ÖZGÜN ARAŞTIRMA ORIGINAL RESEARCH

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EVALUATION OF CONVENTIONAL CYTOGENETIC, MOLECULAR CYTOGENETICS AND MOLECULAR GENETICS RESULTS IN HEMATOLOGICAL MALIGNANCES

HEMATOLOJİK MALİGNİTELERDE KONVANSİYONEL SİTOGENETİK, MOLEKÜLER SİTOGENETİK VE MOLEKÜLER GENETİK SONUÇLARININ DEĞERLENDİRİLMESİ

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Öz

Amaç

Hematolojik maligniteler, kemik iliği kaynaklı hücrelerin neoplazmlarıdır. Epidemiyolojik ve klinik çalışmalar sonucunda somatik hücrelerde meydana gelen mutasyonların, bu malignitelerin çoğunda sayısal ve yapısal kromozomal anomaliler ile spesifik gen düzeyindeki değişiklikler içerdiğini göstermektedir. Oluşan bu değişikliklerin hematolojik malignensilerin tanı ve takibinde aynı zamanda tedavi seçimi ve prognozu belirlemede kritik role sahip olduğu belirlenmiştir. Çalışmamız, hematolojik maligniteye sahip hasta guruplarında kromozomal ve moleküler olarak meydana gelen bu genetik değişikliklerin neler olduğunu tespit etmeyi amaçlamaktadır.

Gereç ve Yöntem

Bu çalışmaya Süleyman Demirel Üniversitesi Tıp Fakültesi Hematoloji Anabilim Dalı'na hematolojik malignite ön tanısı veya tanısı ile başvuran 110 hasta dahil edildi. Akut Miyeloid Lösemi (AML), Kronik Miyeloid Lösemi (KML), Kronik Lenfoblastik Lösemi (KLL), Miyelodisplastik Sendrom (MDS), Kronik Miyeloproliferatif Neoplazm (KMPN), Multipl Myelom (MM)/Diğer Plazma Hücreli Neoplazmlar (DPHN) ve Lenfoma hastalıklarına sahip yedi grubun kemik iliği kültürleri yapılarak üç farklı analiz yöntemi ile incelendi. Sayısal ve yapısal kromozomal değişiklikler sitogenetik kromozom analizi ve Floresan In Situ Hibridizasyon (FISH) yöntemleri ile ve JAK-2 V617F mutasyonu Real-Time PCR (RT-PCR) ile analiz edildi.

Bulgular

Sitogenetik ve FISH analizileri sonucunda, KML için t(9;22) mutasyonu %30,8 ve %22,6 oranlarında, AML için t(15;17) %7,7 oranında pozitif bulundu. Ayrıca, MDS için del (5q) ve del (7q) değerlendirildi ve delesyon saptanmadı. Hastalar del(13q14) ve del(17p13) açısından değerlendirildiğinde, lenfoma (%28,6 ve %71,4) KLL (%50 ve %62,5) ve MPHN (%50 ve %85,7) hastalarında değişen oranlarda bulundu. Ayrıca RT-PCR sonuçlarına göre JAK-2 V617F mutasyonu, KML hasta gurubunda (18.8%) KMPN hasta grubunda ise (81.2%) heterozigot positive olarak bulunmuştur.

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Sonuç
Laboratuvarımızda elde ettiğimiz bulgular ışığında, hematolojik maligniteye sahip hastaların rutinde çalışılan mevcut genetik analizlerine ek olarak belirlediğimiz mutasyonlarında incelenmesi ve hasta gruplarının da genişletilerek çalışılmasının hastalığın tanı ve prognozunun değerlendirilmesine katkıda bulunacağını düşünmekteyiz.

Anahtar Kelimeler: Hematolojik malignite, konvansiyonel sitogenetik, moleküler sitogenetik, moleküler genetik

Abstract

Objective
Hematological malignancies are neoplasms of bone marrow-derived cells. Epidemiological and clinical studies show that the mutations occurring in somatic cells include numerical and structural chromosomal abnormalities and specific gene-level changes in most of these malignancies. It has been determined that these changes have a critical role in the diagnosis and follow-up of hematological malignancies, as well as in the choice of treatment and determining the prognosis. Our study aims to determine what these genetic changes occur as chromosomal and molecular in patient groups with hematologic malignancy.

