CA, USA). Paired primers were produced by Xinbei Biotechnology (Shanghai, China) as follows: hsa_circRNA_092488, forward 5′-GCAGGTTGTTCATCGGGCATTC-3′, reverse 5′-GGCTTACAGC-ACGGAAGTGTTTC-3′; Ki-67, 5′-AGCGGCTCTCTTTAACACAGT-3′, 5′-TGACCCCCAAAGGATACACG-3′; NLRP3, 5′-GGACTGAAGCA-CCTGTTGTGCA-3′, 5′-TCCTGAGTCTCCCAAGGCATTC-3′; GAPDH, 5′-GTCTCCTCTGACTTCAACAGCG-3′, 5′-ACCACCCTGTTGCTGT-AGCCAA-3′; U6, 5′-CTCGCTTCGGCAGCACATA-3′, 5′-AACGATTCA-CGAATTTGCGT-3′. Endogenous U6 or GAPDH was used as the control for the normalization of the expression of miRNA or mRNA. For mRNA expression analysis, untreated RNA samples were used. For circRNA expression analysis, RNA samples treated with RNase R were used to ensure the removal of linear RNA.

Western Blotting

Concentrations of extracted protein were determined using a BCA Kit (Dingguo Biotechnology, Guangzhou, China). The separation of proteins (~40 μg) was carried out by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Subsequently, samples were transferred to a PVDF membrane (Thermo Fisher Scientific, Waltham, MA, USA). The membranes were then blocked with 5% skimmed milk at room temperature for 1 h and then incubated using primary antibodies against NLRP3 (1:1000; Cat No. ab263899; Abcam, Cambridge, UK), caspase-1 (1:500; Cat No. ab207802; Abcam), IL-1β (1:500; Cat No. ab283818; Abcam), p-NF-κB-p65 (1:500; Cat No. 3033; Cell Signaling Technology, Danvers, MA, USA), NF-κB-p65 (1:500; Cat No. 3034; Cell Signaling Technology), P-IκBα (1:1000; Cat No. 2859; Cell Signaling Technology), IκBα (1:1000; Cat No. 4818; Cell Signaling Technology), and GAPDH (1:2000; Cat No. 4967; Cell Signaling Technology) in a cold room overnight. The next day, incubation was performed using anti-mouse IgG (1:2000; Cat No. ab6728; Abcam) or anti-rabbit IgG (1:2000; Cat No. ab6721; Abcam) for 1 h.

CCK-8 Assay

Cell proliferation under various transfection conditions was measured by CCK-8 assay. Transfected cells were inoculated using 96-well plates and then 10 μL of the CCK-8 mixture (Dojindo, Kumamoto, Japan) was added to the cells. Cell proliferation was measured at different time points (days 1, 2, 3, and 4). Further

incubation of the cells was then carried out for an additional 2 h and the absorbance at 450 nm was recorded by microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Transwell Experiment

A transwell kit (ChenGong Biotechnology, Shanghai, China) was used to evaluate cell migration. A cell suspension (~5x10^5 cells) was added to the upper chamber (pore size: 8 µm; Becton Dickinson), which was precoated with Matrigel (Sigma-Aldrich, St. Louis, MO, USA). Subsequently, 500 µL of culture medium supplemented with 10% fetal bovine serum was loaded in the lower chamber. After 48 h, non-migratory cells were discarded using a cotton bud. Fixation of the cells that remained in the lower chamber was carried out using ice-cold methanol for 15 min. Cells were then stained using 0.5% crystal violet solution.

Cell Cycle Analysis and Measurement of Apoptosis

Cells were seeded at a density of 5x10^5 cells/well on 6-well plates. Subsequently, they were spun down at low speed (1000 rpm) at 4 °C for 5 min. The obtained pellets were then washed three times and suspended using PBS, and then fixation of the cells was performed using 70% ice-cold ethanol and samples were stored at 4 °C for 48 h. Lysis of the cells was then performed before flow cytometry and the cells were centrifugated and suspended using PI staining solution with 50 µL/mL PI and 250 µL/mL RNase A (Sigma-Aldrich). The distribution of cell cycle phases was evaluated using a flow cytometry device (Becton Dickinson) and the data were analyzed using FlowJo 7.6 software (FlowJo LLC, Becton Dickinson). For cell apoptosis, the cell suspension was incubated at 4 °C in the dark for 30 min and staining was performed using 5 µL of annexin V-FITC (JingMei Biotech, Beijing, China).

RNA Pulldown Assay

The probes of NLRP3 and the negative control, which were labeled with biotin, were generated by GenePharma (Shanghai, China). The labeling of cell lysates was carried out with Dynabeads M-280 Streptavidin (Thermo Fisher Scientific). Beads conjugated with immobilized NLRP3 were treated using ethylenediaminetetraacetic acid (10 mM).

Assessment of mRNA Stability

To prevent the additional synthesis of RNA, treatment with actinomycin D (5 µg/mL; MedChemExpress, Monmouth Junction, NJ, USA) was applied to the cells. Subsequently, treatment with actinomycin D was carried out at different time points. The extraction of RNA was then performed and samples were subsequently used for qRT-PCR. Remaining levels of NLRP3 RNA at different time points were normalized to the level at the start of the treatment.

Immunofluorescence Analysis

The fixation of cells was conducted with prechilled acetone (Sigma-Aldrich) for 20 min. Subsequently, fixed cells were washed using PBS and further incubated in blocking buffer for 30 min. Incubation with primary anti-Ki67 antibody (1:500; Cat. No. ab15580; Abcam) was then carried out at 4 °C overnight. The following day, samples were washed three times and incubation of the cells was carried out for 60 min with a secondary antibody conjugated with Alexa-Fluor 568 (1:1000, Molecular Probes, Eugene, OR, USA). Subsequently, nuclei were stained with DAPI solution and the stained cells were washed three times and mounted on slides using 10% Mowiol D488 reagent (Calbiochem, Nottingham, UK).

Statistical Analysis

Data obtained in the experiments of this study are shown as mean ± standard error of the mean. Data were analyzed using IBM SPSS Statistics 26 (IBM Corp., Armonk, NY, USA). Statistical significance was determined with Student's t-test or one-way analysis of variance (ANOVA). The post-hoc Tukey test was conducted after ANOVA. The Pearson correlation test was used to examine correlations of relative gene expression. The chi-square test was used to assess significant differences between the low- and high-hsa_circRNA_092488 expression groups. The measurement data conformed to normal distribution.

Results

CircRNA Characterization in Endothelial Progenitor Cells

For the characterization of circRNAs in EPCs, the levels of linear RNAs were measured following the addition of oligo-dT primer, whereas circRNAs were not observed (Figure 1A). Furthermore, linear RNAs were sensitive to digestion induced by RNase R but not circRNAs (Figure 1B).

Upregulation of hsa_circRNA_092488 Revealed in Samples from Patients with Deep Vein Thrombosis

Levels of hsa_circRNA_092488 were remarkably elevated in patients with DVT compared to the healthy controls (Figure 1C). To explore the roles of hsa_circRNA_092488 upregulation in the progression of DVT, function experiments were carried out. Cell models with hsa_circRNA_092488 overexpression and knockdown were respectively generated (Figures 1D and 1E).

Involvement of hsa_circRNA_092488 in Biological Behavioral Changes of Endothelial Progenitor Cells

The results of the CCK-8 assay revealed that overexpression of hsa_circRNA_092488 significantly inhibited the proliferation of EPCs (Figure 2A), while knockdown of hsa_circRNA_092488 remarkably promoted the proliferative activity of the cells (Figure 2B). In addition, levels of Ki-67 were decreased in EPCs with

hsa_circRNA_092488 overexpression (Figures 2C and 2D) and were enhanced in cells treated with sh-hsa_circRNA_092488 (Figures 2E and 2F).

Furthermore, the migration of EPCs was suppressed by the overexpression of hsa_circRNA_092488 (Figures 3A and 3B), while cell migration was elevated after transfection with sh-hsa_circRNA_092488 (Figures 3C and 3D). The results of cell cycle phase distribution suggested the shift of EPCs from the S to the G0/G1 phase following treatment with oe-hsa_circRNA_092488 (Figures 3E and 3F). In contrast, the proportion of cells at the G0/G1 phase was reduced and the proportion at the S phase was elevated after the knockdown of hsa_circRNA_092488 (Figures 3G and 3H).

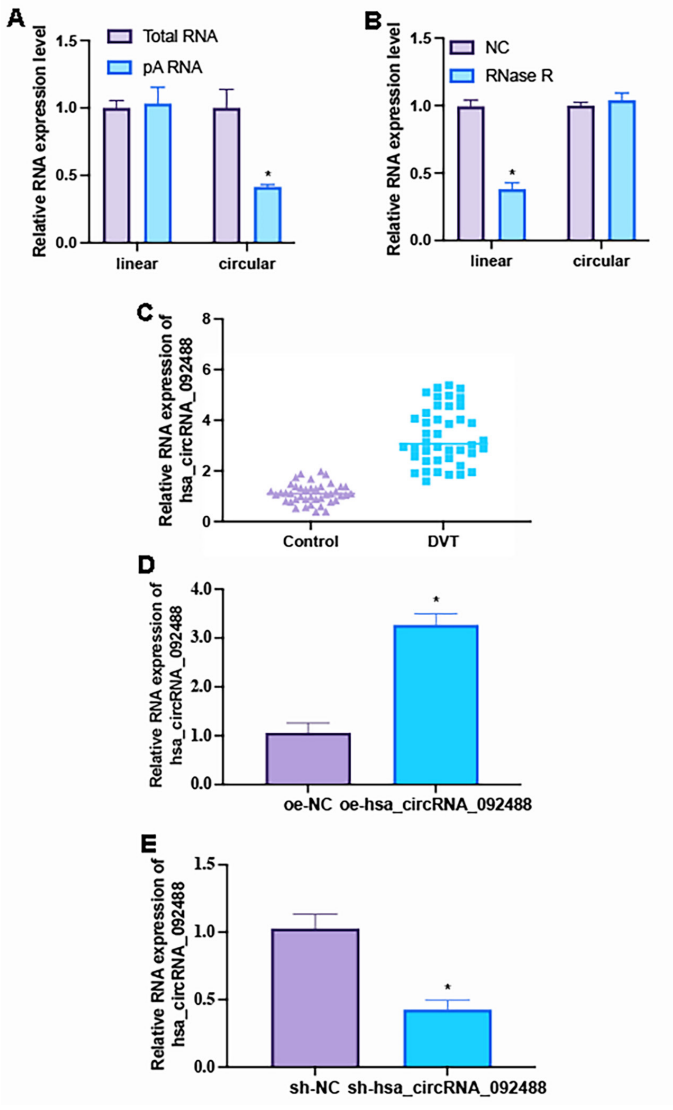

Figure 1. Characterization of circular RNAs (circRNAs) in endothelial progenitor cells and upregulated hsa_circRNA_092488 in deep vein thrombosis. A) Linear RNAs were observed after adding oligo-dT primer while there was no expression of circRNAs. B) The results revealed the resistance of circRNAs to digestion induced by RNase R but not linear RNAs. C) hsa_circRNA_092488 was upregulated in cases of deep vein thrombosis compared to the healthy control group. D, E) Cells with hsa_circRNA_092488 overexpression and knockdown were produced using lentiviral vectors.

*: p<0.05; NC: negative control; DVT: deep vein thrombosis.

Flow cytometry revealed that hsa_circRNA_092488 overexpression triggered cell apoptosis in EPCs (Figures 4A and 4B). In contrast, the apoptosis rate was decreased after transfection of sh-hsa_circRNA_092488 (Figures 4C and 4D).

NLRP3 as the Novel Target of hsa_circRNA_092488

The association of hsa_circRNA_092488 and NLRP3 in EPCs was revealed using RNA pull-down analysis (Figure 5A). The stability of NLRP3 mRNA in EPCs was found to be enhanced after transfection with oe-hsa_circRNA_092488 (Figure 5B). In contrast, the mRNA stability was decreased in EPCs with hsa_circRNA_092488 knockdown (Figure 5C). In addition, NLRP3 expression was enhanced in samples obtained from patients with DVT (Figure 5D), and the levels of hsa_circRNA_092488 and NLRP3 were positively correlated in those samples (Figure 5E; $r=0.602$, $p<0.05$). Western blotting demonstrated that NLRP3 protein levels were increased in EPCs treated with oe-hsa_circRNA_092488 and reduced in cells transfected with sh-hsa_circRNA_092488 (Figures 5F and 5G).

Functioning of hsa_circRNA_092488 Through the NLRP3/NF-κB Pathway

The protein expression of NLRP3/NF-κB-associated molecules was examined (Figure 6). The results of western blotting showed that overexpression of hsa_circRNA_092488 in cells notably elevated the protein expression levels of caspase-1, IL-1β, P-NF-κB-p65/NF-κB-p65, and P-IκBα/IκBα, while the knockdown of hsa_circRNA_092488 significantly reduced the levels of these proteins in EPCs.

Discussion

In previous studies, circRNAs were shown to bind to miRNA to regulate the expression of target genes; thus, they have become a new focus of research as they are involved in various biological processes [6]. For example, circ_0020123 and hsa_circ_0001020 modulated EPC migration and tube formation and regulated the progression of DVT [7,8]. Upregulated hsa_circRNA_092488 was detected in DVT and its involvement in inflammation was reported [9,10]. In the present study, the biological functions of hsa_circRNA_092488 in DVT were explored and the underlying mechanisms were evaluated.

In our experiments, upregulated hsa_circRNA_092488 was confirmed in DVT patients and it inhibited EPC proliferation and migration, triggered the shift of the cell cycle from the S phase to the G0/G1 phase, and induced cell apoptosis. Similarly, upregulation of hsa_circ_0001020 accelerated the

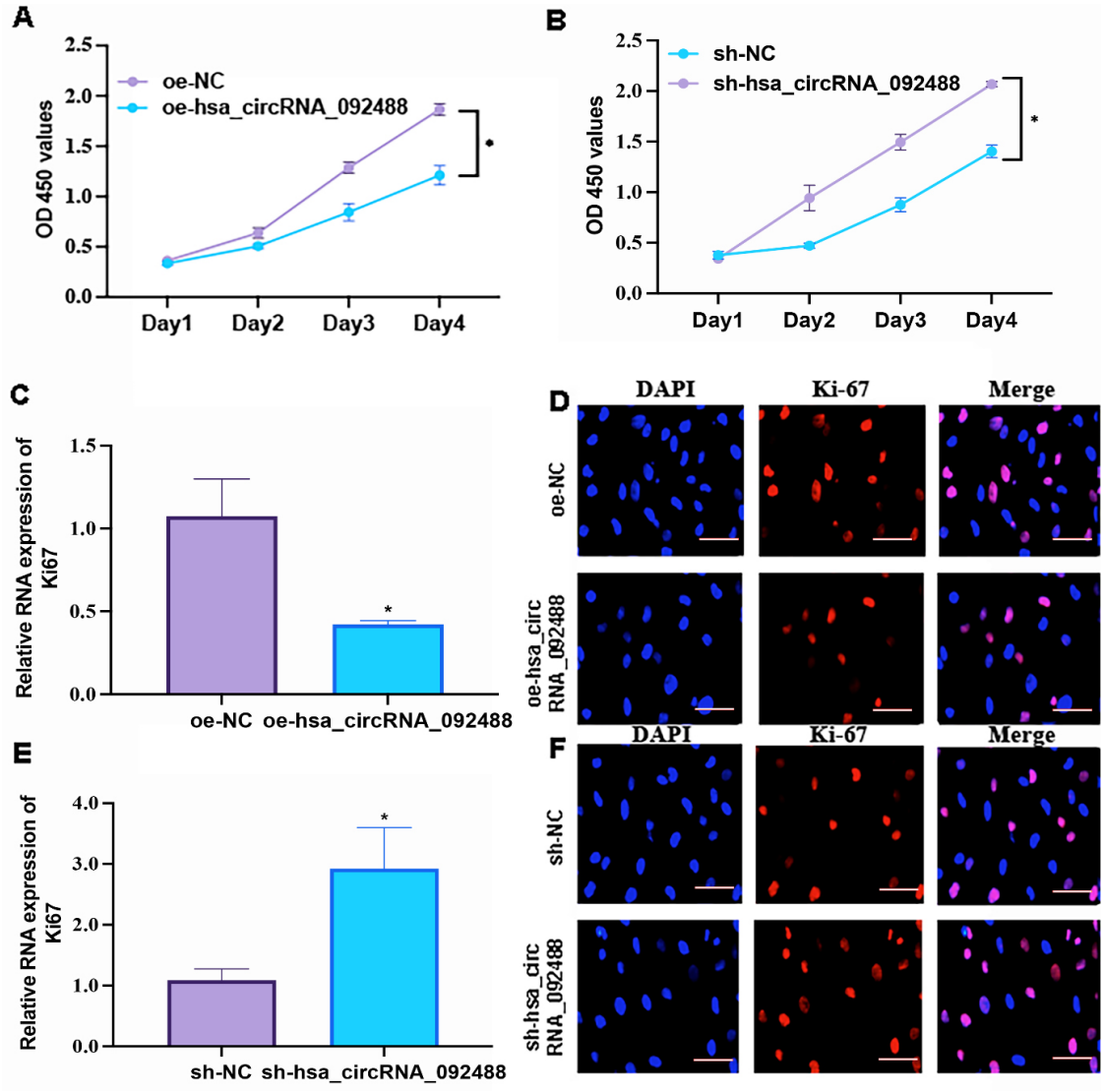

Figure 2. hsa_circRNA_092488 participated in the regulation of biological behaviors of endothelial progenitor cells (EPCs). A) Overexpression of hsa_circRNA_092488 notably downregulated the proliferative activity of EPCs. B) Knockdown of hsa_circRNA_092488 enhanced cell proliferation. C-F) Subcellular staining with Ki-67 was also evaluated in EPCs following treatment with oe-hsa_circRNA_092488 or sh-hsa_circRNA_092488.

*: p<0.05; magnification for (D): 40×; scale bar: 50 μm.

development of DVT by sponging miR-29c-3p to promote the expression of MDM2 [8]. In addition, the long non-coding RNA CRNDE promoted DVT by sequestering miR-181a-5p away from thrombogenic Pcyox1l [11]. In that study, both CRNDE and Pcyox1l levels were enhanced in the blood of mice with DVT and miR-181a-5p was the novel target of CRNDE. Furthermore, the knockdown of CRNDE and restoration of miR-181a-5p both inhibited inflammatory injury, therefore suppressing the formation of thrombi in the mice [11].

NLRP3 has been explored as the putative target of hsa_circRNA_092488. NLRP3 is a protein encoded by the *NLRP3* gene located on the long arm of chromosome 1 [12]. NLRP3 is abundantly detected in macrophages and is a component of inflammasomes. Research has indicated that various inflammatory diseases are associated with NLRP3 inflammasomes [12]. NLRP3 inflammasomes could be activated by a variety of stimuli, such as mitochondrial dysfunction and elevated levels of reactive oxygen species [13]. Dysregulated activation of NLRP3 inflammasomes has been implicated in numerous disorders including diabetes, atherosclerosis, and Alzheimer disease. Small-molecule inhibitors targeting NLRP3 inflammasomes have shown certain therapeutic potential but their clinical feasibility still requires further exploration. Once activated, NLRP3, along with the adaptor protein ASC and the effector protein caspase-1, initiates the assembly

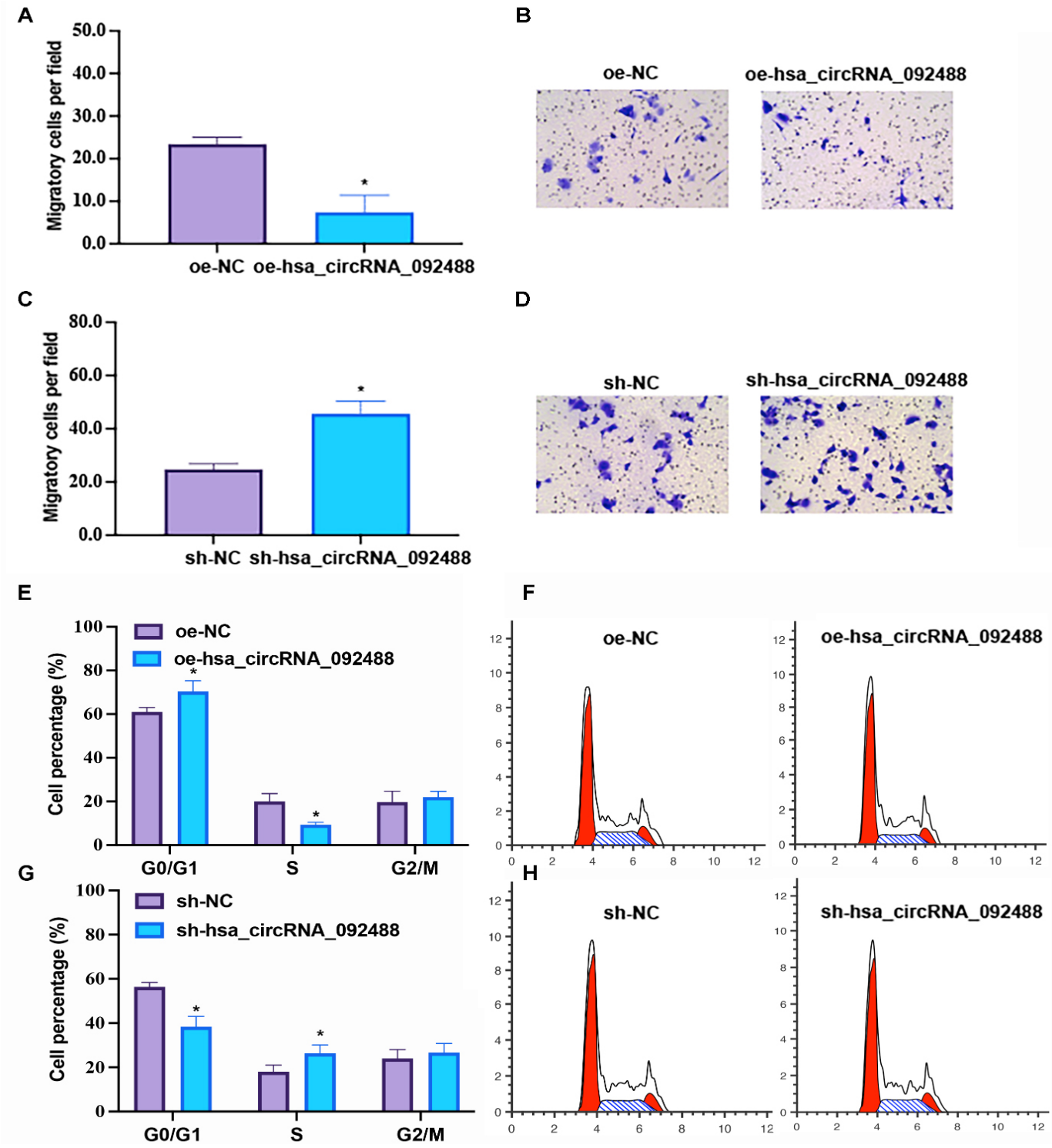

Figure 3. The migration and the cell cycle phase distribution of endothelial progenitor cells (EPCs) were affected by hsa_circRNA_092488. A-D) The migrative ability of EPCs was evaluated following treatment with oe-hsa_circRNA_092488 or sh-hsa_circRNA_092488. E-H) Cell cycle phases of EPCs were also evaluated after transfection with oe-hsa_circRNA_092488 or sh-hsa_circRNA_092488.

*: $p<0.05$.

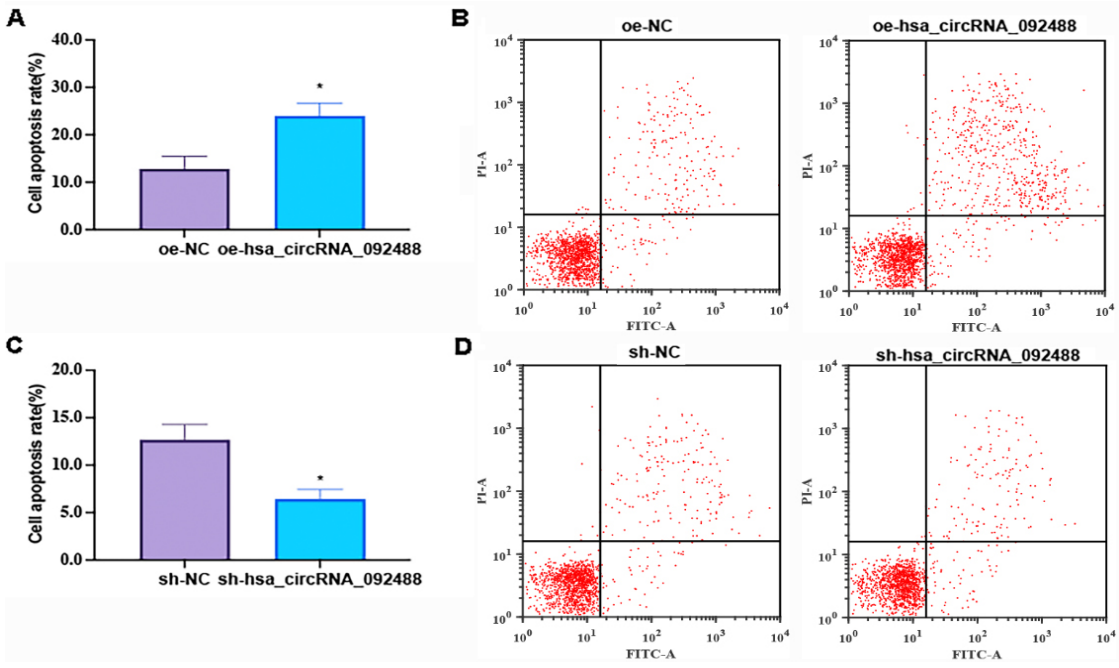

Figure 4. Cell apoptosis was triggered by upregulated hsa_circRNA_092488. A, B) The rate of cell apoptosis was elevated in endothelial progenitor cells transfected with oe-hsa_circRNA_092488. C, D) Apoptosis was inhibited by sh-hsa_circRNA_092488.

*: $p<0.05$.

of inflammasomes, leading to the activation of caspase-1 and subsequent cleavage of interleukin (IL)-1β and IL-18. Aberrant activation of NLRP3 inflammasomes is associated with the pathogenesis of various inflammatory diseases [13]. Upon activation, NLRP3 inflammasomes could promote the activation of NF-κB and the production of cytokines in inflammatory diseases [14,15]. In a previous study, the knockdown of NLRP3 inhibited the activation of NF-κB and secretion of cytokines in both microbially induced and sterile inflammation. NLRP3 not only triggered the activation of caspase-1 post-translationally but also promoted the expression of cytokines in the innate immune system [15]. In line with those findings, our results suggest that hsa_circRNA_092488 can exert its regulatory functions by activating the NLRP3/NF-κB signaling pathway, as overexpression of hsa_circRNA_092488 enhanced the protein levels of caspase-1, IL-1β, P-NF-κB-p65/NF-κB-p65, and P-IκBα/IκBα while its knockdown lowered them.

