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THE 4G/5G POLYMORPHISM IN THE PROMOTER OF THE PLASMINOGEN ACTIVATOR INHIBITOR-1 (PAI-1) GENE IN SUBJECTS WITH CANCER

Urokinase plasminogen activator system includes proteolytic enzymes that may contribute to cancer cell invasion by degrading the surrounding extracellular matrix and dissociate cell-cell or cell-matrix attachments. There is substantial evidence in many types of cancer that the antigen content of one component of the system – plasminogen activator inhibitor-1 (PAI-1) in primary cancer tissue extracts are of strong prognostic value: high level of PAI-1 in tumor predict poor prognosis for the patient. Moreover, it was demonstrated that the level of PAI-1 in metastasis is significantly higher compared to primary tumors. An insertion/deletion polymorphism in the promoter of the PAI-1 gene has been described that relates to plasma PAI-1 levels. We studied this polymorphism in the subjects with cancer. Blood was taken from 53 patients (16 breast cancers, 12 colorectal cancers, 9 gastric cancers, 9 melanomas and 7 head and neck cancers) and 53 matched controls. The frequencies of the 4G and 5G alleles in patients were 0.53 and 0.47, respectively, compared with 0.43 and 0.57 in controls. The genotype distribution differed significantly between the two groups – the 4G/5G genotype was observed more frequently in patients with cancer than in the controls. Further studies are needed to check whether the prevalence of the 4G/5G genotype may influence an individual's plasminogen activation system capacity and thereby contribute in a small way to the cancer risk profile.

Key Words: Plasminogen activator inhibitor-1 (PAI-1), PAI-1 gene, gene polymorphism, cancer

INTRODUCTION

The urokinase plasminogen activation system is not only responsible for the removal of fibrin from the circulation, but is also believed to play a role in other biological processes, such as ovulation, embryogenesis, intima proliferation, atherosclerosis, degradation of the extracellular matrix, tumorigenesis and metastasis [5, 25, 31]. The system includes the urokinase

type plasminogen activator (u-PA), the specific plasminogen activator inhibitors PAI-1 and PAI-2 and the urokinase receptor (u-PAR). PAI-1, an approximately 50-kD glycoprotein belonging to the serine protease inhibitor superfamily, is the major physiological inhibitor of the system. Its biosynthesis is regulated by a number of hormones, cytokines, growth factors, tumor-promoting phorbol esters and other agents [2, 14, 15].

Cancer cell invasion and metastasis are multifactorial processes that include adherence to the basement membrane, secretion of proteolytic enzymes and cell migration into vessels and lymphatic nodes followed by extravasation at distant sites [22]. It has been shown that plasmin bound to tumor cells significantly increases the degradation of basement membrane [4], so PAI-1, like the remaining components of the plasminogen activation system, can be involved in tumor invasion and metastasis. The level of PAI-1 was positively correlated with a more invasive tumor cell phenotype of many types of cancers, so it can be related to prognosis [3].

PAI-1 was shown first to be a prognostic marker in both node-negative and node-positive breast cancer [12, 13, 17]. It was also shown that in gastric cancer, high level of PAI-1 correlated with shortened patient survival [11, 28]. In lung adenocarcinoma, PAI-1 was found to be an independent indicator of prognosis [30]. High levels of PAI-1 predicted shortened survival in patients with advanced ovarian cancer [21]. The feasibility of altering tumor metastasis by disrupting plasminogen activators function by PAI-1 was demonstrated in murine models of prostate carcinoma and intraocular melanoma [1, 32]. Tumor necrosis factor α increased the production of PAI-1 in eight human colon carcinoma cell lines [33].

Changes in PAI-1 biosynthesis is usually preceded by changes in PAI-1 gene transcription and mRNA level [2, 23]. Gene variability could contribute to the level of the PAI-1 biosynthesis [16]. Eight different polymorphism on the PAI-1 gene have been described: (a) two (CA)_n repeat polymorphisms, one in the promoter and one in the intron 4 [7, 24], (b) an *HindIII* restriction fragment length polymorphism [18], (1991); (c) an insertion (5G)/deletion (4G) polymorphism at position -675 of the PAI-1 gene promoter [6]; (d) two G→A substitutions at positions -844 and +9785; (e) two polymorphisms in the 3' untranslated region (T→G substitution at position 11 053 and 9-nucleotide insertion/deletion located between nucleotides +11 320 and +11 345 in a threefold repeated sequence) [16]. In view of the possibly significant role of PAI-1 for tumor spreading, it is important question, whether these polymorphisms can account for the development and/or the progression of cancer.