Materials and Methods
In this study, 110 patients who were admitted to the Department of Hematology of the Süleyman Demirel University Faculty of Medicine with a pre-diagnosis or diagnosis of Hematologic Malignancy were included. Three different analyses applied to the cultured bone marrow tissue samples of seven groups of hematology patients who suffered from Acute Myeloid Leukemia (AML), Chronic Myeloid Leukemia (CML), Chronic Lymphoblastic Leukemia (CLL), Myelodysplastic Syndrome (MDS), Chronic Myeloproliferative Neoplasm (CMPN), Malignant Plasma Cell Neoplasm (MPCN) and Lymphoma diseases. Numerical and structural chromosomal changes were examined by cytogenetic chromosome analysis and Fluorescent In Situ Hybridization (FISH) methods and JAK-2 V617F mutation was analyzed by Real-Time PCR (RT-PCR).

Results
As a result of cytogenetic and FISH analyzes, the t(9;22) mutation was found to be 30.8% and 22.6% positive for CML, and t(15;17) was found to be positive at 7.7% for AML. Also, del(5q) and del(7q) were evaluated for MDS and no deletion was detected. When the patients were evaluated in terms of del (13q14) and del (17p13), varying rates were found in lymphoma (28.6% and 71.4%) CLL (50% and 62.5%) and MPHN (50% and 85.7%) patients. Also, according to the results of RT-PCR, the JAK-2 V617F mutation was found as heterozygous positive in the CML patient group (18.8%) and CMPN patient group (81.2%).

Conclusion
In the light of the findings we have obtained in our laboratory, we think that examining patients with hematological malignancies in addition to the existing genetic analyzes that are routinely studied and studying the patient groups by expanding them will contribute to the evaluation of the diagnosis and prognosis of the disease.

Keywords: Hematological malignancies, conventional cytogenetics, molecular cytogenetics, molecular genetic.

Introduction

Hematologic malignancies are neoplasms that occur in cells derived from bone marrow. The malignant cells in many patients with leukemia, lymphoma, or other malignant hematologic disease have acquired clonal chromosomal abnormalities (1, 2). Besides, specific gene levels in hematologic malignancies changes are used to make the diagnosis and to evaluate the prognosis of the disease (3). Karyotype analysis of chromosomes from bone marrow samples, FISH (Fluorescent In Situ Hybridization) and RT-PCR (Real Time-PCR), which are one of the molecular cytogenetic methods, are among the most frequently used methods in detecting abnormalities in hematological malignancies (4).

Conventional cytogenetic analysis methods allow the analysis of the chromosomal structure in the examined sample without predicting a known genetic change. These methods are superior to FISH and molecular analyzes in that they detect an unfamiliar chromosomal rearrangement and are informed of the entire genome. However, considering the difficulty of obtaining cells from bone marrow, it is more difficult and time-consuming than the other two methods. Also, in case of insufficient combination with other methods is necessary (5).

Fluorescent In Situ Hybridization (FISH) is the most important technique used in cancer genetics because its analysis enables analysis in the interphase nucleus. Besides, it is a faster and more sensitive technique than conventional cytogenetics in terms of working with specific probes suitable for karyotypic rearrangements specific to hematological malignancy type (2). The superiority of the FISH technique is that it is workable in interphase nucleus cells and can yield results from an average of 300 interphase cells. Moreover, FISH permits analysis of proliferating (metaphase cells) and non proliferating (interphase nuclei) cells, and is useful in establishing the percentage of neoplastic cells before and after therapy (minimal residual disease) (6, 7).

Molecular studies on structural and numerical chromosomal abnormalities occurring in hematological malignancies quantitatively detect changes in the target-specific gene region by the RT - PCR method (8, 9). After the first discovery of translocation t(9;22) (q34;q11) which is between the breakpoint cluster region (BCR) gene on chromosome 22 and ABL gene on chromosome 9, many chromosomal aberrations causing fusion genes in cancer have been shown by cytogenetic techniques (10-12). The discovery of molecular techniques has led to the most precise determination of the percentage of cases with certain chromosomal aberrations. Translocation t(9;22) is observed in 95% of CML (chronic myelogenous leukemia) patients, in 2-10% of pediatric AML (acute myeloid leukemia) cases, and in 20-50% of adult ALL (acute lymphoblastic leukemia) cases (13). Chronic myeloproliferative neoplasm (CMPN) is characterized by excessive proliferation of multipotent stem cells in one or more blood cell lines. These diseases include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis. The JAK-2 protein acts as a hematopoietic growth factor in the cell. Therefore, it is thought that it may be important in cancer formation, especially in blood cancers (14). In a study conducted in 2006, they found that 96% of polycythemia vera, 60% of essential thrombocythemias and 69% of patients with myelofibrois were positive for JAK-2 mutation. In another study, the JAK-2 mutation was found to be 77% in Polycythemia Vera, 26% in essential thrombocythemias, and 100% in patients with mlelofibrozos (15, 16).

