The role of certain gene polymorphisms involved in the apoptotic pathways in polycythemia vera and essential thrombocytosis

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None declared

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Abstract

Background. Polycythemia vera (PV) and essential thrombocytosis (ET) are hematological disorders characterized by excessive production of mature and functional blood cells. These cellular disorders are thought to be associated with impaired apoptosis, which is one of the major cellular death mechanisms in hematopoietic cells.

Objectives. In this study, our objective was to examine the association between potential polymorphisms of the Bcl 2, Bax, Fas and Fas Ligand genes involved in apoptosis and the occurrence of PV and ET.

Material and methods. A total of 93 patients diagnosed with PV (n = 38) or ET (n = 55) at the Department of Hematology were included in this study, and 93 healthy individuals served as controls. DNA isolation was performed in blood samples obtained from both groups of subjects to determine the Bcl 2, Bax, Fas, and Fas L genotypes using the real-time PCR method.

Results. No statistically significant differences between controls and patients were found in terms of Fas -670 G > A (rs1800682), Fas -1377 G > A (rs2234767), Fas L IVS2 -124 A > G (rs5030772), Bax -248 G > A (rs4645878) and Bcl 2 -938 C > A (rs2279115) polymorphisms, genotypes, and allele frequency ($p > 0.05$).

Conclusions. The results show that polymorphisms in the Bcl 2, Bax, Fas, and Fas Ligand genes involved in the apoptotic pathways may not play a role in the pathogenesis of PV and ET.

Key words: polymorphism, polycythemia vera (PV), essential thrombocytosis (ET), apoptotic pathway genes

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Clonal myeloproliferative neoplasms (MPN) are classified into 2 major groups. While classical MPNs are represented by chronic Ph-positive myeloid leukemia (CML), carrying the Philadelphia (Ph) translocation and the BCR-ABL (Breakpoint Cluster Region-Abelson proto-oncogene) fusion gene, atypical MPNs consist of Ph-negative CML without the Philadelphia (Ph) translocation or the BCR-ABL fusion gene, polycythemia vera (PV), essential thrombocytosis (ET) and primary myelofibrosis (PMF). They generally occur in adulthood, with an annual incidence rate of 5–10 cases per one million people. Despite extensive, decades-long clinical and laboratory research, the etiology of BCR-ABL-negative myeloproliferative diseases is not exactly known. Although myeloproliferative diseases consist of a heterogeneous group of disorders, they are characterized by the excessive growth of multipotent stem cells in one or several blood cell lines.[1–3] In 2005, the JAK2 V617F mutation was discovered and was present in the majority of PV patients and 40–60% of ET and PMF patients. This type of mutation is referred to as a "Class I" mutation and is generally different from Class II mutations, which cause the MDS syndrome phenotype and occur in the active cellular growth signal mediators, including important molecules involved in the differentiation process. On the other hand, Class III mutations include multiple repetitive somatic mutations in the genes responsible for epigenetic regulation.[4,5]

Apoptotic mechanisms function through 3 pathways: the extrinsic pathway requiring cell surface and death receptors, the intrinsic pathway of mitochondrial origin, and the perforin/granzyme pathway mediated by cytotoxic T-cells. The aberrations in these mechanisms are involved in the pathogenesis of a number of conditions, including degenerative and autoimmune disorders and hematological cancers.[6,7] Deeper insight into the role of apoptosis in malignant conditions will not only shed more light on the pathogenetic mechanisms, but also may provide certain clues on how to develop more effective therapeutic strategies.

Allelic variations in the promoter region of genes may result in qualitative or quantitative changes through their effects on the transcription factor binding site or other regulatory sites of the gene. Many single-nucleotide polymorphisms (SNPs) are known to be required for the development of malignancies or in pathways such as apoptosis, which play an important role in resistance to chemotherapeutic agents.[8,9] Thus, in this study, the role of SNPs such as Fas -670 G > A (rs1800682), Fas -1377 G > A (rs2234767), FasL IVS2 -124 A > G (rs5030772), Bax -248 G > A (rs4645878) and Bcl 2 -938 C > A (rs2279115) involved in the apoptotic pathway was explored in terms of their effects on the molecular pathogenesis of 2 MPNs: PV and ET.