A previous study similarly revealed that NF-κB signaling could modulate proinflammatory and coagulation responses in DVT [16]. That study demonstrated that miRNAs or drugs designed to interfere with NF-κB signaling could offer promising therapeutic approaches to improve thrombosis, but the appropriate dose and possible side effects need to be considered and require further investigation [16]. In another study, the essential regulatory role of NLRP3/HIF-1α signaling within a complex network of coagulation and inflammation during the progression of thrombosis was revealed [17]. Another study indicated that NLRP3 inflammasomes were able to promote the production of IL-1β and it was elevated in the activation of platelets and the formation of thrombi [18]. Therefore, targeted therapies against NLRP3 or IL-1β could also be beneficial for the treatment of inflammation-related thrombosis.

Study Limitations

There are some limitations of the present study. To confirm the findings, the expression of apoptosis-related molecules should be examined in transfected cells in further work. Furthermore, in vivo experiments should be carried out in future work to confirm these findings.

Conclusion

The upregulation of hsa_circRNA_092488 in cases of DVT was able to suppress the proliferation and migration of EPCs, induce cell cycle arrest from the S to the G0/G1 phase, and trigger apoptosis. Moreover, hsa_circRNA_092488 could function by activating the NLRP3/NF-κB signaling pathway, as overexpressed hsa_circRNA_092488 elevated the protein levels of caspase-1, IL-1β, P-NF-κB-p65/NF-κB-p65, and P-IκBα/IκBα while its knockdown lowered them. Therefore, the hsa_circRNA_092488/NLRP3/NF-κB pathway could be associated with the progression of DVT, and this novel signaling pathway may be considered as a putative therapeutic candidate for the treatment of DVT, possibly involving the targeting of circRNAs, the design of drugs

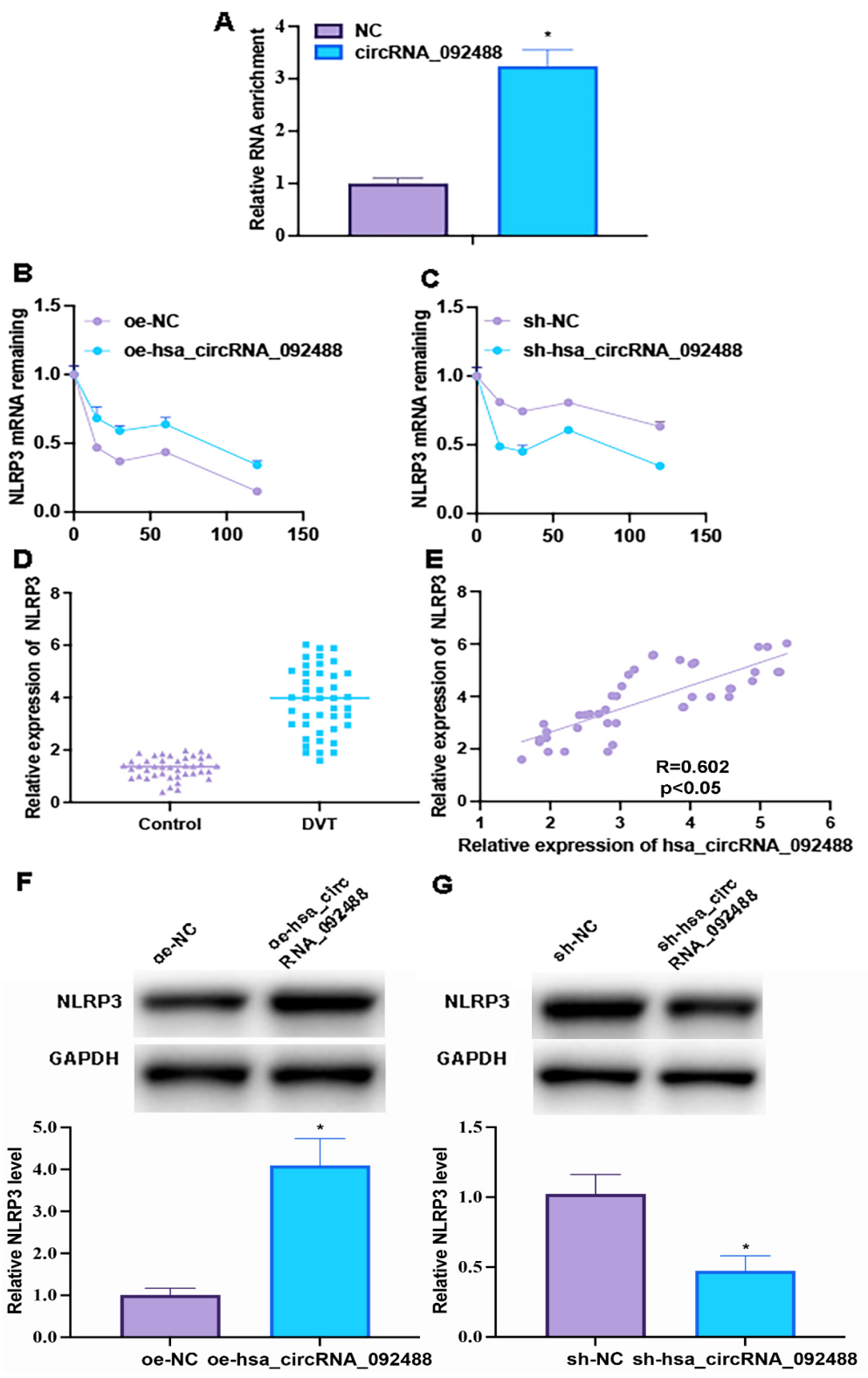

Figure 5. NOD-like receptor protein 3 (NLRP3) was the putative downstream target molecule of hsa_circRNA_092488. A) The interaction of hsa_circRNA_092488 and NLRP3 was revealed by RNA pull-down analysis. B) The stability of NLRP3 mRNA was elevated in endothelial progenitor cells (EPCs) transfected with oe-hsa_circRNA_092488. C) NLRP3 mRNA stability was reduced in EPCs by sh-hsa_circRNA_092488. D) NLRP3 expression was enhanced in cases of deep vein thrombosis (DVT). E) Levels of hsa_circRNA_092488 and NLRP3 were positively correlated in DVT. F, G) NLRP3 protein levels were increased in EPCs treated with oe-hsa_circRNA_092488 and reduced in cells transfected with sh-hsa_circRNA_092488.

*: $p<0.05$.

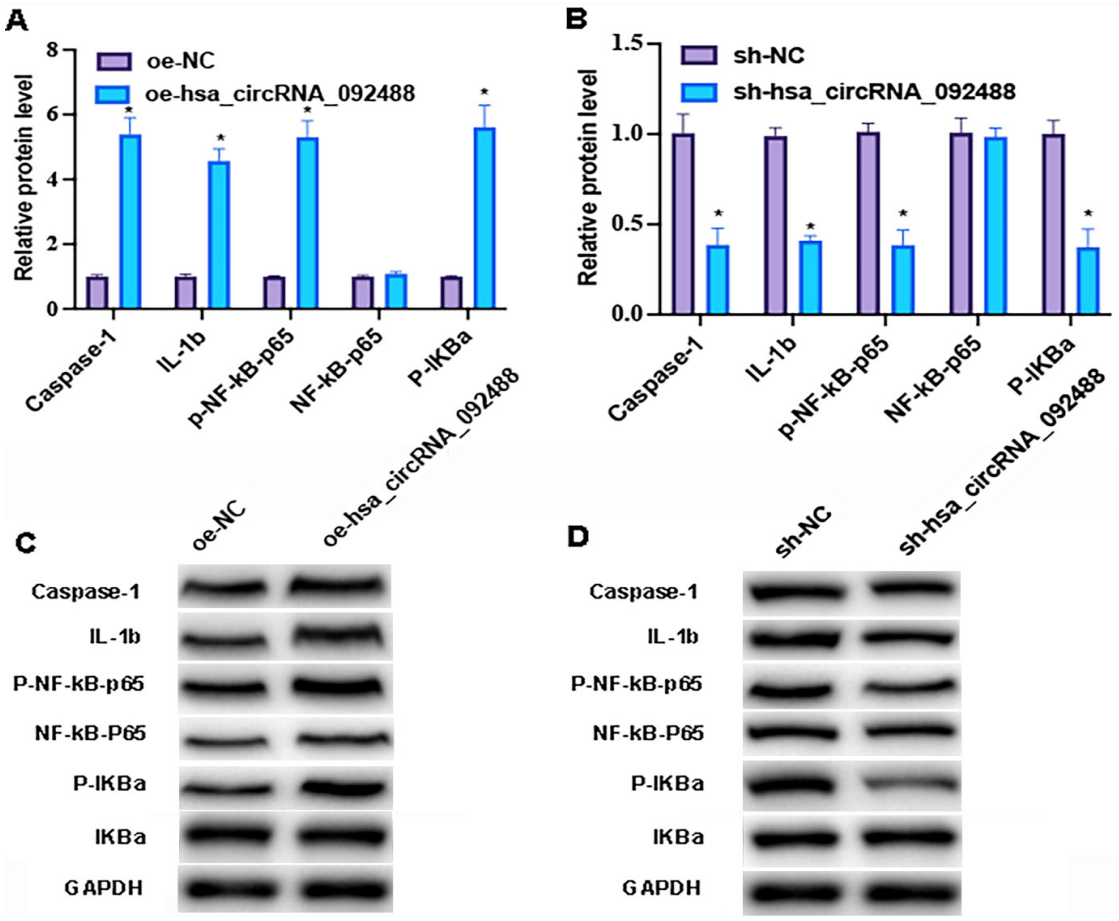

Figure 6. A-D) The levels of NOD-like receptor protein 3/nuclear factor (NF)-κB-associated proteins were affected by hsa_circRNA_092488. Overexpression of hsa_circRNA_092488 in endothelial progenitor cells remarkably increased the protein levels of caspase-1, interleukin-1β, P-NF-κB-p65/NF-κB-p65, and P-IκBα/IκBα while its knockdown lowered these protein levels.

*: $p<0.05$.

to interfere with NF-κB signaling, or targeted therapies against NLRP3 or IL-1β. However, the optimal doses and potential side effects must also be considered. These points require further investigation.

Ethics

Ethics Committee Approval: All procedures were performed in line with the Declaration of Helsinki and the protocols were reviewed by the Ethics Committee of the First Affiliated Hospital of Jinzhou Medical University (decision no: 202047).

Informed Consent: Informed consent was obtained from all patients and controls.

Footnotes

Authorship Contributions

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

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

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RESEARCH ARTICLE

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Turk J Hematol 2025;42:119-135

Efficacy, Safety, and Tolerability of Ferric Carboxymaltose and Iron Sucrose in Iron-Deficiency Anemia: A Systematic Review and Meta-Analysis of Randomized Controlled Trials

Demir Eksikliği Anemisinde Ferrik Karboksimaltoz ve Demir Sükrozun Etkililiği, Güvenirliği ve Tolere Edilebilirliği: Randomize Kontrollü Çalışmaların Sistematik Derlemesi ve Meta-Analizi

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Abstract

Objective: This study comprehensively compares the efficacy, safety, and tolerability of two commonly used intravenous iron preparations, ferric carboxymaltose (FCM) and iron sucrose (IS), in adult patients with iron-deficiency anemia (IDA).

Materials and Methods: A systematic literature search was conducted across the PubMed, Ovid MEDLINE, Web of Science, and Cochrane Library databases up to January 1, 2024, to identify randomized controlled trials directly comparing FCM and IS treatments in adult patients with IDA. The primary outcome of interest was change in hemoglobin (Hb) levels during follow-up. Meta-analyses were conducted with inverse variance random effects models.

Results: Fourteen trials were included in the study, with a total of 4757 patients. FCM resulted in a non-significant increase in Hb levels (mean difference [MD]: 0.45 g/dL, 95% confidence interval [CI]: 0.08 to 0.83, p=0.02) and ferritin levels (MD: 37.32 ng/mL, 95% CI: 18.98 to 55.65, p<0.01) compared to IS. FCM was associated with a higher risk of hypersensitivity reactions compared to IS (relative risk [RR]: 2.97, 95% CI: 1.35 to 6.52, p<0.01) but showed no significant difference in severe adverse events (RR: 1.03, 95% CI: 0.88 to 1.21, p=0.70) and had a non-significant increased risk of hypophosphatemia (RR: 2.84, 95% CI: 0.89 to 9.06, p=0.08).

Conclusion: Ten studies showed some concerns of risk of bias (RoB) and four studies had a high RoB for the change in Hb levels during follow-up. The lack of standardized definitions for hypersensitivity reactions and variability in dosing protocols and follow-up durations across studies may affect the generalizability of our safety findings.

Keywords: Ferric carboxymaltose, Iron sucrose, Iron-deficient anemia, Hypophosphatemia, Hypersensitivity

Öz

Amaç: Bu çalışma, demir eksikliği anemisi (DEA) olan erişkin hastalarda yaygın olarak kullanılan iki intravenöz demir preparatı olan ferrik karboksimaltoz (FCM) ve demir sükrozun (IS) etkililik, güvenirlik ve tolere edilebilirlik açısından kapsamlı bir karşılaştırmasını sunmaktadır.

Gereç ve Yöntemler: DEA tanılı erişkin hastalarda FCM ve IS tedavilerini doğrudan karşılaştıran randomize kontrollü çalışmaları belirlemek amacıyla, 1 Ocak 2024 tarihine kadar PubMed, Ovid MEDLINE, Web of Science ve Cochrane Library veri tabanlarında sistematik bir literatür taraması yapıldı. Birincil çıktı, takip süresince hemoglobin (Hb) düzeyindeki değişiklik olarak belirlendi. Meta-analizler, ters varyanslı random etki modeli kullanılarak gerçekleştirildi.

Bulgular: Çalışmaya toplam 4757 hastayı içeren 14 çalışma dahil edildi. FCM, IS'ye kıyasla Hb düzeylerinde anlamlı olmayan bir artış (ortalama fark [MD]: 0,45 g/dL, %95 güven aralığı [CI] 0,08-0,83, p=0,02) ve ferritin düzeylerinde anlamlı bir artış (MD: 37,32 ng/mL, %95 CI: 18,98–55,65, p<0,01) ile ilişkili bulundu. FCM, IS'ye kıyasla aşırı duyarlılık reaksiyonları açısından daha yüksek bir risk ile ilişkilendirildi (risk oranı [RR]: 2,97, %95 CI: 1,35-6,52, p<0,01); ancak ciddi advers olaylar açısından anlamlı bir fark saptanmadı (RR: 1,03, %95 CI: 0,88 - 1,21, p=0,70). Ayrıca hipofosfatemi açısından FCM ile anlamlı olmayan şekilde artmış bir risk gözlendi (RR: 2,84, %95 CI: 0,89-9,06, p=0,08).

Sonuç: Hb düzeylerindeki değişiklik açısından değerlendirildiğinde yanlılık riskleri 10 çalışmada şüpheli olarak değerlendirilirken 4 çalışmada ise yüksek düzeyde olduğu tespit edildi. Aşırı duyarlılık reaksiyonları için standart tanımların bulunmaması, doz protokollerindeki ve takip sürelerindeki farklılıklar güvenilirlik bulgularımızın genellenebilirliğini sınırlayabilir.

Anahtar Sözcükler: Ferrik karboksimaltoz, Demir sükroz, Demir eksikliği anemisi, Hipofosfatemi, Aşırı duyarlılık reaksiyonları

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Introduction

Iron deficiency anemia (IDA) is a prevalent condition with significant health consequences affecting various patient populations, including individuals with chronic diseases, heavy menstrual bleeding, and gastrointestinal disorders [1,2,3,4]. Intravenous iron therapy is often preferred in cases where rapid iron repletion is necessary or when oral iron formulations are ineffective or poorly tolerated [5,6]. Among intravenous iron therapies, ferric carboxymaltose (FCM) and iron sucrose (IS) are widely used. FCM allows for larger doses in fewer administrations compared to IS, making it more convenient for patients and healthcare providers [7,8,9]. FCM is a colloidal iron(III) hydroxide complexed with carboxymaltose, a carbohydrate polymer that facilitates controlled iron release. This allows for the replenishment of iron stores required for the synthesis of hemoglobin (Hb), myoglobin, and various enzyme systems involved in oxygen transport and cellular metabolism. Unlike dextran-based formulations, FCM enables iron uptake via the reticuloendothelial system without the release of free iron, thereby reducing the risk of oxidative stress. IS is also an iron(III) hydroxide complex with sucrose that undergoes dissociation within the reticuloendothelial system. The released iron contributes to increased serum iron concentrations and is subsequently incorporated into Hb, restoring iron levels in iron-deficient patients [7,8,9].

Previous randomized controlled trials (RCTs) have examined the comparative efficacy and safety of FCM and IS, particularly in the treatment of anemia in various populations [10,11,12,13,14,15,16]. However, the use of FCM and IS in different patient populations and clinical contexts has shown varying efficacy and safety results [6,17,18]. In the REPAIR-IDA trial [15], which included 2584 patients with IDA and chronic kidney disease (CKD), FCM showed a significantly greater increase in Hb levels compared to IS (1.13 g/dL vs. 0.92 g/dL; 95% confidence interval [CI]: 0.13-0.28), with a higher proportion of patients in the FCM group achieving Hb increases of ≥1.0 g/dL (48.6% vs. 41.0%). Importantly, no significant difference was observed between the two treatments regarding cardiovascular safety, including major adverse cardiac events, although FCM was associated with a higher incidence of transient hypertensive episodes.

In a study by Mahey et al. [19] involving 60 women with anemia due to abnormal uterine bleeding, FCM resulted in a more rapid increase in Hb levels at 6 weeks compared to IS (p=0.005), although no significant difference was observed at 12 weeks (p=0.11). Similarly, Lee et al. [20] demonstrated that FCM was as effective as IS in achieving Hb of ≥10 g/dL in women with preoperative anemia due to menorrhagia, with a significantly shorter time to reach this target in the FCM group (7.7 days vs. 10.5 days).

Laso-Morales et al. [21] compared FCM and IS in 104 patients with postoperative anemia following colorectal cancer surgery. Both treatments led to comparable increases in Hb by postoperative day 30 (FCM: 2.5 g/dL vs. IS: 2.4 g/dL), but FCM was associated with a lower infection rate (9.8% vs. 37.2%, p<0.05). In contrast, a study conducted in Japan with patients with IDA due to hypermenorrhea showed the non-inferiority of FCM compared to saccharated ferric oxide, with a mean Hb increase of 3.90 g/dL in the FCM group and 4.05 g/dL in the control group (difference: -0.15 g/dL; 95% CI: -0.35 to 0.04).

A recent trial [22] conducted in China compared the efficacy of FCM and IS in 371 patients with IDA. The primary endpoint of achieving Hb increase of ≥2 g/dL within 8 weeks was met by 99.4% of FCM-treated patients compared to 98.3% of IS-treated patients, confirming non-inferiority (difference: 1.12%; 95% CI: -2.15 to 4.71). Additionally, a higher proportion of FCM-treated patients achieved early Hb response at 2 weeks (85.2% vs. 73.2%; 95% CI: 3.31 to 20.65), and FCM showed a greater increase in transferrin saturation (TSAT) and serum ferritin levels at all time points.

These findings highlight the variability in the efficacy and safety outcomes of FCM and IS across different patient populations and clinical scenarios. To date, there have been no systematic reviews comparing FCM and IS in the management of IDA regardless of etiology. Given the need for more conclusive evidence, we conducted a systematic review and meta-analysis of RCTs to compare the efficacy and safety of FCM and IS in the treatment of IDA.

Materials and Methods

This study was conducted following a predefined protocol registered with the International Prospective Register of Systematic Reviews (PROSPERO; registration number: CRD42022337858).

Eligibility Criteria

We identified RCTs evaluating the efficacy and safety of FCM versus IS in patients with IDA regardless of etiology. We excluded studies that were not RCTs, including observational studies, case reports, case series, narrative reviews, editorials, commentaries, or expert opinions. Studies involving individuals under 18 years of age were also excluded. Additionally, we excluded studies that compared FCM or IS with oral iron, placebos, or other intravenous iron formulations (e.g., ferric derisomaltose or ferric gluconate) without a direct comparison between FCM and IS. Studies that did not report at least one predefined outcome of interest or provided incomplete or unclear data that could not be extracted for meta-analysis and those not published in English were also excluded.

Search Strategy and Study Selection

A systematic search was performed in the Cochrane Central Register of Controlled Trials (Cochrane CENTRAL), Ovid MEDLINE, PubMed, and Web of Science databases up to January 1, 2024. This study was reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [23]. Additionally, the reference lists and citations of included studies from the past 5 years were screened for relevant articles. Only studies published in English were considered. Detailed information about the search strategy is provided in the Supplementary File. References identified through the database searches were imported to EndNote v21.3 (Clarivate Analytics, Philadelphia, PA, USA). After removing duplicates, full-text articles were retrieved if their abstracts were deemed eligible by at least one reviewer. Each full-text article was then independently assessed for final inclusion in this systematic review and meta-analysis, with any disagreements resolved through consensus.

Outcomes

The primary efficacy outcome of interest was the change in Hb level during follow-up, while the primary safety outcome of interest was the risk of serious or severe adverse events (AEs). Secondary outcomes included Hb increase of 2 g/dL during follow-up, achievement of Hb levels of 12 g/dL during follow-up, change in serum ferritin levels from baseline, hypersensitivity reactions, risk of hypophosphatemia, and withdrawals due to AEs.

Data Extraction

Data were extracted independently by two reviewers using a standardized data extraction form. The extracted data included:

- Study characteristics: First author, year of publication, study design, etiology, sample size, intervention details (type of iron preparation [FCM or IS] with cumulative dose), primary outcome, and prespecified secondary outcomes in the protocol.

- Participant characteristics: Number of patients, age, gender, race (white, black or African American, Asian, or other), use of erythropoiesis-stimulating agents, previous iron therapy, baseline Hb value (g/dL), baseline ferritin level (ng/mL), baseline TSAT (%), and baseline estimated glomerular filtration rate (mL/min/1.73 m^2) for each arm in the included studies.

Data were double-checked for accuracy and consistency. In the event of incomplete outcome data, we employed available-case analysis, and if a study reported results graphically, we extracted data using a digital analysis tool [24].

Risk of Bias Assessment

Two reviewers (L.H.T. and A.V.H.) independently assessed the risk of bias (RoB) in the included RCTs using the Cochrane RoB2.0 tool [25], with any disagreements resolved through discussion. The RoB2.0 tool evaluates five domains of bias: the randomization process, deviations from intended interventions, missing outcome data, outcome measurement, and selection of the reported result. A study was considered to have high RoB if at least one domain was rated as "high risk" or was deemed to have "some concerns" if at least one domain raised concerns without any domains being rated as high RoB.

Statistical Analysis

Meta-analyses were primarily conducted using inverse variance random effects models; for rare outcomes with incidence of <10%, the Mantel-Haenszel method was applied. Between-study variance (τ^2) was calculated using the Paule-Mandel method [26], with CIs adjusted using the Hartung-Knapp method [27]. Dichotomous outcomes were presented as relative risks (RRs) with 95% CIs, and continuous outcomes were presented as mean differences (MDs) with 95% CIs. Between-study heterogeneity was assessed using the Cochran Q test and I^2 statistics, with values of <30% indicating low heterogeneity, 30%-60% moderate heterogeneity, and >75% substantial heterogeneity [28]. Publication bias was visually examined using funnel plots and statistical methods, including Egger tests. Sensitivity analyses were conducted by sequentially excluding each study to assess the impact on pooled RR estimates.

All analyses were performed using R version 4.4.1 (www.r-project.org) with the meta and metafor packages. Statistical tests were two-sided with a significance threshold of $p<0.05$. Values for interaction of $p<0.1$ were considered statistically significant for a given subgroup [29]. Subgroup analyses, based on the etiology of IDA and RoB for primary outcome, were conducted to explore potential sources of heterogeneity.

Results

Study Selection

A total of 688 records were identified through database searches, including Cochrane CENTRAL, PubMed, Ovid MEDLINE, and Web of Science. After the removal of 292 duplicates, 396 records remained for screening. Of these, 331 were excluded based on titles and abstracts. Sixty-three full-text articles were assessed for eligibility and 14 were excluded due to irrelevant interventions, 14 due to unsuitable study designs, 10 due to incorrect publication types, and 2 due to wrong populations. After this screening, 14 RCTs involving a total of 4757 participants [11,12,13,14,15,16,19,20,21,22,30,31,32,33] were included in this meta-analysis (Figure 1).

Study Characteristics

Fourteen RCTs were included in this meta-analysis comparing FCM and IS in various populations with IDA. The included

studies were categorized based on the underlying causes of IDA. Detailed summaries of the study characteristics (Table 1) and patient characteristics (Supplementary Table S1) of each included RCT are provided.

Three studies were identified involving patients with gynecological disorders. Mahey et al. [19] compared FCM and IS in women with IDA due to abnormal uterine bleeding and found that FCM was more effective in raising Hb levels with fewer AEs. Ikuta et al. [11] examined Japanese women with hypermenorrhea-induced IDA, demonstrating the non-inferiority of FCM to IS in both efficacy and safety. Lee et al. [20] investigated patients with preoperative anemia due to menorrhagia, finding that FCM led to a faster and higher increase in Hb levels compared to IS.