As a result of the tests to be performed in hematologic malignancies, treatment protocols are shaped according to the positivity-negativity of the response to the drug. At the same time, it is possible to know the presence of new mutations and to reduce and increase the number of drugs used accordingly. Or it can determine the capacity of the disease to transform into other types of malignancy. Also, the most effective treatment protocols can be reached by the clinician in light of these studies.

For these reasons, this study aimed to determine the reliability, accuracy, efficacy, success rate, the applicability of the methods used in the diagnosis of patients with hematological malignancy and to investigate the effectiveness of these methods used in the diagnosis and follow-up of these patients.

Material and Methods

In this study, 110 patients who were admitted to the Department of Hematology of Süleyman Demirel University, Faculty of Medicine, Department of Internal Diseases, who had been diagnosed with hematologic malignancy or who had come with a prediagnosis were included. Blood and bone marrow samples were analyzed in the SDU Medical Faculty Medical Genetics Laboratory. A total of 59 patients was classified into leukemia subgroups, including 31 patients in the CML group, 13 patients in the AML group, 8 patients in the CLL group, and 7 patients in the MDS group. Also, 51 people were classified as 30 patients in CMPN, 14 patients in Malignant Plasma Cell Neoplasm, 5 patients in Hodgkin, No-Hodgkin Lymphoma, and 2 patients in Burkitt Lymphoma. In other words, 98 bone marrow and 12 peripheral blood samples were obtained from 110 patients.

Bone marrow samples taken into heparinized tubes were in vitro cultured for 24-48-72 hours using Hematopoietic Cell Karyotyping Medium (Biological Industries, 944113). Cells were harvested according to routine methods and preparations were prepared by staining the preparations according to the Giemsa-Trypsin method. 20 GTG-banded metaphase sites were analyzed from each patient according to the International System for Human Cytogenetic Nomenclature (ISCN) 2016. When sufficient metaphase was not found, the number of metaphases obtained was analyzed.

Preparations were made with different FISH probes (Cytocell, UK) according to the standard protocol and molecular cytogenetic FISH analyses were performed from the cells prepared for conventional cytogenetics. FISH signals in 100 interphase cells were analyzed for each patient.

As the first for molecular study, DNA was isolated from blood samples taken from EDTA tubes. The obtained DNA samples were lifted to +4ºC to study

JAK-2. (DNA Purification kit, Germany, 1212/003). Samples prepared according to the manufacturer's protocol were placed on the RT-PCR device and studied by selecting the appropriate program. Quantitative values at the molecular level were obtained by studying JAK-2 (JAK–2 Mutation Kit, DZJAK) by RT-PCR (ABI Prism 7500).

Statistical analyses were performed using IBM SPSS Statistics 18 program. Data were given as a number, percentage, mean and standard deviation. Comparisons between the groups were analyzed by Fisher exact for categorical variables, Pearson for continuous variables in correlation, and Spearman correlation tests for categorical variables.

Results

The gender distribution of the 110 patients included in our study was 61 males (55.5%) and 49 females (44.5%). When the distribution of the study population by groups is examined; 13 AML (11.8%), 8 CLL (7.3%), 31 CML (28.2%), 30 CMPN (27.3%), 7 Lymphoma (6.4%), 7 MDS (6.4%) and 14 MPCN (12.7%) distribution was observed. In the study population, the distribution of the subgroups in the CMPN (30 patients) and CML (4 patients) groups; 7 ET (20.58%), 12 PV (35.29%) and 4 Myelofibrosis (11.76%) were detected.

The demographic information of the total of 110 patients in the study group is given below under the 7 groups that we grouped our patients. (Table 1).

Cytogenetic Findings of Patients

Cytogenetic analyses were performed by the System International System for Human Cytogenetic Nomenclature (ISCN, 2016) protocols. Cytogenetic examinations were performed by making bone culture for 24-48-72 hours from bone marrow samples and all cytogenetic chromosomal analyzes were obtained from these samples.

