FAS/FASL gene polymorphisms in Turkish patients with chronic myeloproliferative disorders

Fusun Gediz Ozdemirkiran¹, Sinem Nalbantoglu², Zafer Gokgoz³, Bahriye Kadriye Payzin¹, Filiz Vural⁴, Seckin Cagirgan¹, Afig Berdeli²

¹Department of Hematology, Atatürk Research and Education Hospital, Izmir Katip Çelebi University, Izmir, Turkey
²Department of Molecular Genetics, Faculty of Medicine, Ege University, Izmir, Turkey
³Department of Hematology, Baskent University, Adana, Turkey
⁴Department of Hematology, Faculty of Medicine, Ege University, Izmir, Turkey

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Abstract

Introduction: Chronic myeloproliferative disorders (CMPD) are chronic myeloid hematological disorders, characterized by increased myeloid cell proliferation and fibrosis. Impaired apoptotic mechanisms, increased cell proliferation, uncontrolled hematopoietic cell proliferation and myeloaccumulation may contribute to the pathogenesis of CMPD. The aim of our study was to show the possible role of FAS/FASL gene polymorphisms in CMPD pathogenesis and investigate the association with clinical parameters and susceptibility to disease.

Material and methods: We included 101 (34 polycythemia vera (PV), 23 primary myelofibrosis (PMF), 44 essential thrombocythemia (ET)) CMPD patients diagnosed according to the WHO classification criteria and 95 healthy controls in this study. All the patients and the controls were investigated for FAS/FASL gene expression, allele frequencies and phenotype features, and also FAS mRNA levels were analyzed.

Results: Chronic myeloproliferative disorders patients showed increased FAS-670AG + GG genotype distribution compared with the control group (p < 0.05). While the A allele was more frequent in both groups, AG genotype was more frequent in CMPD patients. There was no association between FAS-670A>G gene polymorphism and some clinical parameters such as splenomegaly and thrombosis (p > 0.05). No statistically significant difference in FASL+843C>T genotype or allele frequency was found between groups (p > 0.05). Moreover, no statistically significant difference was detected in FASL and JAK2V617F mutations (p > 0.05). FAS mRNA expression was 1.5-fold reduced in patients compared to healthy subjects.

Conclusions: According to our findings, FAS/FASL gene expression may contribute to the molecular and immunological pathogenesis of CMPD. More investigations are needed to support these data.

Key words: FAS/FASL, gene polymorphism, chronic myeloproliferative disorders.

Introduction

Chronic myeloproliferative disorders (CMPD) are pluripotent hematopoietic stem cell diseases, characterized by proliferation of one or more
myeloid cell lines (erythroid, granulocytic, megakaryocytic) in the bone marrow, without exhibiting any differentiation and maturation defect [1]. Common properties of these diseases include uncontrolled proliferation of one or more of the myeloid cell lines, relatively normal maturation, hepatosplenomegaly, transformation to acute leukemia in different proportions and development of bone marrow fibrosis [2]. Development, progression and myeloproliferative mechanisms of the disease are not quite clear yet. Molecular mechanisms as well as aberrant expression of the genes that regulate apoptosis are thought to play a role here too, as in other hematologic malignancies [3]. Impairment of the apoptosis mechanisms and aberrant expression of pro-antiapoptotic proteins contribute to the excessive cell proliferation and development of fibrosis [4]. The relationship between the impairment of apoptosis and various hematological malignancies (chronic neutrophilic leukemia, myelodysplastic syndrome (MDS), chronic myeloid leukemia (CML), etc.) has been revealed in the literature [5]. Apoptosis is known as programmed cell death [6, 7]. It occurs through two main mechanisms: either a change in the mitochondrial permeability (intrinsic pathway) or binding of the death receptor and the specific ligand on the cell surface (extrinsic pathway) [8, 9]. FAS (CD95) is a molecule expressed on the cell surface, and it initiates the death signal after binding to its ligand (FASL) [10]. The FAS/FASL pathway is a critical system for hematopoietic cell survival and apoptosis [11–13]. Genetic variations in the promoter region of FAS/FASL may play an important role in the pathogenesis of CMPD by inducing apoptosis. The aim of our study was to investigate the role of FAS/FASL gene polymorphisms in CMPD pathogenesis and their possible association with clinical parameters as well as susceptibility to the disease.

