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Functional characterization of polymorphisms in the human TFPI gene

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ABSTRACT

Tissue factor pathway inhibitor (TFPI) is the primary physiological inhibitor of tissue factor (TF) induced coagulation. Low plasma TFPI levels have been shown to be associated with increased risk of arterial and venous thrombosis. Several clinical studies have reported that single nucleotide polymorphisms (SNPs) in the regulatory regions of the gene, such as the −287T/C, the −399C/T, and the −33T/C SNPs, may affect plasma TFPI levels. However, molecular studies investigating the functionality of the polymorphisms are lacking. In this study, we found that the −287C and −399T alleles affected the activity of the promoter using a reporter gene system. This was also the case for the −33T/C polymorphism. An association regarding the transcriptional activity of the reporter gene was detected between the −287C allele and the −33T/C polymorphism. Analysis of the polymorphic sites with electrophoretic mobility shift assay (EMSA) showed that all three polymorphisms potentially alter DNA–protein interactions. Based on these findings, we speculate that the −287C and the −33C alleles can be associated with lowered risk of thrombosis.

1. Introduction

There are major individual differences in the risk of thrombosis indicating a role of genetic variation. Tissue factor pathway inhibitor (TFPI) is the primary physiological inhibitor of tissue factor (TF) induced clotting [1]. The mature TFPI protein is a ~ 42 kDa Kunitz-type protease inhibitor comprising an acidic N-terminal region, three tandem Kunitz-type serine protease inhibitor domains and a basic C-terminus [2]. TFPI is mainly synthesized by endothelial cells. The majority of TFPI is associated with the vessel wall whereas the remaining TFPI circulates in plasma [3,4]. Accumulating evidence suggests that TFPI has a key role in the regulation of blood coagulation *in vivo* [5–10]. In addition, results from case-control studies indicate that reduced level of plasma TFPI is a risk factor for the development of both venous and arterial thrombosis [11,12].

The TFPI gene is located on chromosome 2, where it spans 70 kb spread on nine exons separated by eight introns [13,14]. Potential binding sites for various transcription factors have been identified in a 1990 bp region upstream for the transcriptional start site [15–18]. Several polymorphisms in the promoter and coding region of the TFPI gene have been reported [19–24], and some have been associated with total TFPI levels in plasma. The C allele of the −287T/C polymorphism was shown to give elevated plasma level of TFPI compared to the T allele, while no differences in plasma TFPI levels could be demonstrated between the C and T alleles for the −399C/T polymorphism [20,22]. The −33T/C polymorphism is located in intron 7 33 bp upstream of the splice acceptor site [21]. The −33C allele has been reported to be associated with elevated total TFPI plasma levels compared to the −33T allele [25,26] and has thus been suggested to be associated with a lowered risk of venous thrombosis [25]. Moreover, a linkage between the −287C and the −33C regarding total TFPI plasma levels has previously been demonstrated [25,27]. At present, no association to deep venous thrombosis has been reported for the −287 and the −399 polymorphisms. However, the −33C and −399T alleles have been shown to be associated with risk for venous thrombosis in the presence of other risk factors such as the antiphospholipid syndrome [28].

Measurement of plasma TFPI levels is complex since the inhibitor exists in free and bound plasma pools as well as the presence of a large endothelial-associated pool, which might be the more important pool from a physiological point of view. This complicates the interpretation of the results from case-control studies concerning the link between polymorphisms and total TFPI levels in plasma. A common way to study functional effects of genetic

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variations is expression analysis using a reporter gene system. The aim of the present work was to determine whether the −287T/C, −399C/T, and −33T/C polymorphisms could affect the transcriptional activity of the TFPI promoter using luciferase reporter gene systems. Additionally, we aimed to examine whether the −33T/C polymorphism in intron 7 could influence the potential effects on the TFPI promoter obtained by the −287T/C and −399C/T polymorphisms. We found that both the −287C and the −399T alleles affected the luciferase activity and a correlation between the −287C and the −33T/C polymorphism was detected. The results obtained with the reporter gene analysis could be due to changes in binding affinity/specificity of nuclear proteins as demonstrated by electrophoretic mobility shift assay (EMSA).