In the present work the frequencies of the variants of the common insertion (5G)/deletion (4G) polymorphism at position -675 of the PAI-1 promoter (4G/5G polymorphism, Fig. 1) in subjects with cancer as compared with healthy controls was studied.

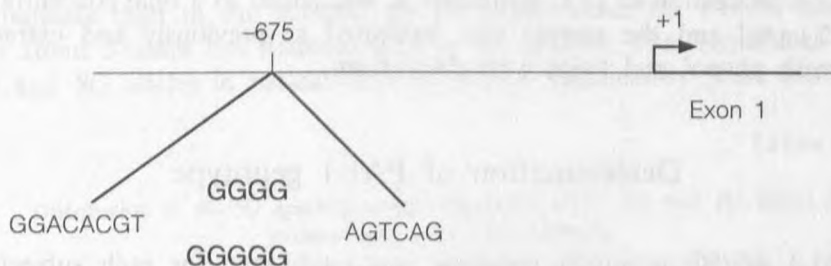


Fig. 1. Schematic representation of the PAI-1 gene showing the location of the 4G/5G polymorphic site and its flanking sequences

MATERIALS AND METHODS

Chemicals

RNAse, proteinase K, Triton X-100 and EDTA were from Sigma Chemicals (St. Louis, MO). Taq polymerase was obtained from Promega (Madison, WI, USA). Specific primers were purchased in ARK Scientific GmbH Biosystems (Darmstadt, Germany). Four deoxyribonucleotides triphosphates were from Boehringer (Mannheim, Germany).

All other reagents were of the highest purity available.

Subjects

The study included blood from 53 healthy volunteers and 53 patients with various cancers – 16 breast cancers, 12 colorectal cancers, 9 gastric cancers, 9 melanomas and 7 head and neck cancers. All patients had biopsy-verified malignant tumors. Blood was collected at the Department of Oncology, Medical Academy of Łódź from patients enrolled to surgery. The control subjects were age- and sex matched.

DNA extraction

Blood was mixed with equal volume of the buffer comprising 1% Triton X-100, 2% sarcosyl, 0.8 M urea, 20 mM EDTA, 0.4 M NaCl, 200 mM

Tris, pH 8.0 and RNase A was added to a final concentration of 100 $\mu\text{g/ml}$. After 2 h incubation at 55°C proteinase K was added to a final concentration of 125 $\mu\text{g/ml}$ and the sample was incubated as previously and extracted once with phenol and twice with chloroform.

Determination of PAI-1 genotype

PAI-1 4G/5G promoter genotype was established for each subject by polymerase chain reaction (PCR) amplification of genomic DNA using the allele specific primers (Fig. 1): insertion 5G allele: 5'-GTC TGG ACA CGT GGG GG-3', deletion 4G allele: 5'-GTC TGG ACA CGT GGG GA-3', each in a separate PCR reaction together with the common downstream primer 5'-TGC AGC CAG CCA CGT GAT TGT CTA G-3' and a control upstream primer 5'-AAG CTT TTA CCA TGG TAA CCC CTG GT-3' to verify the occurrence of DNA amplification in the absence of the allele on the genomic DNA [10]. The PCR was carried out in a Perkin-Elmer thermal cycler, model Gene Amp PCR System 2400 in a final volume of 25 μl containing 10 ng DNA, 13 pmol of specific primers, 1 mM dNTPs and 1 U Taq polymerase together with 2.5 μl 10 \times Taq buffer. The PCR cycle conditions were 94°C for 60 s, 54°C for 30 s then 72°C for 40 s for 35 cycles. The amplified DNA fragments were separated by 5% polyacrylamide gel electrophoresis and, after staining with ethidium bromide, viewed under ultraviolet light. Each subject was classified into one of the three possible genotype groups: 4G/4G, 4G/5G or 5G/5G.

Statistical analysis

Allele frequency was estimated by gene counting and analyzed by χ^2 test. The χ^2 test was also used to compare the observed numbers of each PAI-1 genotype with those expected for a population in Hardy-Weinberg equilibrium.

RESULTS AND DISCUSSION

The distribution of the 4G/5G genotypes in patients with cancers and controls is displayed in Table 1.

The genotype distribution differed significantly between the two groups (Table 1) – the 4G/5G genotype was observed almost twice more frequently in patients than in the control; on the other hand, the 5G/5G genotype was about 5 times less frequent than in the control. The frequencies of the 4G and 5G alleles in patients did not differ significantly from controls.