Material and methods

Study population

A total of 93 individuals between 28 and 85 years of age (mean age of 59.53 ± 15.52) diagnosed with PV and ET between 2010 and 2012 at the Department of Hematology, Medical Faculty of Mersin University were included in this study. The control group consisted of 93 individuals between 40 and 81 years of age (mean age of 51.96 ± 10.73) with no disease history. Thus, blood samples obtained from a total of 186 subjects were analyzed.

Extraction of genomic DNA

After written informed consent was obtained from each of the study subjects, 4–5 mL of peripheral venous blood was placed into centrifuge tubes containing 1 mL of 2% EDTA. The DNA extraction was performed using Miller DNA isolation with a salting out precipitation method.[10]

Genotyping

The genotyping of the Fas -670 G > A (rs1800682), Fas -1377 G > A (rs2234767), FasL IVS2nt -124 A > G (rs5030772), Bax -248 G > A (rs4645878) and Bcl 2 -938 C > A (rs2279115) gene polymorphisms was performed using pre-designed TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, USA). The Assays-on-Demand SNP genotyping kit was used for the Polymerase Chain Reaction (Applied Biosystems). Single nucleotide polymorphism amplification assays were performed according to the manufacturer's instructions. In brief, 25 µL of reaction solution containing 30 ng of DNA was mixed with 12.5 µL of 2X TaqMan Universal PCR Master Mix (Applied Biosystems) and 1.25 µL of pre-developed assay reagent from the SNP genotyping product (C_9578811_10 for Fas gene -670 G > A [rs1800682]; C_12123966_10 for Fas gene -1377 G > A [rs2234767]; C_32334221_10 for FasL gene IVS2nt -124 A > G [rs5030772]; C_27848291_10 for Bax gene -248 G > A [rs4645878]; and C_3044428_30 for Bcl 2 gene -938 C > A [rs2279115], Applied Biosystems) containing two 900 nM primers and two 200 nM MGB TaqMan probes. Reaction conditions consisted of preincubation at 60°C for 1 min and at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. Amplifications and analysis were performed in an ABI Prism 7500 Real-Time PCR System (Applied Biosystems), using the SDS 2.0.3 software for allelic discrimination (Applied Biosystems).

Statistical analysis

The Independent Samples t-test and one-way ANOVA were used to compare continuous variables between groups. Analysis of the association between groups

and genotypes/alleles was done by χ^2 or likelihood ratio tests according to the expected value rule for crosstabs. The Hardy-Weinberg equilibriums were controlled in both control and study groups for all genotypes. Descriptive statistics were presented by mean and standard deviation for continuous variables, and with frequencies and percentages for categorical variables. Statistical analyses were done by SPSS v. 15 statistical package and p-values less than 0.05 were considered statistically significant.

Results

The group of patients with PV or ET consisted of 51 men and 42 women with a mean age of 59.53 ± 15.52 (ranging from 28 to 85 years). Thirty-eight patients (19 women, 19 men) presented with PV, while 55 (32 women, 23 men) patients had ET. There was a statistical difference in terms of the mean age between PV-ET patients and controls (p = 0.001). The Fas -670 G > A (rs1800682), Fas -1377 G > A (rs2234767), FasL IVS2 -124 A > G (rs5030772), Bax -248 G > A (rs4645878) and Bcl 2 -938 C > A (rs2279115) polymorphism distribution in patients with PV or ET was similar to those observed in healthy controls. As a result, there was no association between the Fas -670 G > A (rs1800682), Fas -1377 G > A (rs2234767), Fas L IVS2 -124 A > G (rs5030772), Bax -248 G > A (rs4645878) and Bcl 2 -938 C > A (rs2279115) polymorphisms and PV-ET (Table 1).

Discussion

MPNs are multipotent hematopoietic stem cell disorders that are characterized by the uncontrolled growth of mature blood cells.[11] There are no distinct boundaries between these disorders, which makes them capable of presenting in a number of different disease categories with the ability to evolve into each other.[12]

The allelic variations in the promoter region of the genes may cause qualitative or quantitative alterations through their effects on the transcription factor binding site or other regulatory sites. Many SNPs are known to be required for the development of certain malignant conditions and for pathways, such as apoptosis, which are important in terms of resistance to chemotherapy.[8,9] Thus, our objective was to examine the role of the Fas -670 G > A (rs1800682), Fas -1377 G > A (rs2234767), FasL IVS2 -124 A > G (rs5030772), Bax -248 G > A (rs4645878) and Bcl 2 -938 C > A (rs2279115) SNPs in the molecular pathogenesis of the 2 MPNs: PV and ET.

