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Full Length Article

Functional characterization of annexin A5 gene promoter allelic variants

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ABSTRACT

Background: M1 and M2 haplotypes are defined by 4 consecutive allelic variants in regulatory regions of the annexin A5 gene and have been found to reduce promoter activity. To date, no research has been carried out to investigate differential and individual impact each of the allelic variants has on promoter activity. In the current study, we functionally characterized the M1 and M2 haplotype allelic variants (c.-467G>A, c.-448A>C, c.-422T>C, c.-373G>A). We also characterized two other allelic variants located in the same regulatory region (c.-628C>T, c.-302T>G).

Materials and methods: Their impact on the ANXA5 promoter activity was examined using a luciferase reporter assay in BeWo cells. Electrophoretic mobility shift assay with probes centered around each polymorphism was used to examine the binding ability of the allelic variants to nuclear proteins from BeWo cells.

Results: Only the c.-467G>A and c.-628C>T allelic variants influenced the activity of the ANXA5 promoter, as measured by luciferase activity. Differential specific interactions with nuclear proteins were obtained for all allelic variants, except for the c.-302T>G, indicating that these polymorphisms could have an impact on the ANXA5 expression.

Conclusions: We have functionally characterized allelic variants in the ANXA5 promoter, both alone and in combinations, and the results suggest that combinations of several individual variants contribute to modulate the ANXA5 transcriptional activity, most likely through binding of nuclear factors. These results provide new knowledge and insight into the mechanisms underlying the regulation of annexin A5 levels in healthy controls.

1. Introduction

Annexin A5 is ubiquitously expressed in human tissues [1], but particularly abundant in normal placenta [2]. It acts as an anticoagulant protein, through interfering with phospholipid dependent clotting reactions [3]. Defects resulting in reduced annexin A5 levels are believed to be responsible for hypercoagulability, such as during pregnancy [4]. In women with preeclampsia, weak immunostaining of annexin A5 in placenta has been reported [5]. In mice, infusion of anti-annexin A5 antibodies caused various degrees of fetal absorption simultaneously with thrombosis and necrosis of absorbed embryos [6].

Two novel haplotypes defined as M1 and M2, were previously reported in the 5′-flanking region of the annexin A5 gene (ANXA5) [4]. The M2 haplotype is characterized by 4 single nucleotide substitutions, that, according to the single nucleotide polymorphism (SNP) identification system, are known as rs112782763 (c.-467G>A), rs28717001 (c.-448A>C), rs28651243 (c.-422T>C), and rs113588187 (c.-373G>A). The combination of the c.-448A>C and c.-422T>C SNPs constitutes the M1 haplotype [4]. By using a reporter gene assay in HeLa cells, it was demonstrated that both the M1 and the M2 haplotypes reduced the ANXA5 promoter activity by 60 and 37%, respectively [4]. Two additional SNPs in the ANXA5 promoter have been reported; rs62319820 (c.-628C>T) [11] and rs1050606 (c.-302T>G) [13]. The former SNP has been tightly linked to the M1 haplotype [11], whereas the latter SNP has been suggested as a new risk factor for pregnancy loss in a Japanese population, an association that was found to be independent of the M2 haplotype [12]. Lately, combinations of the M2 haplotype and c.-302G and of the M1 haplotype and the c.-302G and c.-628T SNPs were defined as H3 and H4 haplotypes, respectively [14]. Both haplotypes constitute common combinations in the ANXA5 promoter.

In German women with pregnancy loss an increased prevalence of the M2 has been reported [4] and in an Italian study, the M2 was found to be significantly associated with pregnancy complications [7] and small-for-gestational age newborns [8]. It has also been reported that the placental ANXA5 mRNA levels were dependent on the M2 haplotype carriership [9]. Whether the effects of M1 or M2 on the ANXA5

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expression reflects the annexin A5 levels, remains still questionable, mainly due to observed high variability of the intra-haplotypes [10]. Recently, the association between annexin A5 plasma levels and the SNPs within the ANXA5 promoter was examined [11]. It was concluded that the ANXA5 promoter haplotypes may determine the variability of ANXA5 plasma levels in healthy subjects, although additional studies are needed to add clear evidences on clinical influence of circulating annexin A5 levels. In another study, a correlation between the expression levels of ANXA5 in placenta and the M2 haplotype was reported [12].

Gene expression can be controlled at the transcriptional level through interplay between transcription factors that bind to specific DNA sequences (reviewed in [15]). To our knowledge, the ANXA5 promoter has not been thoroughly studied regarding identification of transcription factors regulating its expression. There are indications that this promoter has a complex regulation with multiple transcriptional start sites. Two canonical Sp1 binding sites and several other segments of the promoter have been identified as important for promoter activity [16].

Due to discordant results from epidemiological studies on ANXA5, our underlying hypothesis for the present study was that single SNPs and combinations of them, can differentially influence the ANXA5 expression. Therefore, we have functionally characterized the various SNPs constituting the M1 and M2 haplotypes and also the c.-302T>G and c.-628C>T SNPs. Using luciferase reporter assay and electrophoretic mobility shift assay (EMSA), we demonstrate that the M1 and M2 related SNPs might differentially and individually contribute to the expression levels of ANXA5, possibly by influencing the binding of transcription factors.