For patients with impaired iron absorption, three trials were evaluated. Evstatiev et al. [14] conducted the FERGIcor trial, focusing on IDA due to inflammatory bowel disease, and concluded that FCM was superior to a placebo in improving Hb values. Laso-Morales et al. [21] compared single-dose FCM with multiple doses of IS in postoperative colorectal cancer patients, showing that FCM was more convenient and effective in correcting postoperative anemia. Struppe et al. [33] conducted a pilot study evaluating the impact of intravenous iron on bone

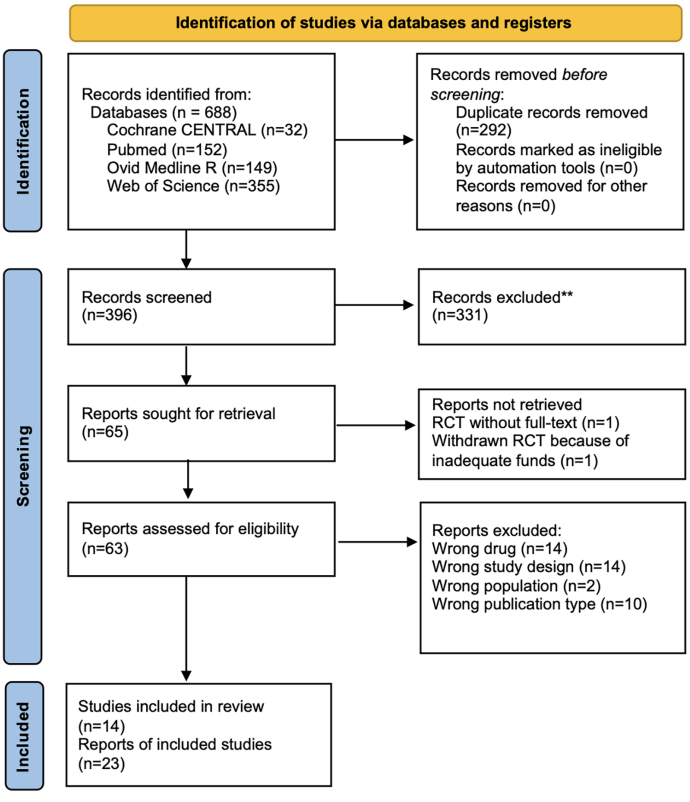

Figure 1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram of eligible studies.

RCT: Randomized controlled trial.

turnover markers and serum phosphate levels, suggesting that FCM had a more favorable safety profile than IS.

Three studies were identified involving patients with impaired renal function. Onken et al. [15] conducted the REPAIR-IDA trial, comparing FCM and IS in patients with IDA and impaired renal function, and found that FCM resulted in a quicker and more sustained increase in Hb levels. Roberts et al. [30] evaluated the effects of intravenous iron on fibroblast growth factor 23 in hemodialysis patients, showing that FCM was associated with better outcomes than IS. Bielesz et al. [12] studied different iron dosing strategies in long-term hemodialysis patients, concluding that FCM was more effective and required fewer doses than IS.

Among the studies involving patients with mixed etiologies, Naqash et al. [13] compared FCM and IS in women with IDA due to various causes, concluding that FCM was more effective and had a better safety profile. Jin et al. [22] conducted a randomized trial with Chinese patients with IDA of mixed etiology and found that FCM was not inferior to IS, with the added benefit of fewer required doses.

For postpartum anemia, two studies were included. Rathod et al. [16] investigated FCM in Indian women with postpartum anemia, showing significant improvement in Hb levels with a single dose. Similarly, Wajid et al. [32] compared FCM and IS in women with postpartum anemia, concluding that FCM was more effective and safer than IS.

Finally, for pregnancy-related IDA, Jose et al. [31] compared FCM and IS in pregnant women and found that FCM provided superior outcomes in terms of Hb improvement and safety profile.

Risk of Bias and Publication Bias

The Cochrane RoB2.0 tool was used to assess the quality of the included studies. Ten studies were classified as having some concerns of RoB and four studies were deemed to have a high RoB for change in Hb levels during follow-up (Figure 2). All studies had some concerns of RoB in the domain of deviations from the intended interventions, mostly because of their open-label study designs.

To evaluate publication bias, a graphical funnel plot was used. Visual inspection of the plot revealed asymmetry, indicating the presence of publication bias for all studies except two small and negative RCTs (Supplementary Figure 1).

Primary Outcome Results

In the overall analysis of 12 RCTs [11,12,13,15,16,19,21,22, 30,31,32,33] involving 4,734 participants, FCM resulted in a significant increase in Hb levels during follow-up compared to IS (MD: 0.45 g/dL, 95% CI: 0.08 to 0.83, I^2: 97%, p=0.02) (Figure 3). The clinical importance of this finding suggests that FCM

may offer modest benefits over IS in raising Hb levels across a broad population of IDA patients.

Subgroup Analysis Results

When stratified by the etiology of anemia, FCM demonstrated a statistically significant improvement in Hb levels specifically in patients with postpartum anemia [16,32] (MD: 1.04 g/dL, 95% CI: 0.75 to 1.33, p<0.01), but inverse results were obtained for hemodialysis patients [12,30] (MD: -0.24 g/dL, 95% CI: -0.53 to 0.04, p<0.01) compared to IS (Supplementary Figure 2). Upon classifying studies based on impaired iron absorption (MD: 0.17 g/dL, 95% CI: -0.34 to 0.69) [14,21,33], impaired renal function (MD: -0.09 g/dL, 95% CI: -0.46 to 0.28) [12,15,30], gynecological disorders (MD: 0.26 g/dL, 95% CI: -0.62 to 1.14) [11,19,20], postpartum anemia (MD: 1.04 g/dL, 95% CI: 0.75 to 1.33) [16,32], and mixed etiology (MD: 1.10 g/dL, 95% CI: -0.36 to 2.56) [13,22], a significant difference was observed among the subgroups in favor of postpartum anemia for FCM (Supplementary Figure 3).

Table 1. Study characteristics of the included trials.

Reference	Study design	Population	Sample size	Intervention, cumulative dose + SD (mg)	Comparator, cumulative dose + SD (mg)	Primary outcome
Evstatiev et al. [14], 2011	Multicenter open-label RCT	IBD-associated IDA	485	FCM, 1377+381	IS, 1160+316	Hemoglobin response rate at week 12
Onken et al. [15], 2014	Multicenter open-label RCT	NDD-CKD-associated IDA	2584	FCM, 1464+158	IS, 963+138	Non-inferiority in the change from baseline to highest hemoglobin levels at day 56
Mahey et al. [19], 2015	Open-label RCT	Uterine bleeding-associated IDA	60	FCM, N/A	IS, N/A	Rise in hemoglobin levels above baseline
Rathod et al. [16], 2015	Double-blinded RCT	Postpartum-associated IDA	300	FCM, N/A	IS, N/A	Changes in hemoglobin and serum ferritin levels at 2 and 6 weeks after treatment
Roberts et al. [30], 2016	RCT	HD-CKD-associated IDA	42	FCM, 200	IS, 200	Change in fibroblast growth factor 23 levels from pre-infusion to day 2 after infusion
Ikuta et al. [11], 2018	Multicenter open-label RCT	Hypermenorrhea-associated IDA	294	FCM, 1349+N/A	IS, 1357+N/A	Mean change in hemoglobin from baseline to highest observed level
Naqash et al. [13], 2018	RCT	Mixed etiology	200	FCM, N/A	IS, N/A	Achievement of target hemoglobin and ferritin levels
Lee et al. [20], 2019	Multicenter open-label RCT	Hypermenorrhea-associated IDA	101	FCM, 923.1+207.3	IS, 939.6+352.3	Proportion of patients achieving hemoglobin levels of ≥10 g/dL within 2 weeks after the first administration
Jose et al. [31], 2019	Open-label RCT	Pregnancy-associated IDA	100	FCM, 1739.6+105.5	IS, 1730.4+121.9	Improvement in hemoglobin and ferritin levels
Wajid et al. [32], 2021	RCT	Postpartum-associated IDA	160	FCM, N/A	IS, N/A	Recovery of normal hemoglobin levels by day 21
Bielesz et al. [12], 2021	Open-label RCT	HD-CKD-associated IDA	142	FCM, N/A	IS, N/A	Change in hemoglobin at week 40 from baseline
Laso-Morales et al. [21], 2022	Open-label RCT	Colorectal cancer surgery-associated IDA	104	FCM, 1000+N/A	IS, N/A	Change in hemoglobin concentration at postoperative day 30
Struppe et al. [33], 2023	Open-label pilot RCT	IBD-associated IDA	20	FCM, N/A	IS, N/A	Longitudinal evaluation of serum phosphate levels after iron substitution therapy
Jin et al. [22], 2024	Multicenter open-label RCT	Mixed etiology	371	FCM, 1521+231	IS, 1464+325	Achievement of hemoglobin response (increase of ≥2 g/dL from baseline) within 8 weeks

SD: Standard deviation; RCT: randomized controlled trial; IBD: inflammatory bowel disease; IDA: iron-deficiency anemia; NDD-CKD: non-dialysis-dependent chronic kidney disease; HD-CKD: hemodialysis-dependent chronic kidney disease; FCM: ferric carboxymaltose; IS: iron sucrose; N/A: not available.

Secondary Efficacy Outcomes

The proportion of patients achieving an increase of ≥2 g/dL in Hb (3 RCTs, 1078 patients) [14,22,30] was comparable between the FCM group (RR: 1.06, 95% CI: 0.93 to 1.20, p=0.38) and IS (Supplementary Figure 4). FCM also showed non-significant superiority in achieving normal Hb levels during follow-up (RR: 1.77, 95% CI: 0.98 to 3.20, p=0.06) (Supplementary Figure 5) [14,16,21,32].

Ferritin levels during follow-up were significantly improved in the FCM group compared to the IS group (MD: 37.32 ng/mL, 95% CI: 18.98 to 55.65, p<0.01) (Supplementary Figure 6) [13,16,19,22,33].

Figure 2. Risk of bias assessment of the included randomized controlled trials in terms of change in hemoglobin levels during follow-up.

Safety Outcomes

The pooled risk for serious or severe AEs was comparable between the FCM and IS groups (RR: 1.03, 95% CI: 0.88 to 1.21, p=0.70) (Figure 4A) [11,12,13,14,15,16,19,20,22,30,31,32]. This finding suggests that both FCM and IS have acceptable safety profiles with no clinically meaningful differences in serious AEs. FCM was associated with a significantly higher incidence of hypersensitivity reactions compared to IS (RR: 2.97, 95% CI: 1.35 to 6.52, p<0.01) (Figure 4B) [11,12,13,14,15,16,19,21,22,30, 31,32]. The occurrence of hypophosphatemia was more frequent in the FCM group, although the difference did not reach statistical significance (RR: 2.84, 95% CI: 0.89 to 9.06, p=0.08) (Figure 4C) [11,14,15,16,22,31]. Similarly, the results of pooled analysis of all AEs did not differ significantly between FCM and IS (RR: 0.89, 95% CI: 0.63 to 1.27, p=0.53) (Supplementary Figure 7) [11,12,13,14,15,16,19,22,31]. No significant difference in withdrawal rates due to AEs was observed between the two groups (RR: 1.53, 95% CI: 0.60 to 3.89, p=0.37) (Supplementary Figure 8) (Table 2) [11,12,14,19,20,21,22,31].

Discussion

Our meta-analysis demonstrated that FCM provides a potential advantage over IS in improving Hb and ferritin levels among patients with IDA. Notably, FCM showed a statistically significant improvement in Hb levels compared to IS, especially in patients with postpartum anemia. This analysis adds to the existing body of evidence by highlighting the differential impacts of FCM and IS across various subpopulations, underscoring FCM's enhanced efficacy in achieving target Hb levels swiftly. Although FCM is associated with

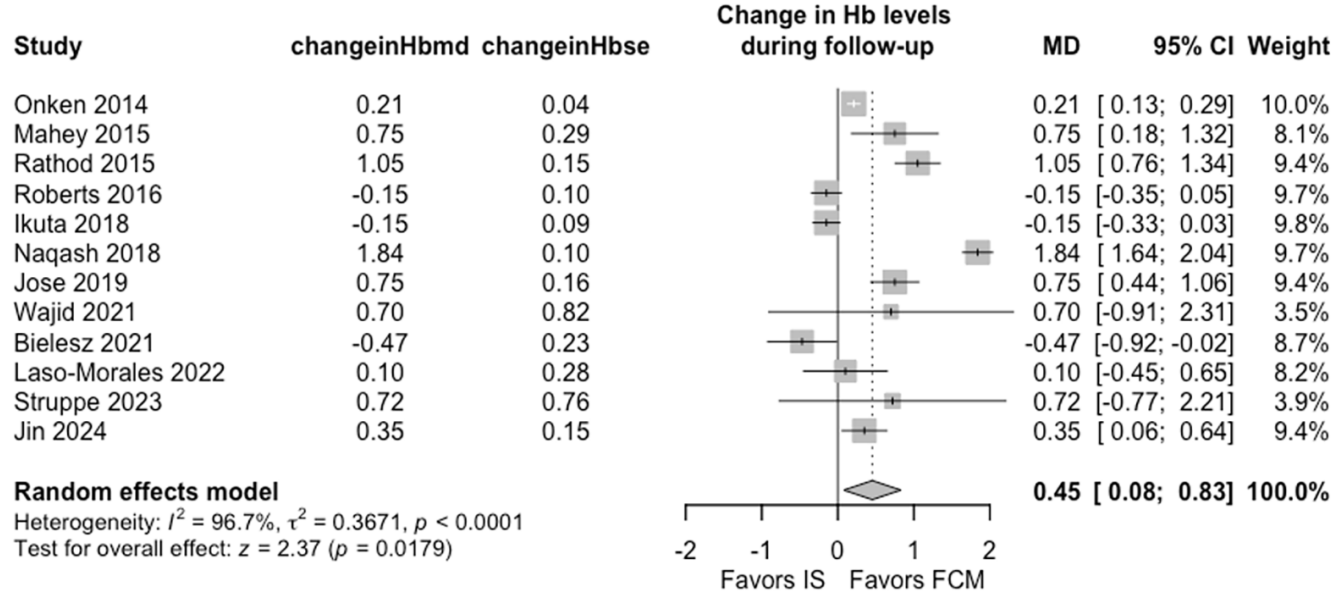

Figure 3. Forest plot of change in hemoglobin levels during follow-up.

Hbmd: Hemoglobin-mean value; Hbse: hemoglobin-standard error; Hb: hemoglobin; MD: mean difference; CI: confidence interval; IS: iron sucrose; FCM: ferric carboxymaltose.

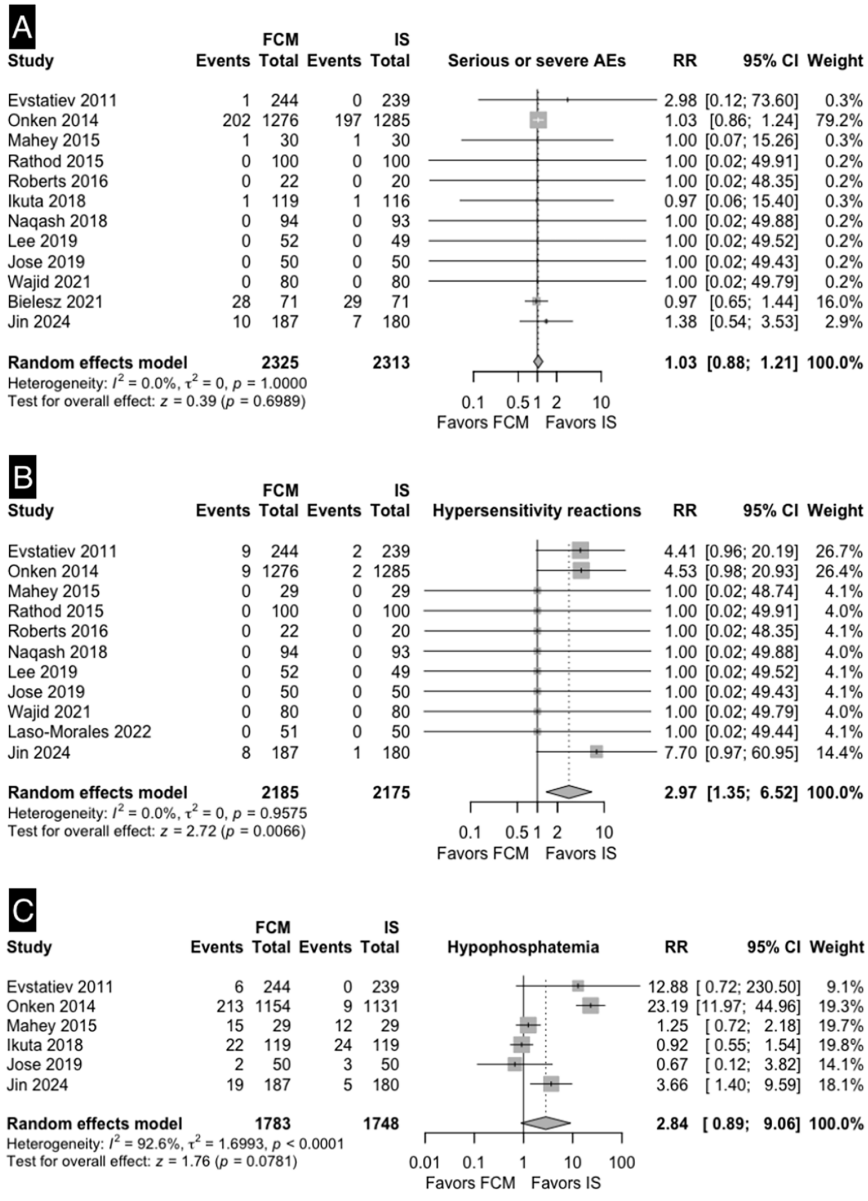

Figure 4. Forest plots of risk of serious or severe adverse events (A), hypersensitivity reactions (B), and hypophosphatemia (C).
FCM: Ferric carboxymaltose; IS: iron sucrose; AE: adverse event; RR: relative risk; CI: confidence interval.

Table 2. Safety of ferric carboxymaltose compared to iron sucrose in anemia patients.

Outcomes	Number of studies	FCM		IS		Pooled effect size RR (95% CI)	p	I^2 (%)
		Number of events	Total number of patients	Number of events	Total number of patients			
Serious or severe AEs	12	243	2325	235	2313	1.03 (0.88 to 1.21)	0.6989	0
Hypersensitivity reactions	11	26	2185	5	2175	2.97 (1.35 to 6.52)	0.0066	0
Hypophosphatemia	6	277	1783	53	1748	2.84 (0.89 to 9.06)	0.0781	92.6
Any AEs	9	470	2169	443	2167	0.89 (0.63 to 1.27)	0.53	47
Withdrawal rate	8	11	804	6	788	1.53 (0.60 to 3.89)	0.37	0

FCM: Ferric carboxymaltose; IS: iron sucrose; RR: relative risk; CI: confidence interval; AE: adverse event.

a significantly increased risk of hypersensitivity reactions and a non-significant increase of hypophosphatemia and serious or severe AEs regardless of the etiology of IDA, our findings suggest the importance of monitoring patients receiving both agents.

IDA represents a significant global health concern due to its widespread prevalence and profound impact on individual health and socioeconomic development. According to the 2021 Global Burden of Disease study [2], the global prevalence of anemia was 24.3%, equating to approximately 1.92 billion cases. Although this marks a decrease from 28.2% in 1990 [34], the absolute number of cases has grown due to population expansion. IDA remains the leading cause of anemia worldwide, constituting 66.2% of total cases, particularly affecting women of reproductive age and children under 5 years of age. The primary etiologies of IDA include dietary iron deficiency, chronic inflammatory diseases, and conditions affecting iron absorption, such as gastrointestinal disorders and CKD [3]. The widespread burden of IDA and its profound effects on quality of life, cognitive function, and physical performance underscore the importance of timely and effective iron repletion, particularly in populations with high physiological demands or significant iron losses [5].

Parenteral iron therapy, such as FCM and IS, is a critical option when oral iron formulations are ineffective, poorly tolerated, or contraindicated, such as in patients with severe IDA, malabsorption syndromes, and CKD or those who cannot adhere to oral regimens due to gastrointestinal side effects [7,35]. FCM offers a practical advantage in delivering higher doses in a single administration, allowing for rapid repletion and improved patient compliance [36]. However, FCM's association with hypersensitivity reactions and hypophosphatemia necessitates careful monitoring [37]. IS, while requiring multiple administrations to achieve adequate iron levels, may be preferable in patients with higher sensitivity to infusion reactions [38]. Thus, the choice of intravenous iron therapy should be tailored to individual patient needs while considering efficacy, safety profiles, and logistical considerations.

Increasing Hb levels in patients with IDA is of paramount importance across diverse subpopulations and etiologies [35,39]. For patients with CKD, there is a consensus that the correction of Hb levels with intravenous iron therapy is linked to improved outcomes in terms of reduced hospitalizations and enhanced quality of life [40]. Additionally, in the obstetric population, correcting Hb in pregnant and postpartum women not only addresses maternal anemia but also reduces the risks associated with postpartum hemorrhage and supports optimal fetal development [41]. Achieving target Hb levels thus has significant implications, serving to mitigate the morbidity associated with anemia and, ultimately, enhance patient-centered outcomes across these varied clinical contexts.

The safety profiles of parenteral iron agents, and particularly those of FCM and IS, are a crucial consideration in clinical practice as they impact adherence, tolerability, and preference in managing IDA. In accordance with our results, FCM has a favorable safety profile with a lower incidence of AEs compared to IS, as also observed in meta-analyses among obstetric and gynecologic populations [10]. FCM's ability to deliver a high dose in a single administration session not only enhances patient adherence by reducing the need for multiple infusions but also aligns well with clinical settings that prioritize efficiency. However, FCM is associated with treatment-emergent hypophosphatemia, especially in cases requiring repeated dosing, which mandates careful monitoring. IS is known to require multiple doses for full iron replenishment in IDA patients and it was shown to carry a higher risk of severe hypersensitivity reactions compared to a carbohydrate-polymer agent [42]. Both agents rarely lead to true anaphylaxis, with most reactions being mild infusion-related responses. The robust safety and tolerability of these agents combined with their low rates of treatment discontinuation due to AEs underscore their suitability and reliability in clinical practice for a range of IDA etiologies.

Shin et al. [10] reported the safety of FCM and IS, which are widely used by obstetric and gynecological IDA patients, in their systematic review. The incidence of AEs was reported to be lower in the FCM group than in the IS group (p=0.003). No serious AEs were reported in either group. In a systematic review and meta-analysis reported by Bharadwaj et al. [43], 26% fewer side effects occurred in the FCM group compared to the IS group (p=0.001). Srimathi et al. [44] reported a meta-analysis of pregnant women aged 15-49 years with IDA who were given FCM or IS. A total of 18 studies were included. Fewer side effects were reported in the FCM group compared to the IS group (p=0.003). In the prospective study conducted by James et al. [45] including 120 pregnant IDA patients, the number of patients given FCM and IS was 60 each. Mild side effects were reported to occur in 7.5% of the patients included in that study.

Hardy and Vandemergel [46] examined the frequency of hypophosphatemia in their retrospective study of the data of patients who received FCM or IS. Fifty-two patients were included in the IS group and 78 patients were included in the FCM group. The phosphate level measured before treatment in the IS group was 1.08±0.23 mmol/L and it was reported not to have changed significantly after IS administration (1.00±0.29 mmol/L; p=0.37). Hypophosphatemia was reported in 22% of the patients after IS infusion, with phosphate levels falling below 0.80 mmol/L, while all had been within the normal range before injection. The mean phosphate level before treatment in the FCM group was 1.08±0.18 mmol/L and it decreased to 0.82±0.29 mmol/L after iron administration (p<0.0001). After FCM administration, 13% of patients had a phosphate level of <0.32 mmol/L and 51% had a phosphate level of <0.80 mmol/L.

It is also important to note that published RCTs lack standardized definitions for hypersensitivity reactions. For instance, Ikuta et al. [11] used MedDRA definitions, which provide a standardized set of terms for hypersensitivity reactions, categorized into five groups and aiding clinicians and researchers in estimating the risk for the general population, whereas Lee et al. [20] used the National Cancer Institute Common Terminology Criteria for Adverse Events (version 4.0) to report AE and safety data. However, both of these studies reported only a few safety outcomes and they did not address hypersensitivity-related AEs. Therefore, our findings on safety parameters, including severe AEs and hypersensitivity reactions, should be interpreted with caution. This uncertainty and heterogeneity in reporting AEs should be considered by guideline developers and policymakers, as this study has provided the most comprehensive data on this subject.

Study Limitations

Our meta-analysis has several strengths, including the large number of patients analyzed across multiple clinical settings and the inclusion of both short-term and long-term efficacy outcomes. However, it is important to acknowledge certain limitations. First, not all trials reported data on key safety outcomes, such as hypophosphatemia or standardized definitions for serious or severe AEs or hypersensitivity, which may limit the generalizability of our findings regarding AEs. Second, while we included a broad range of patient populations, the heterogeneity in dosing protocols, follow-up durations, and etiologies across the included studies may have influenced the observed treatment effects.

Conclusion

This systematic review and meta-analysis has demonstrated the potential advantage of FCM over IS in improving Hb and ferritin levels, particularly among patients with gynecological disorders underlying IDA. While the two iron preparations demonstrated comparable efficacy in the general population, the findings of this review underscore the importance of considering the specific etiology of anemia when choosing between these treatments.

Ethics

Ethics Committee Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent: The article presents a meta-analysis. Therefore, informed consent is not required.

Footnotes

Authorship Contributions

Concept: L.H.T., A.S.; Design: L.H.T., A.S.; Data Collection or Processing: L.H.T., A.S.; Analysis or Interpretation: L.H.T., A.S.; Literature Search: L.H.T., A.S.; Writing: L.H.T., A.S.

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

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Supplementary File

1. Search algorithms

Web of Science

#1(((((((ALL=(iron deficiency anemia)) OR ALL=(anemia)) OR ALL=(iron deficien*)) OR ALL=(iron deplet*)) OR ALL=(anaemia)) OR ALL=(anemic)) OR ALL=(anaemic))

#2(((ALL=(carboxymaltose)) OR ALL=(ferinject)) OR ALL=(injectafer)) OR ALL=(Dextri-Maltose)

#3((((((((((((((ALL=(sucrose)) OR ALL=(saccharated ferric oxide)) OR ALL=(iron sucrose)) OR ALL=(iron saccharate)) OR ALL=(ferric saccharate)) OR ALL=(ferri saccharate)) OR ALL=(iron (iii) hydroxide sucrose complex)) OR ALL=(venofer)) OR ALL=(hippiron)) OR ALL=(ferrisaccharate)) OR ALL=(ferrivenin)) OR ALL=(sucrofer)) OR ALL=(feojectin)) OR ALL=(ferric oxide saccharate)) OR ALL=(sucroferric oxyhydroxide)

#4#1 AND #2 AND #3

Cochrane Central Register of Controlled Trials (Cochrane CENTRAL)

#1MeSH descriptor: [Anemia, Iron-Deficiency] explode all trees

#2MeSH descriptor: [Anemia] explode all trees

#3#1 OR #2

#4 (carboxymaltose OR ferinject OR injectafer OR dextri-maltose):ti,ab,kw

#5 MeSH descriptor: [Ferric Oxide, Saccharated] explode all trees

#6(sucrose OR 'saccharated ferric oxide' OR 'iron sucrose' OR 'iron saccharate' OR 'ferric saccharate' OR 'ferri saccharate' OR 'iron (iii) hydroxide sucrose complex' OR venofer OR hippiron OR ferrisaccharate OR ferrivenin OR sucrofer OR feojectin OR 'ferric oxide saccharate' OR sucroferric oxyhydroxide)):ti,ab,kw

#7#5 OR #6

#8(#3 AND #4 AND #7) in Trials

Ovid Medline (R) (Epub Ahead of Print, In-Process, In-Data-Review & Other Non-Indexed Citations and Daily 1946 to Jan 1, 2024)

1 (exp *Anemia/ or Anemia.mp.) or (exp *Anemia, Iron-Deficiency/ or Anemia, Iron-Deficiency.mp.) {Including Related Terms}

2 exp *Anemia/ or Anemia.mp.

