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Evaluation of the Effect of *NQO1 C609T (rs1800566)* Gene Variations in Philadelphia-negative Myeloproliferative Neoplasms in Turkish Population

Philadelphia-negatif Miyeloproliferatif Neoplazilerde *NQO1 C609T (rs1800566)* Gen Varyasyonlarının Etkisinin Türk Toplumunda Değerlendirilmesi

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ABSTRACT

Introduction: Philadelphia-negative myeloproliferative neoplasm (MPN) is a hematopoietic stem cell disorder consisting of essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF) associated with myeloid cell proliferation without differentiation and maturation defects. It is characterized by hypercellular bone marrow and an increase in one or more cell lines in the peripheral blood. In the hematopoietic stem cell, janus kinase 2 (JAK2), which is a cytoplasmic tyrosine kinase, remains constantly phosphorylated (active) as a result of the V617F somatic mutation in the pseudokinase region. Even if the phosphorylated JAK2 does not receive a stimulus, it performs signal transmission and causes continuous gene expression. This explains the excessive increase in one or more blood cell lines. NAD(P)H quinone oxidoreductase-1 (NQO1) is a phase 1 enzyme that prevents the formation of reactive and toxic semiquinone metabolites by reducing two electrons in one step. The C609T polymorphism of the *NQO1* gene leads to loss of enzyme activity due to the enzyme becoming unstable. While enzyme activity is not observed in individuals with both mutant alleles, moderate activity is observed in heterozygous individuals. Studies have reported a relationship between NQO1 C609T polymorphism and various cancer types. In our study, it was aimed to investigate the possible relationship between NQO1 C609T polymorphism and MPN development.

Methods: Our study group consisted of 119 MPN patients and 122 healthy controls. DNA isolation was performed from peripheral blood taken from the study groups. The JAK2 V617F mutation was detected using Real-time polymerase chain reaction (PCR), and *NQO1 C609T* gene polymorphism was detected using the PCR- restriction fragment length

ÖZ

Amaç: Philadelphia-negatif miyeloproliferatif neoplazi (MPN); farklılaşma ve olgunlaşma kusuru olmaksızın, miyeloid hücre çoğalması ile ilişkili esansiyel trombositemi (ET), polisitemi vera (PV) ve primer miyelofibrozisten (PMF) oluşan hematopoetik kök hücre bozukluğudur. Hipersellüler kemik iliği ve periferik kanda bir veya daha fazla hücre serisinin artması ile karakterizedir. Hematopoietik kök hücrede, sitoplazmik tirozin kinaz olan janus kinaz 2 (JAK2), psödokinaz bölgesindeki V617F somatik mutasyonunun bir sonucu olarak sürekli fosforile (aktif) kalır. Fosforile JAK2 bir uyarıcı almasa bile, sinyal iletimi gerçekleştirir ve sürekli gen ekspresyonuna neden olur. Bu durum bir veya daha fazla kan hücre serisinin aşırı artışını açıklamaktadır. Nikotinamid adenin dinükleotid fosfat kinon oksidoredüktaz-1 (NQO1) tek basamakta iki elektron indirgenmesini sağlayarak reaktif ve toksik semikinon ara metabolitlerinin oluşumunu engelleyen faz 1 enzimidir. *NQO1* geninin C609T polimorfizmi, enzimin kararsız hale gelmesinden dolayı enzim aktivitesinin kaybına yol açar. İki alleli de mutant olan bireylerde enzim aktivitesi görülmezken, heterozigot bireylerde orta düzeyde aktivite gözlenir. Yapılan çalışmalarda NQO1 C609T polimorfizmi ile çeşitli kanser tipleri arasında ilişki olduğu bildirilmiştir. Çalışmamızda NQO1 C609T polimorfizmi ile MPN gelişimi arasındaki olası ilişkinin araştırılması amaçlanmıştır.

Yöntemler: Çalışma grubumuzu 119 MPN hastası ve 122 sağlıklı kontrol oluşturdu. Çalışma gruplarından alınan periferik kandan DNA izolasyonu yapıldı. Janus kinase 2 (JAK2) V617F mutasyonu gerçek zamanlı polimeraz zincir reaksiyonu (PZR), *NQO1 C609T* gen polimorfizmi PZR-sınırlayıcı enzim

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polymorphism method. SPSS 21.0 was used for statistical analysis.

