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Regular paper

Assessment of the frequency of the transforming growth factor beta-1 sequence polymorphisms in patients with alcohol dependence syndrome

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Alcohol abuse is one of the most significant factors in the development of liver fibrosis. The pathomechanism of liver fibrosis is the same regardless of its etiology. Fibrosis is a sign of an imbalance between the synthesis of the extracellular matrix components and their degradation. Among the many cytokines that affect hepatic stellate cell activation it seems that transforming growth factor beta (TGF-β) is the most significant, either as the direct factor stimulating polymerase chain reaction (HSC) proliferation and transformation into myofibroblasts, or as the direct factor causing an increase in the activity of genes responsible for the synthesis of extracellular matrix components. The aim of the study was to reveal possible dependencies and differences between the presence of certain alleles of the TGF-β1 gene and its blood level in the study and control group. Blood samples were obtained from 39 patients, the control group consisted of 21 patients. The results obtained in the course of this study showed no statistically significant differences between the frequencies of particular polymorphisms. In the case of haplotype frequencies, insignificant differences were found for the algorithm Excoffier-Laval-Balding predicted haplotypes while one significant difference between the study and control groups was detected in case of the TC haplotype frequency predicted using the *Expectation-Maximization* algorithm. However, the difference in frequency of TC haplotype predicted by both algorithms was not significant. Genetic analysis of two single nucleotide polymorphisms (SNPs) in exon I of the TGF-β1 gene did not show significant differences between the occurrence of particular polymorphisms and haplotypes in the populations under study.

Key words: transforming growth factor TGFβ-1, alcohol dependence syndrome, TGF-β1 gene

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INTRODUCTION

Alcohol abuse is one of the most significant factors in the development of liver fibrosis. The pathomechanism of liver fibrosis is the same regardless of its etiology. Thus, attempts are being made to monitor liver pathology, otherwise known as fibrosis of this organ in people who abuse alcohol, by indicating the concentration of some of the components of the extracellular matrix (ECM) or their metabolic products present in the blood. Fibrosis is a sign of an imbalance between the synthesis of ECM components and their degradation. The damaging agent activates a cascade of mechanisms leading to the activation of polymerase chain reaction (HSC) and their transformation into myofibroblasts producing the greatest amount of ECM in the liver. Much smaller amounts of these molecules are produced by the hepatocytes and endothelial sinus cells (Kozłowska, 2000a; Kozłowska, 2000b; Kozłowska *et al.,* 2001; Katz *et al.,* 2001; Kim *et al.,* 2003; Lebensztejn, 2003).

Among the many cytokines that affect HSC activation it seems that transforming growth factor beta (TGF-β) is the most significant (Kozłowska, 2000; Flisiak *et al.,* 2002). TGF-β always stimulates this process (Kozłowska, 2000a; Mazur *et al.,* 2003), either as the direct factor stimulating HSC proliferation and transformation into myofibroblasts, or as the direct factor causing an increase in the activity of genes responsible for the synthesis of ECM components (Lebensztejn, 2003).

Permanent exposure to the damaging factor causes an iterative cycle of repair, leading to excessive accumulation of connective tissue elements. The damaging factor initiates a cascade of mechanisms leading to the activation of HSC and their transformation into myofibroblasts producing the greatest amount of ECM in the damaged liver. Several models of HSC activation and the fibrosis process are usually presented. Generally, initiation takes place in the incipient stage of activation; the pre-inflammatory phase is caused mainly by paracrine stimuli from neighboring liver cells which sensitize the HSC to the activity of the cytokines. The consolidation and continuation of the fibrosis process, which is associated with both paracrine and autocrine intracellular transduction, comes next.

As a result of the activation of HSC, ECM components are generated. Growth factors (TGF-β1 mainly) and other cytokines responsible for the storage, distribution and biological activity of ECM proteins, take part in the fibrosis process.

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Abbreviations: TGF-β, transforming growth factor beta; HSC, hepatic stellate cells; ECM, the extracellular matrix; *EM, Expectation-Maximization algorithm; ELB,* Excoffier-Laval-Balding *algorithm;* SNP, single nucleotide polymorphism; PCR, polymerase chain reaction; TβRI, receptor type I; TβRII, receptor type II; TβRIII, receptor type III

Table 1. Primer sequences

	Sequence	%GC	Tm
Primers for the generation of the sequencing template			
Forward	CTACCTTTTGCCGGGAGAC	57.89	52.13
Reverse	TCGATAGTCTTGCAGGTGGA	50.00	52.29
Length of the PCR product: 213 bp.			
Sequencing primer			
Forward	ACCACACCAGCCCTGTTC	61.11	53.66

Previous studies justify the recognition of TGF-β as a progression factor of chronic liver disease; however its diagnostic differentiating utility requires further research.