2. Materials and methods

2.1. Cell culture

The Chinese hamster ovary cell line (CHO-K1) and the human embryonic kidney cell line (HEK293T) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Both cell lines were maintained in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Basel, Switzerland) supplemented with 10% heat inactivated fetal calf serum (Lonza), and 50 U/ml penicillin/50 μl/ml streptomycin (Lonza).

2.2. Plasmid constructs

A fragment spanning the region +45 to −1224 upstream of the TFPI gene was PCR amplified from human genomic DNA isolated from whole blood using the following primers: forward 5′-GCAGG GCATGAGGTAAT-3′ and reverse 5′-AAGTCGCTGCTGTCTGTTAGA-3′. The PCR was performed with the TaqMan® PCR Kit (Qiagen GmBH, Hilden, Germany) using the following conditions: 94 °C for 3 min, 35 cycles of 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 1 min completed with a final extension step at 72 °C for 10 min. A fragment spanning −322/+15 of intron 7/exon 8 (+1 denotes the first base in exon 8) was amplified using the same conditions and primers: forward 5′-AAGAAATTTTCAGCAGTTAC-3′ and 5′-AGGGACCGTGA AATTCTA-3′. The PCR fragments were cloned into the pCR®2.1-TOPO vector (Invitrogen, Carlsbad, CA). The resulting constructs were verified by sequencing and revealed that the haplotypes of the upstream region was −287T/−399C and the genotype of the −33 polymorphism was −33T. The various haplotypic variants of the upstream region and the −33C variant were generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The primers used were: −287C forward 5′-CAAACTGCAA AAAAGCTTATTAACAGTG-3′ and reverse 5′-CACTGTTAATAATCTTTT TTGCAGTTTG-3′; −399T forward 5′-CAATAGATAATGCATTACATTG TCTCACTAAGAGAG-3′ and reverse 5′-CTCTCTTAGTGAGACAATGT AATGCATTATCTATTG-3′; −33C forward 5′-CCACTATCACACATG GCTTACCATG-3′ and reverse 5′-CATGGTAAGCCATTTGTGATAGT GG-3′. The mutations were confirmed by sequencing. The fragments were subsequently subcloned into the luciferase reporter plasmids pGL3 Basic and Promoter (Promega, Madison, WI). Constructs were verified by sequencing.

2.3. Transient transfections and luciferase assay

CHO cells (5×10^4 cells/well) and HEK293T cells (1.5×10^5 cells/well) were plated onto 24-well plates and grown to 50–80% confluency. The cells were co-transfected with the reporter plasmids (0.25 μg) and pRL-SV40 (2.5 ng) (Promega) as an internal transfection control by Lipofectamine LTX (Invitrogen). After 24 h the cells were washed with phosphate-buffered saline and subjected to a dual luciferase assay according to the manufacturer's instructions (Promega).

2.4. EMSA (electrophoretic mobility shift assay)

Nuclear extracts from CHO and HEK293T cells were prepared as previously described [29]. Biotin-labeled and corresponding unlabeled oligonucleotides containing the −287T/C, −399C/T, or −33T/C were synthesized by Eurogentec (Seraing, Belgium). The sequence for −287 was 5′-ACAAACTGCAAAAAAGT/CTTATTAA CAGTGTAAT-3′; for −399 5′-ATGCATTACATTGTC/TTCACTAAGAGA GACC-3′; for −33 5′-GAAATCCACTATCACAT/CATGGCTTACCATGT TT-3′. To make double-stranded probes and competitors, complementary oligonucleotides of equal amounts were heated at 95 °C for 5 min and then annealed by stepwise reducing the temperature to 25 °C during 1 h. The LightShift® Chemiluminescent EMSA Kit (Pierce, Rockford, IL) was used for the EMSA assays. The binding reactions were carried out for 30 min at 20 °C in the presence of 1 μg poly(dI–dC), protease inhibitor cocktail (Sigma, St. Louis, MO), 3 mM $MgCl_2$, 0.05% NP-40, 10 μM $ZnSO_4$, and 100 μM EDTA (only for the −399 probe) in 1× binding buffer using 20 fmol biotin-labeled double-stranded probes and 10 μg and 8 μg nuclear extracts from HEK293T and CHO-K1 cells, respectively. For the competition experiments, 200-fold molar excess of unlabeled double-stranded oligonucleotides was added to the binding reaction 10 min before the addition of the labeled probes. DNA–protein complexes were allowed to form for 30 min at room temperature before they were separated on an 8% native polyacrylamide gel. The complexes were blotted onto a positively charged nylon membrane (Pierce). The membrane was UV cross-linked and the probes were visualized according to the LightShift® Chemiluminescent EMSA Kit (Pierce) and LAS 4000 Mini Imaging System (Fujifilm, Life Science, New Haven, CT, USA).

