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GENETIC POLYMORPHISMS IN PATIENTS WITH MYELODYSPLASTIC SYNDROME*

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Myelodysplastic syndrome (MDS) is a family of clonal disorders characterized by dyshematopoiesis and susceptibility to acute myelogenous leukemia. Tumor necrosis factor-a (TNF-α) and transforming growth factor-β (TGF-β) are cytokines that play key roles in the pathogenesis of MDS. There have been several reports on the presence of genetic polymorphisms in the DNA sequence encoding the leader sequence of the TGF-β1 protein, and in the –308 promoter region of TNF-α. The association between TNF-α and TGF-β1 gene polymorphism and the susceptibility to MDS and the progression of the disease was investigated. As compared with healthy control subjects (n = 74), patients with MDS (n = 55) showed no significant deviations in genotype or allele frequencies of TNF-α. Similarly, there were no differences in the distribution of TNF-α genotypes between the MDS patients with only anemia (mild group) and those with bi- or pancytopenia (severe group). On the other hand the TT homozygosity at codon 10 in exon 1 of TGF-β1 gene was associated with a severe degree of cytopenia [95% CI OR = 4.889, p = 0.0071]. These findings suggest that the investigated genetic polymorphisms do not predispose to the development of MDS, but that TGF-β1 gene polymorphism may affect the disease progression.

Keywords: myelodysplastic syndrome, tumor necrosis factor-alfa, transforming growth factor-beta, gene polymorphism

Introduction

The myelodysplastic syndrome (MDS) comprises a distinct, albeit heterogeneous group of hematopoietic disorders characterized by ineffective hemato-

* Dedicated to Professor Ilona Béládi on the occasion of her 80th birthday.

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poiesis and an increased propensity to marrow failure and leukemic transformation. The natural history of the disease ranges from a chronic course that may span years to a rapid course towards leukemic progression. Clonal proliferation is a consequence of acquired somatic mutation that confers a proliferative advantage to cells. Clinically, patients with MDS present with variable cytopenias due to an ineffective hematopoiesis of unknown etiology. The current classification systems of MDS (FAB and WHO) are based on the morphological features of the bone marrow and the blood [1, 2]. An important recent observation in this regard is the excessive intramedullary apoptotic death seen in the bone marrow biopsies of MDS patients, one mechanism invoked to explain the apparent discrepancy between cellular marrow and peripheral blood cytopenias [3, 4]. Abnormal cytokine production, autoreactive T-lymphocytes and an altered interaction between the progenitor cells and the extracellular matrix can all promote apoptosis. Several cytokines or ligands known to have proapoptotic properties, are upregulated in many patients with MDS. A number of studies have stressed the importance of increased levels of tumor necrosis factor-α (TNF-α) in the serum and bone marrow in promoting apoptosis [5, 6, 7]. Transforming growth factor-β (TGF-beta) is another cytokine which is generally considered to be a key negative regulator of hematopoietic stem and progenitor cells [8, 9, 10].

It has been shown that the blockade of TNF-α or Fas-ligand enhances hematopoietic colony transformation from MDS marrow *in vitro* and improves blood cell counts *in vivo,* thus, one of the main new directions in the treatment of MDS is the use of compounds exerting inhibitory activity on proapoptotic cytokines [11, 12, 13].

The regulatory and coding regions of cytokine genes are relatively polymorphic and may therefore be useful in disease association studies. It has been demonstrated that the production of TNF-α or TGF-β1 varies from individual to individual and partly depends on the polymorphisms of these genes. The most widely investigated polymorphism of the TNF-α gene is the G-to-A transition in the –308 promoter region [14]. It has been shown that an A nucleotide at position –308 is associated with increased transcription and production of TNF-α [15, 16]. Further, several polymorphisms have been described in the TGF-β1 gene, including a T-to-C transition at nucleotide 29, in the region encoding the signal sequence, which results in a leucine-proline substitution at the 10th amino acid [17]. It has been shown that TT homozygous genotypes are high TGF-β1 producers [18, 19, 20]. The correlation between the TNF-α gene polymorphism [21, 22, 23, 24, 25] or the TGF-β1 gene polymorphism [26, 27, 28, 29] and the disease status has been studied in a wide array of different diseases, the results suggesting that the gene

polymorphisms of TNF-α or TGF-β may provide a rational indicator of the disease susceptibility or prognosis. In the present study, we have investigated whether TNF-α gene polymorphism in the –308 promoter region or TGF-β1 gene polymorphism in codon 10 are associated with the development and severity of MDS.