The differences in the concentration of TGF-β1 protein may be due to the increased gene expression in the activation of signalling pathways to promote its expression. However, it cannot be excluded that the sequence of the gene or its promoter may modulate the efficiency of gene expression, contributing to the formation of the observed phenotypic variation.

The TGF-β1 gene is located on the long arm of chromosome 19 (19q13.1, Gene ID: 7040). Its sequence consists of 23 020 bp. and it comprises seven introns, forming a 2217 bp mRNA. Within the TGF-β1 gene itself and in its promoter region the occurrence of a number of single nucleotide polymorphisms (SNPs) some of which may be important for the expression and functioning of the protein encoded by this gene, has been described (Shah *et al.,* 2006).

The aim of the study was to look for possible dependencies and differences between the presence of certain alleles of the TGF-β1 gene and its blood level in the study and control group.

MATERIALS AND METHODS

Genetic analysis. Blood samples were obtained from 39 patients (20 men and 19 women), chosen because of the elevated levels of the TGF-β1 protein in the serum. The control group consisted of 21 patients, who did not demonstrate an elevated level of the TGF-β1 protein. Isolation of DNA from blood samples was poured onto a piece of chromatography paper Whatman 3Chr (Whatman) and dried in a fume hood at room temperature for 24 hours. Small fragments, measuring approximately 0.3 x 0.3 cm, were obtained from the dried blood, and subjected to DNA isolation using DNA IQ kit (Promega) according to the instructions provided in the kit.

Amplification and purification of polymerase chain reaction (PCR) products of the TGF- β1 gene fragment was conducted by means of PCR product sequencing with an internal primer. The sequences of the primers for the amplification of exon 1 of the TGF-β1 gene fragment containing the tested polymorphisms (constituting a sequencing template) and the sequencing primer were designed using Primer–BLAST (web service http://www.ncbi.nlm.nih.gov/tools/primer-blast/), utilizing the complete TGF-β1 gene sequences (GenBank: NG_013364) and the sequences of the two studied polymorphic regions in dbSNP (rs1800470 and rs1800471) as a reference sequence. Primer sequences are shown in Table 1. Genomic DNA samples were amplified under the following conditions: reaction volume: 50 μL, 0.75 mM $MgCl_2$, 0.2 mM DTP, 150 pm of each primer, 2U of GoTaq Flexi polymerase (Promega) per a reaction, PCR buffer (included with Taq polymerase) 1x, DNA template 10 μL. PCR temperature profile: initial denaturation of 2 min, 94°C, 38 cycles of denaturation 94°C 1 min, annealing 55°C 1 min and elongation 72°C 1.5 min, final elongation 72°C 10 min.

PCR products were purified on Amicon Ultra 0.5 columns (Millipore). 45μl of PCR product was put onto an Amicon Ultra 0.5 column (Millipore), 450 ml of dH_2O was added and spun at 14000 rpm for 10 minutes at room temperature. The filtrate was discarded. Washing was repeated twice using the above scheme. After the last centrifugation the column was inverted by 180°, transferred to a new test tube and spun for 2 minutes at 1000 rpm at room temperature.

Sequencing. Sequence reactions were carried out using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. 4 μL of the purified PCR product and the sequencing primer (Table 1) at 10 pmoles per reaction, were used for sequencing. Sequencing products were separated on an ABI PRISM® 3130xl (ABI) sequencer using a 36 cm long capillary and a POP-7 (ABI) polymer according to the instructions supplied with the sequencing kit.

RESULTS

The obtained electrophoregrams were analyzed with the SeqScape 2.5 (ABI) software, using the sequence for the TGF-β1 gene obtained from the GenBank database (NG_013364) as a reference sequence. The obtained sequences were exported to a text file, on the basis of which a batch file for the Arlequin program was prepared. The Arlequin 3.5.1.2 software (Excoffier 2005) was used to assess the allele, genotype and haplotype frequencies. The presence and frequency of particular haplotypes was detected basing on two predictive algorithms implemented in the Arlequin program (ELB -Excoffier-Laval-Balding and EM — *Expectation-Maximization*

Table 2. Summary of the information on the two SNPs loci located within the TGF-β1 gene, which were used in this study.

	nucleotide change	AA change	position on chromosome19 (NC_000019.9)	Position in the gene
rs1800471	G/C	Arg25Pro	41858876	Exon 1
rs1800470	C/T	Pro10Leu	41858921	Exon 1

algorithm) using the default settings. The statistical significance of differences in allele and haplotype frequencies was calculated using a one-sided test of difference between two proportions, implemented in the Statistica 10.0 package (StatSoft). The statistically significant difference assumed for each of the calculations was p-value <0.05.