Material and methods

Subjects

One hundred and one patients with confirmed diagnosis of Philadelphia chromosome negative (Ph–) CMPD according to WHO criteria and 95 healthy volunteers were included in the study. Among 101 patients with CMPD, 34 had polycythemia vera (PV), 23 had primary myelofibrosis (PMF), and 44 had essential thrombocythemia (ET). Chronic myeloproliferative disorders patients who were regularly followed up in the hematology outpatient clinic and healthy volunteers with no history of systemic illness or systemic drug use were informed about the study in detail. The study was approved by the human research ethics committee of the hospital and informed consent was obtained from the patients and healthy volunteers who agreed to participate in the study. The patients’ history, physical examination, clinical and laboratory findings (white blood count, hemoglobin, hematocrit, platelet count, iron, total iron binding capacity, ferritin, erythropoietin levels, cytogenetic and molecular examinations (Ph chromosome, Bcr-abl)) at the first diagnosis were recorded by the scanning hematology outpatient clinic follow-up program.

Molecular methods

Genomic DNA preparation and quantitation

Genomic DNA was extracted from EDTA-anticoagulated whole blood samples using a commercially available genomic DNA purification kit (Qiagen, Ontario, Canada) following the manufacturer’s instructions. DNA concentration was determined spectrophotometrically by a NanoDrop digital spectrophotometer using the manufacturer’s instructions and diluted as 100 ng/µl. The isolated DNA (2 µl) was loaded on 1% agarose gel to detect the quality of the DNA.

Polymerase chain reaction (PCR) amplification was carried out on a GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, CA, USA) in a 25 µl reaction mixture in 0.2 ml thin-walled PCR strip tubes (Axygen Scientific, Inc, Union City, CA, USA) containing 1 µl of genomic DNA solution, GeneAmp Gold Buffer (15 mmol/l Tris HCl, pH 8.0, 50 mmol/l KCl) (PE Applied Biosystems), 1.5 mmol MgCl2, 50 µmol/l each of deoxyGTP (dGTP), deoxyCTP, deoxyATP (dATP), deoxyTTP (dTTP), and deoxyCTP (dCTP) (Promega, Madison, WI), 25 pmol each of forward and reverse primers and 1.0 U AmpliTaq Gold polymerase (PE Applied Biosystems). The cycling conditions were initially 95°C for 10 min, followed by 35 amplification cycles at 95°C for 45 s, 62°C for 45 s, and 72°C for 45 s, and a final extension at 72°C for 7 min.

Primers used for PCR-RFLP

For FAS-670A>G polymorphism the forward primer was 5′-CTA CCT AAG AGC TAT CTA CCG TTC-3′ and the reverse primer was 5′-GTC TGT CCA TGG TGT GGC TGC-3′.

Digestion protocol

The amplified 331 bp PCR product (3 µl) was digested in a 10-µl final reaction volume using 1 µl of Reaction Buffer 2 and 5 units of MvaI restriction enzyme (New England Biolabs, Beverly, MA, USA) at 37°C overnight. Controls of known genotype were included for every set of digestions carried out. After digestion, allele G yielded three fragments of 99, 188, and 44 bp, whereas allele
A yielded two fragments of 99 and 232 bp. Digested fragments were separated on 3% agarose gels and visualized after ethidium bromide staining in the BioDoc-It System (UVP, Upland, CA, USA) Bio-imaging system.

**PCR-RFLP genotyping for −843C/T polymorphism of FASL gene**

Genomic DNA was amplified using PCR carried out on a Gene-Amp PCR System 9700 (PE Applied Biosystems). The cycling conditions were initially 95°C for 10 min, followed by 35 amplification cycles at 95°C for 30 s, 45°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. Forward (5′-CCA TGA AAA TGA ACA CAT TG-3′) and reverse (5′-CCC ACT TTA GAA ATT AGA TC-3′) primers were used according to the published sequence (GenBank accession number AF027385).

**Digestion protocol**

The amplified 114 bp PCR product (7 µl) was digested at 37°C for 3 h in a 20 µl final reaction volume using 2 µl of Reaction Buffer 2 and 5 units of DraIII restriction enzyme (New England Biolabs, Beverly, MA). After digestion allele T yielded two fragments of 98 and 16 bp, whereas allele C was not digested and yielded 114 bp. Digested PCR samples were subjected to electrophoresis on gels containing a mixture of 1.5% agarose (Sigma, St. Louis, MO) and 1.5% NuSieve GTG (BMA, Rockland, ME).