Materials and methods

Patients

Control. A control group of 74 healthy blood donors (age, 41.31 ± 1.47, 21 to 73; 42 men and 32 women) was investigated.

Patients. Between May 2001 and January 2004, a total of 55 cases (15 men and 40 women; age 70.69 ± 1.43, 25 to 87) with MDS documented by marrow biopsy was enrolled. The diagnosis of MDS (n = 55) was based on the FAB criteria [30] (50 of these patients had refractory anemia (RA), while 5 cases were diagnosed as having refractory anemia with an excess of blast (RAEB). Patients with RAEB in transformation (RAEB-T) were considered as having acute leukemia and were excluded. None of the patients had received specific therapeutic agents prior to the study. They were supported only by red blood cell transfusion. Patients were divided into two groups: mild group containing patients with only anemia (hemoglobin<100 g/l) (n = 31), and severe group containing patients with bi- or pancytopenia (neutrophil count <1 G/l, platelet count <100 G/l) (n = 24). Leukemic transformation occurred in 6 patients; another 4 patients died due to the MDS without leukemic transformation.

All patients and controls were of adult Hungarian ethnic origin and resided in Hungary.

This study was performed in accordance with the ethical standards laid down in the most recent version of the 1964 Declaration of Helsinki. The ethical committee of the participating university had approved the study. Informed written consent was obtained in advance from each of the patients.

DNA extraction

Genomic DNA from whole blood containing EDTA was extracted by a standard technique (High Pure PCR Template Preparation Kit, Roche Diagnostic Gmbh, Mannheim, Germany). The analysis of the polymorphisms was based on

polymerase chain reaction (PCR) techniques performed in a thermal cycler (GeneAmp PCR System 2700, Applied Biosystems, Foster City CA, USA).

TNF-α genotype assay

The G-to-A transition at position –308 in the promoter region was analyzed by PCR-RFLP (restriction fragment length polymorphism) [14]. A single base change at the 3' end of primer A1 (underlined) was required for the formation of an *NcoI* (Fermentas, Vilnius, Lithuania) recognition sequence CCATGG (instead of GCATG originally found on the investigated gene)

(primer A1:5'-AGGCAATAGGTTTTGAGGGCCAT-3' and
primer A2:5'-TCCTCCCTGCTCCGAT TCCG-3') [14].

The reaction mixture of 100 µl contained 100 ng of genomic DNA, 20 pmol each of the A1 and the A2 primer, 2.5 U Taq DNA polymerase, 1.5 mM $MgCl_2$, 1 × PCR Taq polymerase buffer + $(NH_4)_2SO_4$ (Fermentas, Vilnius, Lithuania) and 25 mM of each dNTP (Fermentas, Vilnius, Lithuania). The PCR conditions were as follows: initial denaturation at 94 °C for 3 minutes; 36 cycles of 94 °C for 1 minute each, 60 °C for 1 minute and 72 °C for 1 minute, and a final extension at 72 °C for 5 minutes. The amplified product was digested with the endonuclease *NcoI* and analyzed on a 12% polyacrylamide gel. The TNF G allele gives two fragments, of 87 bp and 20 bp, while the TNF A allele gives a single, 107 bp fragment.

TGF-β genotype assay

The defined single-nucleotide polymorphism T^{29}-C in exon 1 of the human TGF-β1 gene was determined by an amplification refractory mutation system (ARMS) with a generic primer (sense),

(5'- TCCGTGGGATACTGAGACACC-3');

and with two allele-specific antisense primers, differing from each other in only one base at the 3' end:

primer C: 5'-GCAGCGGTAGCAGCAGCG-3' and
primer T: 5'- AGCAGCGGTAGCAGCAGCA-3' [31].

The reaction mixture of 50 µl contained 100 ng of genomic DNA, 20 pmol each of the sense and the antisense primer, 1.25 U Taq DNA polymerase, 1.5 mM $MgCl_2$, 1 × PCR Taq polymerase buffer with $(NH_4)_2SO_4$, and 25 mM of each dNTP (Fermentas, Vilnius, Lithuania). The thermocycling procedure was as follows: ini-

tial denaturation at 94 °C for 5 minutes; 35 cycles of 94 °C for 30 seconds each, 60 °C for 30 seconds and 72 °C for 30 seconds, and a final extension at 72 °C for 5 minutes. The PCR products were analyzed by 1.5% agarose (Sigma-Aldrich, St. Luis, MO, USA) gel electrophoresis. The expected size of the specific amplification product was 241 bp. Samples from 2 known homozygotic individuals and 1 heterozygotic individual, confirmed by sequencing, were included in each reaction. Sequencing was performed with an automated sequencer (ABI Prism; Applied Biosystems, CA, USA).