Single nucleotide changes of Fas -670 G > A (rs1800682) and Fas -1377 G > A (rs2234767) occur in the promoter region, at the repeating sequences of the binding sites for STAT1 (signal transducers and activators of transcription 1) and SP1 (Stimulatory Protein 1). These two polymorphisms lead to a reduced expression of Fas and Fas L genes. On the other hand, the Fas L IVS -124 (rs5030772) polymorphism is located in the second intron of the Fas L gene. Our results showed no significant associations between the genotype ratios and allele frequencies of the Fas -670, Fas L -1377 and Fas L IVS -124 polymorphisms between controls and patients with PV or ET. The deregulation of the Fas signal pathway is involved in the mechanisms of immune escape and tumorigenesis, and it is also associated with the differentiation, invasion and metastasis of cancer cells. In adult T-cell leukemia (ATL), where activated T lymphocytes were observed due to human T lymphotrophic virus type 1 (HTLV-1) infection, Farre et al. showed that the presence of the Fas -670 polymorphism was associated with clinical manifestations and survival.[13]

The Bcl 2 gene involved in the mitochondrial pathway of the apoptotic mechanism does play a role not only as a regulator protein for anti-apoptosis, but also as a suppressor of cell growth. It has 3 exons and 3 promoter regions. It is located 1400 bp upstream of the translation initiation site and acts as a negative regulator on P1 (first promoter), which directs the transcription. Bcl 2 not only acts as an anti-apoptosis regulator protein, but also as a proliferation inhibitor. Therefore, Bcl 2 has multiple functional effects in tumorigenesis, probably explaining why Bcl 2 expression is significant in diagnosis and why it differs according to the type of tumor. Polymorphisms altering the function/expression of the Bcl 2 gene affect the apoptotic mechanisms and potentially serve as an important marker to guide targeted treatments.[14,15] In our study, the polymorphisms of the P2 promoter of the Bcl 2 gene which serve as a negative regulator element were explored. In terms of the genotype ratios and allele frequencies of the Bcl 2 -938 C > A (rs2279115) polymorphism, control subjects did not differ significantly from those with PV or ET (p > 0.05). Bcl 2 may be expressed both in normal hematopoietic cells as well as in malignant hematopoietic cells, such as the leukemic blast cells of AML. Accordingly, Moon et al. proposed that a Bcl 2 protein or its gene expression could be used as a diagnostic marker for AML after chemotherapy. Bcl 2 expression is known to be increased upon Bcl 2 induction or selection of cells overexpressing Bcl 2 in patients with AML. Also, AML patients with higher Bcl 2 protein or gene expression have a shorter survival and poor response to chemotherapy. Moon et al. concluded that the Bcl 2 -938 C > A polymorphism was linked to overall survival and remission rates following chemotherapy in patients with leukemia.[16] Nückel et al. in a study involving patients with chronic lymphocytic leukemia (CLL) found that in the Bcl 2 938 C > A polymorphism, the AA genotype was associated with an increased expression of Bcl 2 and that it may be appropriate to use this as a genetic prognostic marker in patients with CLL.[17] Hwan et al. reported that there was a significant association between the Bcl 2-938

Table 1. The frequency of distribution of genotypes and alleles in the patient and control groups