2.5. Statistical analysis

The luciferase activity is expressed as mean ± SEM after adjusting the transfection efficiency by normalization with the pRL-SV40 control value. Differential luciferase expression between wild-type and mutant was determined using non-parametric test in GraphPad Prism Software Version 5.0 (Mann–Whitney). Data were considered statistically significant at $p < 0.05$.

3. Results

3.1. The TFPI 5′ UTR −287C and −399T alleles affect promoter activity

To explore the functional effects of the −287C and −399T alleles on TFPI expression, constructs with the four possible haplotypes of the polymorphic sites were created in the promoterless pGL3 Basic vector upstream of the luciferase reporter gene (Fig. 1A). The inserted fragments consisted of 1269 bp spanning the region +45 to −1224 of the TFPI upstream promoter region. The fragment containing the −287T/−399C haplotype represents the wild-type haplotype. In all experiments, the pGL3 Basic vector served as a negative control. Transient transfections of the various constructs were performed into HEK293T cells and the non-TFPI expressing cell line CHO-K1. To normalize for variations in the transfection efficiency, the constructs were co-transfected with the pRL-SV40 vector. Compared to the wild-type, the other three haplotypic variants displayed 10–40% increase in the luciferase activity when transfected into both HEK293T and CHO-K1 cells (Fig. 1B). The −287C/−399T haplotype showed slightly but statistically significant ($p = 0.0002$) higher luciferase activity in both cell types compared to the −287C/−399C and −287T/−399T haplotypes.

