http://communications.science.ankara.edu.tr

Commun.Fac.Sci.Univ.Ank.Ser. C Biology
Volume 33, Number 2, Pages 67-80 (2024)
ISSN 1303-6025 E-ISSN 2651-3749
DOI: 10.53447/communc.1407463

Research Article; Received: December 20, 2023; Accepted: February 09, 2024

COMPARISON OF SANGER SEQUENCING AND NEXT GENERATION SEQUENCING METHODS FOR INVESTIGATION OF JAK2 EXON 12 MUTATIONS IN FOLLOW-UP OF PATIENTS WITH CHRONIC MYELOPROLIFERATIVE DISEASE AND JAK2 V617F NON-MUTATION

MUSTAFA ARSLAN[1], BÜŞRANUR ÇAVDARLI[2], VEHAP TOPÇU[2], HAKKI TAŞTAN[1]

[1]Department of Biology, Faculty of Science, Gazi University, Ankara, TÜRKİYE
[2]Genetics Clinic of Ankara Bilkent City Hospital, Ankara, TÜRKİYE

ABSTRACT Myeloproliferative neoplasms (MPNs) are clonal disorders of hematopoietic stem cells with increased proliferation and efficient maturation of myeloid cells, leading to peripheral blood leukocytosis and excess erythrocytes or platelets. Mutations of the JAK2 V617F, CALR and MPL genes confirm the diagnosis of myeloproliferative neoplasm (MPN). Mutations in JAK2 have been identified in the majority of patients with PV, ET and PM, highlighting the importance of constitutive activation of JAK2 signaling induced by mutations. In our study, Sanger Sequencing and Next Generation Sequencing methods were used to search for JAK2 Exon 12 mutations in 100 individuals who suffered from Chronic Myeloproliferative Disease and did not have JAK2 V617F mutation by Real-Time PCR method, and the results were examined comparatively. The examination was made with DNA material isolated from peripheral blood samples taken from patients who were referred to Ankara Numune Training and Research Hospital (ANEAH) Genetic Diseases Diagnosis Center. First of all, individuals who have with negative JAK2 V617F RT-PCR test results were selected. PCR was performed by adjusting sufficient amounts and concentrations from the DNA samples obtained from the peripheral blood of these patients. After the PCR process, the JAK2 Exon 12 regions were sequenced and examined using the Sanger sequencing method. A Next Generation Sequencing (NGS) study was performed by creating libraries from the DNA of the patients whose JAK2 Exon 12 region was negative, and the results were analyzed using the database. Some of the studies were conducted at the ANEAH Genetic Diseases Diagnostic Center, and the other 46 patients were performed at the Intergen Genetic Diseases Diagnosis Center within the scope of NGS study service procurement. According to the analyzes made, the results of Sanger Sequencing and Next Generation Sequencing studies showed similarity. Despite the deep bottom readings, a different result could not be obtained from the Sanger Sequencing method in the NGS study.

Keywords Chronic myeloproliferative disease, sanger sequencing, next generation sequencing, Janus Kinas 2, Real-Time PCR

mustafa.arssln@gmail.com- 0000-0001-7427-0941 https://ror.org/01wntqw50
bcavdarli@gmail.com- 0000-0002-0953-6210 https://ror.org/033fqnp11
avehaptopcu@gmail.com- 0000-0001-7224-5697 https://ror.org/033fqnp11
hakkitastangazi@gmail.com- Corresponding author; 0000-0001-9540-2931 https://ror.org/01wntqw50

2024 Ankara University
Communications Faculty of Sciences University of Ankara Series C: Biology
The copyright of the works published in our journal belongs to the authors and these works are published as open access under the CC-BY 4.0 license

1. INTRODUCTION

Myeloproliferative disorders consist of a set of chronic hematological diseases arising from a mutant multipotent hematopoietic stem cell. According to the World Health Organization (WHO) 2016 classification system, Myeloproliferative diseases (MPD); Chronic myeloid leukemia (CML) is divided into seven subcategories: chronic neutrophilic leukemia, polycythemia vera (PV), primary myelofibrosis (PMF), essential thrombocythemia (ET), chronic eosinophilic leukemia, and unclassified MPH [1].