Gene			PV (n : 38) n(%)	ET (n : 55) n(%)	PV + ET (n : 93) n(%)	Control (n : 93) n(%)
Fas-670 G > A rs1800682	genotype	GG	9 (23.7)	13 (23.6)	22 (23.6)	23 (24.7)
		GA	18 (47.4)	23 (41.8)	41 (44.1)	49 (52.7)
		AA	11 (28.9)	19 (34.6)	30 (32.3)	21 (22.6)
	allel	G	36 (47.4)	49 (44.5)	85 (45.7)	95 (51.1)
		A	40 (52.6)	61 (55.5)	101 (54.3)	91 (48.9)
Fas-1377 G > A rs2234767	genotype	GG	28 (75.7)	41 (74.5)	69 (75.0)	68 (73.1)
		GA	8 (21.6)	14 (25.5)	22 (23.9)	24 (25.8)
		AA	1 (2.7)	0 (0.0)	1 (1.1)	1 (1.1)
	allel	G	64 (86.5)	96 (87.3)	160 (87.0)	160 (86.0)
		A	10 (13.5)	14 (12.7)	24 (13.0)	26 (14.0)
FasL IVS2-124 A > G rs5030772	genotype	GG	27 (71.0)	37 (67.3)	8 (8.6)	57 (61.3)
		GA	9 (23.7)	12 (21.8)	21 (22.6)	31 (33.3)
		AA	2 (5.3)	6 (10.9)	64 (68.8)	5 (5.4)
	allel	G	63 (82.9)	86 (78.2)	37 (19.9)	145 (78.0)
		A	13 (17.1)	24 (21.8)	149 (80.1)	41 (22.0)
Bax-248 G > A rs4645878	genotype	GG	29 (76.3)	43 (78.2)	72 (77.4)	78 (83.9)
		GA	8 (21.1)	10 (18.2)	18 (19.4)	13 (14.0)
		AA	1 (2.6)	2 (3.6)	3 (3.2)	2 (2.1)
	allel	G	66 (86.8)	86 (87.3)	162 (87.1)	169 (90.9)
		A	10 (13.2)	14 (12.7)	24 (12.9)	17 (9.1)
Bcl2-938 C > A rs2279115	genotype	CC	8 (21.0)	15 (27.3)	23 (24.7)	20 (21.5)
		CA	21 (55.3)	24 (43.6)	45 (48.4)	49 (52.7)
		AA	9 (23.7)	16 (29.1)	25 (26.9)	24 (25.8)
	allel	C	37 (48.7)	54 (49.1)	91 (48.9)	89 (47.8)
		A	39 (51.3)	56 (50.9)	95 (51.1)	97 (52.2)

polymorphism and disposition to CML, which is a clonal myeloproliferative disorder.[18]

The proapoptotic function of the Bax gene involved in the induction of apoptosis and the regulation of the apoptosis pathway by many other genes, such as Bcl 2 and p53, through their interaction with the Bax gene (at least partially), have given rise to an increased interest in the Bax gene for cancer research. Recent studies have shown that the Bax gene is a tumor suppressor. Deletions of the gene have been shown to be associated with lymphoid hyperplasia and have also been found to possess a significant negative growth function from a hematopoietic aspect.[19]

The genotype ratios and allele frequencies of Bax-248 G > A (rs4645878) polymorphisms, which occur in the 5' untranslated region (UTR) of the Bax gene and which cause a reduction in the expression of the gene, did not differ significantly between control subjects and patients with PV or ET. However, Saxena et al. observed that the Bax -248 polymorphism occurring in the Bax promoter was associated with a decreased expression of Bax in CLL, as well as with the failure to achieve a complete response to conventional therapy.[20] Skogsberg et al., however, concluded that the Bax-248 polymorphism had no role as a marker of survival and prognosis in CLL.[21]

The exact cause of MPNs is unknown. However, molecular-genetic studies suggest that the majority of this type of neoplasm may be the result of acquired clonal genetic events. Therefore, this group of disorders represents a good candidate for molecular diagnostic studies. Particularly in this type of hematological malignancy, a better understanding of the apoptotic mechanisms responsible for homeostasis during the growth and differentiation of hematopoietic blood cells will not only allow us to gain deeper insights into the pathogenesis of these disorders, but will also guide us in our treatment decisions. Our results showed that the Fas -670 G > A (rs1800682), FAS 1377 G > A (rs2234767), Fas L IVS2 -124 A > G (rs5030772), Bax -248 G > A (rs4645878) and Bcl 2 -938 C > A (rs2279115) polymorphisms, which are involved in extrinsic and intrinsic apoptotic pathways, are not associated with the development of PV or ET. Although SNPs in apoptosis-associated genes, and changes in the gene and protein expression have been the subject of extensive research in many cancer types, to our knowledge, no studies examining the polymorphisms of the genes involved in the apoptotic pathway in patients with PV or ET, which are classified as hematological malignant conditions, have been carried out. We believe that further studies involving a larger patient series may better elucidate these associations. Also, the possibility that the occurrence of these diseases may be associated with variants of the genes examined should also be borne in mind. Furthermore, our findings indicate the need to examine other molecular changes of the apoptotic processes in patients with PV or ET.