Results: No statistically significant difference was found between the patient and control groups in terms of NQO1 genotype distributions (p>0.05). When cases with ET, PMF, and PV were compared in terms of frequency of JAK2 V617F mutation, the rate in PV was higher (respectively; 62.5%, 61.5%, 78.6%). There was no relationship between JAK2 mutation positivity and NQO1*2 polymorphism.

Conclusion: According to the results obtained from our study, there is no relationship between NQO1*2 polymorphism and MPN. Variants of the NQO1 enzyme, which is essential in detoxification and activation of procarcinogens, did not increase the formation of the JAK2 V617F mutation, which is common in MPN patients. It is thought that the results should be supported by increasing the number of patient and control groups.

Keywords: Myeloproliferative neoplasm, MPN, JAK2 V617F mutation, *NQO1 C609T* gene polymorphism

parça uzunluk çeşitliliği yöntemi kullanılarak tayin edildi. İstatistiksel analizlerde SPSS 21.0 paket programı kullanıldı.

Bulgular: Hasta ve kontrol grubu arasında NQO1 genotip dağılımları açısından istatistiksel olarak anlamlı fark bulunmamıştır (p>0,05). ET, PMF ve PV tanılı olgular JAK2 V617F mutasyonunun sıklığı açısından karşılaştırıldığı zaman PV'deki oran daha yüksek saptanmıştır (sırasıyla; %62,5, %61,5, %78,6). JAK2 mutasyonu pozitifliği ile NQO1*2 polimorfizmi arasında ilişki saptanmamıştır.

Sonuç: Çalışmamızdan elde ettiğimiz sonuçlara göre, NQO1*2 polimorfizmi ile MPN arasında ilişki bulunmamaktadır. Prokarsinojenlerin detoksifikasyonu ve aktivasyonu için gerekli olan NQO1 enziminin varyantları, MPN hastalarında yaygın olan JAK2 V617F mutasyonunun oluşumunu artırmamıştır. Sonuçların hasta ve kontrol grubu sayısının artırılmasıyla desteklenmesi gerektiği düşünülmektedir.

Anahtar Kelimeler: Miyeloproliferatif neoplazi, MPN, JAK2 V617F mutasyonu, *NQO1 C609T* gen polimorfizmi

Introduction

Philadelphia-negative (Ph-) myeloproliferative neoplasms (MPNs) are a group of clonal multipotential hematopoietic stem cell diseases characterized by overproduction of mature and fully functional blood cells in one or more cell types, as well as thrombotic events and leukemia transformation. They consist mainly of polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) (1).

Our knowledge of the pathogenesis of MPNs has been dramatically expanded since the janus kinase 2 (JAK2) V617F mutation was discovered in 2005. In the period after the JAK2 V617F mutation was found, more critical fundamental deviations were identified, such as repetitive changes in the thrombopoietin receptor (MPL) gene, exon 12 mutations in the *JAK2* gene, and finally different types of mutations in the calreticulin gene. Advanced studies have shown that the JAK2 V617F mutation is higher than 95% in PV and approximately 50-60% in ET or PMF (2-4).

The JAK2 V617F mutation, the somatic single-point mutation in the *JAK2* gene in patients with myeloproliferative neoplasia, is a tyrosine kinase mutation, and this mutation occurs in 50-95% of Ph- MPN cases (5). In 2006, mutations in the 515th codon of MPL were identified in 3-8% of cases diagnosed with ET and PMF (6). In 2007, an additional mutation was identified in JAK2 exon 12 in several cases with PV that did not carry the JAK2 V617F allele (7). All of these mutant alleles cause the gain of function due to the activation of tyrosine kinase-dependent cellular signal pathways, especially the JAK-STAT pathway (8). However, activation in the JAK-STAT pathway has also been proven in cases that do not carry the JAK2 or MPL mutation. This evidence indicates that there are still unknown mutations in other genes associated with this pathway, and this is still of great interest to researchers. Also, the cellular and molecular mechanisms involved in the pathophysiology of MPNs have not been fully elucidated yet. Although these mutations cannot fully explain the phenotypic heterogeneity of MPNs, further genetic changes still await identification in approximately 20% of ET and PMF cases (7,8).