JAK2 (Januskinase 2) gene has tyrosine kinase activity and is a gene located in the JAK-STAT pathway and has an important role in cell proliferation. JAK2 V617F mutation occurs by substituting the amino acid valine to phenylalanine at position 617 of the 14th exon of the gene. It causes increased cell proliferation and sensitivity to cytokines and is frequently seen in PV, ET and PMF diseases classified as Philadelphia negative MPN [1,2]. However, mutations of the JAK2 V617F, CALR and MPL genes confirm the diagnosis of MPN [2]. Although rarer mutations in the 12th exon of the JAK2 gene can also be seen in patients with normal JAK2 V617F [2]. In these clonally proliferating diseases, the same mutation is not expected to be seen in all lymphocytes in the peripheral blood. In a review published by Tefferi and Vardiman in the journal Nature in 2008 [3], it was emphasized that current testing systems are not standardized for JAK2 mutations, there are no allele-specific tests in peripheral blood samples, and false negative test results are inevitable due to low mutant allele densities. For this reason, a search has been made for a method to be used to detect low mutations. New generation testing systems and informatics technologies that develop with technology allow us to eliminate such doubts [3].

Next generation sequencing; It has the potential to significantly accelerate biological research as it makes comprehensive analysis of the genome, transcriptome, and DNA-protein interactions cheap, routine, and widespread [4].

In addition, since it shows mutation rates for clonal diseases, it provides highly sensitive results in cancer diagnosis and treatment follow-up. PV accompanied by JAK2 mutations in 95% of cases; It is an MPH characterized by polycythemia (thrombocytosis, leukocytosis, erythrocytosis) and splenomegaly [5].

If the JAK2 V617F mutation is detected as positive at the time of diagnosis in these patients, it is tested quantitatively at regular intervals because it is an important marker in the selection of treatment and evaluation of response to treatment. In patients with negative JAK2 V617F mutation, JAK2 exon 12 mutations are investigated by Sanger sequencing method [6, 7].

However, although the clinical findings were clear, the fact that no mutation was found in this study suggested that the clonal nature of the disease and the possibility of a false negative result being missed due to technical reasons due to the low mutation load. Our aim in this study is to make a methodological evaluation by comparing the results of JAK2 exon 12 mutations in patients whose results were found to be normal and those whose PV diagnosis could not

be ruled out clinically and hematologically, and those who had the same test repeated using the new generation sequencing method the JAK2 V617F mutation is detected as positive at the time of diagnosis in these patients, it is tested quantitatively at regular intervals because it is an important marker in the selection of treatment and evaluation of response to treatment. In patients with negative JAK2 V617F mutation, JAK2 exon 12 mutations are investigated by Sanger sequencing method [8, 9].

However, although the clinical findings were clear, the fact that no mutation was found in this study suggested that the clonal nature of the disease and the possibility of a false negative result being missed due to technical reasons due to the low mutation burden. Our aim in this study is to make a methodological evaluation by comparing the results of JAK2 exon 12 mutations in patients whose results were found to be normal and those whose PV diagnosis could not be ruled out clinically and hematologically, and those who had the same test repeated using the new generation sequencing method [10, 11].

JAK2 gene

It is a gene that makes a protein that sends signals to cells to promote cell growth and helps control the number of red blood cells, white blood cells, and platelets made in the bone marrow. Mutated (changed) forms of the JAK *gene* have been found in some blood conditions, including PV, ET and PMF [10]. This gene encodes a non-receptor tyrosine kinase that plays a central role in cytokine and growth factor signaling. The primary isoform of this protein has an N-terminal FERM domain that is required for erythropoietin receptor association, an SH2 domain that binds STAT transcription factors, a pseudokinase domain, and a C-terminal tyrosine kinase domain. Cytokine binding induces autophosphorylation and activation of this kinase. This kinase then recruits and phosphorylates signal transducer and activator of transcription (STAT) proteins [11].

JAK-STAT signaling pathway

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway is considered one of the central communication nodes in cell function. More than 50 cytokines and growth factors have been identified in the JAK/STAT signaling pathway, such as hormones, interferons (IFNs), interleukins (ILs), and colony-stimulating factors. JAK/STAT-mediated downstream events vary and include hematopoiesis, immune fitness, tissue repair, inflammation, apoptosis, and adipogenesis. Loss or mutation of JAK/STAT components is associated with many diseases in humans [12]. The JAK/STAT signaling pathway has profoundly influenced recent understanding of human health and disease. Many studies have been published reporting the importance of this pathway in malignancies and autoimmune diseases. The JAK/STAT signaling pathway was first discovered while studying how IFNs lead to activation of a transcription factor. The JAK/STAT pathway is a highly conserved signal transduction pathway. It regulates multiple cellular mechanisms associated with various disease development [13]. Dysregulation of

the JAK/STAT pathway is associated with various diseases. For example, the JAK2 V617F mutation frequently occurs in MPH [12, 13, 14]. The JAK-STAT pathway is important for functional hematopoiesis, and several activating mutations in JAK proteins have recently been identified as underlying causes of blood disorders [15-16]. One of the best studied examples is the JAK2 V617F mutant, which is found in 95% of patients with polycythemia vera and 50% of patients affected by essential thrombocythemia and primary myelofibrosis [17]. Much work has been done to understand how JAK2 V617F affects hematopoietic stem cell (HSC) renewal and lineage differentiation [18]. Because convincing evidence has been found supporting the idea that the mutation is acquired at the HSC level [12-19].