2. Materials and methods

2.1. Cell cultures

The human placental trophoblastic cell line BeWo derived from a choriocarcinoma (ATCC® CCL-98™, American Type Culture Collection, Rockville, MD, USA) [17] was maintained in a humidified atmosphere at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Lonza Group Ltd., Basel, Switzerland) supplemented with 10% (vol/vol) heat inactivated fetal bovine serum (Lonza Group Ltd.), 100 U/ml penicillin, 100 μg/ml streptomycin (Lonza Group Ltd.) and 0.6 mM l-proline (Sigma-Aldrich, St. Louis, MO, USA).

2.2. Plasmid construction

Using human genomic DNA isolated from whole blood of a subject with wild-type genotypes, a fragment of the ANXA5 promoter was amplified. The fragment covered the nucleotides no. 4623 to 5402 of the NCBI Reference sequence NG_0320421. The following primers were used: forward 5′-CCCACGCACTATGTTGAGCA-3′ and reverse 5′-TGGAAGCGATGTCCCCAAAG-3′. The amplification reaction was performed on 100 ng of genomic DNA in a total volume of 50 μl using the HotStarTaq® *Plus* DNA Polymerase kit (Qiagen GmBH, Hilden, Germany). A typical PCR cycling protocol was optimized and outlined as follows: 95 °C for 5 min, 35 cycles at 94 °C for 1 min, 63 °C for 55 s, 72 °C for 1 min, and a final extension of 10 min at 72 °C. The PCR fragment was cloned into the promoterless firefly luciferase reporter pGL3-Basic Vector (Promega, Madison, WI, USA). The resulting construct ($pGL3ANXA5_{wt}$) was verified by sequencing. The various genotypes were generated with $pGL3ANXA5_{wt}$ as a template using site-directed mutagenesis (QuickChange Site-directed Mutagenesis kit, Stratagene, La Jolla, CA, USA). Primers for the mutagenesis were designed by the QuikChange® Primer Design Program and are reported in Table 1. The mutations were verified by sequencing.

2.3. Cell transfection and luciferase assays

BeWo cells (4.0×10^4 cells/well) were plated in 24-well plates, grown to 50–80% confluence, and then transiently transfected using the Lipofectamine® LTX Reagent (Invitrogen, Carlsbad, CA, USA). Cells were co-transfected with a total of 0.5 μg per well of the experimental reporter plasmids or the promoterless pGL3-Basic vector and 5 ng per well of Renilla-luciferase vector pRL-SV40 (Promega, Madison, WI, USA) as an internal transfection control. Twenty-four hours after transfection, the cells were washed with phosphate-buffered saline, lysed in passive lysis buffer (Promega) and subjected to a dual luciferase assay according to the manufacturer's instructions (Dual-Luciferase® Reporter Assay System DLR™, Promega). Luciferase activity was assessed using a GloMax® 96 Microplate Luminometer with Dual Injectors (Promega). All samples were normalized against pGL3-Basic before calculating the ratios between wild type and mutant genotypes.

2.4. Electrophoretic mobility shift assays (EMSA)

Nuclear extracts from BeWo cells were prepared as previously described [18]. Biotin-labelled and corresponding unlabelled oligonucleotides containing the various genotypes were synthesized by Eurogentec (Seraing, Belgium). The sequences of the oligonucleotides are listed in Table 2. To make double-stranded probes and competitors, equal amounts of complementary oligonucleotides were heated at 95 °C for 5 min and then annealed by stepwise reducing the temperature to 25 °C during 1 h. EMSA was performed using the LightShift® Chemiluminescent EMSA Kit (Pierce, Rockford, IL). The binding reactions were carried out for 20 min at 20 °C in the presence of 1 μg poly(dI-dC) and protease inhibitor cocktail (Sigma-Aldrich) in binding buffer using 20 fmol biotin-labelled double-stranded probes and 3 μg nuclear extracts from BeWo cells. In addition, 30 mM $MgCl_2$ or 1 mM EDTA was present in the binding reactions for the -628C>T and -302T>G probes, respectively. For competition, 200-fold molar excess of unlabelled double-stranded oligonucleotides was added to the binding reaction 10 min prior to the addition of the labelled probes. DNA-protein complexes were

Table 1
Primers designed for site-directed mutagenesis.

SNP tag number	Mutation		Primer sequence
rs62319820	c.-628C>T	Sense	5′-gagcggcgcctcttcctggttccag-3′
		Antisense	5′-ctggaaccaggaagaggcgccgctc-3′
rs112782763	c.-467G>A	Sense	5′-gcctgcggttgaggccctggcgg-3′
		Antisense	5′-ccgccagggcctcaaccgcaggc-3′
rs28717001	c.-448A>C	Sense	5′-gcgggggtgggccgggccaagcc-3′
		Antisense	5′-ggcttggcccggcccaccccgc-3′
rs28651243	c.-422T>C	Sense	5′-agggccggggcggggccgctg-3′
		Antisense	5′-cagcggccccgccccggccct-3′
rs113588187	c.-373G>A	Sense	5′-taggtgcagctgccagatccttcagcgtc-3′
		Antisense	5′-gacgctgaaggatctggcagctgcaccta-3′
rs1050606	c.-302T>G	Sense	5′-gctctcccgggggttcggggcactt-3′
		Antisense	5′-aagtgccccgaacccccgggagagc-3′