**RNA isolation from peripheral blood leukocytes**

Total RNAs were prepared from peripheral blood leukocytes (PBLs) by the Qiagen RNA Blood Mini Isolation Kit (Qiagen GmbH, Hilden, Germany). According to the manufacturer’s instructions, 20 µl of the obtained total RNA was denatured for 10 min at 70°C to prevent dimerization, then reverse transcribed in a total volume of 50 µl containing 50 mM Tris HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM dNTPs, 15 µg/ml of random primers (Promega), 40 units of RNAsin (Promega), and the RNA sample. Samples were incubated for 1 cycle in the thermal cycler for 10 min at 26°C, 60 min at 42°C, and 5 min at 95°C, and then finally cooled to 4°C. Quantity and quality assessment of the obtained cDNA molecule was evaluated by the above method. Also, conventional PCR was performed with the newly designed RT-PCR primers for confirmation of the sequences.

**FAS gene mRNA expression quantitation by real-time PCR**

Relative quantification describes the change in expression of the target gene relative to the internal reference gene. Reactions were performed in a 50 µl volume of diluted cdNA sample, with 0.5 µM specifically designed primers (NM.000043.4), and 4 mM MgCl₂ by the Qiagen Quantitect Probe RT-PCR kit using ABI PRISM 7000. Cycling conditions consisted of 2 min at 50°C, and 45 cycles of 10 min at 95°C, 15 s at 95°C, and 1 min at 65°C. A primer-probe set designed for human RNase P, a housekeeping gene, was added to each sample to allow normalization to the total RNA content co-amplifying with FAS as an internal control.

All of the genotyping was performed blinded with respect to subject characteristics, and blinded quality control samples were inserted to validate genotypes. One sample for each of the three possible genotypes had formerly been confirmed by sequencing and served as standards in the restriction analysis. Additionally, the genotypes for FAS and FASL genes identified by the PCR restriction fragment length polymorphism (PCR-RFLP) method were confirmed by DNA sequencing, and sequences were compared with the published FAS and FASL gene DNA sequences. Concordance for blinded samples was 100%.

**Statistical analysis**

The Hardy-Weinberg principle [14] was used to test whether the patient and the control groups were balanced. Then, using the t-test and Pearson’s χ² tests, the patients’ and subjects’ genotype distribution and allele frequency were evaluated. The association of the genotype and allele frequency with the disease parameters was assessed by χ² and one-way ANOVA tests. The statistical analysis was performed using SPSS and p < 0.05 was considered statistically significant.

**Results**

A total of 101 CMPD patients who were diagnosed according to 2008 WHO criteria and were under the follow-up in Ege University Department of Hematology and 95 healthy volunteers (46 female/49 male, median age 49.4 years) were included in the study. Fifty of the patients were female and 51 were male, and the median age of the patients was 50.6 years. Age and gender features were homogeneous in the patient and the control groups (Table I). The demographic and clinical data according to each of the three CMPD groups is shown in Table II.

**FAS gene −670A>G polymorphism**

When comparing FAS gene −670A>G polymorphism genotype distribution between CMPD patients and the healthy control group, a statistically significant increase was found in AG and
GG genotypes in the patient group \((p = 0.003, \text{ Table III})\).

In the CMPD group, AG genotype was more prevalent, while the A allele was detected more frequently in both groups. FAS gene \(-670A\rightarrow G\) polymorphism and clinical findings such as splenomegaly and venous thrombosis showed no correlation \((p = 0.082, p = 0.412, \text{ respectively})\) (Table IV). In the comparison between ET, MF and PV groups, no significant differences were detected regarding FAS gene \(-670A\rightarrow G\) polymorphism \((p > 0.05)\) (Figure 1). Also, no statistically significant difference was found between FAS gene \(-670A\rightarrow G\) polymorphism and JAK2V617F mutation \((p > 0.05)\). FAS mRNA expression was 1.5-fold reduced in patients compared to healthy subjects and it was statistically significant \((p = 0.003)\).