Statistical analysis

Statistical analyses for comparison of allele and genotype frequencies between groups were made by using the χ^2 test, and Fisher's exact test if one cell had $n<5$. A level $p<0.05$ was considered statistically significant. For comparison of age and sex between the patients and the controls, the Mann–Whitney *U* test and the Fisher's exact test was used. Statistical calculations were performed with the GraphPad Prism4 statistical program.

The genotype frequencies for each polymorphism were tested for deviation from the Hardy-Weinberg equilibrium by means of the χ^2 test, with one degree of freedom used.

Results

The distributions of genotypes and alleles for the different polymorphisms were consistent with those predicted by the Hardy-Weinberg equilibrium in the patients and in the controls. The two groups were matching according to sex (Fisher's exact test $p = 0.058$), but there was a significant difference in the mean age between the controls and the patient group (Mann-Whitney *U* test $p<0.01$).

The –308 promoter polymorphism of TNF-α

The genotypic distribution of the TNF-α gene –308 promoter G-to-A polymorphism is shown in Figure 1. There were no significant differences in the TNF-α -308 promoter genotypic distribution between the patients with MDS [GG, GA, AA = 63.6%, 30.9%, 5.5%] and the healthy controls [GG, GA, AA = 68.9%,

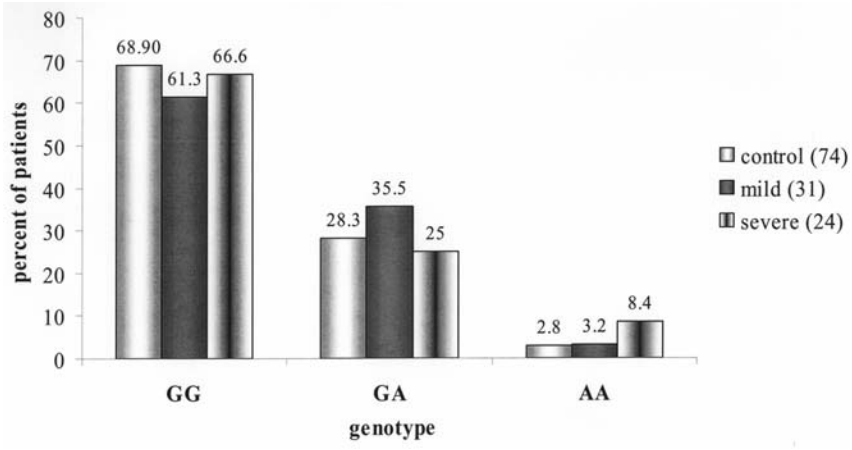

Figure 1. Distribution of TNFα (–G308A) genotypes between the patients with myelodysplastic syndrome (MDS) and the healthy controls. Chi-square test: $p>0.05$ comparisons between the mild group, the severe group and the controls. Mild group: patients with only anemia; severe group: patients with bi- or pancytopenia

28.3%, 2.8%]. Similarly, there were no differences in the distribution of TNF-α –308 G-to-A genotypes between the MDS patients with only anemia (mild group) [GG, GA, AA = 61.3%, 35.5%, 3.2%] and those with bi- or pancytopenia (severe group) [GG, GA, AA = 66.6%, 25.0%, 8.4%].

The T^{29}-C polymorphism in codon 10 of TGF-β1

Figure 2 depicts representative results relating to the TGF-β1 genotyping. To detect the nucleotide swap, ARMS was used. By means of the two allele-specific primers, the homozygote mutant (CC), the homozygote wild (TT) and the heterozygote (TC) variants (336 bp long product) were well distinguishable.