3 1 or 2

4 exp Ferric Oxide, Saccharated/ or Ferric Oxide, Saccharated.mp.

5 (exp Ferric Oxide, Saccharated/ or Ferric Oxide, Saccharated.mp.) {Including Related Terms}

6 Sucrose/ or sucrose.mp.

7 saccharated ferric oxide.mp.

8 iron sucrose.mp.

9 iron saccharate.mp.

10 ferric saccharate.mp.

11 iron (iii) hydroxide sucrose complex {Including Related Terms}

12 venofer.mp.

13 hippiron.mp.

14 ferrisaccharate.mp.

15 ferrivenin.mp.

16 ferric oxide saccharate.mp.

17 sucroferric oxyhydroxide.mp.

18 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17

19 Carboxymaltose/ or carboxymaltose.mp.

20 ferinject.mp.

21 injectafer.mp.

22 Dextri-Maltose.mp.

23 19 or 20 or 21 or 22

24 3 and 18 and 23

Pubmed

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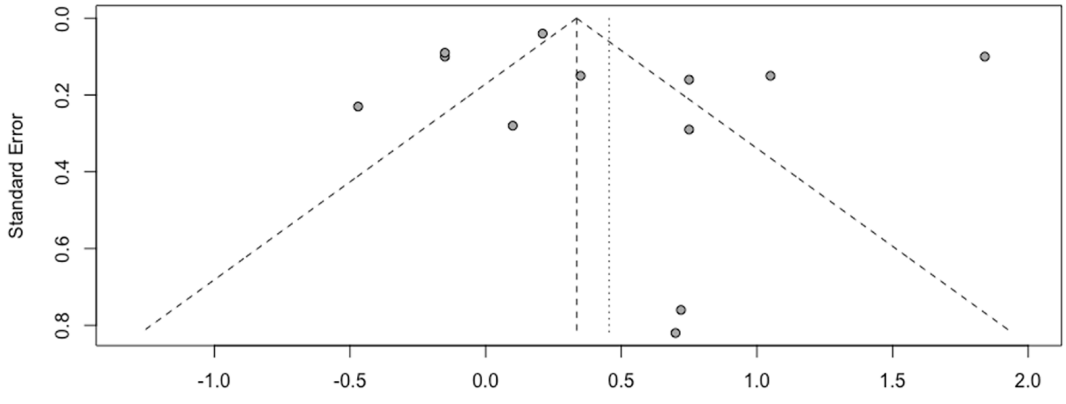
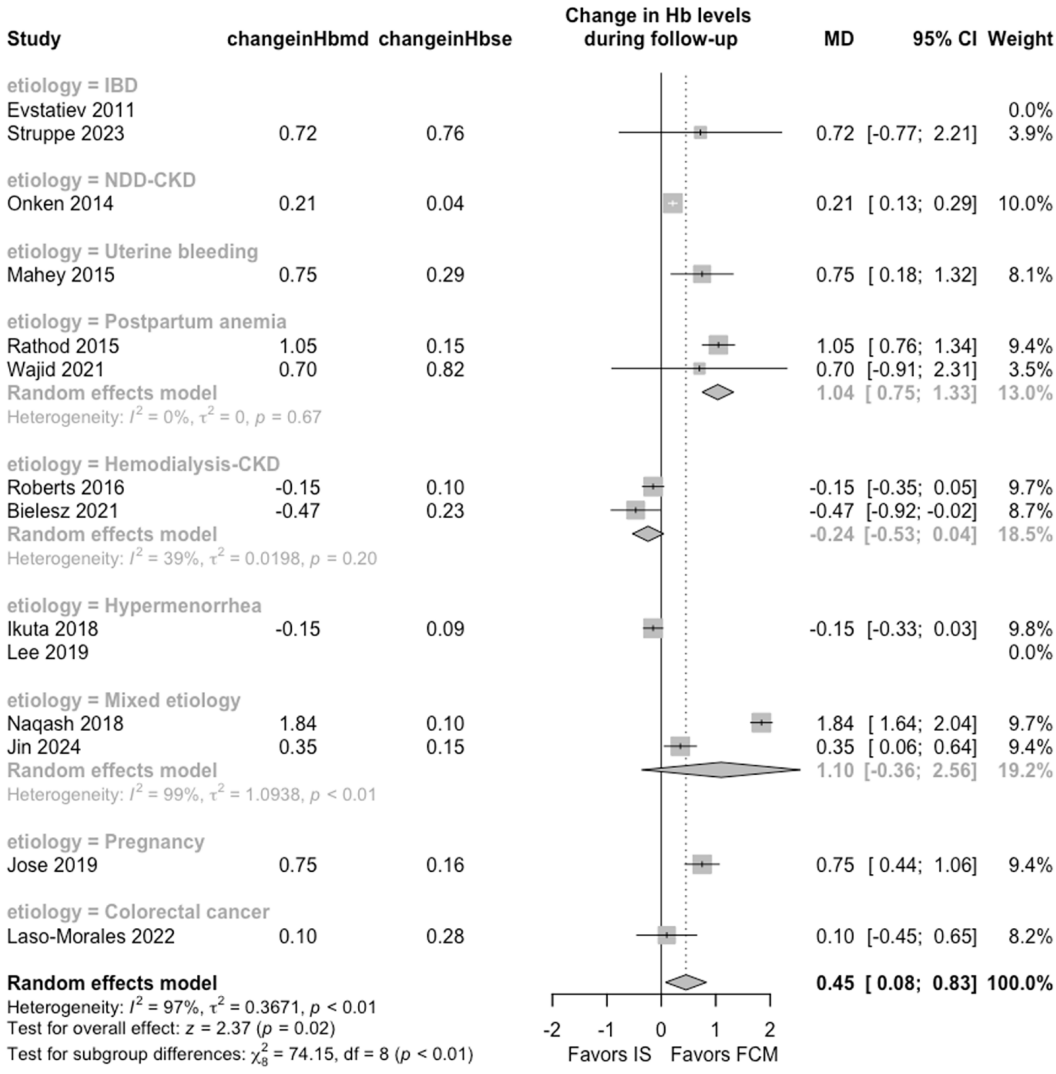
#4#1 AND #2 AND #3

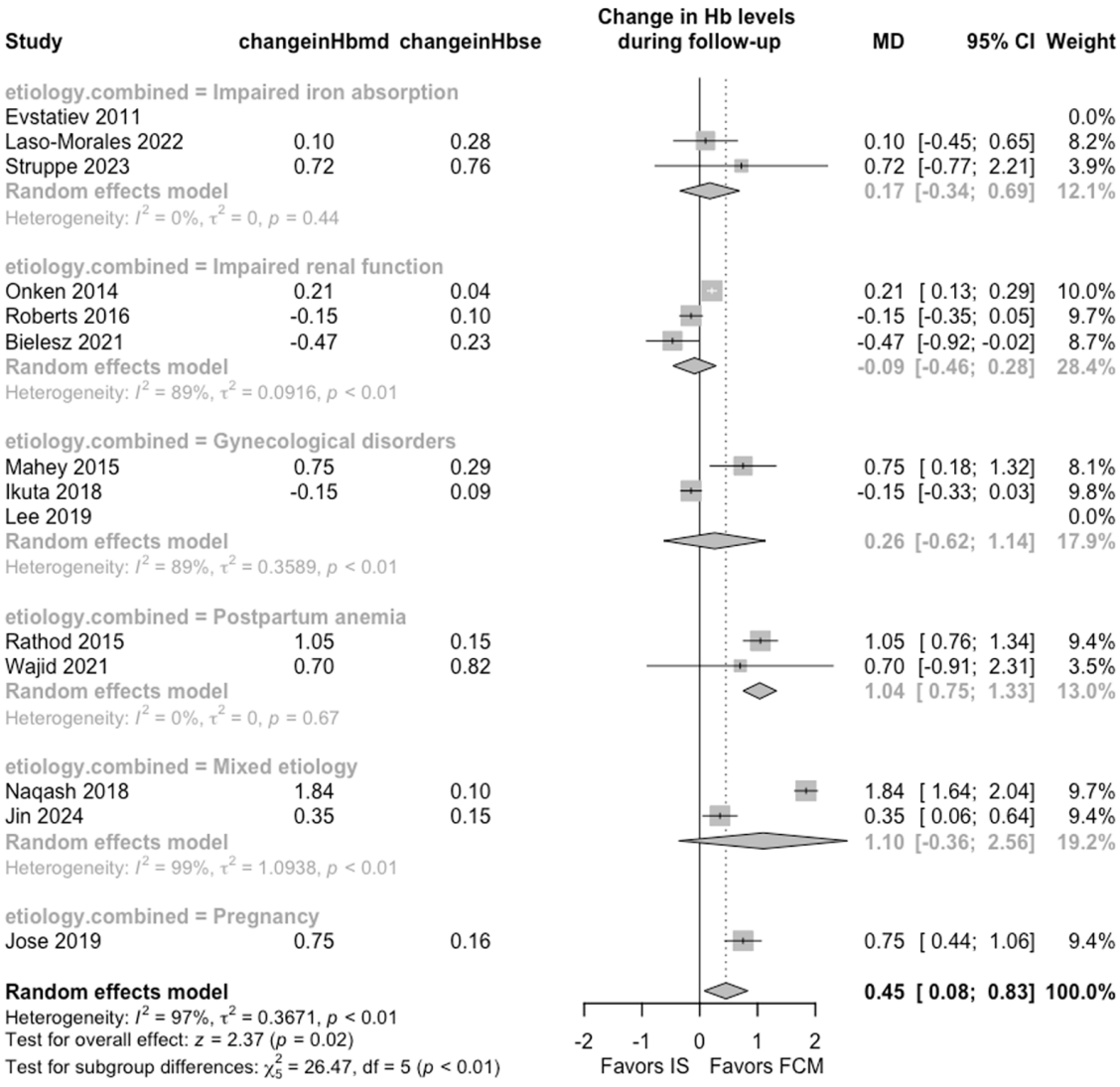


Supplementary Figure 1. Funnel plot with 95% confidence limits of the pooled proportion of change in hemoglobin levels during follow-up.



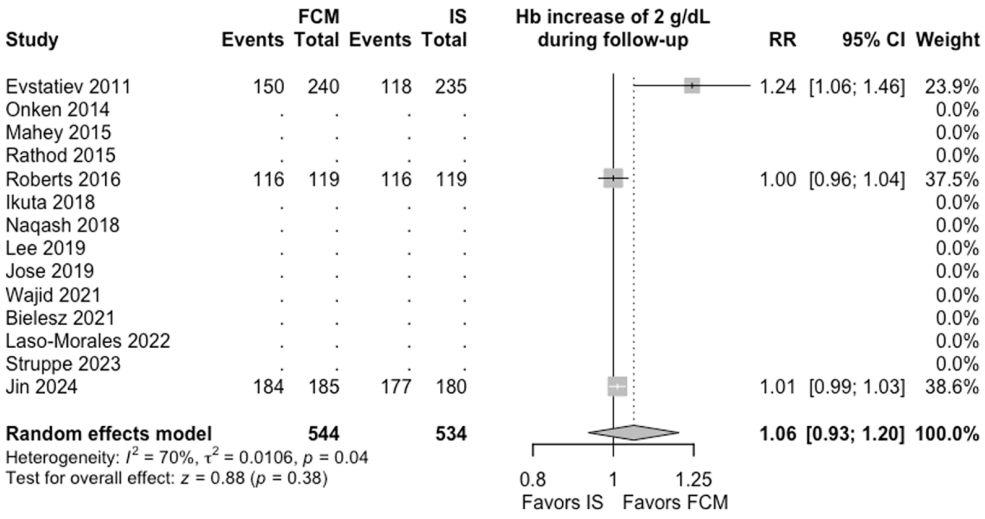
Supplementary Figure 2. Forest plot of subgroups by etiology of change in hemoglobin levels during follow-up.

IBD: Inflammatory bowel disease; NDD-CKD: non-dialysis dependent chronic kidney disease; FCM: ferric carboxymaltose; IS: iron supplementation; Hb: hemoglobin; MD: mean difference; CI: confidence interval.



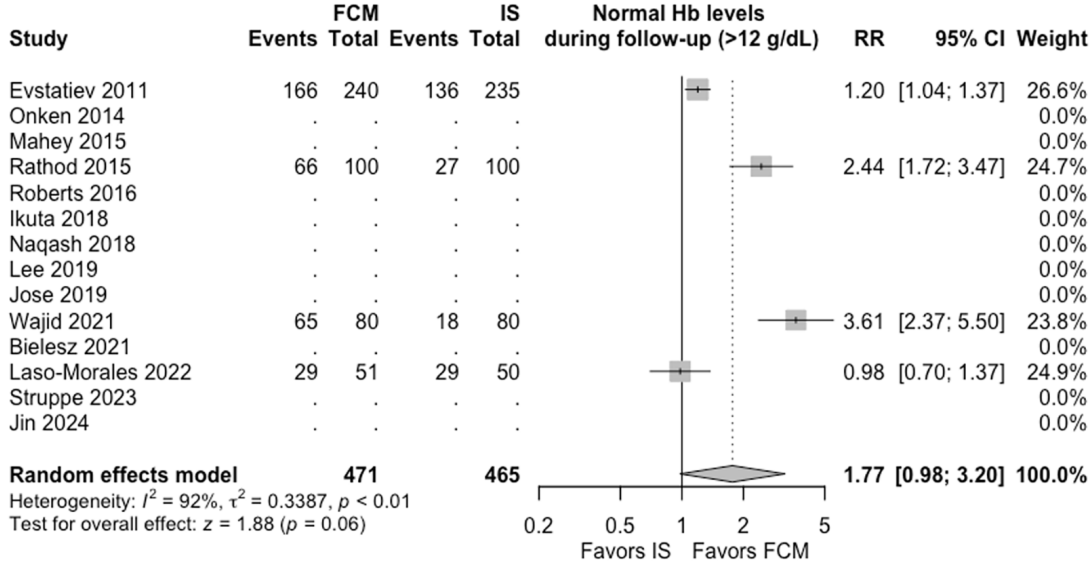
Supplementary Figure 3. Forest plot of subgroups by combined etiology of change in hemoglobin levels during follow-up.

FCM: Ferric carboxymaltose; IS: iron supplementation; Hb: hemoglobin; MD: mean difference; CI: confidence interval.



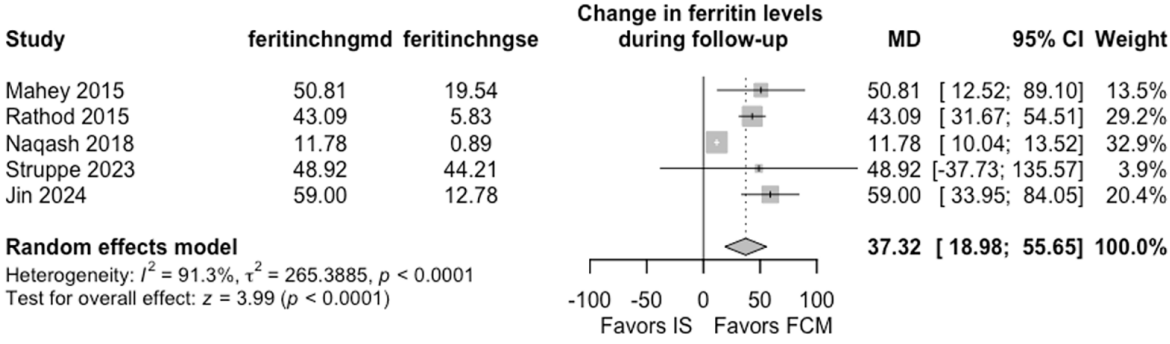
Supplementary Figure 4. Forest plot of hemoglobin increase of 2 g/dL during follow-up.

FCM: Ferric carboxymaltose; IS: iron supplementation; Hb: hemoglobin; RR: relative risk; CI: confidence interval.



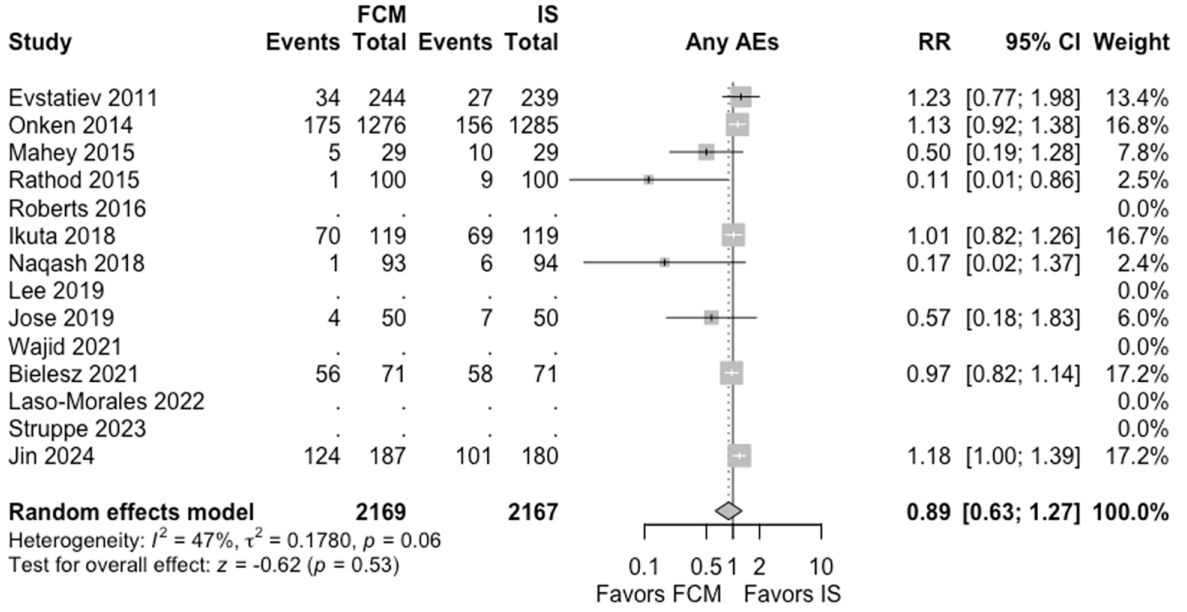
Supplementary Figure 5. Forest plot of risk of achievement at normal hemoglobin levels.

FCM: Ferric carboxymaltose; IS: iron supplementation; Hb: hemoglobin; RR: relative risk; CI: confidence interval.



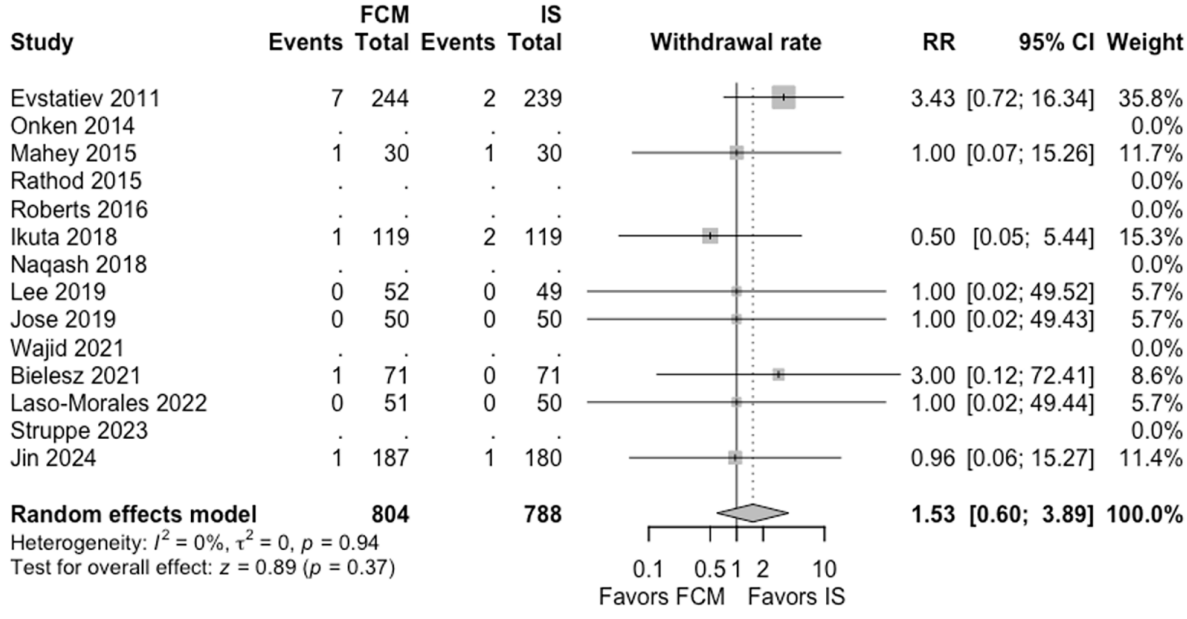
Supplementary Figure 6. Forest plot of change in ferritin levels during follow-up.

MD: Mean difference; CI: confidence interval; FCM: ferric carboxymaltose; IS: iron supplementation.



Supplementary Figure 7. Forest plot of risk of any adverse events.

FCM: Ferric carboxymaltose; IS: iron supplementation; RR: relative risk; CI: confidence interval; AEs: adverse events.



Supplementary Figure 8. Forest plot of risk of withdrawal due to adverse events.

FCM: Ferric carboxymaltose; IS: iron supplementation; RR: relative risk; CI: confidence interval.

Supplementary Table S1. Patient baseline characteristics of the included trials.

Study ID	Arm	Sample size	Age, mean (SD or range), years	Female, n (%)	White, n (%)	Black or African American, n (%)	Asian, n (%)	Other, n (%)	ESA use, n (%)	Previous iron therapy, n (%)	Baseline Hb, g/dL	Baseline ferritin, ng/mL	Baseline TSAT (SD), %	Baseline eGFR, mL/min/1.73 m^2
Evstatiev et al. [14]	FCM	240	39.5 (18-81)	146 (59.8%)	240 (100%)	0 (0%)	0 (0%)	0 (0%)	-	-	10.1 (1.5)	14.8 (24.6)	9 (9.1)	-
	IS	235	38 (18-78)	138 (57.7%)	235 (100%)	0 (0%)	0 (0%)	0 (0%)	-	-	10.3 (1.5)	17.8 (27.6)	9.6 (9.5)	-
Onken et al. [15]	FCM	1276	67.5 (13)	810 (63.5%)	676 (53%)	334 (26.2%)	20 (1.6%)	12 (0.9%)	230 (18%)	696 (54.5%)	10.31 (0.833)	73.01 (64.6)	19.79 (7.78)	32.5 (14.7)
	IS	1285	67.2 (13)	818 (63.7)	693 (53.9%)	325 (25.3%)	21 (1.6%)	10 (0.8%)	228 (17.7%)	693 (53.9%)	10.32 (0.826)	75.05 (64.1)	19.56 (7.4)	32.27 (14.9)
Mahey et al. [19]	FCM	30	36.3 (9)	30 (100%)	0 (0%)	0 (0%)	0 (0%)	30 (100%)	-	-	7.42 (1.23)	10 (3.9-28)	-	-
	IS	30	35.2 (7.5)	30 (100%)	0 (0%)	0 (0%)	0 (0%)	30 (100%)	-	-	7.73 (1.2)	8.8 (2.3-20)	-	-
Rathod et al. [16]	FCM	100	25.9 (3.57)	100 (100%)	0 (0%)	0 (0%)	0 (0%)	100 (100%)	-	-	7.71 (1.17)	35.52 (20.2)	-	-
	IS	100	26 (3.66)	100 (100%)	0 (0%)	0 (0%)	0 (0%)	100 (100%)	-	-	8.05 (1.07)	38.39 (19.79)	-	-
Roberts et al. [30]	FCM	22	70.9 (66.7-76.8)	6 (27%)	22 (100%)	0 (0%)	0 (0%)	0 (0%)	16 (73%)	-	11.1 (0.9)	21.1 (13.3)	29 (9)	-
	IS	20	75.1 (56.1-79.8)	6 (30%)	20 (100%)	0 (0%)	0 (0%)	0 (0%)	11 (55%)	-	11.5 (1)	18.7 (13)	27 (12)	-
Ikuta et al. [11]	FCM	119	41.3 (6.2)	119 (100%)	0 (0%)	0 (0%)	119 (100%)	0 (0%)	-	-	9.2 (1.15)	-	-	-
	IS	119	41.4 (6.1)	119 (100%)	0 (0%)	0 (0%)	119 (100%)	0 (0%)	-	-	9.25 (1.08)	-	-	-
Naqash et al. [13]	FCM	100	30.41 (7.99)	100 (100%)	0 (0%)	0 (0%)	0 (0%)	100 (100%)	-	-	7.82 (0.75)	18.29 (2.16)	7.67 (1.97)	-
	IS	100	27.32 (4.15)	100 (100%)	0 (0%)	0 (0%)	0 (0%)	100 (100%)	-	-	7.64 (0.72)	18.13 (1.67)	7.02 (1.61)	-
Lee et al. [20]	FCM	52	44 (5.7)	52 (100%)	0 (0%)	0 (0%)	52 (100%)	0 (0%)	-	-	8.4 (1.4)	5.8 (5.7)	6.1 (7.3)	-
	IS	49	43.4 (5)	49 (100%)	0 (0%)	0 (0%)	49 (100%)	0 (0%)	-	-	8.4 (1.1)	5.7 (3.9)	4.8 (5.9)	-
Jose et al. [31]	FCM	50	27.5 (3.9)	50 (100%)	0 (0%)	0 (0%)	0 (0%)	50 (100%)	-	-	8.57 (0.89)	7.9 (0.4-22.3)	8 (0.4-30.5)	-
	IS	50	26.2 (3.6)	50 (100%)	0 (0%)	0 (0%)	0 (0%)	50 (100%)	-	-	8.67 (0.86)	9 (0.94-23)	12.5 (0.03-19.1)	-
Wajid et al. [32]	FCM	80	26.86 (4.32)	80 (100%)	0 (0%)	0 (0%)	0 (0%)	80 (100%)	-	-	-	-	-	-
	IS	80	23.16 (5.17)	80 (100%)	0 (0%)	0 (0%)	0 (0%)	80 (100%)	-	-	-	-	-	-
Bielesz et al. [12]	FCM	71	60 (15)	21 (30%)	69 (97%)	0 (0%)	2 (3%)	0 (0%)	65 (92%)		11.1 (0.9)	210 (109-403)	17 (13-24)	-
	IS	70	57 (14)	20 (28%)	68 (96%)	0 (0%)	3 (4%)	0 (0%)	67 (96%)		11.2 (1.1)	270 (131-414)	21 (13-27)	-
Laso-Morales et al. [21]	FCM	50	73 (10)	31 (62%)	50 (100%)	0 (0%)	0 (0%)	0 (0%)	-	-	11.2 (1.6)	102.8 (262.8)	10.6 (5.5)	-
	IS	51	71 (12)	26 (51%)	51 (100%)	0 (0%)	0 (0%)	0 (0%)	-	-	11.4 (1.4)	89.6 (194.7)	13.4 (9)	-
Struppe et al. [33]	FCM	10	48 (34-57)	5 (50%)	10 (100%)	0 (0%)	0 (0%)	0 (0%)	-	-	10.8 (9.2-11.5)	50 (28.25-211.25)	-	-
	IS	10	63 (46-72)	6 (60%)	10 (100%)	0 (0%)	0 (0%)	0 (0%)	-	-	9.65 (8.78-11.8)	30 (10.5-141.5)	-	-
Jin et al. [22]	FCM	187	39.9 (9.9)	173 (92.5%)	0 (0%)	0 (0%)	187 (100%)	0 (0%)	-	18 (9.6%)	7.74 (1.49)	4.47 (2.15)	4.82 (1.9)	-
	IS	180	38.9 (8.7)	169 (93.9%)	0 (0%)	0 (0%)	180 (100%)	0 (0%)	-	19 (10.6%)	8.06 (1.45)	4.93 (6.1)	4.92 (2.91)	-

FCM: Ferric carboxymaltose; IS: iron sucrose; ESA: erythropoiesis-stimulating agent; Hb: hemoglobin; TSAT: transferrin saturation; eGFR: estimated glomerular filtration rate; SD: standard deviation.