2. MATERIALS AND METHODS

Included 100 patients who were sent from the Hematology outpatient clinic of Ankara Numune Training and Research Hospital to the Genetic Diseases Diagnosis such as genetic testing between January 2016 and March 2018. In this study; Patients diagnosed with MPH, without JAK2 V617F mutation, without any mutation detected by JAK2 *exon 12* Sanger sequencing, and who agreed to participate in this study, were investigated for JAK2 exon 12 mutations by next-generation sequencing method. This study was found ethically appropriate by Ministry of Health Turkish Public Hospitals Institution; Ankara Province 1st Regional Public Hospitals Association General Secretariat SBÜ Ankara Sample was found ethically appropriate by the SUAM Clinical Research Ethics Committee with the number E.Board-E-18-1956.

DNA Isolation

After the blood in EDTA tubes taken from the patients is recorded in the sample acceptance unit, with the help of commercial kits were used for DNA isolation and it was shown during the our study. Invitrogen™ PureLink™ Genomic DNA Mini Kit was used for DNA isolation. High quality DNAs with an A260/A280 ratio between 1.7 and 1.9 were included in this study [20, 21].

Polymerase Chain Reaction

It is a revolutionary method developed by Kary Mullis in the 1980s. PCR is based on exploiting the ability of DNA polymerase to synthesize a new DNA strand that is complementary to the presented template strand. Because DNA polymerase can only add a nucleotide to a pre-existing 3'-OH group, it needs a primer to which it can add the first nucleotide [22]. This requirement makes it possible to delineate a specific region of template sequence that the researcher wishes to expand. At the end of the PCR reaction, the specific sequence will be collected in billions of copies (amplicons) [23, 24]. In our study, the Polymerase Chain reaction was performed using the GML® SeqFinder Sequencing System JAK-2 Kit.

Polymerase Chain Reaction

Gel electrophoresis is a laboratory method used to separate DNA, RNA or protein mixtures based on molecular size. In gel electrophoresis, the molecules to be separated are pushed through a gel containing small porous inserts with the help of an electric field. Molecules move through the pores in the gel at a speed inversely proportional to their length [21, 22]. Agarose gel using a microwave oven, 1% agarose was placed in 1X TAE- and boiled until the agarose was completely dissolved. After the gel was left to cool for a while, it was poured into the gel plate and allowed to polymerize [23] (Figure 1).

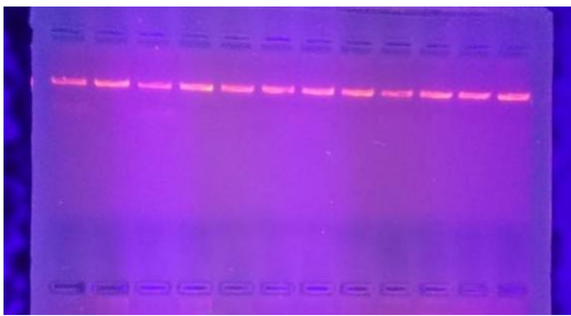

FIGURE 1. Appearance of amplicons containing JAK2 gene included Exon 12 regions on agarose gel

Sanger Sequencing

The method was developed in 1975 by Frederick Sanger, who was later awarded the Nobel Prize in Chemistry in 1980. By using this method it has to understanding which DNA sequences occurs. Sanger sequencing uses the SBS approach, in which a DNA polymerase produces DNA reads from a template to be analyzed of the DNA. The nature of the nucleotide at a particular position is now determined using special dyes. Although Sanger sequencing is too laborious and expensive for WGS, it continues to be used routinely when specific genes or gene fragments need to be sequenced, for example for viral or bacterial genotyping or for resistance testing. Modern Sanger sequencing typically uses fluorescently labeled dideoxynucleotides detected by a laser after capillary electrophoresis to generate an array chromatogram with fluorescence peaks corresponding to the inclusion of four different fluorescent dyes bound to ddATP, ddCTP, ddGTP and ddTTP [24, 25] (Figure 2).

FIGURE 2. Patient example with Sanger Sequencing of the JAK2 Exon 12 region

Next Generation Sequencing

Next-generation sequencing (NGS), massively parallel or deep sequencing are related terms that describe a DNA sequencing technology that is revolutionizing genomic research. Using NGS, the entire human genome can be sequenced in a single day [26]. Millions to billions of sequences from individual strands of DNA are analyzed separately but simultaneously. This technology allows the entire human genome to be fully analyzed in a few days for a few thousand dollars. Undoubtedly, as technology advances, speed will increase and cost will decrease; such that it will become practical to obtain full sequence analysis of cancers and matching germline for each patient. Hybridization, and real-time product detection with Sanger sequencing are being replaced by a new technology (NGS) [27].