The genotypic distribution of the T^{29}-C polymorphism of the TGF-β1 gene is shown in Figure 3. No significant difference in genotypic distribution was found between the MDS patients overall and the healthy controls. A significant difference was observed, however, when the genotypes were taken into consideration within the two subgroups of MDS ($\chi^2 = 10.98$, $p = 0.0268$). To elucidate the reason for this significance, we compared the number of TT homozygotes (high TGF-β-producing phenotype) and C carriers (CT and CC) among the patients with mild and severe cytopenia forms of MDS and the healthy controls (Table I), and a sig-

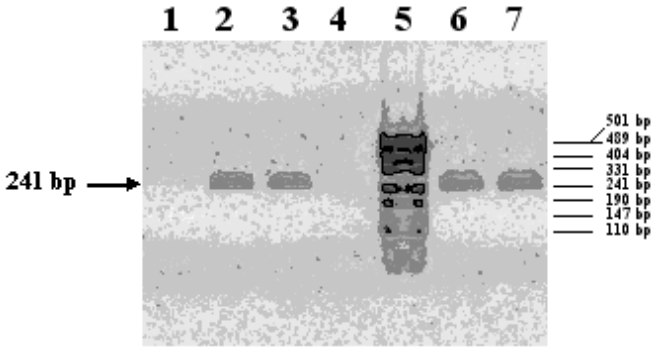

Figure 2. PCR/ARMS analysis of the transforming growth factor (TGF-β) T^{29}-C polymorphism. By means of the two allele-specific primers, the homozygote mutant CC (lanes 1 and 2), the homozygote wild TT (lanes 3 and 4) and the heterozygote TC (lanes 6 and 7) variants (336 bp long product) were well distinguishable

nificant difference was observed ($\chi^2 = 7.75$ p = 0.0207). There was also a considerable difference in frequency of the TT genotype between the two subgroups of MDS (Table I). This was due to overpresentation of the TT genotype in the patients with bi- or pancytopenia (severe group) [TT = 66.7% (severe) vs. TT = 29.0% (mild)] (p = 0.0071, OR = 4.889, 95% CI 1.548 to 15.44). Alternatively, the C carrier status (representing the CT and CC genotypes) was markedly more frequent among the patients with only anemia (mild group).

Table I

Distribution of TT homozygosity and C-carrier status of transforming growth factor (TGF-β) T^{29}-C (Leu10Pro) genotypes in the patients with myelodysplastic syndrome (MDS) with mild or severe cytopenia and the healthy controls

	MDS patients		Healthy controls
	mild cytopenia[1] (n = 31)	severe cytopenia[2] (n = 24)	(n = 74)
TGF T^{29}-C (Leu10Pro) genotypes			
TT	29.0% (9)[ab]	66.7% (16)[ab]	44.6% (33)[b]
C-carrier	71.0% (22)[ab]	33.3% (8)[ab]	55.4% (41)[b]

[a] Fischer exact test: OR = 4.889, p = 0.0071; statistical analysis was performed between the TT and CT+CC genotypes; comparison between the mild group and the severe group.
[b] Chi-square test: $\chi^2 = 7.75$, df = 2, p = 0.0207; statistical analysis was performed between the TT and CT+CC genotypes; comparisons between the mild group, the severe group and the controls.
[1] Patients with only anemia.
[2] Patients with bi- or pancytopenia.

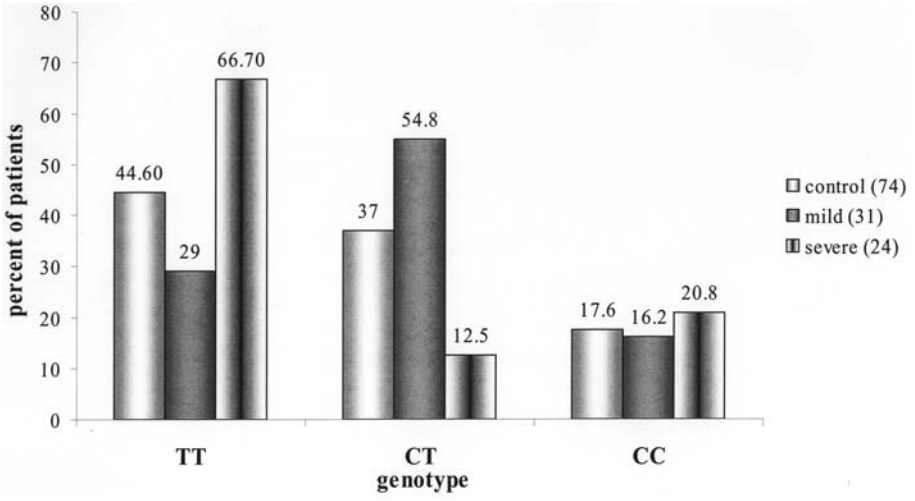

Figure 3. Distribution of transforming growth factor (TGF-β) T^{29}-C (Leu10Pro) genotypes between the patients with myelodysplastic syndrome (MDS) and the healthy controls. Chi-square test: $\chi^2 = 10.98$, df = 4; p = 0.0268 comparisons between the mild group, the severe group and the controls. Mild group: patients with only anemia; severe group: patients with bi- or pancytopenia