BRIEF REPORT

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Retrospective Evaluation of Clinical and Follow-Up Outcomes in Primary Cutaneous CD30+ Lymphoproliferative Disorders

Primer Kutanöz CD30+ Lenfoproliferatif Hastalıklarda Klinik ve Takip Sonuçlarının Retrospektif Değerlendirilmesi

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Abstract

This study evaluated the demographic data, clinical characteristics, treatment approaches, and treatment responses of 43 patients with primary cutaneous CD30+ lymphoproliferative disorders. Lymphomatoid papulosis (LyP) was characterized by predominantly papular (94.1%) and generalized (70.6%) lesions, while primary cutaneous anaplastic large-cell lymphoma (pcALCL) presented with tumoral (77.8%) and solitary (77.8%) lesions (p<0.001). Common treatments for LyP included methotrexate (response rate: 78.5%), topical corticosteroids (response rate: 83.3%), and phototherapy (response rate: 85.8%), but relapse rates were high. In pcALCL, complete remission was achieved with all treatments, with no relapses after brentuximab vedotin (BV). Secondary malignancies were noted in 20.6% of LyP cases. Both LyP and pcALCL had a 100% 5-year disease-specific survival rate, although two LyP patients (5.9%) died of secondary malignancies. In conclusion, LyP and pcALCL are both indolent lymphomas, with LyP being more prone to relapse. BV is effective for resistant pcALCL. LyP patients need long-term monitoring due to the risk of secondary malignancies.

Keywords: Primary cutaneous CD30+ lymphoproliferative disorders, Lymphomatoid papulosis, Primary cutaneous anaplastic large-cell lymphoma

Öz

Bu çalışmada, primer kutanöz CD30+ lenfoproliferatif hastalık tanılı 43 hastanın demografik verileri, klinik özellikleri, aldığı tedaviler ve tedavi yanıtları incelendi. Lenfomatoid papülozis (LP), genellikle papüler (%94,1) ve generalize (%70,6) lezyonlarla karakterize iken; primer kutanöz anaplastik büyük hücreli lenfomada (pkABHL) sıklıkla tümoral (%77,8) ve soliter (%77,8) lezyonlar izlendi (p<0,001). LP'de en sık verilen tedaviler metotreksat (yanıt oranı: %78,5), topikal kortikosteroidler (yanıt oranı: %83,3) ve fototerapiydi (yanıt oranı: %85,8), ancak nüks oranları yüksekti. pkABHL hastalarında ise tüm tedavilerle tam remisyon sağlanmıştı ve brentuksimab vedotin (BV) tedavisinden sonra ise nüks izlenmedi. LP olgularının %20,6'sında sekonder maligniteler saptandı. LP ve pkABHL hastalarında 5 yıllık hastalığa özgü sağkalım oranı %100'dü, ancak LP hastalarının ikisi (%5,9) sekonder maligniteler nedeniyle hayatını kaybetti. Sonuç olarak, LP ve pkABHL indolent lenfomalardır; ancak LP'de nüks daha sık izlenmektedir. Dirençli pkABHL olgularında BV etkili bir tedavi seçeneğidir. LP hastalarında sekonder malignite riski nedeniyle uzun dönem takip gereklidir.

Anahtar Sözcükler: Primer kutanöz CD30+ lenfoproliferatif hastalıklar, Lenfomatoid papülozis, Primer kutanöz anaplastik büyük hücreli lenfoma

Introduction

Primary cutaneous CD30+ lymphoproliferative disorders (LPDs), including lymphomatoid papulosis (LyP) and primary cutaneous anaplastic large-cell lymphoma (pcALCL), are rare conditions characterized by cutaneous involvement and an overall excellent prognosis. The 5-year survival rates are 99% for LyP and 95% for pcALCL [1].

Although both diseases express CD30, they differ significantly in clinical presentation and management. LyP typically manifests as multiple recurrent papulonodular lesions, while

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pcALCL usually presents as solitary or localized nodules/tumors [2]. Asymptomatic LyP often requires no treatment, but symptomatic cases are typically managed with phototherapy or methotrexate. For pcALCL, localized lesions are treated with surgery or radiotherapy, and chemotherapy is utilized for cases with extracutaneous involvement [3].

LyP patients are at an increased risk of developing secondary malignancies, especially mycosis fungoides (MF), highlighting the need for long-term monitoring [2].

This study aims to evaluate the clinical features, treatment strategies, and outcomes of primary cutaneous CD30+ LPDs at a tertiary care center.

Materials and Methods

We conducted a retrospective review of 43 patients diagnosed with primary cutaneous CD30+ LPDs in the Ankara University Faculty of Medicine from January 2006 to July 2023. Ethical approval (no: 19-1190-17, date: 27.11.2017) was obtained from the Ankara University Faculty of Medicine, Clinical Research Ethics Committee, and informed consent was provided by all patients.

Diagnoses were confirmed through clinical assessment, histopathological examination, and immunohistochemistry. Systemic anaplastic large-cell lymphoma was excluded through clinical, laboratory, and imaging investigations. Clinical data including patient demographics, lesion types, lesion distribution, and extent were documented according to the criteria of the European Organization of Research and Treatment of Cancer and the International Society for Cutaneous Lymphomas [4].

Histopathological examinations involved hematoxylin and eosin staining, with immunohistochemical testing performed using antibodies against CD2, CD3, CD4, CD5, CD7, CD8, CD20, and CD30 and additional tests for CD56, TIA-1, and cytotoxic markers anaplastic lymphoma kinase (ALK), perforin, and granzyme B as needed. Fluorescence in situ hybridization analyses were performed using an *IRF4-DUSP22* break-apart probe (Kreatech, Leica Biosystems, Deer Park, IL, USA) for the study of 6p25.3 rearrangement.

Treatment responses were categorized as complete response (CR), partial response, or no response. Patients achieving CR but later relapsing were considered as relapsed [3]. Spontaneous regression of individual lesions within weeks or months, regardless of whether new lesions appeared, was defined as self-healing. Survival data for overall survival (OS) and disease-specific survival (DSS) were recorded until July 1, 2023.

Statistical Analysis

Data were analyzed using IBM SPSS Statistics 25.0 (IBM Corp., Armonk, NY, USA) and statistical comparisons were made using chi-square tests, independent t-tests, and Mann-Whitney U tests with the significance threshold set at $p<0.05$.

Results

Demographic and Clinical Findings

Out of 43 patients, 34 were diagnosed with LyP and 9 with pcALCL. The mean follow-up duration was 54 months (range: 4-180 months). Patients with LyP predominantly presented with papular lesions (94.1%), while pcALCL was more commonly associated with tumoral lesions (77.8%) ($p<0.001$). LyP lesions were often generalized (70.6%), whereas pcALCL lesions were typically solitary (77.8%) ($p<0.001$) (Figure 1). Self-healing occurred in all cases of LyP but only 11.1% of pcALCL cases ($p<0.001$). Extracutaneous involvement, specifically inguinal lymph node involvement, was observed in one pcALCL patient by fluorodeoxyglucose positron emission tomography-computed tomography. The demographic characteristics and clinical data of the patients are summarized in Table 1.

Histopathological and Immunophenotypic Features

Histologically, LyP was predominantly type A (67.6%), followed by type C (23.5%), type E (5.9%), and type D (2.9%). No type B lesions were observed. Both LyP and pcALCL were negative for ALK expression. CD56 positivity was found in 27.3% of LyP cases.

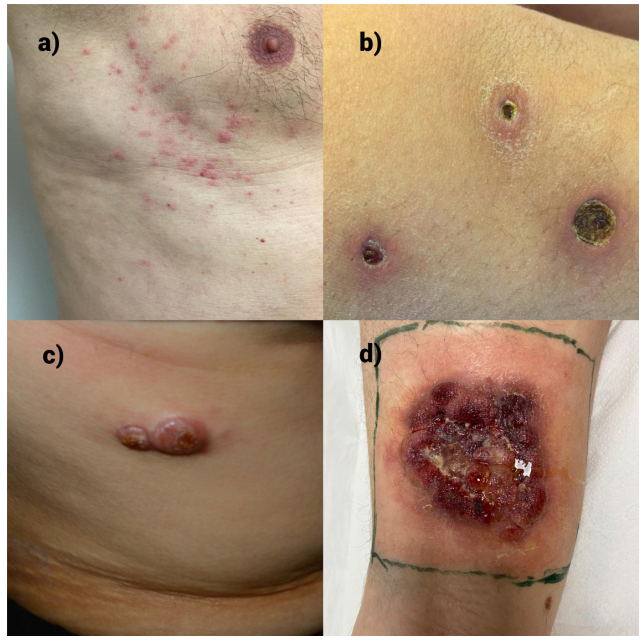

Figure 1. a) Papules on the trunk in a patient with LyP; (b) ulcerated papulonodular lesions covered by necrotic crusts in a patient with LyP; (c, d) ulcerated tumoral lesions in patients with pcALCL.

LyP: Lymphomatoid papulosis; pcALCL: primary cutaneous anaplastic large-cell lymphoma.

Cytotoxic markers were detected in 1 of 7 LyP patients and 2 of 5 pcALCL patients. *DUSP22* rearrangements were identified in 2 of 4 pcALCL cases.

Treatment and Treatment Responses

Treatment strategies for LyP included methotrexate, psoralen and ultraviolet A (PUVA) therapy, and topical corticosteroids (TCs). Methotrexate (<20 mg/week) had an overall response rate of 78.5%, while PUVA and TCs showed response rates of 85.8% and 83.3%, respectively. However, relapse rates for all treatments were high. For pcALCL, 2 patients treated with brentuximab vedotin (BV) achieved CR. One of these patients who received BV treatment had lymph node involvement, while the other patient experienced relapses following treatment with radiotherapy, CHOP, and ESHAP protocols (Tables 2 and 3).

Secondary Malignancies

Secondary lymphomas developed in 7 LyP patients (20.6%), including 4 cases of MF, with 2 of the patients in the early stages and 2 in advanced stages. One patient developed Hodgkin lymphoma, one patient had pcALCL, and another had non-Hodgkin lymphoma (Burkitt lymphoma). No significant associations were found between the occurrence of secondary lymphoma and patient demographics or LyP subtypes (p>0.05).

Survival Outcomes

The mean follow-up duration for LyP patients was 52.3 months, with 16 patients alive with disease, 15 patients alive without disease, and 3 deaths (2 from secondary lymphomas, 1 from another cause). The mean follow-up duration for pcALCL patients was 54.1 months. One patient died from septic shock and 7 were alive without disease. The 5-year DSS rates were 100% for both LyP and pcALCL. The 5-year OS rates were 90.9% for LyP and 83.3% for pcALCL.

Discussion

LyP and pcALCL are distinct entities within the spectrum of primary cutaneous CD30⁺ LPDs, with generally favorable prognoses but differing in clinical presentation and histopathology [5]. In our study, the male-to-female ratio among LyP cases (1.41) was consistent with prior studies, while the ratio for pcALCL cases (0.8) was lower than expected [6]. The mean age at diagnosis for LyP was 48.4 years, while for pcALCL it was 54.5 years. These findings align with prior reports, although a few pediatric cases were observed in our study, which is unusual for these conditions [6,7,8,9,10,11].

Histologically, type A LyP lesions predominated, which is consistent with other studies [12], and we observed no type B

Table 1. Demographic characteristics and clinical findings of patients with primary cutaneous CD30⁺ lymphoproliferative disorders.

	LyP (n=34)	pcALCL (n=9)	p
Male/female ratio	23/11	4/5	0.257
Mean age at diagnosis, years, mean ± SD	48.4±18.69	54.5±24.23	0.415
Age at diagnosis, years <18 years old, n (%) >60 years old, n (%)	 2 (5.9) 8 (23.5)	 1 (11.1) 4 (44.4)	 0.515 0.237
Type of skin lesions, n (%)	Papule: 32 (94.1) Plaque: 10 (29.4) Nodule: 9 (26.5) Papulonodular: 8 (23.5) Tumor: 2 (5.9)	Papule: 2 (22.2) Plaque: 1 (11.1) Nodule: 2 (22.2) Papulonodular: 1 (11.1) Tumor: 7 (77.8)	<0.001
Distribution of lesions, n (%)	Head-neck: 5 (14.7) Trunk: 16 (47.1) Upper extremity: 18 (52.9) Lower extremity: 18 (52.9)	Head-neck: 3 (33.3) Trunk: 1 (11.1) Upper extremity: 2 (22.2) Lower extremity: 4 (44.5)	0.199
Lesion pattern, n (%)	Generalized: 24 (70.6) Localized: 9 (26.5) Solitary: 1 (2.9)	Generalized: 1 (11.1) Localized: 1 (11.1) Solitary: 7 (77.8)	<0.001
Subjective symptoms Pruritus, n (%) Pain, n (%) Asymptomatic, n (%)	 18 (52.9) 2 (5.9) 12 (35.3)	 3 (33.3) 1 (11.1) 3 (33.3)	 0.457 0.515 1.000
Ulcer, n (%)	6 (17.6)	2/5 (40)	0.268
Self-healing, n (%)	34 (100)	1 (11.1)	<0.001
Extracutaneous disease, n (%)	-	1 (11.1)	0.209

LyP: Lymphomatoid papulosis; pcALCL: primary cutaneous anaplastic large-cell lymphoma; SD: standard deviation.

Table 2. Treatments and treatment responses in patients with primary cutaneous CD30+ lymphoproliferative disorders.

	Partial remission, n (%)	Complete remission, n (%)	No response, n (%)	Relapse after treatment*, n (%)
LyP patients (n=34)				
Methotrexate (n=14)	3 (21.4)	8 (57.1)	3 (21.4)	6 (75)
Topical corticosteroids (n=12)	4 (33.3)	6 (50)	2 (16.7)	5 (83.3)
Phototherapy (n=7)	3 (42.9)	3 (42.9)	1 (14.3)	2 (66.7)
Follow-up without treatment (n=7)	6 (85.7)	1 (14.3)	-	-
Peginterferon alfa-2a (n=4)	2 (50)	2 (50)	-	1 (50)
Systemic steroids (n=3)	1 (33.3)	1 (33.3)	1 (33.3)	1 (100)
Intralesional interferon alfa-2a (n=1)	-	1 (100)	-	-
Surgical excision (n=1)	-	1 (100)	-	1 (100)
pcALCL patients (n=9)				
Local radiotherapy (n=5)	-	5 (100)	-	2 (40)
Surgical excision (n=4)	-	4 (100)	-	1 (25)
Brentuximab vedotin (n=2)	-	2 (100)	-	-
Multi-agent chemotherapy (n=2)	-	2 (100)	-	2 (100)

*: Only for patients who achieved complete response; LyP: lymphomatoid papulosis; pcALCL: primary cutaneous anaplastic large-cell lymphoma.

Table 3. Distribution of sequential treatments with responses in patients with lymphomatoid papulosis (n=34).

	Initial treatments	Second treatments	Third treatments
Methotrexate (n=14)	n=11 (PR: 2, CR: 6, NR: 3)	n=1 (CR: 1)	n=2 (PR: 1, CR: 1)
Topical corticosteroids (n=12)	n=12 (PR: 4, CR: 6, NR: 2)	-	-
Phototherapy (n=7)	n=4 (PR: 1, CR: 3)	n=2 (PR: 1, NR: 1)	n=1 (PR: 1)
Follow-up without treatment (n=7)	n=5 (PR: 4, CR: 1)	n=2 (PR: 2)	-
Peginterferon alfa-2a (n=4)	-	n=4 (PR: 2, CR: 2)	-
Systemic steroid (n=3)	n=1 (PR: 1)	n=2 (CR: 1, NR: 1)	-
Intralesional interferon alfa-2a (n=1)	-	n=1 (CR: 1)	-
Surgical excision (n=1)	n=1 (CR: 1)	-	-

PR: Partial remission; CR: complete remission; NR: no response.

lesions in our cohort. *DUSP22* rearrangements were found in two pcALCL cases, a finding that aligns with the literature, as these rearrangements are more common in pcALCL compared to LyP. The prognostic significance of *DUSP22* rearrangements in pcALCL remains unclear but they have been associated with an indolent clinical course in other settings [13,14,15].

Treatment for LyP typically involves a watch-and-wait approach for limited lesions, with phototherapy and methotrexate providing effective treatment for generalized cases [3]. Methotrexate was effective in 78.5% of our cases, though the high relapse rate indicates the need for ongoing management. Phototherapy, and especially PUVA, was effective in our cohort, with relapse rates consistent with the literature [3,16,17,18].

pcALCL treatment often involves radiotherapy or surgery for localized lesions, with chemotherapy and BV used for relapsed or advanced cases [3,19,20,21]. The use of BV in our study showed promising results, particularly in treatment-resistant cases. Relapse after BV was not observed in our cohort during a 3.5-year follow-up period, suggesting its potential as a viable treatment for resistant pcALCL.

The development of secondary malignancies, particularly MF, is a well-established risk in cases of LyP [22,23,24]. In our

study, 20.6% of LyP patients developed secondary lymphomas, underscoring the need for vigilant long-term follow-up. No significant associations were found between demographic factors or LyP subtype and the occurrence of secondary malignancies.

Conclusion

LyP and pcALCL are distinct primary cutaneous CD30+ LPDs with favorable prognosis. Although treatment modalities often lead to remission, the chronic and recurrent nature of LyP and the risk of secondary malignancies necessitate ongoing management. pcALCL patients can benefit from localized treatments including surgery and radiotherapy, with BV emerging as a promising option for resistant cases. Future research should focus on identifying factors that influence relapse and secondary malignancy risk to refine treatment and follow-up strategies.

Ethics

Ethics Committee Approval: Ethical approval (no: 19-1190-17, date: 27.11.2017) was obtained from the Ankara University Faculty of Medicine, Clinical Research Ethics Committee.

Informed Consent: Informed consent was provided by all patients. Patient consent was obtained for the photographs.

Footnotes

Authorship Contributions

Surgical and Medical Practices: H.Ş., A.T.A., İ.K.Y., A.H., I.K., A.K., A.B., B.N.A.; Concept: H.Ş., A.T.A., İ.K.Y., A.H., I.K., A.K., A.B., B.N.A.; Design: H.Ş., A.T.A., İ.K.Y., A.H., I.K., A.K., A.B., B.N.A.; Data Collection or Processing: H.Ş., A.T.A., İ.K.Y., A.H., I.K., A.K., A.B., B.N.A.; Analysis or Interpretation: H.Ş., A.T.A., İ.K.Y., A.H., I.K., A.K., A.B., B.N.A.; Literature Search: H.Ş., A.T.A., İ.K.Y., A.B., B.N.A.; Writing: H.Ş., A.T.A., İ.K.Y., A.B., B.N.A.

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

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Acute Myeloid Leukemia with *NUP98::LNP1* Fusion Mimicking Chronic Myeloid Leukemia

Kronik Miyeloid Lösemiyi Taklit Eden *NUP98::LNP1* Füzyonu ile Seyreden Akut Miyeloid Lösemi

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**These authors contributed equally to this work.*

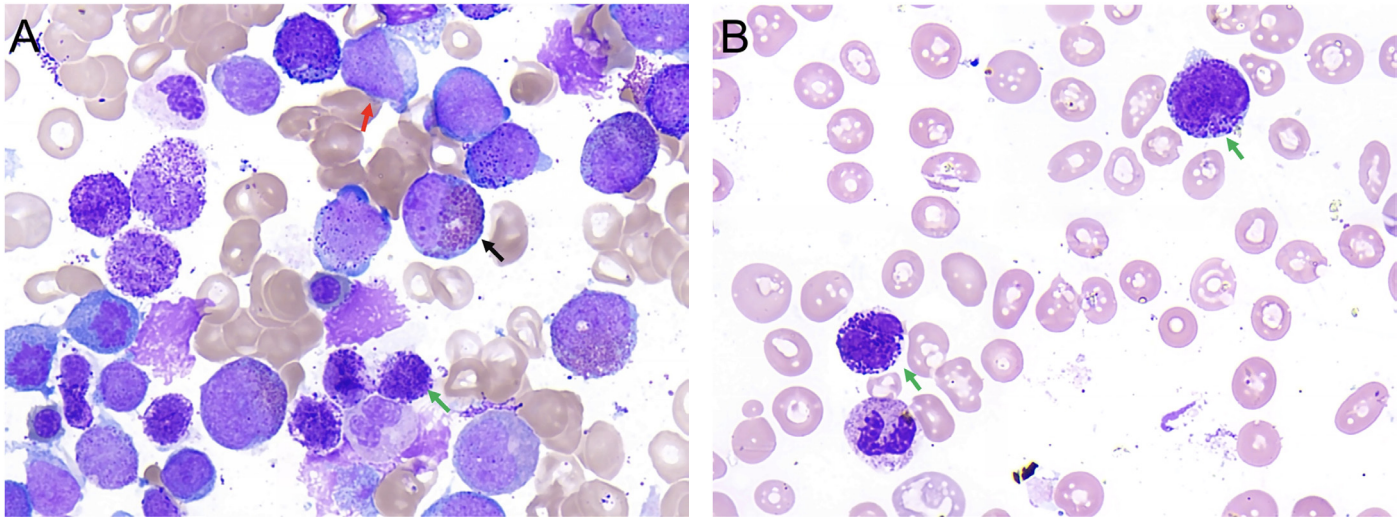

Figure 1. A, B) The red arrow points to a myeloblast, the black arrow points to an eosinophil, and the green arrows point to basophils. Original magnification of 1000^{x}, Wright-Giemsa stain.

A 60-year-old female patient presented with a history of hepatitis, tuberculosis, and gastric cancer. She had developed recurrent fever during the treatment for gastric cancer. Peripheral blood (PB) examination showed white blood cell count of 47.7x10^{9}/L, with 1% blasts, 1% promyelocytes, 2% neutrophilic myelocytes, 5% neutrophilic metamyelocytes, 13% neutrophils, 8% lymphocytes, 3% monocytes, 3% eosinophils, and 64% basophils. Hemoglobin was 87 g/L and platelet count was 75x10^{9}/L. A bone marrow (BM) smear revealed active proliferation of granulocytes with 10% myeloblasts (Figure 1A). The proportions of eosinophils and basophils in the BM were also notably increased, accounting for 9% and 24% of nucleated cells, respectively (Figure 1A). Additionally, the percentage of basophils in the PB was significantly elevated, reaching 52%

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(Figure 1B). The morphological features of the BM and PB closely mimicked chronic myeloid leukemia in the chronic high-risk phase, characterized by a significant elevation in basophils. Interestingly, both quantitative reverse-transcription polymerase chain reaction and fluorescence in situ hybridization indicated negativity for the *BCR::ABL1* fusion gene. The chromosomal karyotype was 46,XX,t(3;11)(q12.2;p15.4). Meanwhile, RNA sequencing identified only the *NUP98::LNP1* fusion transcript, with no detectable mutations in other genes such as *ASXL1*, *NRAS*, *KRAS*, *SRSF2*, *TET2*, *CBL*, *CSF3R*, *JAK2*, *ETNK1*, or *SETBP1*. Ultimately, a diagnosis of acute myeloid leukemia (AML) with *NUP98* rearrangement was established, as AML with *NUP98* rearrangements is exempt from the historical threshold of 20% blasts according to the 2022 guidelines of the World Health Organization [1,2].

AML with *NUP98::LNP1* fusion transcripts is extremely rare. To date, only one case has been reported in which blasts exceeded 20% [3]. The present case is the first reported instance of this specific morphological presentation in AML with *NUP98::LNP1* fusion transcripts. Numerous studies have demonstrated that *NUP98* rearrangements are associated with unfavorable prognosis, highlighting the critical importance of their identification [4,5,6].

Keywords: Acute myeloid leukemia, *NUP98*::*LNP1*, Morphological presentation

Anahtar Sözcükler: Akut myeloid lösemi, *NUP98::LNP1*, Morfolojik görünüm

Ethics

Informed Consent: Written informed consent was obtained from the individual for the publication of any potentially identifiable images or data included in this article.