The distribution of cytogenetic chromosome analysis regardless of the distribution of the general study population by groups, whether or not anomaly was detected; In 15 patients, 13.6% of the anomalies were detected, while 59 patients (53.6%) could not detect any anomaly. The number of patients who could not obtain metaphase for chromosome analysis was 5 (4.5%) and 31 patients (28.2%) were never studied (due to lack of chromosome analysis in diagnosis and follow-up). Cytogenetic analysis results of patients grouped according to diagnosis were determined (Table 2).

Molecular Cytogenetic (FISH) Findings of Patients

For FISH analysis, FISH probes of the most common chromosomal rearrangements were used according to the clinical picture of patients coming to our department. Distribution according to groups in the general study population; t(4;14) were positive 1 patient (7.1%) in the MPCN patient group We found the t(8;14) translocation positive in the lymphoma patient group (14.3%). t(8;21) was found to be positive only in 1 patient (7.7%) in the AML patient group. t(9;22) were positive in 7 patients (22.6%) in the CML patient group. t(11;14) was positive in 1 patient (28.6%) in the lymphoma patient group. t(14; 18) were studied in 9 patients and no positive results were found. t(15;17) were positive only in 1 AML patients (7.7%). IGH 14 chromosome fracture site probe was positive in 1 patient (12.5%) in CLL patient group and 3 pa-

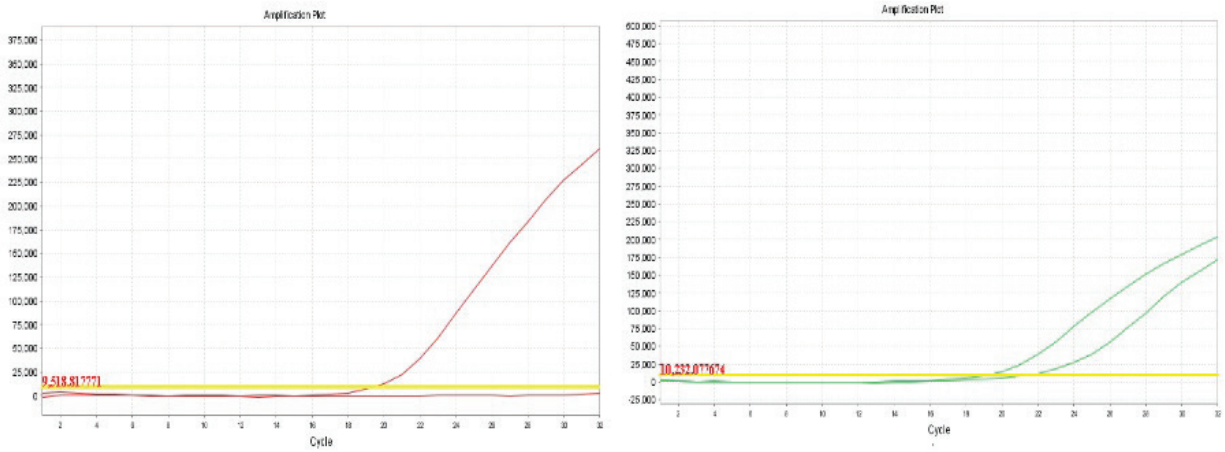

Figure 1
JAK-2 negative and heterozygous positive patient peaks studied by RT-PCR

Table 1 The demographic characteristics of patients divided into seven groups according to their diagnosis

Diagnosis	Number (%)	Age (Mean±SD)	Gender (M/F)	WBC (10^3/µl) (Min-Max)	PLT (10^3/µl) (Min-Max)
CML	31 (28.2)	57.48± 16.27	20/11	2.8-437	38-1323
AML	13 (11.8)	57.38± 17.37	7/6	2.3-148	29-243
CLL	8 (7.3)	68.63±16.60	4/4	23-135	19-311
CMPN	30 (27.3)	60.86±16.74	14/16	3.9-74	5-1629
Lymphoma	7 (6.4)	59.29±12.34	5/2	1.2-70	8-296
MDS	7 (6.4)	70.00±16.01	3/4	3.1-18	17-527
MPCN	14 (12.7)	62.15±12.27	8/6	1.8-96	19-414