Frequencies of the two selected TGF-β1 polymorphisms in the study group and the control group.

An assessment of the frequency of the two selected TGF-β1 sequence polymorphisms in the study group and the control group was carried out. For the purposes of this pilot study two polymorphisms, identified in the database as dbSNP rs1800470 and rs1800471, located in the distal part of exon 1 of the TGF-β1 gene, 45-bp from each other (Table 2), were selected. The polymorphisms are responsible for the change in the N-terminal sequence of the encoded protein and seem to be associated with the occurrence of cancer and cardiovascular disease (Wick, 1999; Wiliams, 2006).

Table 3 shows the basic statistical parameters obtained for the analyzed SNPs in both the study and control groups. There were no statistically significant differences in the level of heterozygosity and in the allele frequencies in the two groups. Also, the observed heterozygosity in the exact test was not significantly different from the expected heterozygosity, which means that the tested SNP systems remained in Hardy-Weinberg equilibrium.

Table 4 shows basic statistical parameters for the haplotypes predicted using two different algorithms (ELB and EM) based on genotypes obtained for the test and control group. The haplotypes are defined as two nucleotides, the first of which is an allele present in the rs1800470 system and the second is an allele present in the rs1800471 system. Therefore the CG haplotype should be read as rs1800470C: rs1800471G. The applied algorithms indicated the possibility of the presence of a TC haplotype in the control group, whose presence was not detected in the study group. However, frequency difference for this haplotype between the two groups is statistically insignificant in our data. It should also be noted that the TC haplotype frequency in the control group is higher for the EM algorithm than for the ELB algorithm, although the difference is not statistically significant.

The results obtained in the course of this study showed no statistically significant differences between the frequencies of particular polymorphisms. In the case of haplotype frequencies, non-significant differences were found for the ELB predicted haplotypes while one significant difference between the study and control groups was detected in case of the TC haplotype frequency predicted using the EM algorithm. However, the difference in frequency of TC haplotype predicted by both algorithms was not significant.

Table 3. The basic parameters of the two studied SNPs.

		Group under study	Control group
	n	39	21
	N	78	42
rs1800470	Allele frequency		
	Allel C	0.5	0.47619
	Allel T	0.5	0.52381
	p(A)	0.4017	
	Heterozygosity		
	Observed	0.53846	0.57143
	Predicted	0.50649	0.51103
	p(H)	0.75415	0.67581
rs1800471	Allele frequency		
	Allel C	0.089744	0.119048
	Allel G	0.910256	0.880952
	p(A)	0.3049	
	Heterozygosity		
	Observed	0.12821	0.2381
	Predicted	0.1655	0.21487
	p(H)	0.25508	1

n, number of respondents; N, number of chromosomes tested (= 2n); p(A), p-value for the differences in allele frequencies between populations. p(H), p-value for the difference between the observed and expected heterozygosity calculated using an exact test.

DISCUSSION

TGF-β plays a prominent role in the activation of lipocytes is the main mediator in the development of alcoholic liver disease leading to fibrosis (Chen *et al.,* 2002; Amani *et al.,* 2004; von Linsingen *et al.,* 2005). It is a dimer in protein, secreted as an inactive complex; the free form of TGF-β (the result of the separation of the LAP protein from the dimer through the action of proteolytic enzymes) is biological active. Active TGF-β1 has a specific receptor, through which it acts on lipocytes and myofibroblasts causing the transformation of lipocytes into myofibroblasts as well as stimulating the synthesis of their ECM components (such as collagens, glycoproteins and proteoglycans) and reducing the production of matrix macromolecule degrading enzymes (collagenases and other metalloproteinases), contributing in this way to the development of pathological fibrosis in the liver (Flisiak *et al.,* 2000; Forns *et al.,* 2002; Myers *et al.,* 2003; Poynard *et al.,* 2003; Rosenberg, 2003; Patel *et al.,* 2004; Le Calvez *et al,* 2004; Berg *et al.,* 2004; Rosenberg *et al.,* 2004; Kelleher *et al.*, 2005; Schuppan *et al.,* 2008).