Footnotes

Authorship Contributions

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

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Extranodal NK/T-Cell Lymphoma, Nasal Type

Ekstranodal NK/T-Hücreli Lenfoma, Nazal Tip

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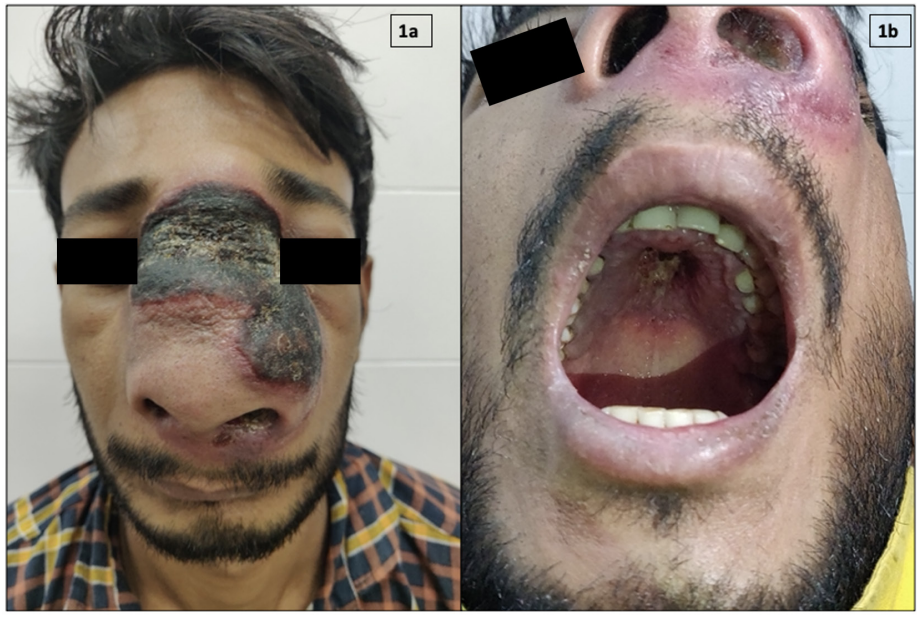

Figure 1. Examination revealed a large necrotic mass on the patient's nose (a) and a perforating ulcer on the hard palate (b).

A 26-year-old man presented with a 3-month history of pain and swelling over the nose without any fever, weight loss, or night sweats. Examination revealed a large necrotic mass on his nose (Figure 1a) and a perforating ulcer on the hard palate (Figure 1b). His blood counts were normal. A biopsy from the nasal lesion revealed large atypical cells with brisk mitosis. Immunohistochemistry was positive for CD2, CD3, CD7, CD56, perforin, and granzyme but negative for CD20, CD5, CD4, CD8, ALK, and CD30. Epstein-Barr encoding region in situ hybridization was positive according to the biopsy, confirming the diagnosis of extranodal NK/T-cell lymphoma, nasal type (ENKTCL-NT). Epstein-Barr virus DNA was detected in the peripheral blood (90,900 IU/mL). Positron emission tomography-computed tomography scanning confirmed stage IV disease. The patient initially responded to SMILE chemotherapy (steroids, methotrexate, ifosfamide, L-asparaginase, and etoposide) but later died due to hemophagocytic lymphohistiocytosis.

ENKTCL-NT is an aggressive subtype of non-Hodgkin lymphoma (NHL) caused by the Epstein-Barr virus. It is prevalent in some Asian (China, Japan, Thailand, Korea, Taiwan) and Latin American (Mexico, Peru, Brazil, Guatemala) countries [1,2]. Unlike other NHLs, anthracycline-containing regimens are ineffective for the treatment of ENKTCL-NT. Due to its high efficacy, asparaginase is the cornerstone in the management of ENKTCL-NT [2,3].

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Anahtar Sözcükler: NK/T hücreli lenfoma, Ebstein-Barr virusü, SMILE kemoterapi

Ethics

Informed Consent: Written informed consent was obtained from the patient prior to publication of any material pertaining to the case.

Footnotes

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

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LETTER TO THE EDITOR

Turk J Hematol 2025;42:146-147

Large and Multi-Nuclei Blasts in Acute Myeloid Leukemia with the Hypotriploid Karyotype and *TP53* Mutation with P210 *BCR::ABL1* Transcript

Hipotriploid Karyotip ve P210 *BCR::ABL1* Transkripti ile *TP53* Mutasyonu Olan Akut Miyeloid Lösemide Büyük ve Çok Çekirdekli Blastlar

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To the Editor,

A 62-year-old woman with no significant past medical history presented to the general practice clinic complaining of fatigue for 2 weeks. The doctor promptly referred her to the hematology department due to the initial complete blood count test indicating leukocytosis ($20.8x10^9$/L), anemia (hemoglobin of 79 g/L), and thrombocytopenia ($7x10^9$/L). Abdominal ultrasound did not reveal splenomegaly. The peripheral blood smear revealed 2% blasts and no basophils. Bone marrow cytology showed hypercellularity, with 12% myeloblasts and 31% large cells containing two or more nuclei (Figures 1A-1I). Leukemic cells were positive for CD34, HLA-DR, CD13, CD33, CD38, CD123, and cMPO. These results confirmed that the large cells were myeloblasts. Karyotyping analysis showed 46,XX,t(9;22)(q34; q11.2)[8]/60~65<3n>,XXX,-3,-4,-5,+6,-7,+8,-9,t(9;22)(q34;q11.2)×2,-10,+11,-12,+13,-16,-17,-18,+19,+20,+21,-22[cp5]/46,XX[7] (Figures 1J and 1K). A comprehensive analysis of 20 karyotypes revealed the presence of three cell lines, two of which were abnormal.

The P210 *BCR::ABL1* transcript was detected in bone marrow samples, revealing P210 *BCR::ABL1*/ABL1 100.0 and BCR-ABL P210 ($\%^{IS}$) 29.4% (Figure 1L). Mutation analysis revealed a primary missense mutation in *TP53* at exon six of c.584T>A p.I195N, with a variant abundance of 62.89%. The diagnosis was acute myeloid leukemia (AML) with a hypotriploid karyotype and *TP53* mutation with the P210 *BCR::ABL1* transcript. After one round of the VA regimen (azacitidine at 100 mg daily on days 1-7 combined with venetoclax at 100 mg on day 1, 20-300 mg on day 2, and 40-300 mg on days 3-7) together with imatinib at 600 mg, the BCR-ABL ($\%^{IS}$) value decreased to 11% (Figure 1L) and the platelet count increased to $51x10^9$/L. After 3 weeks of continuous oral administration of imatinib at 60 mg three times daily, BCR-ABL ($\%^{IS}$) declined further to 5.6% (Figure 1L). No *TP53* gene mutation was detectable and the chromosome analysis was consistent with the initial diagnosis. Bone marrow cytology showed hypercellularity with 22% myeloblasts. The patient resumed antitumor therapy with imatinib at 600 mg and venetoclax at 100 mg. This time, the patient was unable to tolerate chemotherapy, fell into a coma, and passed away 3 months later due to the family's decision to forgo further treatment.

In this case, the absence of antecedent leukocytosis, basophils, or splenomegaly supported a diagnosis of P210 *BCR::ABL1*-positive AML. Genetic risk stratification categorizes AML with *BCR::ABL1* fusion, complex karyotypes such as triploidy, -5/5q, and -7/7q as being of higher risk [1]. Blast cells and stem cells in AML require BCL-2 for survival, and preclinical studies have demonstrated the efficacy of the BCL-2 inhibitor venetoclax in treating AML [2]. Combining venetoclax and tyrosine kinase inhibitors (TKIs) may be especially beneficial for patients with Philadelphia chromosome-positive clones predominating without other significant coexisting drivers [3]. One potential reason for this is that chromosomal instability can lead to the emergence of subclones harboring the Philadelphia chromosome in advanced leukemia, and TKI administration may not yield further advantages if *BCR::ABL1* fusion is not the primary oncogenic driver [3]. Unlike chronic myeloid leukemia (CML), *BCR::ABL1* fusion may confer a proliferative advantage in AML because it is unlikely to serve as the primary mutational driver [4]. Thus, the venetoclax and TKI combination regimen is a feasible treatment option for Philadelphia chromosome-positive myeloid leukemia, potentially presenting a particular advantage for patients with CML of the blast phase due to its targeting of the primary driver, *BCR::ABL1* fusion [3,5].

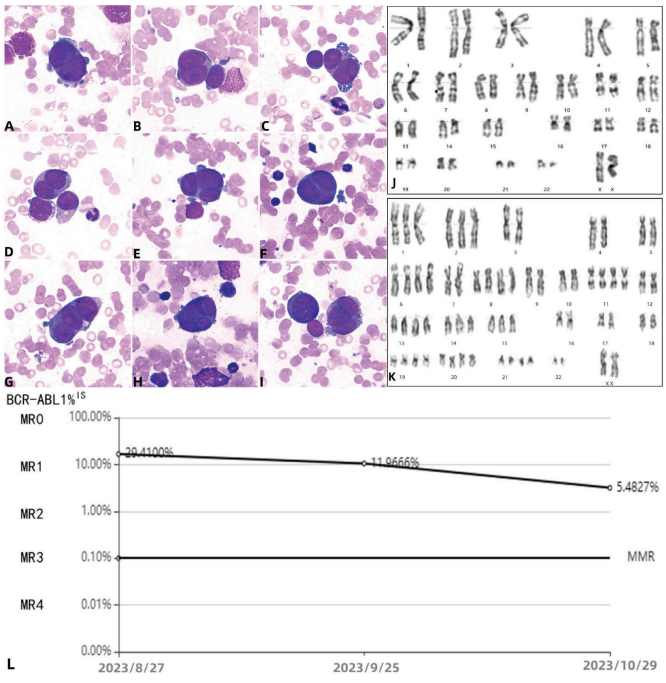

Figure 1. Bone marrow morphology and chromosome karyotype (A-K): the bone marrow aspirate showed binucleated leukemia cells (A-D) and multinucleated leukemia cells (E-I), with basophilic and vacuolated cytoplasm; Wright-Giemsa staining, 1000^{x}. Eight cells showed translocation between chromosomes 9 and 22, with breakage and rejoining at 9q34 and 22q11.2, 46,XX,t(9;22)(q34;q11.2)[8] (J). The second observed abnormality, hypotriploidy, involves multiple abnormalities in chromosome number. Specifically, there are two groups of t(9;22) present, denoted as 60~65<3n>,XXX,-3,-4,-5,+6,-7,+8,-9, t(9;22)(q34;q11.2)×2,-10,+11,-12,+13,-16,-17,-18,+19,+20,+21,-22[cp5] (K). A change in *BCR-ABL1* % was achieved during treatment (L).

Keywords: Acute myeloid leukemia, P210 *BCR::ABL1* transcript, Hypotriploid karyotype

Anahtar Sözcükler: Akut miyeloid lösemi, P210 *BCR::ABL1* transkripti, Hipotriploid karyotip

Ethics

Informed Consent: Informed consent was obtained from the patient's family.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Y.H., X.C.; Concept: Y.H., X.C.; Design: Y.H., X.C.; Data Collection and Processing: Y.H., X.C.; Analysis or Interpretation: Y.H., X.C.; Literature Search: Y.H., X.C.; Writing: Y.H., X.C.

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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LETTER TO THE EDITOR

Turk J Hematol 2025;42:148-149

Basophils in Acute Promyelocytic Leukemia: Clonality or Reactiveness?

Akut Promiyelositik Lösemide Bazofiller: Klonalite mi, Reaktivite mi?

Xue Li, Qingqing Yang, Pengfei Qin, Baodan Yu

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To the Editor,

A 19-year-old female patient presented with ecchymoses of 2-3 cm in diameter on the skin of the right knee and right elbow without injury. On admission, a routine complete blood count showed a leukocyte count of $1.38x10^9$/L, hemoglobin of 63 g/L, and platelet count of $24x10^9$/L. A peripheral blood smear showed abnormal promyelocytes representing 13% of the nucleated cells (Figure 1A). A bone marrow aspirate smear showed 71% abnormal promyelocytes without Auer bodies (Figure 1B) and 5% basophils (Figures 1C and 1D), demonstrating positivity for myeloperoxidase staining (Figure 1E). The presumptive diagnosis was acute promyelocytic leukemia (APL).

Immunophenotyping of the bone marrow aspirate revealed 82% myeloid-derived leukemic cells expressing CD117, CD33, CD9, cMPO, and CD13, consistent with APL. Furthermore, we observed an increase of 4.78% in basophils expressing CD9, CD203, CD11b, CD13, CD33, and CD123. Subsequently, both reverse-transcription polymerase chain reaction and fluorescence in situ hybridization (FISH) confirmed the presence of the *PML::RARA* fusion gene. The karyotyping result was 46,XX,t(15;17) (q24;q21) [18]/46,XX[2]. Next-generation sequencing identified the p.S386* mutation in the *WT1* gene. FISH revealed the following signal patterns across the 62 cells analyzed for the identified *PML::RARA* fusion gene: 2G2R, 8.0%; 1G1R1F, 25.8%; 1G1R2F, 58.1%; 2G2R1F, 6.5%; and 1G2R, 1.6%. To ascertain whether the elevated basophils were clonal or reactive, we isolated basophils from bone marrow samples and subsequently evaluated them utilizing the *PML::RARA* probe. The final diagnosis was APL with clonal basophilia (Figure 1F). The patient received induction chemotherapy comprising all-trans retinoic acid (ATRA), arsenic trioxide (ATO), idarubicin, and venetoclax, followed by ATRA and ATO for consolidation therapy upon achieving molecular remission.

Various myeloid neoplasms have the potential to progress to acute myeloid leukemia (AML) with basophilia, such as chronic myeloid leukemia, atypical chronic myeloid leukemia, myeloproliferative neoplasms without *BCR::ABL1* fusion, and myelodysplastic syndrome. Furthermore, 44% of AML cases with *DEK::NUP214* exhibit basophilia [1]. Concomitant basophilia does not appear to have a specific correlation with the type of APL (i.e., whether classic or a variant). Some studies have shown that ATRA could hinder the differentiation of promyelocytes into eosinophils and basophils, whereas ATO does not show such an effect [2,3]. However, there have also been reports indicating basophilia among patients with APL following induction therapy with ATRA or ATO. In our case, the patient did not show basophilia after induction therapy. The relationship between differentiation-inducing therapy and eosinophil-basophil differentiation remains unclear, necessitating further investigation.

Another concern is whether the basophils present in APL are clonal or reactive. Clonal basophilia in APL diagnosis was previously supported by bone marrow findings (22% basophils, 28% promyelocytes) and FISH-confirmed t(15;17) in 44.5% of nuclei [4], with our study providing direct evidence of its clonal origin despite the heterogeneity that may exist in the origins of basophils after APL treatment [5,6]. It is worth noting that in all pertinent cases, the basophil count returned to normal following hematological remission.

Keywords: Basophils, Acute promyelocytic leukemia, Clonality, Reactiveness

Anahtar Sözcükler: Bazofiller, Akut promiyelositik lösemi, Klonalite, Reaktivite

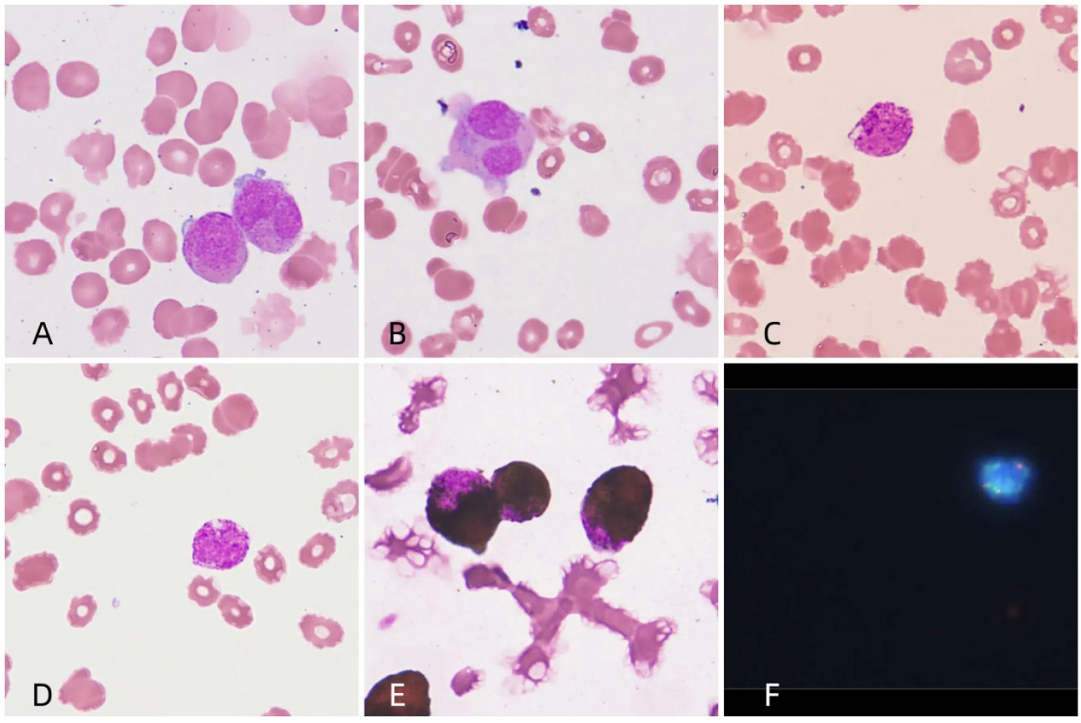

Figure 1. A, B) Abnormal promyelocytes are visible in the peripheral blood (A) and bone marrow aspirate (B), Wright-Giemsa (WG), 1000x. C, D) Basophils are present in bone marrow aspirate (WG, 1000x). E) Positivity for myeloperoxidase staining was observed (1000x). F) Basophils isolated from bone marrow samples were evaluated utilizing the *PML::RARA* probe (1000x).

Ethics

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

Footnotes

Authorship Contributions

Surgical and Medical Practices: P.Q.; Concept: B.Y.; Design: B.Y.; Data Collection or Processing: X.L., Q.Y., P.Q.; Analysis or Interpretation: X.L.; Literature Search: X.L., B.Y.; Writing: X.L., Q.Y., B.Y.

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LETTER TO THE EDITOR

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What Happened Suddenly - Acute Abdomen? A Difficult Case of ATRA-Related Pneumatosis Cystoides Intestinalis

Aniden Ne Oldu: Akut Abdomen? ATRA İlişkili Pnömatozis Sistoides İntestinalis Gelişen Zor Olgu

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To the Editor,

Acute promyelocytic leukemia (APL) is a hematological malignancy characterized by the accumulation of atypical promyelocytes in bone marrow and peripheral blood. This condition is frequently associated with disseminated intravascular coagulation [1], necessitating immediate medical intervention. All-trans retinoic acid (ATRA) is a key therapeutic agent for managing this condition. However, ATRA treatment may lead to differentiation syndrome, a potentially fatal complication accompanied by peripheral blood leukocytosis, which occurs in approximately 15% of patients [2,3]. Pneumatosis cystoides intestinalis (PCI) is a rare disorder characterized by gas-filled cysts within the intestinal wall. Its multifactorial etiology includes high intraluminal pressure, bacterial gas production, chemotherapy, and connective tissue diseases [4,5]. We present the first reported case in the literature of ATRA-induced PCI together with its clinical management.

A 49-year-old man with no known systemic diseases presented to our hospital with fatigue. Laboratory tests revealed white blood cell count of 1490/mm^3, neutrophil count of 540/mm^3, hemoglobin of 5.8 g/dL, and platelet count of 34,000/mm^3. Renal and liver function test results were within normal limits. Coagulation parameters revealed international normalized ratio of 1.3, fibrinogen of 1.63 g/dL, activated partial thromboplastin time of 23.5 s, and D-dimer of 32.2 mg/L. Blastic promyelocytes were observed in peripheral blood smears. Based on flow cytometry findings, a diagnosis of APL was established. Treatment with idarubicin and ATRA was initiated immediately, even before the bone marrow biopsy results were obtained. The patient subsequently developed differentiation syndrome, necessitating intensive care unit admission and temporary discontinuation of ATRA. Arsenic trioxide could not be administered because of the persistent QT prolongation observed on electrocardiography. Remission was not achieved after first induction. Daunorubicin, cytarabine, and ATRA were given as second-line therapy. On day 9 of the therapy, the patient experienced abdominal pain. A microbiological stool examination was performed for the etiology of the patient's abdominal pain and we did not detect any microorganisms. There was dilatation in the transverse colon on direct abdominal radiography. Ileus was not excluded. Since the superior mesenteric artery, superior mesenteric vein, and main vascular structures were seen to be open on abdominal contrast computed tomography (CT), mesenteric ischemia was excluded. No obstruction or mechanical events were detected by tomography. Abdominal CT revealed diffuse air densities in the transverse colon wall, which were identified as PCI. Since the patient's C-reactive protein levels continued to increase during the follow-up period, empirical meropenem and teicoplanin were started for bacterial translocation. Antibiotics were stopped on the 7th day since there was no growth in blood and catheter cultures taken simultaneously. The patient's acute-phase reactants continued to increase under antibiotic treatment but gradually decreased and normalized after ATRA was stopped. These findings led to the discontinuation of ATRA. A colonoscopy was not performed because of deep neutropenia and high perforation risk. In evaluations for differential diagnosis, differentiation syndrome was not considered due to the patient having only abdominal complaints, the absence of supportive findings such as weight gain or renal failure, and the patient being in hematological remission at the time of the event. Drug-related PCI was primarily considered for this patient based on the radiological evidence, particularly in the absence of colonoscopic evidence. The abdominal pain resolved within 1 week of discontinuing ATRA and subsequent CT showed complete resolution of air densities near the transverse colon (Figure 1).

There are three hypotheses on PCI pathogenesis: 1) the mechanical theory, which involves an increase in intraluminal pressure that causes mechanical damage and mucosal rupture of the intestinal wall, leading to the migration of gas from the gastrointestinal cavity to the intestinal wall [6]; 2) the pulmonary theory, which states that chronic lung diseases such as chronic obstructive pulmonary disease, asthma, and interstitial pneumonia lead to alveolar rupture, causing mediastinal emphysema and release of gas along the aorta and mesenteric blood vessels into the intestinal wall [7]; and 3) the bacterial theory, which states that aerogenic bacteria penetrate the intestinal mucosal barrier, ferment in the intestinal wall, and cause gas production. Upon reviewing the literature, we identified four previous patients with hematological malignancies who experienced complications during the neutropenic period. In three of these cases, the issues were associated with etoposide, while in one case, mitoxantrone was implicated. The complications resolved after discontinuing the relevant medication and providing supportive therapies. The mechanism of gas accumulation due to mucosal damage was also explored [8]. In our case, the neutropenic period was the period in which the patient's conventional chemotherapy had ended and he was receiving only ATRA. The patient had a history of dexamethasone exposure with suspicion of differentiation syndrome and was thought to be at risk of intestinal mucosal damage, but he improved after the drug was stopped. He later received conventional chemotherapies other than ATRA and did not have PCI recurrence despite neutropenic periods, which supports our view that the case was related to ATRA. Thus, the present case report highlights an unusual and serious adverse event associated with ATRA therapy. ATRA-associated ulcerations in different organ systems have been reported in many publications before [9,10,11,12,13]. Although murine experiments suggested that retinoic acid derivatives reduced gut inflammation [14], a study of patients with ulcerative colitis found that retinoic acid levels were higher in tissues with high inflammation [15]. In light of all the data, it is obvious that there is a need for more prospective studies on the intestinal effects of retinoic acid derivatives. We hypothesized that ATRA use causes colonic ulcerations, allowing gas to pass through the colon wall and form cysts. Initial antibiotic therapy and bowel rest did not alleviate the symptoms. When the literature was reviewed, no such side effects were previously reported with ATRA or retinoid derivatives. Therefore, it was concluded that this was the first case of its kind and the causal mechanism was thought to be related to mucosal damage in light of other similar studies in the literature.

Rapid improvement following ATRA discontinuation suggests a drug-related etiology for PCI. Awareness of this rare complication is critical for timely diagnosis and appropriate treatment to minimize patient morbidity.

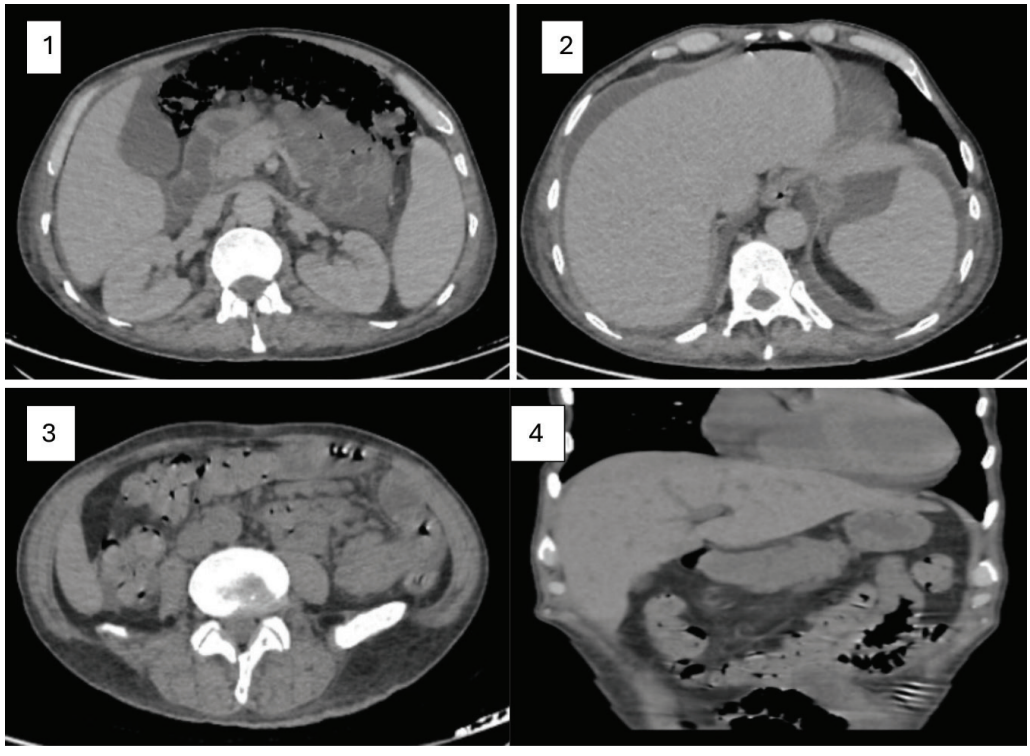

Figure 1. 1) Extensive air densities, consistent with pneumatosis cystoides intestinalis, were noted along the transverse colon up to the level of the splenic flexure, appearing to involve the wall of the transverse color. 2) Free air was observed anterior and posterior to the transverse colon in the abdominal cavity. 3, 4) In the patient's examination in December 2023, the air densities previously observed adjacent to the transverse colon in November 2023 appeared to have resolved.

Keywords: Acute promyelocytic leukemia, Pneumotosis cystoides intestinalis, ATRA, All-trans retinoic acid, ATRA-related toxicity

Anahtar Sözcükler: Akut promiyelositik lösemi, Pnömatozis sistoides intestinalis, ATRA, All-trans retinoik asit, ATRA ilişkili toksisiteler

Ethics

Informed Consent: No photograph revealing the patient's identity is included, and informed consent has been obtained from the patient for clinical sharing purposes.