**FASL gene 843 C/T polymorphism**

CMPD patients and the healthy control group displayed no statistically significant differences in terms of FASL 843C\(\rightarrow T\) genotype distribution or allele frequency \((p = 0.144)\) (Table V). In both groups, CT genotype (46.5% and 45.3%) and the C allele (61.9% and 52.1%) were more common. FASL gene 843C\(\rightarrow T\) polymorphism and clinical findings such as splenomegaly and venous thrombosis showed no correlation \((p > 0.05)\). Moreover, no statistically significant difference was detected for FASL and JAK2V617F mutations \((p > 0.05)\). Statistical data of the correlation of FAS and FASL gene polymorphisms with clinical features in CMPD patients are shown in Table VI.

**Table I. Demographic and clinical features of patients with CMPD and control group**

| Features                          | Patients (N = 101) | Control group (N = 95) |
|-----------------------------------|--------------------|------------------------|
| Age (mean) [years]                | 50.6               | 49.4                   |
| Gender (F/M)                      | 50/51              | 46/49                  |
| Essential thrombocytopenia (N)    | 44                 |                        |
| Polycythemia (N)                  | 34                 |                        |
| Primary myelofibrosis (N)         | 23                 |                        |

**Table II. Comparison of clinical and demographic features according to the three diagnoses**

| Clinical and demographic features | Myelofibrosis | Essential thrombocytopenia | Polycythemia vera |
|-----------------------------------|---------------|----------------------------|-------------------|
| Number of patients                | 23            | 44                         | 34                |
| Median age                        | 55.17         | 48.09                      | 50.79             |
| Gender                            |               |                            |                   |
| Male                              | 11            | 18                         | 22                |
| Female                            | 12            | 26                         | 12                |
| JAK2V617F mutation                |               |                            |                   |
| Negative                          | 10            | 31                         | 5                 |
| Homozygote                        | 1             | –                          | 1                 |
| Heterozygote                      | 12            | 13                         | 28                |
| Splenomegaly                      |               |                            |                   |
| Negative                          | 4             | 42                         | 10                |
| Positive                          | 19            | 2                          | 24                |
| Thrombosis                        |               |                            |                   |
| Negative                          | 21            | 32                         | 22                |
| Positive                          | 2             | 12                         | 12                |
| Hepatomegaly                      |               |                            |                   |
| Negative                          | 13            | 40                         | 26                |
| Positive                          | 10            | 4                          | 8                 |
| Mean LDH                          | 856.91        | 426.22                     | 502.32            |

**Table III. Genotype distribution and allele frequency of FAS gene 670A\(\rightarrow G\) polymorphism in CMPD patients and healthy controls**

| Group   | Genotypes | Allele |
|---------|-----------|--------|
|         | AA        | AG     | GG     | G       | A       |
| CMPD    | 32 (31.7%)| 54 (53.5%)| 15 (14.9%)| 84 (41.6%)| 118 (58.4%)|
| Control  | 52 (54.7%)| 30 (31.6%)| 13 (13.7%)| 56 (29.5%)| 134 (70.5%)|
In our study, increased Fas-670A>G promoter polymorphism was found in the CMPD group compared to healthy controls. Also we investigated the possible relationship between Fas-670 gene polymorphism and clinical manifestations of CMPD. We did not find any correlation between the evaluated clinical findings (splenomegaly, thrombosis) and FAS gene –670A>G polymorphism. No significant difference between CMPD and healthy control groups was found in terms of FASL gene 843C>T genotype and allele distributions.

In addition, FASL-843 gene polymorphism showed no association with the clinical parameters of disease. Comparing the patients with the control group, Fas mRNA expression was found to be 1.5-fold decreased among the patients. Apoptosis is a biological process which maintains homeostasis. Furthermore, abnormal regulation and dysfunctions mechanisms play an important role in disease.

### Table IV. Clinical and laboratory findings of CMPD patients according to FAS gene –670 A>G distribution

| Variable         | AA (N = 32) | AG + GG (N = 69) | P-value |
|------------------|-------------|------------------|---------|
| Splenomegaly     | 18 (56.3%)  | 27 (39.1%)       | 0.082   |
| Thrombosis       | 10 (31.3%)  | 16 (23.2%)       | 0.412   |

### Table V. Genotype distribution and allele frequency of FASL+843C>T in CMPD patients and healthy controls

| Group          | Genotypes  | Allele |
|----------------|------------|--------|
|                | CC         | CT     | TT    | C    | T    |
| CMPD (N = 101) | 39 (38.6%) | 47 (46.5%) | 15 (14.9%) | 125 (61.9%) | 77 (38.1%) |
| Control (N = 95) | 28 (29.5%) | 43 (45.3%) | 24 (25.3%) | 99 (52.1%) | 91 (47.9%) |