Discussion

During the past few years, major progress has been made toward the understanding of the pathological processes of MDS. Genetic abnormalities or altered gene expressions leading to the suppression of hematopoiesis may also be responsible for the development of cytopenia. One of the genetic factors may be an aberration of cytokine regulation and cytokine production, which can lead to an increased apoptosis of hematopoietic progenitor cells, and aberrant hematopoiesis. Several lines of experimental evidence show that erythropoiesis can be severely disturbed by inflammatory mediators such as TNF-α, as TNF suppresses proliferation of erythroid progenitor cells in human marrow cultures [32, 33]. The excessive production of growth inhibitory cytokines such as TNF-α in patients with MDS has been described [5, 6, 34]. Recent data indicate that TGF-β inhibits myeloid, erythroid, megakaryocyte and multilineage colony formation [8, 9, 10]. Accordingly, it appeared plausible to hypothetise that polymorphisms of the TNF-α or TGF-β1 genes may be correlated with MDS or the severity of the disease.

In our study, however, no significant difference in –308 TNF-α polymorphism was observed as regards the G or A alleles when any of the forms of MDS were compared with each other or with the controls.

TNF-α also has an apoptotic effect toward hematopoietic cells. However, the distribution of TNF-308 gene polymorphism was similar among the patients with a mild or a severe degree of cytopenia in MDS. Comparison of the genotype frequencies in patients with MDS suggests that the –308 G/A polymorphism of TNF-α does not influence the susceptibility to the disease. There are several potential reasons for failing to observe any association between the TNF-α –308 polymorphism and MDS. The TNF-α –308 polymorphism would appear to occur at a transcription factor binding site in the TNF-α promoter and hence affects protein binding, thereby altering the transcriptional activity. The transcriptional and translational regulation and the prosttranslational protein processing are major steps involved in the protein expression. The TNF protein expression is probably not regulated exclusively at the transcriptional level. In complex biological systems, the effect of a single gene polymorphism in determining cytokine secretion may be minimized through the interaction of other factors.

TGF-β isoforms are translated as prepropeptide precursors with an N-terminal signal peptide followed by the prodomain and mature domain, and are secreted as biologically latent forms. The Leu10Pro-(T869C) polymorphism in exon 1 of the TGF-β1 gene is located in the signal peptide sequence that is cleaved from the TGF-β1 precursor at the level of codon 10 [35]. This polymorphism at codon 10, within the hydrophobic α-helix of the signal sequence, involves the replacement of a hydrophobic leucine residue with a small, neutral proline residue. Such a change would alter the overall hydrophobicity of the core transport sequence and disrupt the α-helical structure of the region, thereby altering its ability to direct protein transport across the endoplasmatic reticulum [17]. The T29C polymorphism of the TGF-β1 gene does not result in a difference in the mature TGF-β1 protein; merely the TGF-β1 protein level is reduced both *in vitro* and *in vivo* [18, 19, 20].

On the other hand, Yokota et al. reported that the T allele of T^{29}-C polymorphism was linked to a lower production of TGF-β1 [36]. The influence of the allele at codon 10 on TGF-β production is possibly more variable, due to the cross-modulating interfaces between the signaling networks within the cells. In this study, we have shown that in patients with MDS the TGF-β "high-producer genotype" TT causes a 4.9-fold risk of bi- or pancytopenia as compared with the C-carriers. Patients carrying the C allele, with a concomitantly lower TGF-β1 production, have

only anemia. As TGF-β1 is a member of the proapoptotic cytokines, it is very likely that patients with the low-producing genotypes (CC and CT) may have a better chance of a less severe cytopenia in MDS. Although we could not find genotypic differences between the MDS patients overall and the healthy controls, we observed that the genotype of the TGF-β1 gene differed significantly between the severe and mild groups of patients. These findings indicate that the T^{29}-C polymorphism of the TGF-β gene itself is not directly involved in the susceptibility to MDS, but is rather relevant in the outcome of the disease.

Overall, the polymorphism of the TNF-α gene in the –308 promoter region does not have a role in the regulation of the outcome of MDS. In contrast, our findings of an association between the severe form of MDS and TT homozygosity suggest that future studies should focus on the role of TGF-β in MDS. Following these pilot experiments, further investigations on the polymorphisms of the TGF-β1 gene should be included in these examinations.

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