Footnotes

Authorship Contributions

Surgical and Medical Practices: M.E.E., E.İ.K., F.C.; Concept: M.E.E., G.Ö., S.D.; Design: M.E.E., G.Ö., S.D.; Data collection and Processing: M.E.E., A.C., E.Ö.; Analysis or Interpretation: M.E.E.; Literature Search: M.E.E., A.C.; Writing: M.E.E., A.C.

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LETTER TO THE EDITOR

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A Rare Case of Non-Hodgkin Lymphoma Presenting as a Penile Mass

Peniste Kitle Şeklinde Ortaya Çıkan Nadir Bir Non-Hodgkin Lenfoma Olgusu

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To the Editor,

Penile tumors are generally rare and most commonly present as squamous cell carcinoma. Histopathological early diagnosis is crucial for the success of treatment [1]. Lymphoma originating from the male reproductive system is extremely rare, constituting less than 5% of extranodal lymph node cases. The most common subtype among these cases is diffuse large B-cell lymphoma [2]. Among those cases, penile lymphoma is usually reported in the literature as case reports. Symptoms include penile ulcer, phimosis, dysuria, and perineal swelling [1,3].

A 24-year-old man was referred to our clinic with a 1-month history of a palpable mass in his penis (Figure 1). On physical examination, a 2-cm lesion was detected on the left side of the distal penis. The patient did not have symptoms such as fever, night sweats, or weight loss. Penile ultrasound was performed and revealed a 2-cm mass at the distal corpus cavernosum. This lesion was not associated with the skin or subcutaneous tissue. On penile magnetic resonance imaging (MRI), a lesion was observed starting from the distal corpus cavernosum of the penis and extending to the proximal corpus cavernosum, involving the bilateral corpus cavernosum and causing apparent diffusion coefficient-limiting expansion in an area of 55x20 mm (Figure 2). After informed consent was obtained from the patient, excisional biopsy was performed from the distal left corpus cavernosum under general anesthesia. The patient was discharged the day after surgery and there were no postoperative complications or adverse events. Histopathological examination of the excision material revealed malignant cells forming a diffuse infiltration. Non-Hodgkin lymphoma with a high-grade B-cell phenotype was considered due to CD3 negativity and CD20 positivity. The findings were consistent with diffuse large B-cell lymphoma with a post-germinal center cell phenotype (Figure 3). In additional examinations, CD5 (-), CD10 (-), BCL6 (+), MUM1 (+), BCL2 (+), c-MYC (50%), CD30 (-), and EBV-LMP (-) were evaluated. After sperm cryopreservation, the patient underwent systemic evaluation. In fluorodeoxyglucose (FDG) positron emission tomography-computed tomography (PET-CT) imaging, an intensely hypermetabolic focus consistent with the known primary malignancy in the penis and hypermetabolic lymph nodes in the left inguinofemoral area, primarily suggesting involvement of the primary disease, were detected. After receiving four cycles of R-CHOP (rituximab, cyclophosphamide, doxorubicin hydrochloride, vincristine sulfate-oncovin, and prednisone), the patient underwent further PET-CT imaging. The results were reported as a whole-body PET-CT examination showing no pathological FDG uptake. The patient is currently being monitored at regular intervals. In accordance with the National Comprehensive

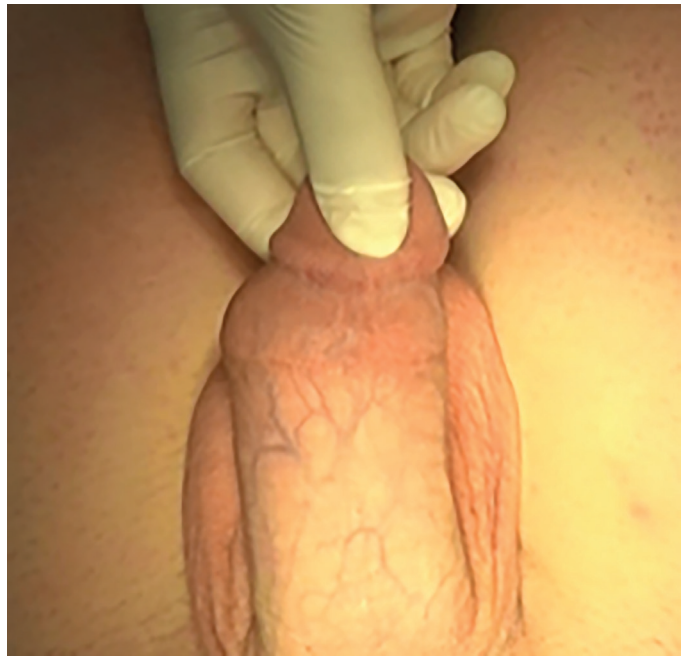

Figure 1. Penile mass on the left side.

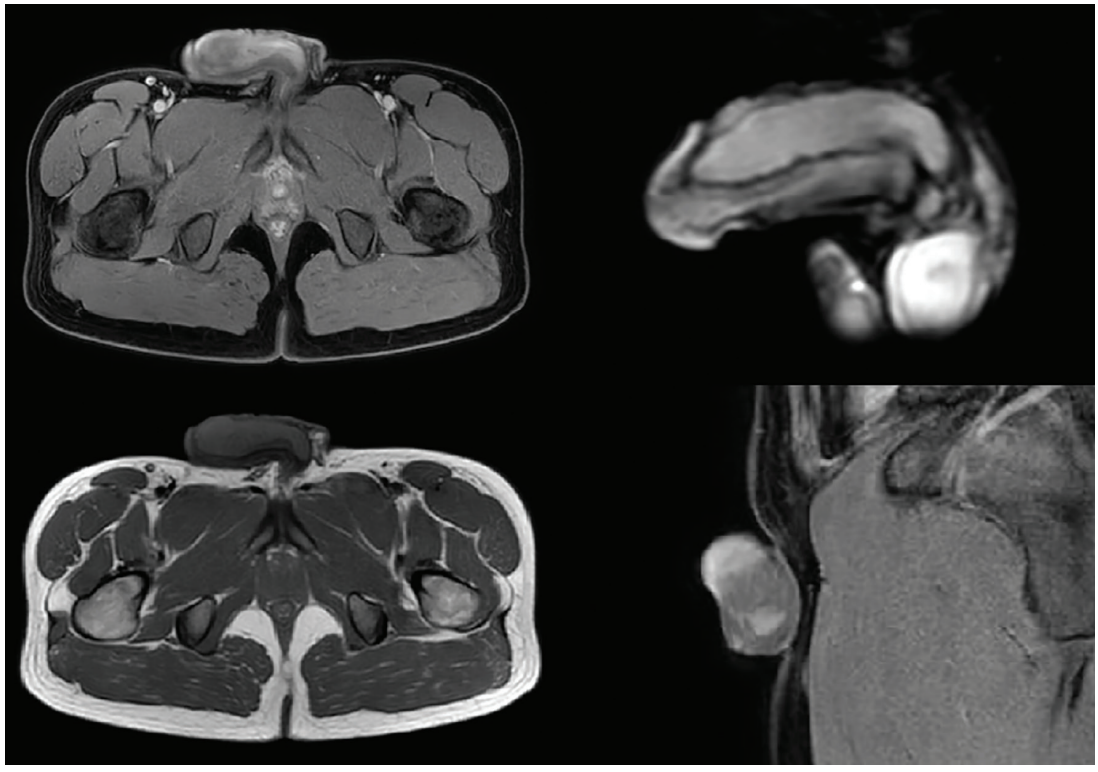

Figure 2. Penile magnetic resonance imaging of a lesion starting from the distal corpus cavernosum of the penis and extending to the proximal corpus cavernosum.

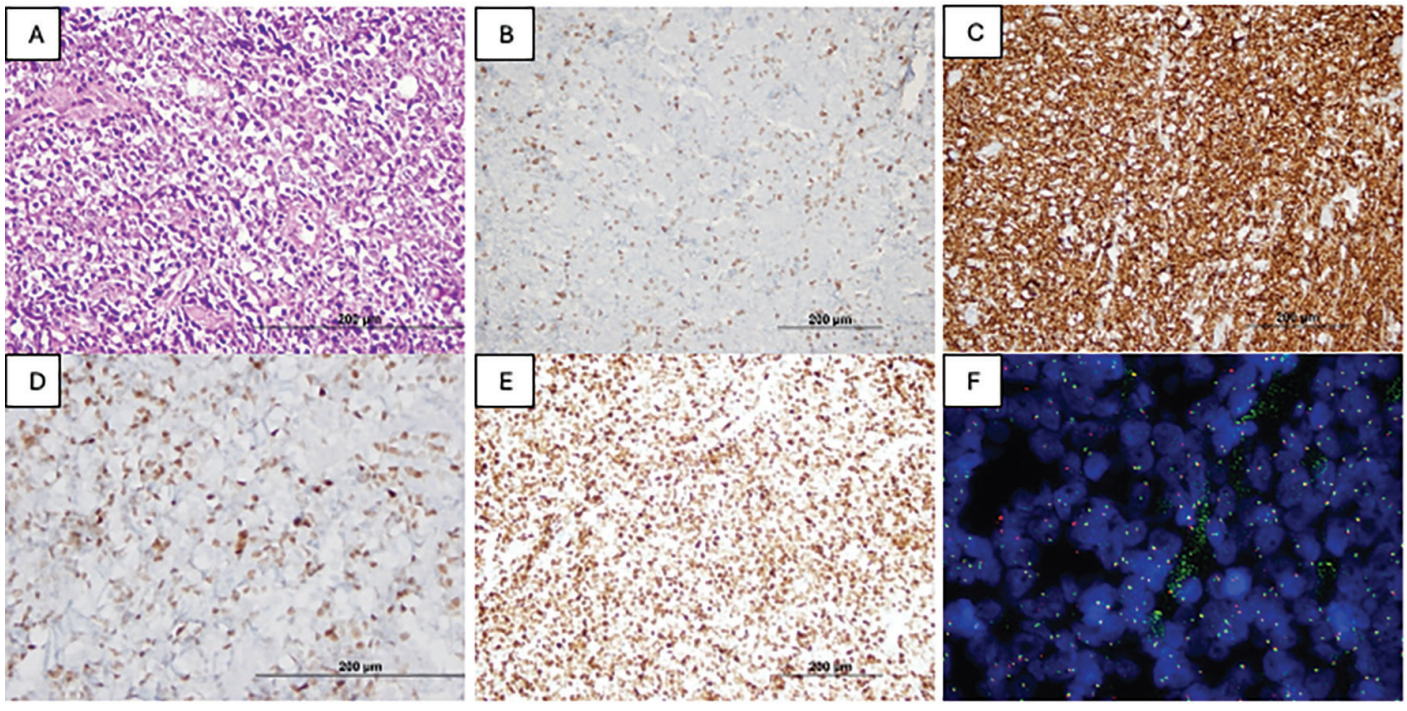

Figure 3. A) Diffuse high-grade neoplastic lymphoid infiltration; hematoxylin and eosin, 400x. B) Absence of expression in neoplastic infiltration with CD3 immunohistochemistry; anti-CD3 antibody, 200x. C) Diffuse expression in neoplastic infiltration with CD20 immunohistochemistry; anti-CD20 antibody, 200x. D) Increased c-MYC expression in neoplastic cells; anti-c-MYC antibody, 400x. E) Proliferation score of 95% in neoplastic cells with Ki-67 antibody; Ki-67 antibody, 400x. F) No rearrangement detected in the *MYC* gene for neoplastic cells; fluorescence in situ hybridization, *MYC* FISH break-apart probe, 1000x.

Cancer Network guidelines for B-cell lymphoma, the case was staged as I-E. The revised International Prognostic Index (R-IPI) score for diffuse large B-cell lymphoma was calculated as 1 because only extranodal involvement was present. This R-IPI score reflects an overall survival rate of 79% and progression-free survival rate of 80%. The central nervous system (CNS)-IPI score was calculated as 1 and CNS prophylaxis was not given as there was no involvement of the adrenal glands, kidneys, or testes.

Non-Hodgkin lymphoma occurs in extranodal sites in 50% of patients but is extremely rare in the penis [3]. Although the main manifestation of primary penile lymphoma has been reported to be a painless mass and ulcer on the corpus cavernosum of the penis, glans penis, and penile skin together with symptoms such as itching or dysuria [2,4], our patient presented with a painless lesion that was not associated with the skin or urethra. Imaging findings of primary penile lymphoma show no characteristic changes and ultrasonography often shows a vascularized mass or ulcer on the penile skin [5]. Since our patient did not have any cutaneous or systemic findings, corpus cavernosum thrombosis was included in the differential diagnosis and MRI was performed. Due to the low incidence of penile lymphoma, a standardized diagnosis plan is yet to be established. A review of the literature reveals that management decisions for primary penile lymphoma are primarily dependent on the stage of the disease, the age of the patient, and the patient's performance status. Since malignant lymphoma is a systemic disease with the potential to metastasize hematogenously, and since treatment with chemotherapy and radiotherapy can preserve penile function, radical surgery appears to be contraindicated. It should only be employed in cases where other modalities have failed. Systemic chemotherapy is a favorable treatment option as it preserves erectile function and prevents deformity. The traditional chemotherapy regimen is CHOP, but alternative regimens, including those containing rituximab, such as R-CVP (rituximab, cyclophosphamide, vincristine, and prednisone), have also been used [2]. Further developments include the potential for radioimmunological conjugates and oblimersen sodium (B-cell lymphoma-2 antisense oligonucleotide) to serve as effective therapeutic agents for the treatment of low-grade malignant lymphomas [4]. Definitive diagnosis is made by biopsy and immunohistochemical examinations [6]. In conclusion, although it is rare in patients presenting with a penile mass, the possibility of lymphoma involvement should be kept in mind.

Keywords: Penile lymphoma, Penile mass, Non-Hodgkin lymphoma, Peyronie's disease

Anahtar Sözcükler: Penil lenfoma, Penil kitle, Non-Hodgkin lenfoma, Peyronie hastalığı

Ethics

Informed Consent: Written informed consent was obtained from the patient for publication of this case report and any accompanying images.

Footnotes

Authorship Contributions

Surgical and Medical Practices: M.D., A.K.; Concept: M.D., A.H.S.; Design: M.D., A.K.; Data Collection or Processing: A.H.S., İ.T., Ö.H., A.A., Z.C.; Analysis or Interpretation: A.H.S., İ.T., Ö.H., A.A., Z.C.; Literature Search: A.H.S., İ.T., M.D.; Writing: A.H.S., İ.T.

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LETTER TO THE EDITOR

Turk J Hematol 2025;42:156-157

Peripheral T-Cell Lymphoma, Not Otherwise Specified, Diagnosed from Prostate Tissue: A Rare Case

Prostat Dokusundan Tanı Konulan, Periferik T-Hücreli Lenfoma, Başka Türlü Sınıflandırılmamış: Nadir Bir Olgu

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To the Editor,

Mature T lymphocytes give rise to an uncommon subtype of non-Hodgkin lymphoma called T-cell lymphoma (TCL). Many cases of peripheral T-cell lymphoma (PTCL), a diverse collection of neoplasms, are classified as PTCL, not otherwise specified (PTCL-NOS), because they cannot be classified [1]. Excluding Asia, where extranodal natural killer/TCL has become the most frequent subtype, PTCL-NOS is the most common subtype worldwide [2]. The median age at diagnosis is 60 years and most patients are adults. The prevalence of the diagnosis is higher among men than women at a ratio of nearly 2:1 [3,4]. According to a worldwide database of 340 cases of PTCL-NOS, 38% of patients have nodal disease alone, 49% have nodal plus extranodal disease, and 13% have extranodal disease without nodal involvement [4]. In the same study, bone marrow was implicated in 20% of the cases, while hepatomegaly and splenomegaly were observed in 17% and 24%, respectively [4]. One-third of patients reported having systemic B symptoms such as fever, night sweats, and weight loss [4]. Extranodal illness is most frequently observed in the skin and gastrointestinal system. Cases of lung, salivary gland, and central nervous system involvement are less common [5,6]. Serum lactate dehydrogenase is high in half of these cases, while hypergammaglobulinemia is seen in 14%. Leukemic presentations are uncommon, but circulating lymphoma cells may be seen [4]. In this report, we present a rare case of PTCL-NOS diagnosed from prostate tissue with bone marrow involvement. This patient, a 65-year-old man, consented to the publication of his case in an academic journal. He presented to the urology clinic with complaints of difficulty urinating for 4 months and was found to have elevated total and free prostate-specific antigen levels of 13 and 1 ng/mL, respectively. There was no history of urinary tract infection. The patient also had B symptoms. Peripheral blood smear revealed normochromic normocytic anemia (hemoglobin: 7.1 g/dL), severe neutropenia ($0.17x10^9$/L), and thrombocytopenia ($18x10^9$/L). Bone marrow aspiration revealed an increased proportion of large lymphocytes, reaching up to 60%. A prostate biopsy confirmed the diagnosis of PTCL-NOS with bone marrow involvement. Positron emission tomography/computed tomography revealed diffuse bone marrow, lymph node, and prostate involvement (stage IV), as depicted in Figure 1. Chemotherapy was initiated with cyclophosphamide, doxorubicin, vincristine, etoposide, and prednisolone. Anthracycline-based chemotherapy is the cornerstone of immunotherapy for CD30-negative PTCL [7]. The biopsy of the patient presented here was negative for CD30. Currently, considering the balance between toxicity and survival outcomes in patients with CD30-positive PTCL, the BV+CHP regimen (brentuximab vedotin, cyclophosphamide, doxorubicin, and prednisone) is recommended over other chemotherapy regimens [8]. In the literature, PTCL-NOS diagnosed from prostate tissue is very rare. In cases of elevated prostate-specific antigen, lymphomas other than solid tumors of the prostate should be considered. Rapid and definitive diagnosis and treatment are vital because TCLs are aggressive and expand rapidly.

Keywords: Lymphomas, T-cell lymphoma, Non-Hodgkin lymphoma, Prostate

Anahtar Sözcükler: Lenfoma, T-hücreli lenfoma, Non-Hodgkin lenfoma, Prostat

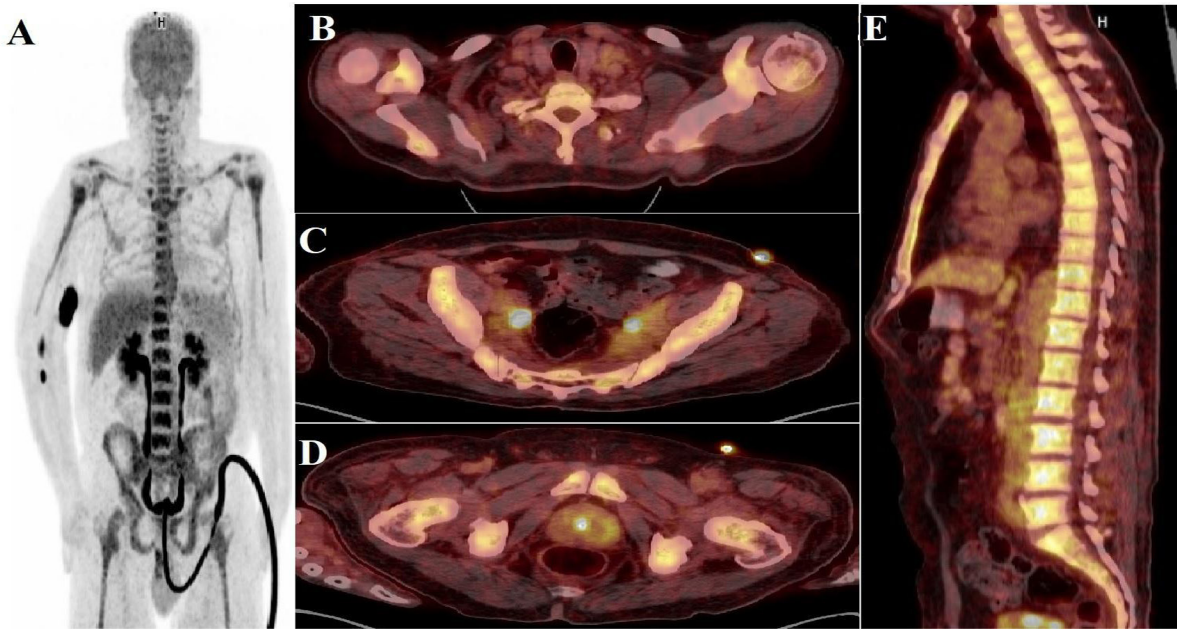

Figure 1. Fluorodeoxyglucose (FDG) positron emission tomography-computed tomography imaging showed increased FDG uptake in the left supraclavicular area (B) and bilateral paraaortic-parailiac lymph nodes in the abdomen (C). Additionally, diffusely increased FDG uptake was observed in the prostate gland parenchyma (D) and bone marrow (A, E).

Ethics

Informed Consent: Informed consent was obtained from the patient.

Footnotes

Authorship Contributions

Surgical and Medical Practices: R.Ç., H.Ö., C.S.; Concept: R.Ç.; Design: R.Ç.; Data Collection or Processing: R.Ç., H.Ö., C.S.; Analysis or Interpretation: R.Ç.; Literature Search: R.Ç.; Writing: R.Ç.

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LETTER TO THE EDITOR

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Successful Treatment of Childhood Hodgkin Lymphoma and Secondary Myelofibrosis Resistant to Intensive Therapy, Including Allogeneic Transplantation

Tedaviye Dirençli Hodgkin Lenfoma ve Sekonder Miyelofibrozisin Allojenik Transplantasyonu İçeren Başarılı Tedavisi

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To the Editor,

Myelofibrosis is a rare complication in children with Hodgkin lymphoma (HL) and presents significant treatment challenges due to the disease's low tolerance to chemotherapy. This condition often necessitates dose reductions, which can negatively impact patient outcomes and prognosis [1,2].

An 8-year-old boy was admitted to the Pediatric Hematology and Oncology Outpatient Clinic at Erciyes University with a 6-month history of malaise, anorexia, weight loss, and nocturnal leg pain. Physical examination revealed dyspnea, tachycardia, supraclavicular lymphadenopathy of 3 cm, and hepatosplenomegaly. Laboratory tests showed leukopenia (white blood cell count: 1940/mm^3), neutropenia (absolute neutrophil count: 950/mm^3), lymphopenia (absolute lymphocyte count: 740/mm^3), thrombocytopenia (platelet count: 85,000/mm^3), and severe anemia (hemoglobin: 4.9 g/dL). His erythrocyte sedimentation rate was elevated at 83 mm/h. Thoracic computed tomography revealed multiple mediastinal lymph nodes.

Axillary lymph node excision and bone marrow biopsy were performed based on clinical findings. Histopathological examination of the excised axillary lymph node and bone marrow biopsy specimen confirmed a diagnosis of the mixed-cell type of classical HL. Additionally, the bone marrow biopsy revealed the presence of myelofibrosis (Figure 1). A positron emission tomography-computed tomography (PET-CT) scan showed diffuse conglomerate lesions with hypermetabolic activity in the mediastinal lymph nodes as well as osteosclerotic lesions in cortical bones. Screening results indicated diffuse nodal and extranodal involvement of lymphoma as the primary disease, affecting the liver, spleen, bones, and bone marrow (Figure 2).

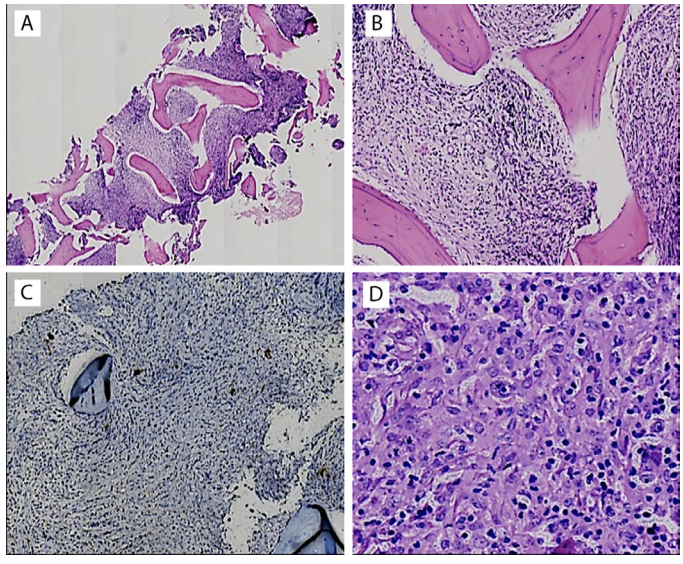

Figure 1. A) Bone marrow biopsy showing extensive fibrosis and hypercellularity (H&E stain, 100^x). B) Higher magnification of the bone marrow biopsy material revealed fibroblastic proliferation in infiltration areas together with rare atypical cells (H&E stain, 200^x). C) Lymph node biopsy showing CD30-positive transformed cells (immunoperoxidase stain, 200^x). D) Lymph node biopsy depicting a mixed inflammatory milieu with single-celled inflammatory cells, including Reed-Sternberg and mononuclear Hodgkin cells (H&E stain, 200^x).

H&E: Hematoxylin and eosin.

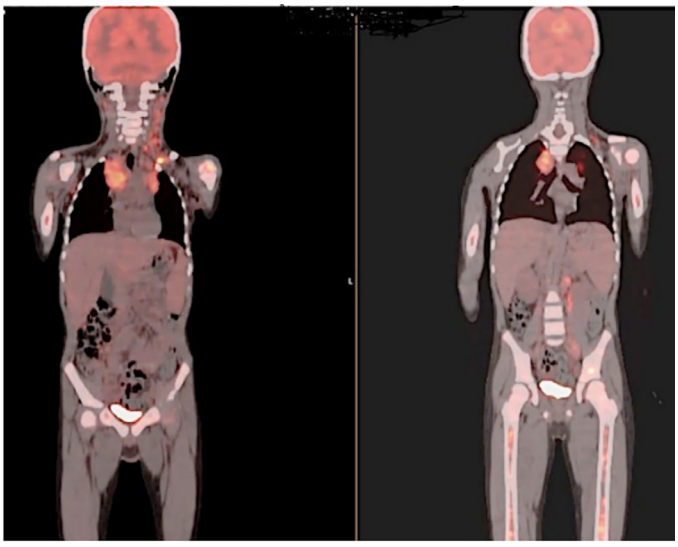

Figure 2. Illustration of the correlation between the clinical manifestations of lymphoma and the distribution of affected lymph nodes within the lymphatic system and other organs.