Statistical Tests Used

Data analysis was done with SPSS 26.0 and worked with a 95% confidence level. Chi-square test, Mann Whitney and Spearman correlation were used in this study. Mann Whitney; It is a testing technique used to compare two independent groups in terms of a quantitative variable. Chi-square test; It is used to determine the relationship between two categorical variables. Spearman correlation; it is a testing technique used to determine the direction and strength of the relationship between two quantitative variables. Mann Whitney analysis was used to compare measurements according to groups, Chi-square analysis was used to compare the reference categories of measurements with grouped variables, and Spearman correlation analysis was used to compare the measurements [28].

3. RESULTS AND DISCUSSION

Sanger Sequencing

As a result of our studies at Ankara Numune Training and Research Hospital Genetic Diseases Diagnosis Center, Sanger Sequencing results of 100 errors were observed to be normal in terms of the JAK2 Exon 12 Gene region.

Next Generation Sequencing

55 of 100 patients with negative Sanger Sequencing results were studied at ANEAH Genetic Diseases Diagnosis Center and evaluated as negative by Dr. Büşranur ÇAVDARLI and Dr. Vehap TOPÇU. The remaining 45 patients were studied by the Intergen Genetic Diseases Evaluation Center within the scope of service procurement and the results were found to be negative. It has been confirmed by quality control methods that the DNAs have sufficient quality and concentration for next-generation sequencing technology. A library was created from DNAs of sufficient quality with the help of IonAmpliSeqTMLibrary Kit 2.0.

The files of the data obtained from this study were taken from the device in BAM (BinaryAlignment/Map) and VCF (VariantCalling Format) formats. The

accuracy of the variants in the VCF file was confirmed by visually evaluating the BAM files in the IGV (BroadInstitute) program. The ANNOVAR program was used to determine the frequency information, in silico prediction tools and other information of the detected variants. When evaluating whether the variants were disease-causing, the mutation evaluation guide published by the American College of Medical Genetics and Genomics (ACMG) in 2015 was taken as reference. Based on this reference, variants are grouped as “pathogenic,” “possibly pathogenic,” “unknown significance,” “possible benign,” and “benign.” To evaluate the impact of the detected variants, disease databases and in silico prediction tools such as HGMD, ExAC, Pubmed, Provean, SIFT, Polyhen-2, ClinVAR, MutationTaster, Varsome, Uniprot, GERP, PhyloP, Human SplicingFinder were used (Figure 3).

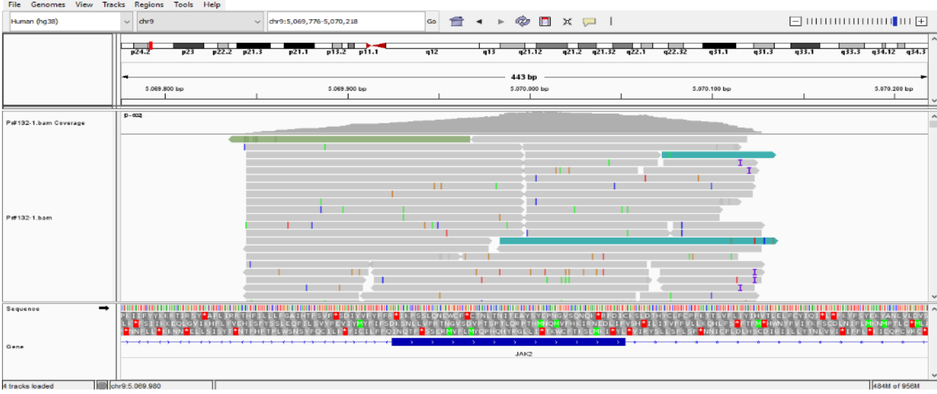

FIGURE 3. Patient example with Next Generation Sequencing of the JAK2 Exon 12 region

Statistical Data

TABLE 1. Testing normality of measurements and descriptive statistics

	Max-Min	Median	Mean± sd	Kolmogorov Smirnov	p
WBC	65.6-4	8.70	9.7±6.31	0.210	0.000
RBC	7.75-4.06	6.03	5.95±0.58	0.116	0.002
PLT	1487-124	241.00	265.46±151.77	0.240	0.000
HGB	22.5-11.1	17.30	17.15±1.66	0.106	0.007
MCV	99-28.3	87.35	85.73±9.34	0.233	0.000
MCH	34-21.3	29.00	28.74±2.07	0.127	0.000
HCT	70.4-38	52.00	51.47±4.81	0.113	0.003

(*p<0.05 normal distribution (-), p>0.05 normal distribution (+), KS test)

Descriptive statistics of the measurements are given. Kolmogorov Smirnov was used to test the normality of measurements. According to the results, WBC, RBC, PLT, HGB, MCV, MCH, HCT measurements do not show normal

distribution ($p<0.05$). Accordingly, non-parametric methods were used in the analyses (Table 1).