Table 4. The haplotype frequencies obtained from the tested SNPs systems using two prediction algorithms.

rs1800470	rs1800471	Study group	Control group	p
The haplotype frequency according to the ELB algorithm				
C	G	0.410	0.381	0.3785
C	C	0.090	0.095	0.4639
T	G	0.500	0.500	0.5000
T	C	0.000	0.024	0.0847
The haplotype frequency according to the EM algorithm				
C	G	0.410	0.411	0.4958
C	C	0.090	0.065	0.3163
T	G	0.500	0.470	0.3769
T	C	0.000	0.054 (p=0.2388)	0.0191

There are 3 types of TGF-β: TGF-β1, TGF-β2 and TGF-β3. This protein is synthesized by Kupffer cells, but can also be released by the platelets, and also by the myofibroblasts themselves, or the activated lipocytes. It is secreted in an inactive form. Among the functions of TGF- β in the body, the most important are:

— the impact on the composition of the ECM matter connected with the fibrosis process.

— the modulation of other cytokine effects by acting on their receptors,

— the stimulation of hepatocyte apoptosis, reducing the activity of metalloproteinases (Arias, 1994 ; Kozłowska, 2000a; Kozłowska, 2000b; Wu & Zern, 2000; Sternlicht & Werb, 2001; Chen *et al.,* 2002; Iredale, 2003; Lebensztejn, 2003; Mazur *et al.,* 2003; Kmieć, 2003; Amani *et al.,* 2004; Shek & Benyon, 2004; Zafrani, 2004; Migita *et al.,* 2005; Tamizifar *et al.,* 2008).

The hepatocytes of patients with alcoholic cirrhosis had a higher concentration of TGF-β than that of healthy liver tissue. The influence of TGF-β on the development of alcoholic liver disease has been acknowledged (Kozłowska, 2000b; Battaler & Brenner, 2005). Mazur *et al.* (2003) confirm elevated levels of TGF-β in patients with chronic hepatitis. However, the assessment of the advancement of liver disease seems to be indefinable using the concentration of TGF-β as an indication — diagnostic parameter.

Studies carried out by Yong-Ku *et al. (*2009) show that the level of TGF-β in patients addicted to alcohol was higher than that of healthy patients. Ogata et al. (1992) found that the level of TGF-β was higher in those individuals addicted to alcohol without a damaged liver than of those in the control group with a damaged liver. It is possible to make the presumption that TGF-β is a predictor, a promoter of alcohol-induced liver disease (Chen *et al.,* 2003).

Independent research demonstrated a significantly higher level of TGF-β1 in patients addicted to alcohol than of that in healthy patients, both in the whole group of patients and in the group of men and women analyzed separately to the healthy patients.

The results obtained during the sequence analysis of the fragment of exon 1 of the TGF-β1gene selected for this study showed no evident, statistically significant differences between the group under study and the control group, as far as the polymorphisms' frequencies and the predicted haplotype frequencies were regarded. It should be noted, however, that our results suggest that such a difference could exist in case of TC haplotype, that was present in control group and absent in the group under study. Both the study and the control group were relatively small, which may have had an impact on the effectiveness of the methods used for detecting differences in allele and haplotype frequencies. It can be expected that with increasing the number of individuals in the study group and the control group in future genetic testing it will be possible to verify the significance of this observation. Our aim is to extend the analyzed SNP system panel using other polymorphisms located in the promoter and other exons of the TGF-β1 gene.

Understanding the function of enzymes and the mechanisms of activation underlies the diagnostic and therapeutic possibilities of many pathophysiological conditions. The genetic characteristics of individuals addicted to alcohol, which would be associated with the increased levels of the abovementioned biochemical, have not, to date, been strictly determined. Such knowledge would allow identifying patients in need of more intensive diagnosis in order to verify the possibility of liver fibrosis or provide preventive measures in order to reduce the risk of them developing cirrhosis.

CONCLUSIONS

Genetic analysis of two SNP polymorphisms in exon I of the TGF- β1 gene, did not show significant differences between the occurrence of particular polymorphisms and haplotypes in populations under study. However, given the pivotal role of the gene under study in the pathological changes in liver it seems necessary to extend the TGF- β1 SNP panel using other polymorphisms located in the promoter region as well as in other regions of the gene.

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The authors declare that they have no conflict of interest.

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