### Table VI. Correlation of FAS/FASL gene polymorphism with clinical features in CMPD patients

| Clinical and demographic features | FAS gene –670A>G polymorphism | FASL gene –843C>T polymorphism |
|-----------------------------------|--------------------------------|--------------------------------|
| Gender                            | p > 0.05                       | p > 0.05                       |
| Male                              | p > 0.05                       | p > 0.05                       |
| Female                            |                                |                                |
| Age                               | p > 0.05                       | p > 0.05                       |
| JAK2V617F mutation                | p > 0.05                       | p > 0.05                       |
| Negative                          |                                |                                |
| Homozygote                        |                                |                                |
| Heterozygote                      |                                |                                |
| Hepatomegaly                      | p > 0.05                       | p > 0.05                       |
| Positive                          |                                |                                |
| Negative                          |                                |                                |
| Splenomegaly                      | p > 0.05                       | p > 0.05                       |
| Positive                          |                                |                                |
| Negative                          |                                |                                |
| Thrombosis                        | p > 0.05                       | p > 0.05                       |
| Positive                          |                                |                                |
| Negative                          |                                |                                |
| Diagnosis                         | p > 0.05                       | p > 0.05                       |
| Myelofibrosis                     |                                |                                |
| Essential thrombocytemia          |                                |                                |
| Polycythemia vera                 |                                |                                |
the development of many hematologic and solid malignancies [15]. A possible polymorphism in the Fas/Fasl gene, which has an important task in this process, can result in hematological malignancies. Tognon et al. included 26 ET, 12 PV and 11 PMF patients in their study examining expression of different apoptosis-related genes in patients with CMPD, and analyzed apoptotic genes, as well as the JAK2V617F mutation [16]. They detected increased expression of FAS, FASL, DR4, FAIM, and c-FLIP I in the CD34 positive cells of the CMPD group, compared with controls. Considering disease subgroups, they demonstrated increased FAS expression in PMF patients, compared to the ET group. Again, they investigated the relationship between the expression of apoptotic genes and JAK2V617F mutation and detected a positive relationship between FAS and DR5 expression and JAK2V617F mutation in patients with PV, whereas no statistically significant result was obtained in patients with ET and PMF. Zeuner et al. observed increased expression of FLIP in erythroid precursor cells of PV patients [17]. In the same study, they also determined the presence of the JAK2V617F mutation in PV erythroblasts. In patients with PV, resistance mechanisms against death receptor-mediated apoptosis have been claimed to have an important role in the pathogenesis of the disease. In another study supporting the effect of resistance mechanisms, they analyzed expression of proapoptotic (FAS, FASL, Bax, Bad) and anti-apoptotic (Bcl-2, Bcl-XL, hTERT) molecules. Increased Bcl-XL expression was detected in the megakaryocytes of patients with ET, whereas increased expression of Bax and Bad was determined in megakaryocytes of PMF. FAS/FASL expression was not detected, while the mitochondrial pathway was emphasized to be critical for megakaryocyte apoptosis in both patient groups. Based on these results, increased expression of apoptotic molecules can support the fibrotic process in PMF, while the anti-apoptotic profile seen in ET was correlated with better prognosis. The role of Fas gene polymorphisms in the pathogenesis of different hematological malignancies was reported previously. Fare et al. investigated the FAS gene promoter 670A>G polymorphism in adult T-cell leukemia and compared the data with the control group, and identified AA genotypes more frequently among the patients [20]. Again, AA genotype has been associated with aggressive disease and poor prognosis, and Fas promoter polymorphism was considered to be associated with disease susceptibility, clinical signs, and survival in adult T-cell leukemia. Zhang et al. investigated the correlation between FAS promoter polymorphisms and cancer risk and retrospectively reviewed 11,461 cases of cancer in the electronic environment, and concluded that there was a high cancer risk related to FAS-1377 AA genotype and smoking but no relationship with FAS-670 GG genotype [21].

In conclusion, we observed significantly higher FAS-670A>G polymorphism frequency in patients with CMPD compared with the control group. This polymorphism should be investigated in larger patient groups in terms of its role in the pathogenesis of CMPD and its susceptibility to the disease.

Conflict of interest

The authors declare no conflict of interest.

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