TABLE 2. Indication, gender distribution

		n (%)
Indication	Polycythemia Vera	37 (37)
	Secondary Polycythemia	63 (63)
Gender	Woman	24 (24)
	Male	76 (76)

The indication of 63.0% of the patients is Secondary Polycythemia, 76.0% of them are male (Table 2).

TABLE 3. Indication, gender distribution

		n (%)
WBC	not within normal range	26 (26)
	within normal range	74 (74)
RBC	not within normal range	68 (68)
	within normal range	32 (32)
PLT	not within normal range	8 (8)
	within normal range	92 (92)
HGB	not within normal range	51 (51)
	within normal range	49 (49)
MCV	not within normal range	7 (7)
	within normal range	93 (93)
MCH	not within normal range	9 (9)
	within normal range	91 (91)
HCT	not within normal range	56 (56)
	within normal range	44 (44)

(WBC: White Blood Cell, RBC: Red Blood Cell, PLT: Platelet, HGB: Hemoglobin, MCV: Mean red blood cell volume, MCH: Mean cell hemoglobin, HCT: Hematocrit)

WBC value of 74.0% of the patients, RBC value of 32.0%, PLT value of 92.0%, HGB value of 49.0%, MCV value of 93.0%, 91.0% The MCH value of 44.0% and the HCT value of 44.0% are within the normal range (Table 3).

TABLE 4. Relationship of reference groups of measurements to gender

		Woman	Male	P.
WBC	not within normal range	8 (33.3)	18 (23.7)	0.501
	within normal range	16 (66.7)	58 (76.3)	
RBC	not within normal range	13 (54.2)	55 (72.4)	0.157
	within normal range	11 (45.8)	21 (27.6)	
PLT	not within normal range	4 (16.7)	4 (5.3)	0.092
	within normal range	20 (83.3)	72 (94.7)	
HGB	not within normal range	12 (50)	39 (51.3)	0.999
	within normal range	12 (50)	37 (48.7)	
MCV	not within normal range	2 (8.3)	5 (6.6)	0.999
	within normal range	22 (91.7)	71 (93.4)	
MCH	not within normal range	2 (8.3)	7 (9.2)	0.999
	within normal range	22 (91.7)	69 (90.8)	
HCT	not within normal range	20 (83.3)	36 (47.4)	0.004*
	within normal range	4 (16.7)	40 (52.6)	

(*p<0.05 there is a relationship, p>0.05 there is no relationship, Chi-square test)

There is no statistically significant relationship between the gender of the patients and the reference groups of WBC, RBC, PLT, HGB, MCV, MCH measurements (p>0.05). The relationship with HCT measurement is statistically significant (p<0.05). The rate of HCT measurement being within the reference range is higher in men (52.6%) (Table 4).

TABLE 5. Relationship of reference groups of measurements to indication

		Polycythemia Vera	Secondary Polycythemia	P.
WBC	not within normal range	12 (32.4)	14 (22.2)	0.375
	within normal range	25 (67.6)	49 (77.8)	
RBC	not within normal range	24 (64.9)	44 (69.8)	0.769
	within normal range	13 (35.1)	19 (30.2)	
PLT	not within normal range	4 (10.8)	4 (6.3)	0.463
	within normal range	33 (89.2)	59 (93.7)	
HGB	not within normal range	16 (43.2)	35 (55.6)	0.326
	within normal range	21 (56.8)	28 (44.4)	
MCV	not within normal range	6 (16.2)	1 (1.6)	0.010*
	within normal range	31 (83.8)	62 (98.4)	
MCH	not within normal range	7 (18.9)	2 (3,2)	0.012*
	within normal range	30 (81.1)	61 (96.8)	
HCT	not within normal range	18 (48.6)	38 (60.3)	0.354
	within normal range	19 (51.4)	25 (39.7)	

(*p<0.05 there is a relationship, p>0.05 there is no relationship, Chi-square test)

There is no statistically significant relationship between the indication type of the patients and the reference groups of WBC, RBC, PLT, HGB, HCT measurements (p>0.05). The relationship with MCV and MCH measurements is statistically significant (p<0.05). In patients with Secondary Polycythemia indication type, MCV (98.4%) and MCH (96.8%) measurements are more likely to be within the reference range (Table 5).