The detoxification enzyme NAD(P)H quinone oxidoreductase 1 (NQO1) is a phase 1 enzyme expressed in a wide range of tissue, including the bone marrow in the human body, including the epithelium of various organs. The most important feature of this enzyme is to prevent the formation of reactive and toxic semiquinone intermediate metabolites by reducing two electrons in a single step (9). NQO1 protects cells from free radicals and toxic oxygen metabolites from single-electron reductions catalyzed by cytochrome P450 and other enzymes (10). Also, NQO1 has been shown to play a direct role in protecting against oxidative stress by preventing the redox cycle and free radical formation (11). There are more than 93 single nucleotide polymorphisms (SNPs) discovered in the *NQO1* gene located on the chromosome in 16q22.1. A change in the amino acid sequence of the protein occurs with the C/T change (C609T) (rs1800566) at position 609, the most studied SNP of the NQO1 cDNA encoding the NQO1 enzyme (P187S) (10). Compared to the original type, heterozygous individuals (C/T or NQO1*1/*2) have intermediate activity, while homozygotes (T/T or NQO1*2/*2) are insufficient in NQO1 activity (12). In the presence of this polymorphism, the enzyme's ability to detoxify carcinogens is reduced, which may increase the likelihood of malignant changes in susceptible individuals.

Described as an anti-cancer enzyme, NQO1 plays a protective role in carcinogenesis by modifying internal exposure to bioactive carcinogens. In some studies, NQO1 C609T polymorphism has been associated with the risk of childhood and adult acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) (13-16).

Based on all this information, in our study, we aimed to investigate whether there is a relationship between NQO1 C609T polymorphism and MPN development.

Methods

The study included 119 patients (64 ET, 13 PMF, and 42 PV) who were diagnosed at the İstanbul University İstanbul Faculty of Medicine, Department of Hematology between 1995-2013 and met the 2008 "World Health Organization (WHO)" criteria (17) and 122 healthy controls. The control group consisted of volunteers without hematological

malignancy in their family and with a mean age similar to the patient group. In this study, which was accepted by the Ethics Committee of İstanbul University İstanbul Faculty of Medicine (decision no: 2012/634-1037), an informed consent form was obtained from the subjects before the study. The study was supported by the İstanbul University Scientific Research Fund (project no: BAP/23924). DNA was isolated from the peripheral blood samples with two cc EDTA taken from patients during their routine check-ups between 2012 and 2013 (MagnaPure Compact DNA Isolation Kit, Roche). JAK2 V617F mutation was determined using real-time-polymerase chain reaction (RT-PCR), and *NQO1*2* gene polymorphism was determined using PCR -restriction fragment length polymorphism method.

JAK-2 V617F Real-time-Polymerase Chain Reaction Analysis

The JAK2 V617F assay was performed using the manufacturer's recommended protocol ("JAK2 MutaScreenTM Kit Reference scale" Ipsogen, Luminy Biotech, Marseille, France).

NQO1 Gene Region Analysis

For *NQO1* gene region

Forward primer: 5'-AGTGGCATTCTGCATTTCTGTG-3',

Reverse primer: 5'-GATGGACTTGCCCAAGTGATG-3',

PCR protocol: Following the initial denaturation at 94 °C for 2 minutes; 35 cycles including 45 seconds denaturation at 94 °C, 45 seconds binding at 58 °C, 45 seconds elongation at 72 °C, and finally 5 minutes at 72 °C (18). Genotyping was performed by imaging the obtained PCR products at 37 °C with Hinf1 restriction enzyme and imaging under UV in 2% agarose gel.

Statistical Analysis

The results obtained from the patient and control groups were statistically evaluated with SPSS 21.0 program (SPSS Inc., Chicago, IL, USA). The differences of the parametric data between the two groups were evaluated with the Student's t-test, and the parametric differences between the three groups were evaluated by one-way analysis of variance. The rates were compared with the chi-square test. The results are shown as mean ± standard deviation (SD). The 95% confidence interval, odds ratio, and p value were determined. Results with a p value of <0.05 were considered significant. Fisher's exact test was used when the minimum expected value (MEV) was ≤5, and Pearson's chi-square was used when MEV was ≥25.