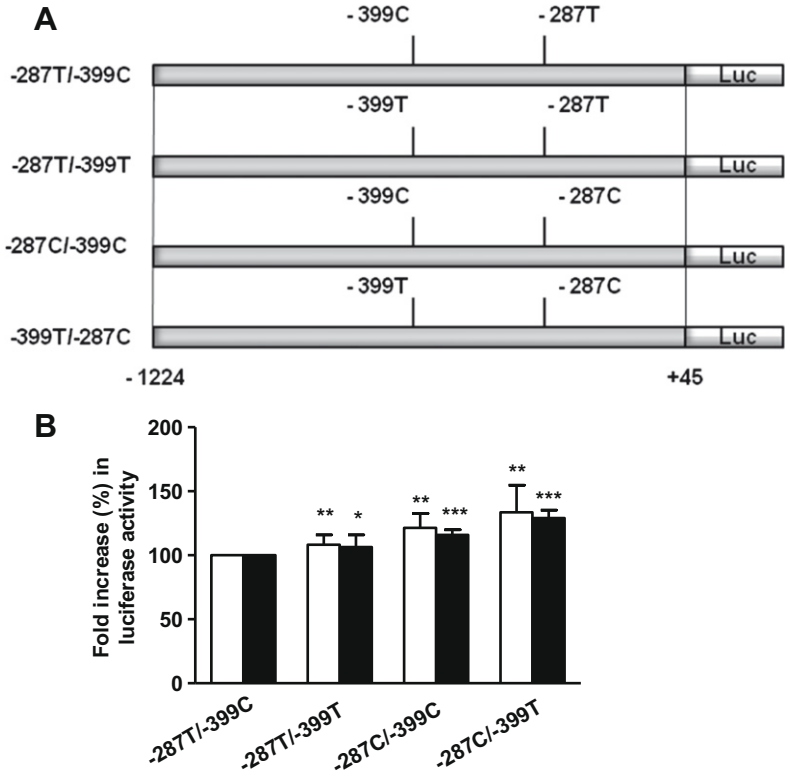

Fig. 1. Transcriptional activity of haplotypes in the TFPI 5′ upstream region. (A) Schematic representation depicting the localization of the polymorphisms in 5′ upstream region of the TFPI gene. Constructs containing the −1224 to +45 fragment of the TFPI 5′ upstream region with various combinations of the −399/−287 haplotypes were created in the promoterless luciferase reporter vector pGL3 Basic. The −399C/−287T construct represents the wild-type haplotype. (B) Luciferase activity of the constructs with the various haplotypes in HEK293T (white bars) and CHO-K1 (black bars) cells co-transfected with pRL-SV40 to normalize for transfection efficiency. The results have been corrected for background represented by empty vector. Fold change in luciferase activity was calculated relative to the activity of the wild-type haplotype and are presented as mean + SEM. HEK293T, $n = 12$ samples. CHO-K1, $n = 8$ samples. $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.0005$ relative to the −287T/−399C construct.

3.2. Functional effects of the intron 7 −33T/C alleles

To examine whether the intron 7 −33T/C polymorphism could have any effects on transcriptional regulation, a fragment spanning −322 to +15 of the intron 7/exon 8, was cloned into the pGL3 Promoter and Basic vectors. One construct with each of the −33 polymorphic alleles was made in each vector (Fig. 2A). Transient transfections of the pGL3 Promoter constructs into the cells revealed that the −33T allele did not affect the transcriptional activity of the promoter in either cell lines (Fig. 2B). The −33C allele, however, showed reduced levels of luciferase activity in HEK293T cells but not in CHO-K1 cells.

3.3. Effects of intron 7 −33T/C polymorphism on the −287C and −399T polymorphisms

Since an association between the intron 7 −33T/C polymorphism and the haplotypes in the 5′ upstream region has been proposed [25,27], we aimed to explore this by co-transfecting the pGL3 Basic 5′ constructs with the two intron 7 −33T/C pGL3 Basic constructs in both HEK293T and CHO-K1 cells. Relative to the luciferase activity obtained with the 5′ pGL3 Basic constructs alone (broken line as 100% in Fig. 3), co-transfections with the −33C allele resulted in reduced luciferase activities for all 5′ haplotypic variants in both cell lines except with the wild-type −287T/−399C in HEK293T cells (Fig. 3A). Similar results were obtained with the −33T allele (Fig. 3B). Except for the −287C/−399C/−33C haplotype, the reduction in luciferase activity was substantially higher in CHO-K1 cells compared to HEK293T cells ($p < 0.005$). The haplotypes containing the −287C allele, had significantly lower expression compared to the −287T allele in both cell types. When co-transfected with the −33T allele only the haplotypes containing the −287C allele displayed reduced luciferase activity in HEK293T cells while in CHO-K1 cells, all haplotypic variants showed reduced luciferase activity. As for the HEK293T cells, only the haplotypes containing the −287C allele, had statistically significant reduction of luciferase activity compared to the haplotypes with the −287T allele. Comparing the results from the co-transfections with the −33C and the −33T allele, the −33C allele resulted in stronger effects then the −33T allele ($p = 0.02$–0.007).

3.4. Differential binding of proteins to the polymorphic variants

To study whether the −287T/C, −399C/T, and −33T/C SNPs could affect DNA–protein interactions, we performed EMSA with allele-specific probes. As shown in Fig. 4, EMSA with both HEK293T and CHO-K1 nuclear extracts revealed that several complexes were formed with both the −287T and C alleles. Competition with 200× molar excess of competitors showed two specific bands for the −287C allele when incubated with nuclear extracts from the HEK293T cell line and one specific band for the CHO-K1 cells (indicated by arrowheads). For the −399T and C alleles, three specific bands were detected for the C allele with nuclear extracts from CHO-K1 cells. Specific complexes of different size were seen for both the T and the C alleles of the −33 polymorphism with CHO-K1 extracts while only the C allele was found to bind specifically to nuclear extracts from HEK293T cells.