TABLE 6. Comparison of measurements by gender

	Woman		Male		P.
	Median	Mean± sd	Median	Mean± sd	
WBC	8.75	11.8±11.88	8.65	9.03±2.71	0.495
RBC	5.58	5.51±0.58	6,10	6.1±0.51	0.000*
PLT	257.50	325.75±276.88	240.00	246.42±73.23	0.147
HGB	16.00	15.85±1.46	17.60	17.56±1.51	0.000*
MCV	87.40	85.25±13.1	87.30	85.88±7.91	0.446
MCH	29.45	28.91±1.99	28.90	28.69±2.11	0.493
HCT	47.70	48.13±4.18	52.70	52.52±4.53	0.000*

There is no statistically significant difference between female patients and male patients in terms of WBC, PLT, MCV, MCH measurements (p>0.05). The difference for RBC, HGB, HCT is statistically significant (p<0.05). RBC (6.1), HGB (17.60), HCT (52.70) measurements are higher in men (Table 6).

TABLE 7. Comparison of measurements by indication

	Polycythemia Vera		Secondary Polycythemia		P.
	Median	Mean± sd	Median	Mean± sd	
WBC	9.60	10.06±3.07	8.40	9.48±7.61	0.018*
RBC	6.03	5.92±0.54	6.03	5.98±0.61	0.999
PLT	254.00	297.81±219.1	234.00	246.46±88.95	0.078
HGB	17.00	16.64±1.75	17.50	17.45±1.54	0.039*
MCV	85.10	83.51±10.42	87.80	87.03±8.47	0.010*
MCH	28.40	27.87±2.35	29.40	29.26±1.71	0.002*
HCT	50,70	50.39±4.57	52.60	52.1±4.87	0.156

(*p<0.05 there is a relationship, p>0.05 there is no relationship, Mann Whitney test)

There is no statistically significant difference in RBC, PLT, HCT measurements between patients with indication type Polycythemia Vera and patients with Secondary Polycythemia (p>0.05). The difference for WBC, HGB, MCV, MCH is statistically significant (p<0.05). While WBC (9.6) measurement is higher in those with indication type Polycythemia Ver, HGB (17.5), MCV (87.8), MCH (29.4) measurements are higher in those with Secondary Polycythemia (Table 7).

TABLE 8. Relationship of measurements

		WBC	RBC	PLT	HGB	MCV	MCH	HCT
WBC	r	one	-0.083	.257 **	0.018	0.001	0.046	0.081
	p		0.413	0.010	0.860	0.992	0.651	0.420
RBC	r		one	-0.179	.628 **	-.372 **	-.454 **	.699 **
	p			0.074	0.000	0.000	0.000	0.000
PLT	r			one	-.380 **	-.290 **	-.238 *	-.341 **
	p				0.000	0.003	0.017	0.001
HGB	r				one	0.170	.223 *	.904 **
	p					0.092	0.026	0.000
MCV	r					one	.726 **	.197 *
	p						0.000	0.050
MCH	r						one	0.079
	p							0.434
HCT	r							one
	p							

(*p<0.05 there is a significant relationship, p >0.05 there is no significant relationship, 0<r<0.299 is weak, 0.300<r<0.599 is moderate, 0.600<r<0.799 is strong, 0.800<r<0.999 is very strong; sperman correlation test)

There is no statistically significant relationship between the patients' WBC measurements and RBC, PLT, HGB, MCV, MCH, HCT measurements (p>0.05). There is a positive relationship between RBC measurement and HGB (r=0.628), HCT (r=0.699) measurements, and a negative, statistically significant relationship between MCV (r=-0.372), MCH (r=-0.454) measurements (p<0.05). There is a negative, statistically significant relationship between PLT measurement and HGB (r=-0.380), MCV (r=-0.290), MCH (r=-0.238), HCT (r=-0.341) measurements (p<0.05).

There is a positive, statistically significant relationship between HGB measurement and MCH (r=0.223), HCT (r=0.904) measurements (p<0.05). There is a positive, statistically significant relationship between MCV measurement and MCH (r=0.726), HCT (r=0.197) measurements (p<0.05). Other relationships are not significant (p>0.05) (Table 8).

Retrospective study aimed at conducting a confidence test. JAK2 mutations have become a target in Polycythemia vera patients diagnosed with chronic myeloproliferative diseases. Genus kinases have been associated with CALR and MPL genes in the literature; however, current testing systems have not been updated for JAK2 mutations. In this study, the characteristics of the disease-causing variants were evaluated in detail through a retrospective analysis. As a result of the literature research, it was seen that intronic mutations can also be associated with Myeloproliferative diseases. In fact, in some publications, possible mutations in the JAK2 Exon 13 region have been emphasized. Its relationship with patient hematological data was examined. JAK2 vary in size

between 120-140 kDa and contain seven regions called Janus homology domain 1-7 (JH 1-7) [17]. There are publications showing that JAK2 Exon 12 mutations are more common in women than in men [28]. Since these mutations are associated with splenomegaly, if ignored, they will negatively affect the patient's quality of life. Since making a molecular diagnosis will enable a more careful hematological follow-up, elucidating the underlying mechanism is very important in preventing malformations that may develop in the patient's future periods.