SD: Standard deviation, M: Male, F: Female, WBC: White Blood Cell, PLT: Platelet

Table 2 Cytogenetic analysis results of patients grouped according to diagnosis

Diagnosis	Number of patients	Results
CML	7	46, XY or 46, XX
	3	46, XX, t(9;22)(q34;q11)
	1	46, XY, del(4q?), del(10q?)
	1	46, XY, inv(9)(p11q12)
	1	48, XY, t(9;22)(q34,q11), +8, +19, i(17)(q10)
	18	Not analyzed
AML	6	46, XX or 46, XY
	1	47, XY, +8
	1	47, XX, +1, der(1;7)(q10;p10), +1q, +7q
	3	Not analyzed
	2	Metaphase not found
CLL	5	46, XX or 46, XY
	1	Not analyzed
	1	Metaphase not found
	1	45, X, -Y
CMPN	22	46, XX or 46, XY
	2	Metaphase not found
	3	Not analyzed
	1	46, XX, del (11q23)
	1	46, XY, del(16q22)
	1	46, XX, t(9;22)
Lymphoma	4	46, XX or 46, XY
	1	46, XY, inv(9)(p11q12)
	2	Not analyzed
MDS	4	46, XX or 46, XY
	1	46, XX, inv(9)(p11q12)
	2	Not analyzed
MPCN	10	46, XX or 46, XY
	1	46, XX, inv(9)(p11q12)
	1	45, X, -X
	2	Not analyzed

tients (42.9%) in the lymphoma group. There was no positivity in patients studied with 5q and 7q deletion probes. Del 13q FISH probes; It was found positive in 4 patients (50%) in the CLL patient group, 2 patients (28.6%) in lymphoma, and 7 patients (50%) in the MPCN patient group. 17p deletion was positive in 5 patients (62.5%) in CLL, 5 patients (71.4%) in lymphoma and 12 patients (85.7%) in MPCN. The high rate of 17p deletion in the MPCN patient group was thought to be due to the low number of patients.

In addition to the pathologies mentioned above, some pathological FISH signal images were detected considering the centromeric or locus probes of the probes studied and other clinical features of the patient. In the AML patient group; 4% t(8;21), 12% t(4;14), 24% cmyc amplification and 66% trisomy 8 were positive. We determined 72% trisomy 9,38% monosomy 9,5% tetraploid in CML patients (9.7%). Monosomy 9 was found in 5% of the CMPN patient group. 28% tetraploidy was positive in the group with lymphoma (14.3%).

Molecular Findings of Patients

In the general study population, 50 patients underwent JAK-2 analysis using the RT-PCR technique. Heterozygous JAK-2 and negative JAK-2 mutations were evaluated according to integral peaks by RT-PCR technique (Figure 1). While 34 (30.9%) of the patients had a negative JAK-2 mutation, 16 (14.5%) had heterozygous positivity.

AML, CLL, ALL, and MPCN patient groups were studied in 1 patient and no mutation was detected. In the CML patient group, 12(80%) negative and (20%) heterozygous positive JAK-2 were detected. 15 (53.6%) patients with CMPN were identified as JAK-2 negative and 13 (46.4%) patients were identified as JAK-2 heterozygote positive. Four patients were found to be negative in the MDS patient group, and no heterozygote was detected in any patient. The JAK-2 analysis was not studied in the lymphoma group (Table 3).

Discussion

Conventional Cytogenetics, Fluorescent In Situ Hybridization (FISH), and Real-Time PCR (RT-PCR) techniques; In the diagnosis and follow-up of patients with hematologic malignancies, sometimes 3 techniques are used alone (17). Molecular cytogenetic techniques (FISH) allow the detection of complex, cryptic, and submicroscopic rearrangements that cannot be determined or resolved by conventional cytogenetic analysis (18, 19). Also, conventional cytogenetic analyses help us to know the chromosomal structure of the sample being examined without predicting any known genetic changes. Conventional cytogenetic analysis is accepted as the gold standard in the diagnosis and follow-up of CML patients (20, 21). The main advantage that distinguishes this method from the other two techniques is that the method is not directed.

The fact that we detected trisomy in 47,XY, +8 and 47, XY, +1, der (1;7) (q10; p10), 1q and 7q in 2 patients in the AML patient group in the patient population supports the above explanations. In the patient population, we identified 47, XY, +8 and 47, XY, +1, der (1;7) (q10; p10), 1q and 7q trisomy in 2 patients in the AML patient group. This showed us that different cytogenetic anomalies may exist in different patient groups and that the above explanations are accurate. Bain BJ detected both numerical and structural chromosomal abnormalities in a conventional cytogenetic study in adult AML patients. This rate is 50-70% and in our study, it is 15.4%. (22). Although the results we have found in our studies are similar, the small number of our patients makes it difficult to make comparisons. Trisomy 8 is a very characteris-