Results

Our study cohort included a total of 119 MPN patients, including 64 MP, 13 PMF, and 42 PV patients, and 119 healthy controls. One of our ET patients (CC genotype) and one of our PMF patients (CT genotype) had AML transformation. Clinical information of our patient group is given in Table 1. The mean age of diagnosis of the patients was 55.44 (SD: 14.25), and the age of the patients was higher in the PMF group than the ET group ($p<0.05$). Blood samples were taken for a mean of 6.18 (SD: 5.66) years after the diagnosis. The mean leukocyte count of patients was $10.99x10^9$/L (SD: 5.61), Hemoglobin (Hb) level was 14.34 g/dL (SD: 3.42), platelet count was $744.07x10^9$/L (SD: 421.25), and LDH level was 511.27 U/L (SD: 264.97). Leukocyte count was statistically significantly higher in the PMF group and PV group than in the ET group ($p<0.001$ and $p=0.014$, respectively). Hb level was higher in the ET group than the PMF group ($p<0.001$) and was significantly higher in the PV group than ET

Table 1. Characteristic features of patients diagnosed with essential thrombocythemia, polycythemia vera, and primary myelofibrosis

	ET	PMF	PV	Total
n (%)	64 (53.80%)	13 (10.90%)	42 (35.30%)	119 (100%)
Gender				
Female (%)	27 (42.19%)	8 (61.54)	20 (47.62%)	55 (46.22%)
Male (%)	37 (57.81%)	5 (38.46%)	22 (52.38%)	64 (53.78%)
Mean age (mean ± SD)	53.97±14.31	63.15±11.32[a]	55.29±14.51	55.44±14.25
Disease duration, years (mean ± SD)	6.32±5.64	6.07±6.17	5.96±5.69	6.18±5.66
Leukocyte ($X10^9$/L) (mean ± SD)	9.65±4.26	14.24±9.35[b]	12.30±4.89[c]	10.99±5.60
Hb (g/dL) (X ± SD) (mean ± SD)	13.63±2.02[b]	10.66±2.40	17.79±3.39[b]	14.34±3.42
Platelet ($X10^9$/L) (mean ± SD)	931.66±372.93[b]	551.44±430.27	436.34±274.93	744.07±421.25
LDH (U/L) (mean ± SD)	421.76±181.68	776.00±392.16	566.80±244.93	511.27±264.97
Splenomegaly group				
Splenomegaly (-) (%)	67.40%	0	27.60%	44.80%
Splenomegaly (+) (%)	32.60%	100%[b]	72.40%[b]	55.20%
Bone marrow reticulin fibrous degree	100%	100%	100%	100%
0	20.93%	0	4.54%	12.90%
1	58.14%	0	54.56%	48.10%
2	20.93%	8.33%	18.18%	18.20%
3	0	75%	18.18%	16.90%
4	0	16.67%	4.54%	3.90%
Use of hydroxyurea (%)	82.80%	76.90%	76.20%	79.80%

[a]: $p<0.05$, [b]: $p<0.001$, [c]: $p=0.014$, ET: essential thrombocythemia, PMF: primary myelofibrosis, PV: polycythemia vera, SD: standard deviation, Hb: hemoglobin, LDH: lactate dehydrogenase

and PMF groups (p<0.001). Platelet count was statistically significantly higher in the ET group than PMF and PV groups (p<0.001 and p<0.001, respectively). Reticulin fibrosis levels were higher in the PMF group than in ET and PV groups. It was observed that reticulin fibrosis levels were increased in ascending order in ET, PV, and PMF groups, respectively. When the PMF group was compared with the ET group, the frequency of splenomegaly was observed to be at high frequency (Fischer's exact test, p<0.001). All PMF patients had splenomegaly. The frequency of splenomegaly was higher in the PV group than in the ET group (Pearson X^2=11.291; p<0.001). There was no difference between the patient groups in terms of hydroxyurea use (p>0.05). JAK2 V617 mutations were detected in 40 (62.5%) ET patients, 8 (61.5%) PMF patients and 33 (78.6%) PV patients (Table 2). Although JAK2 mutation was higher in the PV group than the PMF group, no significant difference was observed (p=0.08). JAK2 V617 mutation distribution was similar between PV and ET and PMF and ET patient groups (p>0.05).