4. Discussion

The TFPI level in plasma is considered as a risk factor for thrombotic events. Although some clinical studies have suggested that

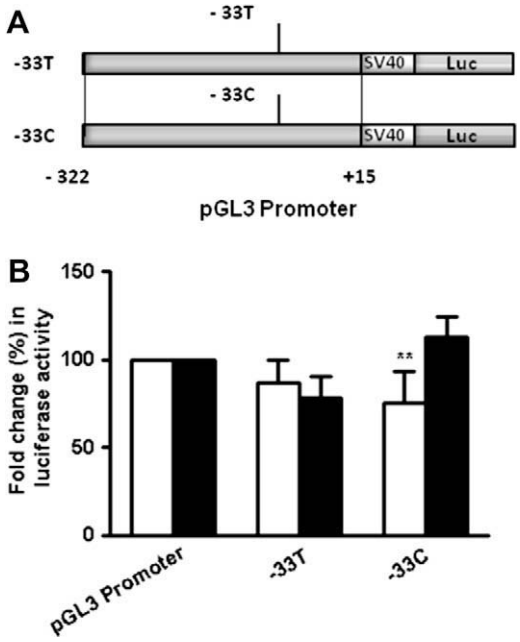

Fig. 2. Transcriptional activity of the TFPI intron 7 −33T/C polymorphism. (A) Schematic representation depicting the localization of the TFPI intron 7 polymorphism. Constructs containing the −322 to +15 fragment from intron 7/exon 8 were created in the pGL3 Promoter vector. The −33T construct represents the wild-type construct. +15 denotes nucleotide 15 in exon 8. (B) Luciferase activity of the intron 7 −33T/C constructs with the various haplotypes in HEK293T (white bars) and CHO-K1 (black bars) cells co-transfected with pRL-SV40 to normalize for transfection efficiency. Fold change in luciferase activity was calculated relative to the activity obtained with empty vector and are presented as mean + SEM. HEK293T, $n = 12$ samples. CHO-K1, $n = 8$ samples. $^{**}p < 0.005$ relative to the empty vector.

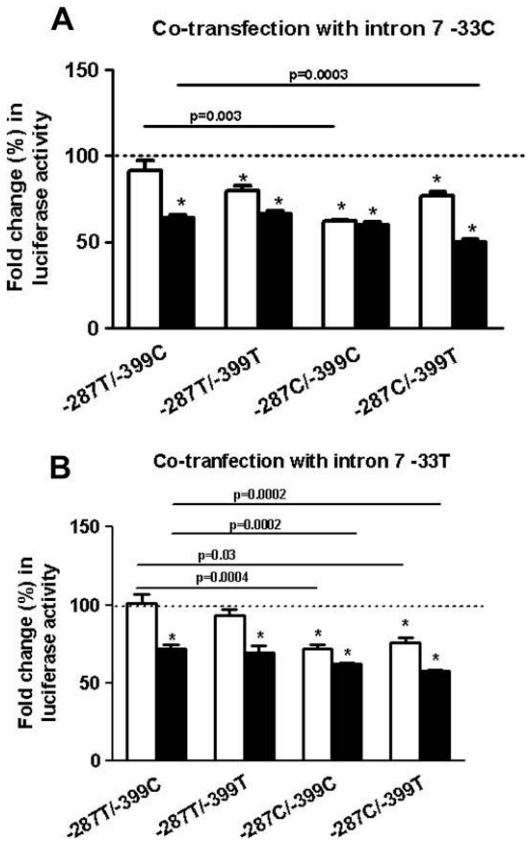

Fig. 3. The influence of the intron 7 −33T/C polymorphism on the transcriptional activity of the 5′ haplotypes. The various 5′ constructs created in the pGL3 Basic vector were co-transfected with either the −33C construct (A) or the −33T construct (B) created in the same vector, into HEK293 (white bars) or CHO-K1 (black bars) cells. Fold change in luciferase activity was calculated relative to the activity of the respective 5′ haplotype constructs alone (represented by the broken line as 100%). The results are presented as mean + SEM. Variations in transfection efficiency were corrected for by co-transfection with pRL-SV40. HEK293T, $n = 12$ samples. CHO-K1, $n = 8$ samples. $^{*}p < 0.05$ relative to the respective 5′ haplotype construct alone.