Table 3 Distribution of Jak-2 parameter in groups

	Negative	heterozygote positive	Not analyzed	Total
CML	12	3	16	31
AML	1	0	12	13
CLL	1	0	7	8
CMPN	15	13	2	30
MDS	4	0	3	7
MPCN	1	0	13	14

tic numerical anomaly and can be detected by both cytogenetic and FISH techniques. Linenberger et al. found 40.8% trisomy 8 in their study (23). In our study, we found Trisomy 8 structure in cytogenetic analysis and it gives information that the diagnosis of the patient may be AML or chronic myeloproliferative neoplasm. Based on the literature information, numerical chromosomal anomalies must be observed in the rate of AML alone and the most common of these are detected on chromosomes 4, -5, -7, 8, 9, 11, 13, 21, 22, -Y respectively. The presence of chromosomal rearrangements of chromosomes 8 and 7 in 2 patients with anomalies coincides with the literature (24). In our study, chromosome analysis was performed in 6 of 8 CLL patients, and 45, X, -Y was found in 1 patient. Standard chromosome analysis shows cytogenetic abnormalities in approximately half of the cases. The most common disorder is trisomy 12. del(13q) and del(14q) disorders are also common (25).

In our study, in 6 patients in the CML group, We detected chromosomal abnormalities in 46, XY, del (4q?), del(10q?), 46, XY, t (9; 22), 48, XY, t(9; 22) (q34, q11), +8, +19, i (17) (q10), 46, XX, t(9; 22) (q34, q11) and 46, XY, inv(9)(p11q12). As stated in the literature, secondary chromosomal abnormalities develop in 70-80% of CML patients in the blastic phase (2. Ph, trisomy 8, i (17)(q10), +19, and Y loss)(26). Our study is consistent with the literature. 46, XY, inv (9)(p11q12) were found in 1 lymphoma patient. Inv (9) is considered to be a normal polymorphic structure and is not clinically important. Almost all of the cytogenetic anomalies observed in MDS cases are those described in AML. There is no cytogenetic abnormality specific to AML or MDS. In MDS cases del (5q), monosomy 7, trisomy 8, del 8, 20q-, -Y, and del (7q) anomalies are frequently observed (27, 28). The MDS patient group and the MPCN patient group had 46, XX, inv (9)(p11;q12) and 46, XX, inv (9)(p11;q12) chromosomal rearrangements respectively. These are considered to be normal polymorphic structure is not clinically important.

In the study conducted by McNeil et al. reported that both interphase and metaphase cells are a powerful complementary analysis to conventional cytogenetic analysis because of the FISH technique (29). We found that our studies were compatible with the literature.

The classification of chronic myeloid neoplasms was regulated by the WHO in 2008. The diseases in this group of disorders, formerly known as Chronic Myeloproliferative Neoplasm (CMPN), were renamed as myeloproliferative neoplasms. Along with this classification, CML has been classified as a separate disease with the presence of Philadelphia (Ph) chromosome and bcr/abl translocation and its distinctive clinical features (30). In our study, by this principle, CML patients and (Ph) chromosome-negative Chronic Myeloproliferative Diseases (Polycythemia Vera, Essential Thrombocytosis, Myelofibrosis) were divided into groups. In our study, the JAK-2 V617F mutation was detected in 11 (92%) of 12 patients with a pre-diagnosis of PV. In previous studies, it has been shown that this rate varies between 65-97% on average (31-33). In the first study conducted by Karkucak et al. in 2012, 70 of 148 patients were diagnosed as PV and 80% of the JAK-2 V617F mutations were detected (34). In our study, we found that 43% of JAK-2 mutations were positive in 3 out of 7 patients with the diagnosis of ET and we found that it was consistent with the literatüre. Also, we found 67% JAK-2 mutations in 2 of 4 patients with a pre-diagnosis of myelofibrosis. In the study of Çetinkaya, 66.7% of JAK-2 mutation were positive in 4 of 6 cases with myelofibrosis (35). Our results are especially similar to the study of Çetinkaya. We also think that increasing the number of patients will improve the quality of evaluation.

As a result, our findings show that indicated mutations should also be added to the routine genetic analyses of patients with hematological malignancies to improve the evaluation of diagnosis and prognosis of these diseases. In addition, as a result of this study, we believe that it will be more beneficial to increase the number of patients in future studies and to evaluate the groups within themselves.

Statement of Ethics

Our study was approved by Isparta Süleyman Demirel University Faculty of Medicine Clinical Research Ethics Committee with decision number 120 dated 05.12.2012.

Disclosure Statement

The authors have no conflicts of interest to declare

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Author Contributions

All authors contributed at every stage.

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