In the statistical evaluation, no relation was found between the presence of splenomegaly and the patient subgroups in terms of the presence of the JAK2 mutation. When NQO1 genotype distribution was examined, rapid metabolizer CC genotype was observed in 42 ET patients (65.6%), 9 PMF (69.2%) patients, and 25 PV (59.5%) patients. Intermediate metabolizer CT genotype was detected in 20 ET (31.3%) patients, four PMF (30.8%) patients and 12 PV (28.6%) patients. The slow metabolizer TT genotype was observed in two ET (3.1%) patients and five PV (11.9%) patients but was not found in PMF patients. When the patients were compared in terms of NQO1*2 polymorphism, no difference was observed between the ET, PMF, and PV groups in terms of genotypes (p>0.05). CC genotype was observed in 85 (69.7%) controls. CT genotype was found in 35 (28.7%) controls, and the TT genotype was found in two (1.6%) controls. When the entire patient group and the control group were examined in terms of genotype distributions, there was no statistically significant difference (p>0.05) (Table 3). In the entire patient group and patient subgroups, it was observed that NQO1 alleles were not related to age, gender, disease duration, leukocyte, platelet, Hb, and bone marrow reticulin fibrosis levels (p>0.05). In the PV group, the platelet count was significantly higher in those carrying the NQO1 T allele (p=0.047). No difference was observed in other patient subgroups.

According to the distribution of NQO1 polymorphism in patient subgroups and total patient group, the presence of JAK2 mutation was examined (Table 4). In terms of the co-existence of NQO1 polymorphism and JAK2 mutation, the patient and control groups were similar (p>0.05). No difference was observed among the patient subgroups (p>0.05).

Discussion

Classic Ph- MPNs are a family of clonal chronic hematological malignancies, including PV, ET, and PMF (19). Transformation of

Table 2. JAK2 V617F mutation distribution of patients diagnosed with essential thrombocythemia, primary myelofibrosis, and polycythemia vera

	ET, n (%)	PMF, n (%)	PV, n (%)	Total, n (%)
JAK2 V617F Mutation				
Yes	40 (62.5%)	8 (61.5%)	33 (78.6%)	81 (68.1%)
No	24 (37.5%)	5 (38.5%)	9 (21.4%)	38 (31.9%)

ET: essential thrombocythemia, PMF: primary myelofibrosis, PV: polycythemia vera, JAK2: janus kinase 2

Table 3. Distribution of NAD(P)H quinone oxidoreductase-1 (C>T) polymorphism genotypes between patient and control groups

Genotype	MPN group (n=119)				Control group (n=122)
	ET (n=64)	PMF (n=13)	PV (n=42)	Total (n=119)	
RM (CC)	42 (65.6%)	9 (69.2%)	25 (59.5%)	76 (63.9%)	85 (69.7%)
IM (CT)	20 (31.3%)	4 (30.8%)	12 (28.6%)	36 (30.2%)	35 (28.7%)
SM (TT)	2 (3.1%)	0 (0%)	5 (11.9%)	7 (5.9%)	2 (1.6%)

MPN: myeloproliferative neoplasm, ET: essential thrombocythemia, PMF: primary myelofibrosis, PV: polycythemia vera, RM: Rapid metabolizer, IM: intermediate metabolizer, SM: slow metabolizer

Table 4. Relationship between NAD(P)H quinone oxidoreductase-1 (C>T) polymorphism and JAK2 V617F mutation in patient groups