Whole-exome analysis performed on a peripheral blood sample and *JAK2*, *CALR*, and *MPL* pathological variant studies performed on a bone marrow aspiration sample yielded normal results. The patient was diagnosed with HL of stage IVB and secondary myelofibrosis. Treatment included eight cycles of chemotherapy, with four cycles of doxorubicin, bleomycin, vinblastine, and dacarbazine followed by four cycles of cyclophosphamide, vincristine, procarbazine, and prednisone. Post-treatment bone marrow analysis was normal.

Four months after completing chemotherapy, the patient presented with persistent fever and lymph node swelling. A biopsy of a suspected cervical lymph node confirmed the recurrence of HL. The patient received two cycles of a combination regimen comprising brentuximab and etoposide, carboplatin, and ifosfamide. However, PET-CT imaging showed no response to treatment. Consequently, an alternative two-cycle regimen containing brentuximab, prednisolone, ifosfamide, vinorelbine, and gemcitabine was initiated.

Given the diagnosis of relapsed/refractory HL and the impossibility of collecting autologous stem cells, the patient underwent haploidentical transplantation from his healthy mother. The procedure involved cyclophosphamide after the transplant following whole-body irradiation and a conditioning regimen with fludarabine and rituximab. On day 54 after the transplant, PET-CT revealed lymphoma progression despite the achievement of complete chimerism.

Progression was confirmed and four cycles of dexamethasone, gemcitabine, and vinorelbine were administered. Post-treatment PET-CT demonstrated a complete metabolic response and the follow-up bone marrow biopsy showed normal findings. At 10 months after the transplant, the patient remains in remission with complete chimerism.

A literature review indicates that cases of HL associated with myelofibrosis are relatively rare. The first documented case in a pediatric patient was reported in 1900, involving a patient who developed myelofibrosis following pancytopenia treatment [3]. Among 10 patients with a follow-up duration of approximately 16 years, Arya et al. [1] reported that 2 patients had primary myelofibrosis and 8 had secondary myelofibrosis. Notably, only 4 of those 8 patients had HL, with Reed-Sternberg cells (RSCs) detected in the biopsy of just one case. No RSCs were observed in the bone marrow of the other patients [1].

In the present case of HL with secondary myelofibrosis, RSCs were detected by bone marrow biopsy. Another study reported that median survival was 9-11 years in patients without bone marrow involvement but it was reduced to 3-4 years in those with bone marrow involvement [4]. The present case involved pancytopenia, bone marrow myelofibrosis, and RSC invasion of the bone marrow.

Although the relationship between HL and myelofibrosis is not fully understood, HL is recognized as a rare cause of myelofibrosis [5]. The pathogenesis of fibrotic changes in the bone marrow of patients with HL is a subject of increasing medical interest. Fibroblasts play a critical role in stromal proliferation, primarily driven by platelet-derived growth factor (PDGF), which is converted into transforming growth factor-beta (TGF-β). Additionally, fibroblasts are stimulated by essential fibroblast growth factors and other cytokines [6]. PDGF promotes fibroblast proliferation, while TGF-β, fibronectin, and type I and III collagen proteins contribute to the synthesis and accumulation of extracellular matrix components [7]. Elevated plasma TGF-β levels have also been reported in patients with malignant fibrous histiocytoma, correlating with peripheral T-cells, cytotoxic T-cells, and periductal lymphoma infiltrates [8,9].

A literature review suggests that the co-occurrence of HL and myelofibrosis in our patient is a unique clinical scenario. Notably, this is the first documented instance of pediatric bone marrow transplantation in such a context.

In conclusion, pediatric myelofibrosis should be considered as a differential diagnosis in children with HL. Further research is needed to better understand the pathogenesis of myelofibrosis and to identify the immunological abnormalities associated with lymphomas. Such studies will be crucial for developing more effective and targeted therapeutic strategies for these coexisting conditions.

Keywords: Allogeneic transplantation, Hodgkin lymphoma, Myelofibrosis

Anahtar Sözcükler: Allojenik transplantasyon, Hodgkin lenfoma, Miyelofibrozis

Ethics

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

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LETTER TO THE EDITOR

Turk J Hematol 2025;42:161-162

Fatty Precipitation in Donor Bone Marrow Caused by Overnight Cold Preservation in a Refrigerator

Verici Kemik İliğinde Buzdolabında Bir Gecelik Soğuk Muhafazanın Neden Olduğu Yağ Çökelmesi

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To the Editor,

Japan experiences many typhoons every year. In the period from summer to autumn, when the risk of typhoons is particularly high in Japan, the risk of delays in bone marrow aspirate transportation increases because public transportation is canceled when typhoons arrive. No domestic or national guidelines have been established regarding the storage of bone marrow aspirate material after collection and the storage conditions are determined by the policies of each transplant facility. However, considering cell viability, the physicians at many stem cell transplantation centers consider it desirable to store bone marrow aspirates at 4 °C if they are to be preserved overnight or longer [1]. Although the standard practice is thus usually bone marrow aspirate storage at 4 °C, we experienced an exceptional case of solidified bone marrow aspirate after 24 h of storage at 4 °C.

In this case, bone marrow aspirate material was collected from a 45-year-old man in another institute on 23 August 2024 and was to be transported to our institute for a 65-year-old female recipient. Transportation of the bone marrow aspirate was scheduled to occur on the same day as the collection but was canceled due to an approaching typhoon. It was subsequently transported by a commercial transporter (Nippon Express, Japan) on 24 August 2024, the next day after collection, after having been stored overnight at 4 °C at the collection center. The amount of bone marrow aspirate collected was 1200 mL and the total absolute number of all nucleated cells (ANC) was 2.29×10^{10} (3.08×10^{8}/kg body weight).

On the day of transportation, 24 August 2024, the bone marrow aspirate material arrived at our institute at 12:00 PM (noon). The ANC had decreased to 1.26×10^{10} (1.70×10^{8}/kg body weight) at the time of arrival to our institute, constituting a 55% reduction. Removal of erythrocytes and plasma was to be performed because of major incompatibility between the donor's blood type (AB+) and the patient's blood type (B+). However, the lipid component in the bone marrow aspirate had solidified (Figure 1), making it highly viscous. This made it impossible to inject the bone marrow aspirate into the apheresis bag. The lipid component required time to dissolve, and then the bone marrow aspirate was safely infused with an ANC recovery value of 75.9% and a mononuclear cell recovery value of 69.1% based on post-processing evaluations. We determined cell viability to be 97.1% using the 7-amino-actinomycin D staining assay. The triglyceride concentration in the bone marrow aspirate was 304 mg/dL, which was higher than that of the peripheral blood

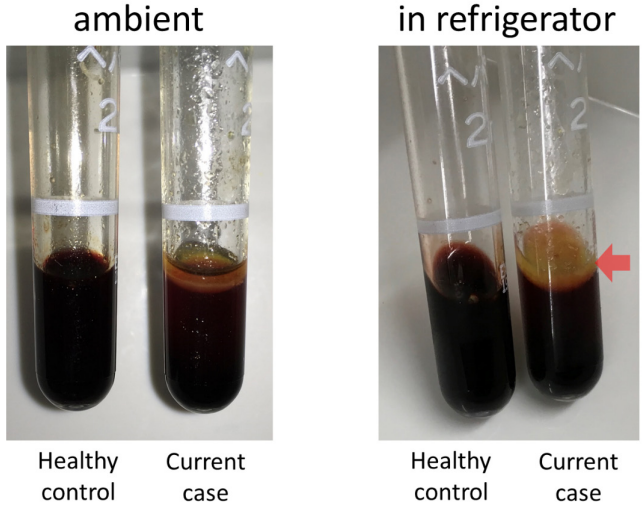

Figure 1. The donor's solidified bone marrow aspirate material. The left panel shows that the bone marrow aspirate material from the donor and that of a healthy control were both liquefied at room temperature. The right panel shows that the lipid component of the donor's bone marrow aspirate material solidified (red arrow) during overnight preservation in a refrigerator. The bone marrow aspirate material from the healthy control was not solidified.

(201 mg/dL). The recipient was engrafted with neutrophils on day 21 and with both erythrocytes and platelets on day 30.

If the delivery of bone marrow aspirate material is unusually delayed for reasons such as weather or natural disasters, storage at room temperature may be desirable in cases where the bone marrow aspirate contains a significant amount of fat. Nevertheless, it should be emphasized that this is an exceptional case and that standard practice generally supports storage at 4 °C.

Keywords: Stem cell transplantation, Bone marrow transplantation, Donor

Anahtar Sözcükler: Kök hücre nakli, Kemik iliği nakli, Verici

Ethics

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

Footnotes

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Surgical and Medical Practices: O.I., T.K.; Concept: O.I.; Design: O.I., T.K. M.U.; Data Collection or Processing: O.I., T.K.; Analysis or Interpretation: O.I., T.K. M.U.; Literature Search: O.I., T.K.; Writing: O.I., T.K.

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LETTER TO THE EDITOR

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Hair Re-Pigmentation After Nilotinib

Nilotinib Sonrası Saç Repigmentasyonu

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To the Editor,

Hair pigmentation in humans is caused by melanin, a pigment produced by melanocytes in the hair follicle. The type and amount of melanin, including eumelanin (black/brown) or pheomelanin (red/yellow), determine hair color. As we age, melanin production decreases, leading to gray or white hair. While hair graying is commonly associated with aging, genetic factors, stress, drugs, and nutritional deficiencies can also accelerate the process [1]. Drugs usually cause hypopigmentation of the hair, but in rare cases, some medications can induce re-pigmentation or hyperpigmentation. We present a case of chronic myeloid leukemia (CML) where the patient's hair returned to its original color after treatment, as it is a rare occurrence.

A 62-year-old male patient was diagnosed with CML in December 2020. The patient also had a history of hypertension and chronic obstructive pulmonary disease. Initially, the patient was treated with imatinib for CML. The patient achieved molecular response with 400 mg of imatinib; however, due to frequent hospitalizations for shortness of breath and edema, and based on the recommendation of the pulmonology department, imatinib treatment was discontinued in August 2022. Bosutinib was started, but due to lack of response after approximately 1 year, bosutinib was discontinued. In September 2023 nilotinib was initiated. After 6 months of nilotinib therapy, in March 2024, the patient noticed a change in hair color with gradual reversal from gray to the original shade (Figures 1 and 2). Drug-induced hyperpigmentation or re-pigmentation of the original hair color may occur with minoxidil, oral retinoids, hormonal therapies, targeted immunotherapies, or immunomodulatory drugs. A systematic review of these drugs reported more than 130 cases of drug-induced re-pigmentation of gray hair [2]. That study noted that although many of these drugs have been used by millions of patients, only a small minority are known to experience hair re-pigmentation. The study also pointed out that this might be due to the lack of reported cases or the unclear mechanisms involved [2].

Hair color changes related to nilotinib and imatinib have been rarely reported in the literature [3,4]. In a retrospective study of patients with CML treated with imatinib, 7% of the 133 patients experienced re-pigmentation of gray hair between the 2nd and 14th months of treatment [4]. The exact mechanism through which tyrosine kinase inhibitors affect hair pigmentation remains unknown. In our case, the patient had a history of using both imatinib and nilotinib. Since hair re-pigmentation occurred after the initiation of nilotinib, it was considered to be associated with nilotinib. However, given that most of the data on this topic come from case reports with limited numbers of patients, the overall level of evidence remains low. More meaningful data may be obtained as additional cases are reported over time.

Figure 1. Patient's hair color before nilotinib.

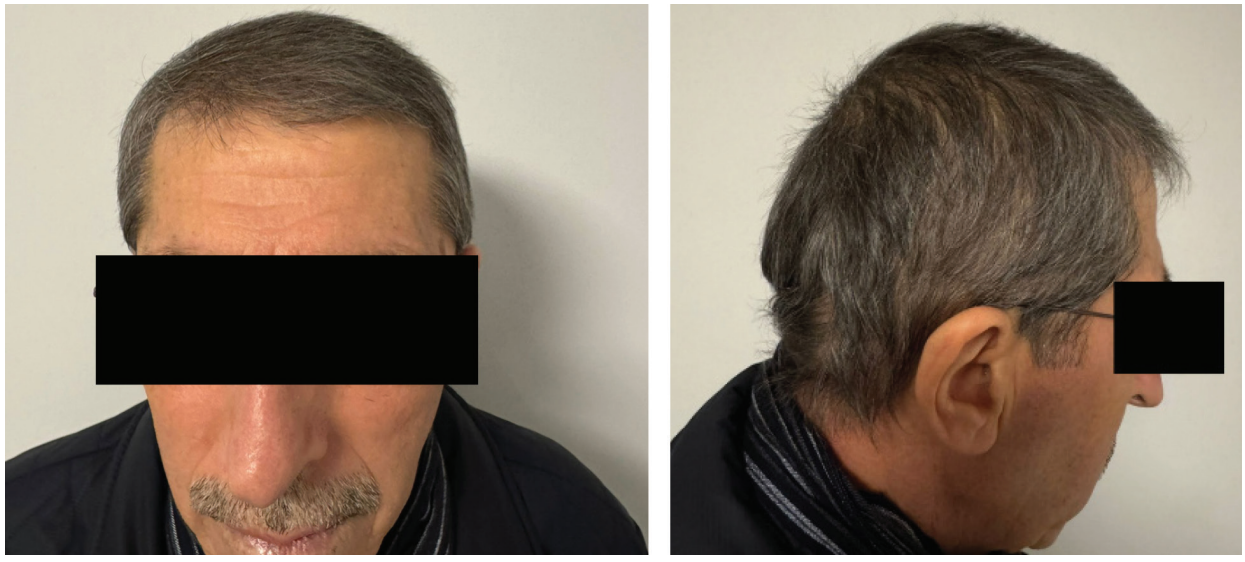

Figure 2. Patient's hair color after nilotinib.

Keywords: Chronic myeloid leukemia, Hair re-pigmentation, Nilotinib

Anahtar Sözcükler: Kronik miyeloid lösemi, Saç repigmentasyonu, Nilotinib

Ethics

Informed Consent: Written informed consent was obtained from the patient for the publication of this case report.

Footnotes

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Surgical and Medical Practices: M.T.; Concept: M.T.; Design: Ö.A.; Data Collection or Processing: Ö.A.; Analysis or Interpretation: Ö.A.; Literature Search: Ö.A.; Writing: Ö.A.

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LETTER TO THE EDITOR

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Optical Genome Mapping as a New Approach to Detecting Cytogenetic Abnormalities: Why Is It Difficult in Multiple Myeloma?

Sitogenetik Anomalilerin Tespitinde Yeni Bir Yaklaşım Olarak Optik Genom Haritalama: Multipl Miyelomda Neden Zor?

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To the Editor,

Multiple myeloma (MM), the second most common type of hematological neoplasm worldwide, is a plasma cell (PC) neoplasm [1]. According to 2022 statistics, the age-standardized incidence rate is 1.8 with a total of 188,000 new MM cases worldwide. Taking into account population growth and aging criteria, the number of newly diagnosed MM cases is expected to increase by 71% and the mortality rate by 79% by 2045 [2]. Genetic abnormalities are important in determining the prognosis of the disease and fluorescence in situ hybridization (FISH) is the preferred method to detect these abnormalities [1]. Since the proportion of PCs in the bone marrow (BM) varies between 10% and 30%, FISH testing of enriched CD138-positive PCs is recommended [3]. Therefore, highly sensitive methods may be more useful in prognostication. Recently, the highly sensitive method of optical genome mapping (OGM) has been used as a new cytogenomic method integrating karyotyping, FISH, and microarrays to increase the detection rate of abnormalities in hematological neoplasms [4,5,6].

For our OGM study, heparinized BM samples of 3-6 mL were collected from MM patients. Mononuclear cells were isolated via the Ficoll-Paque protocol. PCs were separated by flow cytometry using CD56, CD38, CD138, CD45, and CD19 antibodies (BD Pharmingen, San Diego, CA, USA) labeled with different fluorophores. Cells were sorted using a FACSAria II device (BD Biosciences, Franklin Lakes, NJ, USA) and snap-frozen. The OGM procedure was performed in three steps that included the isolation, labeling, and staining of high-molecular-weight DNA as the first step, followed by the loading and running of samples on a Bionano Saphyr device (Bionano Genomics, San Diego, CA, USA) and then data analysis. First, frozen cells were thawed and counted. DNA isolation was applied for samples with ≥1.5 million cells. Genomic DNA was isolated using the Bionano Prep SP-G2 Frozen Cell Pellet DNA Isolation Kit. Only samples containing ≥40 ng/µL of good-quality DNA were loaded onto a chip and run on the Bionano Saphyr device. Data were analyzed using Bionano Access software.

OGM can identify almost all types of structural variation, including translocations, deletions, duplications, insertions, inversions, aneuploidies, and complex chromothripsis with 5% variant allele frequency [5]. The Bionano Rare Variant Pipeline was used with default settings to analyze the data obtained in this study. All variants were filtered to <1% according to the internal control database.

Obtaining the number of PCs required for OGM was challenging, especially in cases of patients with <20% PCs. CD138-negative cells were added to samples with ≤800,000 PCs to reach 1.5 million cells. Although the samples were shipped with adequate dry ice, long-distance transport was also problematic. PCs were frozen at -20 °C for 2 hours and then stored at -80 °C. Longer storage at -80 °C led to more cell loss and a maximum of one night at -80 °C gave the best results. This is probably due to the fragile nature of PCs, which are unsuitable for long-term transfer and storage. In addition, these cells should be processed slowly in wet laboratories. This is necessary to avoid cell destruction and DNA fragmentation. In our study, the PCs were separated by flow cytometry for 25 patients. For 14 patients, the number of PCs was <1 million (100,000 to 950,000). For 11 patients, 1-6 million PCs were obtained. Six of the 11 patients with ≥1 million cells had enough cells after transfer to be included in

the OGM study, while 5 had fewer than 800,000 PCs. Overall, all patients had cell loss of two-thirds after transfer. The PC counts obtained by morphological assessment and flow cytometry were discordant.

It has been predicted that OGM will contribute to the development of therapeutic targets by enabling the detection of new biomarkers that cannot be detected by conventional methods due to their low sensitivity [7]. In MM, the combination of OGM and high-throughput DNA sequencing will enable more comprehensive genomic analysis in the near future [8]. However, the heterogeneity of the number of PCs in MM, the necessity of obtaining more than 800,000 cells, difficulties in working with and transporting PCs, lack of equipment support, and high costs still hinder the widespread use of OGM in MM. If these difficulties are overcome, it seems that the detection of abnormalities in MM by OGM will contribute to improvements that will increase survival, allowing OGM to usurp the role of FISH in diagnosis and prognosis.

Keywords: Multiple myeloma, Genomic structural variation, Chromosome mapping

Anahtar Sözcükler: Multipl miyelom, Genomik yapısal varyasyon, Kromozom haritalama

Footnotes

Authorship Contributions

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

Conflict of Interest: Mehmet Burak Mutlu works for a genetic diseases evaluation company that is the distributor of OGM Technology in Türkiye. The other authors declare no conflicts of interest.

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LETTER TO THE EDITOR

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Psychiatric Manifestations in Polycythemia Vera: A Case of Refractory Delirium and Psychosis Responding to Hematological Treatment

Polisitemia Verada Psikiyatrik Belirtiler: Hematolojik Tedaviye Yanıt Veren Dirençli Deliryum ve Psikoz Olgusu

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To the Editor,

Neuropsychiatric symptoms are among the less common manifestations of polycythemia vera (PV), a myeloproliferative neoplasm characterized by increased red blood cell mass and associated complications. While depression, delirium, and psychosis have been reported in PV, our understanding of their pathophysiology and optimal management remains limited [1,2,3,4,5]. The hypothesized mechanisms for psychiatric symptoms in PV include cerebral hypoperfusion due to hyperviscosity, reduced brain metabolism, and the development of microischemic areas [6]. Previous reports suggest that these symptoms are often resistant to conventional psychiatric treatments and are better managed by addressing the underlying hematological condition [5]. In this letter, we present a 68-year-old man with PV who developed refractory neuropsychiatric symptoms, emphasizing the need for multidisciplinary collaboration.

A 68-year-old retired man with a prior stable work history presented with a 4-month history of left-hand weakness, followed by progressive cognitive decline over the past month. His symptoms included disorientation, worsening forgetfulness, suspiciousness toward his wife (jealous delusions), grandiose delusions (claiming to be the president), and escalating verbal and physical aggression. These symptoms prompted an initial admission to the neurology department for further investigation.

The patient had been diagnosed with PV 7 years prior, confirmed by *JAK2* mutation positivity. He had been treated with phlebotomy and hydroxyurea for 2 years, but he discontinued treatment 5 years ago. He had no prior psychiatric history or significant medical conditions until the onset of the aforementioned symptoms and had not been taking any medications. Brain magnetic resonance imaging conducted during his admission revealed chronic ischemic hyperintensities in the bilateral white matter and an encephalomalacic area in the right parieto-occipital lobe. Laboratory tests showed elevated hemoglobin levels of 19.4 g/dL, hematocrit of 65%, white blood cell count of $16.5 \times 10^3/\mu L$, and platelets of $791 \times 10^3/\mu L$. On psychiatric evaluation, the patient scored 28/30 on the Delirium Rating Scale (DRS) and 7/30 on the Mini Mental State Examination (MMSE). Based on his clinical presentation and test results, the patient was diagnosed with delirium secondary to PV.

Initial treatment with haloperidol at 5 mg/day was initiated but yielded no significant improvement in symptoms. Hematology consultation was sought and alternate-day phlebotomy was started. After five sessions, the patient's hematocrit levels decreased to 49%. Given his age and history of stroke, the patient was classified as high risk and started on hydroxyurea and aspirin therapy along with phlebotomy [7]. Following hematological intervention, there was a marked improvement in his psychotic symptoms and resolution of delirium (DRS score improved to 6/30; Figure 1). However, cognitive dysfunction persisted, with only partial improvement noted (MMSE score improved to 15/30; Figure 1). After 13 days of haloperidol treatment, the patient's psychiatric medication was switched to risperidone at a dose of 2 mg/day. The patient was discharged with ongoing hematological treatment and risperidone at 2 mg/day for residual psychiatric symptoms.

The mechanisms underlying neuropsychiatric symptoms in PV remain unclear. Cerebral hyperviscosity and reduced brain metabolism due to slowed cerebral circulation have

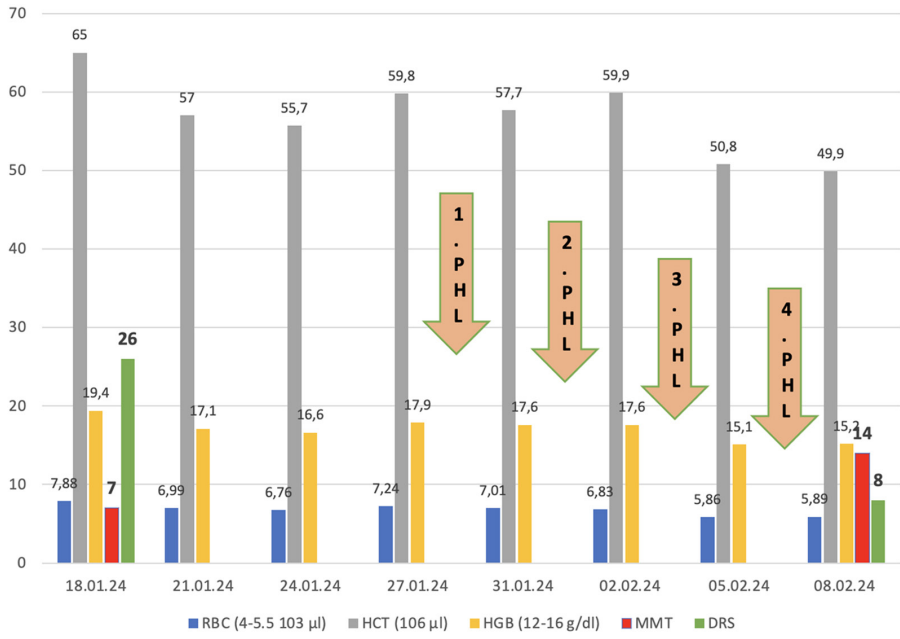

Figure 1. Temporal changes in hematological parameters and neuropsychiatric scale scores during sequential phlebotomy sessions.
RBC: Red blood cells; HCT: hematocrit; HGB: hemoglobin; MMT: Mini-Mental Test; DRS: Delirium Rating Scale; PHL: phlebotomy.

been proposed as possible contributors [6,8]. The presence of microischemic areas may also play a role. Consistent with prior reports, our patient's psychiatric symptoms were resistant to conventional treatments but showed significant improvement with hematological management [1,2,3,5]. This supports the hypothesis that such symptoms are a result of the disease's progression and are reversible with appropriate hematological intervention.

Our findings align with studies suggesting that psychiatric manifestations in PV may indicate advanced disease or progression to myelofibrosis [3]. However, the persistence of cognitive impairment despite the improvement in delirium in our patient supports hypotheses suggesting an uncertain relationship between the reduction in hematocrit levels and the recovery of cognitive functions [9]. While PV treatment may reverse the decreased cerebral blood flow caused by increased blood viscosity and improve certain cognitive functions [10], in some cases the permanent cognitive deficits may be explained by lacunar infarcts resulting from prolonged elevated hematocrit levels [11], although no such infarcts were observed in our patient. Early recognition and collaboration with hematologists are crucial for managing these cases effectively and preventing unnecessary psychotropic interventions.

In patients with no prior psychiatric history, late-onset, atypical, and treatment-resistant psychiatric symptoms should prompt consideration of PV as an underlying cause. Psychiatric manifestations during the course of PV require immediate interdisciplinary collaboration, with hematological management prioritized to address the root cause of symptoms. Furthermore, from a hematological perspective, monitoring and assessing psychiatric symptoms during the course of PV can provide valuable insight into disease severity and guide timely interventions.

Keywords: Polycythemia vera, Delirium, Psychosis, Phlebotomy

Anahtar Sözcükler: Polisitemia vera, Deliryum, Psikoz, Flebotomi

Ethics

Informed Consent: Written informed consent was obtained from the patient and the patient's legal guardian for the publication of this case report and any accompanying images.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Y.E.Y., H.I.E., E.G., N.Ç.T., Ö.D.B.; Concept: Y.E.Y., H.I.E., E.G., N.Ç.T., Ö.D.B.; Design: Y.E.Y., H.I.E., E.G., N.Ç.T., Ö.D.B.; Data Collection or Processing: Y.E.Y., H.I.E.; Analysis or Interpretation: Y.E.Y., H.I.E., E.G., N.Ç.T., Ö.D.B.; Literature Search: Y.E.Y., H.I.E.; Writing: Y.E.Y., H.I.E., E.G., N.Ç.T., Ö.D.B.

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