Both testing systems are reliable in themselves. However, they have advantages over each other. Sanger Sequencing produces cost-effective solutions for low numbers of samples and the workflow is easy. NGS, on the other hand, provides higher sequencing depth and higher sensitivity. All the procedure working is complicated by winter Sanger sequencing. NGS offers higher mutation resolution. It is more focused on discovery and sample yield high in NGS. It offers obtaining more data with the same amount of sample [15].

Although the clinical findings were clear, the fact that no mutation was found in this study. It suggested that the clonal nature of the disease and the possibility of a false negative result being missed due to technical reasons to get the low mutation load. There are publications showing that JAK2 Exon 12 mutation is more common in women than in men [16]. No such finding was found in our study. During the literature review, it was emphasized that possible intronic mutations in the JAK2 gene region and possible mutations in the Exon 13 region should also be investigated.

Acknowledgement Our study was supported by Gazi University Scientific Research Projects Coordination Unit with ID number 5850. I would like to express my endless gratitude to my very valuable teacher, Prof. Dr. Hakkı TAŞTAN, who guided me by sharing his valuable knowledge and experiences in this study. In addition, I would like to thank my esteemed laboratory supervisor, Specialist Dr. Büşranur ÇAVDARLI, and Specialist Dr. Vehap TOPÇU, who provided me with laboratory equipment and valuable information and guided me in our research on the disease that is the subject of this study. I would also like to thank Laboratory Technician Handan ÖCALAN, employee of the Genetic Diseases Diagnosis Center Laboratory of Ankara Numune Training and Research Hospital, and my friend, Molecular Biologist Fatma Nisa ESEN, for their unwavering support during our study.

Author Contribution Statements MA-organization and execution of the experiments in the article, article editing, BÇ- project development, VT-data collection, management, HT- project development, data analysis, article writing and article editing.

Declaration of Competing Interests The authors declare no conflict of interest.

REFERENCES

[1] Tiziano, B., Jürgen, T., Heinz, G., The 2016 WHO classification and diagnostic criteria for myeloproliferative neoplasms: document summary and in-depth discussion, *Blood Cancer Journal*, 8 (15) (2018), 15-21. https://doi:10.1038/s41408-018-0054

[2] Zulkeflee, R. H., Zulkafli, Z., Johan, M. F., Husin, A., Islam, A., Hassan, R., Clinical and laboratory features of JAK2 V617F, CALR, and MPL mutations in Malaysian patients with classical Myeloproliferative Neoplasm (MPN), *International Journal of Environmental Research and Public Health*, 18 (14) (2021), 7582. https://doi:10.3390/ijerph18147582

[3] Tefferi, A., Vardiman, J. W., Classification and diagnosis of myeloproliferative neoplasms: The 2008 World Health Organization criteria and point-of-care diagnostic algorithms, *Leukemia*, 22 (1) (2008), 14-22. https://doi:10.1038/sj.leu.2404955

[4] Üstek, D., Abacı, N., Sırma, S., Çakiris, A., New generation DNA sequencing, *Journal of Experimental Medicine Research Institute*, 1 (1) (2011), 11-18.

[5] Chloé, J., Valérie, U., A unique clonal JAK2 mutation leading to constitutive signaling causes polycythaemia vera, *Nature*, 434 (2005), 1144–1148. https://doi.org/10.1038/nature03546

[6] Tefferi, A., The history of myeloproliferative disorders: before and after Dameshek, *Leukemia*, 22 (1) (2008), 3-13. https://doi.org/10.1038/sj.leu.2404946

[7] Harrison, C.J., Philadelphia Chromosome, *Encyclopedia of Genetics*, 23 (2001), 1449-1450. https://doi.org/10.1006/rwgn.2001.0991

[8] Karakuş, S., Chronic neutrophilic leukemia BCR-ABL negative chronic myeloid leukemia and other atypical myeloproliferative diseases, *Blood*, 2-1, (2012), 122-130.