NQO1 (C>T)		JAK2 V617F mutation		Total, n (%)
		(+) n (%)	(-) n (%)	
ET	CC	17 (70.8%)	25 (62.5%)	42 (65.6%)
	CT+TT	7 (29.2%)	15 (37.5%)	22 (34.4%)
PMF	CC	2 (40%)	7 (87.5%)	9 (69.2%)
	CT+TT	3 (60%)	1 (12.5%)	4 (30.8%)
PV	CC	5 (55.6%)	20 (60.6%)	25 (59.5%)
	CT+TT	4 (44.4%)	13(39.4%)	17 (40.5%)
Total	CC	24 (63.2%)	52 (64.2%)	76 (63.9%)
	CT+TT	14 (36.8%)	29 (35.8%)	43 (36.1%)

NQO1: NAD(P)H quinone oxidoreductase-1, JAK2: janus kinase 2, ET: essential thrombocythemia, PV: polycythemia vera, PMF: primary myelofibrosis

MPNs into AML is characterized by a 20% blast in the bone marrow or peripheral blood, according to the WHO, and is one of the most feared complications of MPN. Approximately 5-10% of all MPNs progress to AML within ten years after diagnosis, which occurs in 1% of ET cases, 4% of PV cases, and 20% of PMF cases. Although the risk factors leading to this transformation have not been entirely determined, advanced age (>60 years) and exposure to chemotherapy are known to increase the risk of transformation. The molecular basis of this progression has not yet been clarified and remains attractive as a current research area (20,21).

In our study, it was aimed to investigate the prevalence of NQO1 C609T polymorphism, which causes loss of enzyme activity and which may be a risk factor in the etiology of specific hematopoietic malignancies in the Turkish community of MPN patients.

In *NQO1* gene defective mice, the increase in myeloid cells in the peripheral blood, bone marrow, and granulocytes significantly causes myeloid cell hyperplasia (22). NQO1-/-g-radiation mice have also been shown to develop the myeloproliferative disease, including loss of spleen follicular structure and bone marrow hypercellularity due to the infestation of granulocytes and megakaryocytes in the blood granulocytes and bone marrow myeloid cells. These results provide direct evidence that mice with NQO1 deficiency are highly prone to developing the myeloproliferative disease (23). The association between NQO1 C609T polymorphism and the risk of acute leukemia has been reported from the studies conducted since 2000, but these data are still insufficient. In their study with 82 patients with different types of hematopoietic malignancies and 99 healthy controls in 2011, Lozic et al. (24) reported that NQO1 C609T polymorphism was more common in adult patients with myeloid disorder compared to adult controls (p=0.0267). The results of the meta-analysis study conducted by Cuiping Li and Yang Zhou (25). in 2014 show that NQO1 C609T polymorphism is a significant genetic risk factor in ALL. As a result of another meta-analysis study, NQO1 C609T polymorphism was found to be associated with increased ALL risk (26). According to the study results of Ouerhani et al. (27), the NQO1 C609T polymorphism has been reported concerning the increased risk of ALL in the Tunisian population. According to the results of Smith et al. (28), null or low NQO1 activity caused by the inheritance of the C609T allele has been reported to be associated with an increased risk of de novo acute leukemia in adults. It has also been reported that there is a statistically significant relationship between NQO1 C609T polymorphism and the risk of chronic myelocytic leukemia (29).

As a result, in most of the studies, NQO1 C609T polymorphism has been associated with increased hematopoietic malignancies. However, the data of the only study conducted by Sirma et al. (30) in the Turkish population on this issue provide evidence that the NQO1 C609T polymorphism does not play a role in the etiology of *de novo* pediatric acute leukemia. We think that these different results between studies may result from differences in ethnic origin and sample sizes.

Conclusion

Our study is the first study to examine the relationship between NQO1 C609T polymorphism and MPN in the Turkish population. Our results are that there is no relationship between NQO1*2 polymorphism and MPN. It is also inferred that the NQO1 enzyme variants, which are important in the activation of detoxification and procarcinogens, do not increase the formation of the JAK2 V617F mutation, which is common in MPN patients. We think that the results should be supported by increasing the number of patient and control groups.

Ethics Committee Approval: In this study, which was accepted by the Ethics Committee of İstanbul University İstanbul Faculty of Medicine (decision no: 2012/634-1037).

Informed Consent: Informed consent form was obtained from the subjects before the study.

Peer-review: Externally peer-reviewed.

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