polymorphisms located in the 5′ upstream region and in intron 7 of the TFPI gene might be involved in the regulation of the plasma TFPI levels, to date there has been no comprehensive investigation of these polymorphisms at the transcriptional level. In this study, we have investigated the possible influence of the −287T/C, −399C/T and the intron 7 −33T/C SNPs on the TFPI levels using a luciferase reporter gene system. Compared to the wild-type haplotype, both the −287C and −399T alleles resulted in increased luciferase activity in both HEK293T and CHO-K1 cells. For the −399T allele, these findings are in contrast to recent clinical studies where no significant differences in plasma TFPI levels with the C or T variants of the −399 SNP were detected [20]. Conflicting results have been reported for the −287T/C polymorphism. In a study from Sayer et al., no significant differences in plasma TFPI levels were found for the two alleles [26], while an *in vitro* study by Amini-Nekoo and Iles found that the expression from the −287C allele was significantly lower than the −287T [18]. Our results are in line with a report from Moatti et al. who found that the presence of the −287C allele resulted in higher plasma TFPI levels [22]. When the intron 7 −33T or C alleles were cloned into the pGL3 Promoter vector, only the −33C allele showed a statistical significant reduction in the luciferase activity in HEK293T cells. This is in contrast with previous clinical studies reporting that the −33C allele was found to be associated with higher levels of plasma TFPI compared to the −33T allele [25,26].

The −287C allele has been shown to be in linkage disequilibrium with the −33C allele [25,27]. To examine this more thoroughly, co-transfections with the −33C and haplotypic variants of the 5′ polymorphism were performed. In HEK293T cells, the haplotypic variants −287T/−399T/−33C, −287C/−399C/−33C, and −287C/−399T/−33C gave reduced luciferase activity compared to the luciferase activities obtained with the 5′ haplotypic variants alone. In CHO-K1 cells all haplotypic variants affected the luciferase activity. When comparing the luciferase activity obtained for −287T/−399T/−33C, −287C/−399C/−33C, and −287C/−399T/−33C with the −287T/−399C/−33C, only the haplotype with the −287C variant was significantly different from the −287T/−399C haplotype. Similar results were obtained for co-transfections with −33T. This strongly suggests that there is an association between the −287C allele and the −33T/C polymorphism. However, this association seems to be independent of the presence of the T or C allele in position −33.

Differences in expression can result from altered binding affinities/specificities of transcription factors to DNA. When we examined the binding of nuclear proteins to oligonucleotides containing the various alleles, several complexes were formed. Using competitor analysis with unlabeled oligonucleotides, specific complexes were detected for the −287C allele in both cell types. This could be a result of a sequence specific transcription factor binding to the C variant, thus increasing the expression. The slightly, although statistically significant increase of luciferase activity with the −399T allele could be explained by the specific complexes observed with the −399C allele in CHO-K1 cells, indicating binding of a sequence specific inhibitor. No specific complexes were found with extracts from the HEK293T cells. A specific complex was also detected for the −33C allele in both cell types, while for the T allele, a specific complex was only detected with nuclear proteins from CHO-K1 cells.