[9] Arslan, S., Screening of BCR/ABL fusion gene mutations in patients diagnosed with CML and ALL, *American Journal of Hematology*, 93 (3) (2014), 442-459. https://doi.org/10.1002/ajh.25011

[10] Tefferi A., Primary myelofibrosis: 2021 update on diagnosis, risk-stratification, and management, *American Journal of Hematology*, 96 (1) (2021), 801-821. https://doi.org/10.1002/ajh.26050

[11] Hu, X., Li, J., Fu, M., Zhao, X., Wang, W., The JAK/STAT signaling pathway: from bench to clinic, *Signal Transduction and Targeted Therapy*, 6 (1) (2021), 402. https://doi.org/10.1038/s41392-021-00791-1

[12] Godfrey A. L., Myeloproliferative neoplasms (MPNs), *Blood Reviews*, 42 (2020), 100717. https://doi.org/10.1016/j.blre.2020.100717

[13] Baumeister, J., Chatain, N., Sofias, A. M., Lammers, T., Koschmieder, S., Progression of myeloproliferative neoplasms (MPN): Diagnostic and therapeutic perspectives, *Cells*, 10 (12) (2021), 3551. https://doi.org/10.3390/cells10123551

[14] Sawyers, C.L., Chronic myeloid leukemia, *New English Journal of Medicine*, 340 (17) (1999), 1330-1340. https://doi.org/10.1056/NEJM199904293401706

[15] El-Sharkawy F., Margolskee E., Pediatric myeloproliferative neoplasms, *Clinical Laboratory Medicine,* 41 (3) (2021), 529-540. https://doi.org/10.1016/j.cll.2021.04.010

[16] Liu, J., Wang, F., Luo, F., The role of JAK/STAT pathway in fibrotic diseases: Molecular and cellular mechanisms, *Biomolecules,* 13 (1) (2023), 119. https://doi.org/10.3390/biom13010119

[17] Barbui, T, Barosi, G, Birgeggard, G, et al., Philadelphia-negative classical myeloproliferative neoplasms: Critical concepts and management recommendations from european leukemianet, *Journal Clinical Oncology*, 29 (6) (2011), 761-770. https://doi.org/10.1200/jco.2010.31.8436

[18] Anía, BJ, Suman, VJ, Sobell, JL, Codd, MB, Silverstein, MN, Melton LJ, Trends in the incidence of polycythemia vera among Olmsted County, Minnesota residents, 1935-1989, *American Journal of Hematology*, 47 (2) (1994), 89-93. https://doi.org/10.1002/ajh.2830470205

[19] Shawky, A.M,, Almalki, F.A,, Abdalla, A.N,, Abdelazeem, A.H, Gouda A.M., A comprehensive overview of globally approved JAK inhibitors, *Pharmaceutics*, 14 (5) (2022), 1001. https://doi.org/10.3390/pharmaceutics14051001

[20] Godfrey, A.L., Green, A.C., Harrison, C.N., Essential thrombocythemia: challenges in clinical practice and future prospects, *Blood*, 141 (16) (2023), 1943-1953. https://doi.org/10.1182/blood.2022017625

[21] Tefferi, A., Pardanani, A., Essential thrombocythemia, *New England Journal of Medicine*, 381 (22) (2019), 2135-2144. https://doi.org/10.1056/NEJMcp1816082

[22] Gianelli, U., Thiele, J., Orazi, A., Gangat, N., Vannucchi, A.M., Tefferi, A., Kvasnicka, H.M., International consensus classification of myeloid and lymphoid neoplasms: myeloproliferative neoplasms, *Virchows Archiv*, 482 (1) (2023), 53-68. https://doi.org/10.1007/s00428-022-03480-8

[23] Gotlib, J., Chronic eosinophilic leukemia/hypereosinophilic syndrome, *Cancer Treatment Research*, 142 (2008), 69-106. PMID: 18283783

[24] Green, M. R., Sambrook, J., The basic polymerase chain reaction (PCR), *Cold Spring Harbor Protocols,* (2018), 436-456. https://doi.org/10.1101/pdb.prot095117

[25] Crossley, B. M., Bai, J., Glaser, A., Maes, R., Porter, E., Killian, M. L., Clement, T., Toohey-Kurth, K., Guidelines for sanger sequencing and molecular assay monitoring, *Journal of Veterian Diagnostic Investigation*, 32 (6) (2020), 767-775. https://doi.org/10.1177/1040638720905833

[26] Levy, S. E., Boone, B. E., Next-Generation sequencing strategies, *Cold Spring Harbol Perspectives Medicine*. 9 (7) (2019), 791. https://doi.org/10.1101/cshperspect.a025791

[27] Hu, T., Chitnis, N., Monos, D., Dinh, A., Next-generation sequencing technologies: An overview, *Human Immunology*, 82 (11) (2021), 801-811. https://doi.org/10.1016/j.humimm.2021.02.012

[28] Schnittger, S., Bacher, U., Haferlach, C., et al., Detection of JAK2 exon 12 mutations in 15 patients with JAK2V617F negative polycythemia vera, *Haematologica*, 94 (3) (2009), 414-418. https://doi.org/10.3324/haematol.13223