References

1. Kralovics R, Skoda RC. Molecular pathogenesis of Philadelphia chromosome negative myeloproliferative disorders. *Blood Reviews*. 2005;19:1–13.
2. Tefferi A, Gary GD. Oncogenes in myeloproliferative disorders. *Cell Cycle*. 2007;6(5):550–566.
3. Fanny BM, Hajer M, Christophe D, et al. Expression level and differential JAK2-V617F–binding of the adaptor protein Lnk regulates JAK2-mediated signals in myeloproliferative neoplasms. *Blood*. 2009;116:5961–5971.
4. Spivak JL. Polycythemia vera: Myths, mechanisms, and management. *Blood*. 2002;100(13): 4272–4290
5. Aaron DV, Ross LL. Genetics of myeloproliferative neoplasms. *The Cancer Journal*. 2014; Volume 20, Number 1, January/February.
6. Khurum HK, Montserrat B, L Rhoda M. Cancer therapeutics: Targeting the apoptotic pathway. *Critical Reviews in Oncology/Hematology*. 2014.
7. Wong RS. Apoptosis in cancer: From pathogenesis to treatment. *Wong Journal of Experimental & Clinical Cancer Research*. 2011;30(87):1–14.
8. Lena FK, Gisela WS, Heike D, et al. Polymorphisms in the apoptotic pathway gene BCL-2 and survival in small cell lung cancer. *J Thorac Oncol*. 2011;6:183–189.
9. Irina B, Eva G, Wen-Yu C, et al. Analysis of single nucleotide polymorphisms in the FAS and CTLA-4 genes of peripheral T-cell lymphomas. *J Hematopathol*. 2008;1:11–21.
10. Miller SA, Dykes DD, Polesky H. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res*. 1988;16:1215.
11. Wanlong M, Hagop K, Xi Z, et al. JAK2 Exon 14 Deletion in Patients with Chronic Myeloproliferative Neoplasms. *PLoS One*. 2010;5:1–7.
12. Provan D, Gribben J. *Molecular Haematology* 2th Edition. UK. Blackwell Publishing 2005;P90.
13. Farre L, Bittencourt AL, Silva-Santos G, et al. FAS 670 promotor polymorphism is associated to susceptibility clinical presentation, and survival in adult Tcell leukemia. *J Leukocyte Biol*. 2008;83:220–222.
14. Hagen SB, Lukas CH, Klaus JS, et al. Regulatory BCL2 promoter polymorphism (2938C>A) is associated with adverse outcome in patients with prostate carcinoma. *Int J Cancer*. 2011;129:2390–2399.
15. Ning Z, Xiaoyan L, Kai T, et al. BCL-2 (-938C > A) polymorphism is associated with breast cancer susceptibility. *BMC Medical Genetics*. 2011;12:48.
16. Joon HM, Sang KS, Myung HL, et al. BCL2 gene polymorphism could predict the treatment outcomes in acute myeloid leukemia patients. *Leukemia Research*. 2010;166–172.
17. Holger N, Ulrich HF, Maja B, et al. Association of a novel regulatory polymorphism (-938C>A) in the *BCL2* gene promoter with disease progression and survival in chronic lymphocytic leukemia. *Blood*. 2007;290-297.
18. Kim DH, Xu W, Ma C, et al. Genetic variants in the candidate genes of the apoptosis pathway and susceptibility to chronic myeloid leukemia. *Blood*. 2009;(113):11.
19. Peng H, Aiello A, Packham G, Isaacson PG, Pan L. Infrequent bax gene mutations in B-cell lymphomas. *J Pathol*. 1998;186:378–382.
20. Anurag S, Oksana M, Koravangattu S, Sathiyanarayanan V, David PS. Association of a novel single nucleotide polymorphism, G(2248)A, in the 50-UTR of BAX gene in chronic lymphocytic leukemia with disease progression and treatment resistance. *Cancer Letters*. 2002;187:199–205.
21. Skogsberg A, Tobin G, Kröber A, et al. The G(-248)A polymorphism in the promoter region of the Bax gene does not correlate with prognostic markers or overall survival in chronic lymphocytic leukemia. *Leukemia*. 2006;20,77–81.