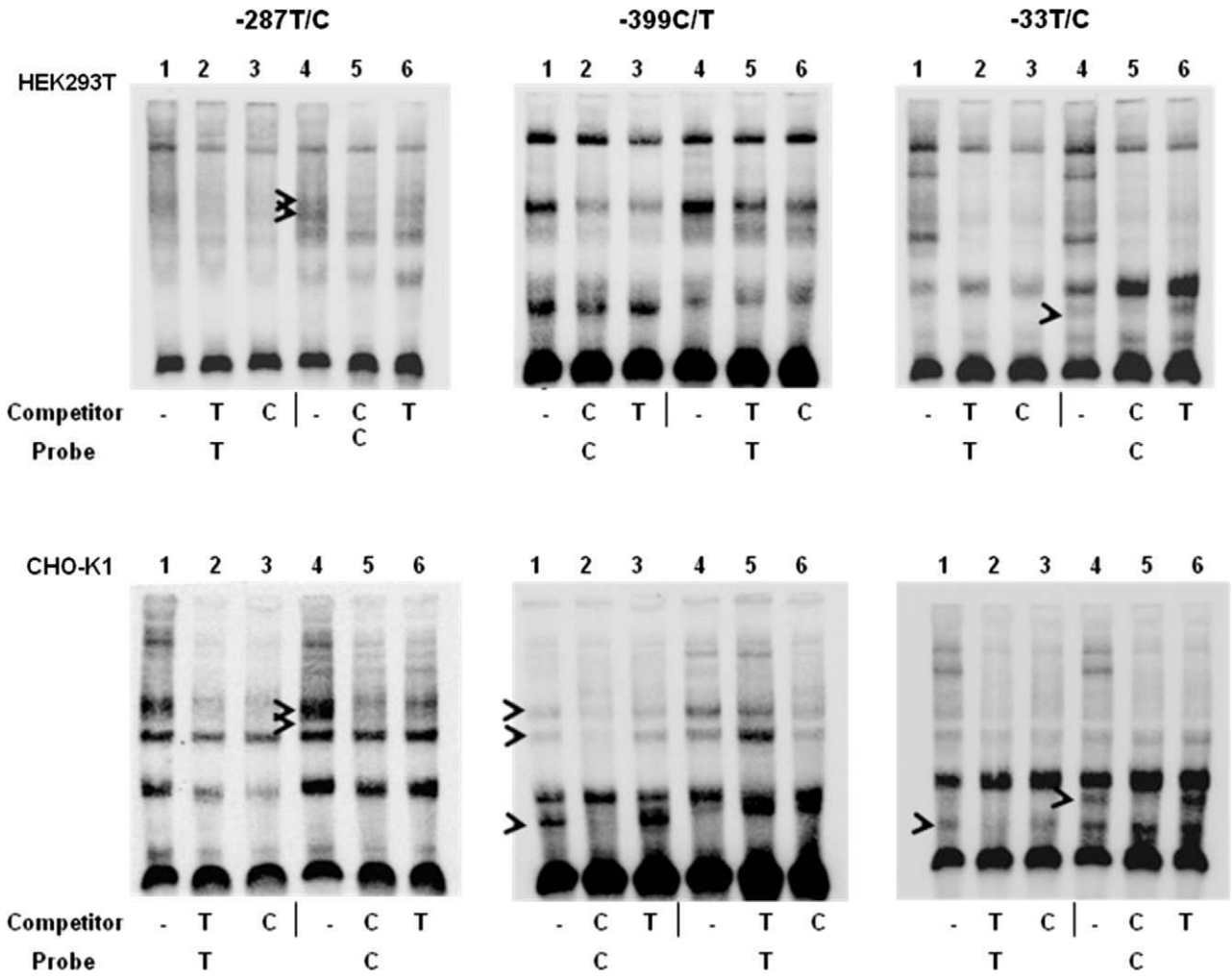

Fig. 4. Interactions of nuclear proteins with the various polymorphisms. Electrophoretic mobility shift assays were performed using nuclear extracts from HEK293T (~10 μg) or CHO-K1 (~8 μg) cells. Nuclear extracts were incubated with biotin-labeled double-stranded oligonucleotides. Assays were performed in the absence or presence of 200-fold molar excess of unlabeled oligonucleotides. Arrowheads indicate specific complexes.

In this study, we have used both TFPI expressing and non-expressing cell lines without detecting any major differences in the luciferase experiments. In EMSA, cell specific complexes were obtained with the −399C and the −33T alleles indicating a possible role of the SNPs in the tissue specific regulation of TFPI. However, this needs to be addressed in further studies. The results we obtained with the reporter gene system do not support the results from *in vivo* clinical studies. However, a direct comparison between these studies and our reporter gene studies is not appropriate. The power of the referred studies is not very strong since the number of individuals in the different groups was rather low and also the number of homozygotes of the polymorphic variant was very low. Additionally, the normal reference range of TFPI in plasma is very wide so that small differences in plasma concentrations due to polymorphic variants would probably not be detected. In a report from Amini-Nekoo and Iles [18], it was demonstrated in a reporter gene system that the −287C allele was associated with lowered luciferase activity. This is in contrast to the present findings and could be due to differences in the regulation of TFPI in HEK293T cells and human microvascular endothelial cells that were used in the report mentioned above.

In summary, we have demonstrated effects of the −287T/C and −399C/T polymorphisms on the transcriptional activity of the TFPI promoter in a dual reporter gene assay system. In addition, we have shown a possible association between the −287C allele and the −33T/C polymorphism. EMSA analysis showed that the single base pair changes alter protein–DNA interactions in all three polymorphisms and further investigations are required to determine the identity of the proteins in these complexes, and thus add more knowledge to how TFPI expression is regulated.

Acknowledgments

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