Table 1

Distribution of 4G/5G genotypes and frequencies of the 4G and 5G alleles in patients with cancer and controls

	Patients ^a		Controls ^a	
	Number	Frequency	Number	Frequency
4G/4G genotype	7	0.14	11	0.22
4G/5G genotype	42	0.79 ^b	23	0.42
5G/5G genotype	4	0.07 ^b	19	0.36
4G allele	56	0.53	45	0.43
5G allele	50	0.47	61	0.57

^a n = 53;

^b p < 0.05.

With the presented data it was shown, that there were different distributions of 4G/5G genotypes in small populations of patients with cancers and controls.

Idea that PAI-1 is an etiological factor in some diseases, first of all of cardiovascular type [18], caused that human PAI-1 promoter was searched for alterations that may affect the level of plasma PAI-1 activity [9]. Evidence that genetic factors can be important for PAI-1 activity comes from clinical studies. Initially, a 3' *HindIII* restriction fragment length polymorphism and an intronic dinucleotide (CA) repeat were associated with elevated PAI-1 levels [6]. More recently 4G/5G polymorphism has been related to circulating level of PAI-1 – levels are the highest in subjects with the 4G/4G genotype and generally about one-third higher than subjects homozygous for the 5G allele [7, 9, 29].

Although levels of PAI-1 are correlated significantly with environmental factors [19, 27] the variants cannot be excluded. The genetic 4G/5G polymorphism seems to be especially interesting because subjects with the 4G allele have higher plasma level of PAI-1 than individuals with the 5G allele [8] and the 4G allele was more common in patients who survived an myocardial infarction [9].

The association of the 4G/5G polymorphism of the PAI-1 promoter with altered levels of plasma PAI-1 activity suggests a differential binding of proteins regulating the transcriptional activity of the gene. It was shown

that both alleles bound a common factor, while the 5G allele bound an additional factor [9]. In an *in vitro* studies it was also demonstrated that the 4G and 5G sites differentially bound enhancer/repressor [7, 9], that suggests a functional role of the 4G/5G polymorphism.

Elevated PAI-1 may contribute also to vascular disease in diabetes mellitus. Pima Indians have a low incidence of cardiovascular disease despite of having a high prevalence of non-insulin-dependent diabetes mellitus which in this population is not associated with elevated PAI-1 activity [26]. In Pima Indians population the frequencies were 23.0% for 4G/4G, 49.8% for 4G/5G and 27.2% for 5G/5G compared to 35.4%, 50.8% and 13.8%, respectively, previously reported in Caucasian with non-insulin-dependent diabetes mellitus. The difference in the frequencies of genotypes may indicate functional difference in the PAI-1 gene in this two populations associated with reduced cardiovascular risk.

Obtained results can be treated as a preliminary report, but they suggest that the problem of the possible correlation between genetic constitution of the PAI-1 gene and possibly other genes of the plasminogen activator system is worth further studying. Our study involved a relatively small number of subjects with cancer. Moreover, the population was heterogeneous in age, sex and the type of cancer. Further studies should consider also these aspects of research. The other problem to discuss is the association between genotype and circulating PAI-1 level. As mentioned above such an association has been established, but it must be taken into account that this relationship can be hold only for healthy individuals. In a pathological state, like in cancer, variation in the level of PAI-1 may, at least in part, be attributed to undefined influences acting on PAI-1 at the time of the disease.

Although there was no difference in allele frequency between subjects with cancer and controls, the prevalence of the 4G/5G genotype may influence an individual's plasminogen activation system capacity and thereby contribute in a small way to the cancer risk profile, probably by interaction with other genetic factors.

Further research are needed to elucidate the relationship between the PAI-1 gene, circulating PAI-1 levels and cancer.

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POLIMORFIZM 4G/5G OBSZARU PROMOTOROWEGO GENU INHIBITORA AKTYWATORÓW PLAZMINOGENU TYPU 1 (PAI-1) U CHORYCH NA NOWOTWORY ZŁOŚLIWE

Urokinazowy układ aktywacji plazminogenu zawiera enzymy proteolityczne mogące brać udział w inwazji komórek rakowych poprzez degradację macierzy zewnątrzkomórkowej i przerywaniu połączeń pomiędzy komórkami i macierzą. Wyniki znaczącej liczby eksperymentów wskazują, że zawartość przeciwciał dla jednego ze składników układu aktywacji plazminogenu inhibitora aktywatorów plazminogenu typu 1 (PAI-1), w ekstraktach z fragmentów pierwotnych guzów nowotworowych może mieć duże znaczenie prognostyczne: wysoki poziom PAI-1 źle rokuję dla pacjenta. Zademonstrowano także, że poziom PAI-1 w metastazie jest wyższy niż w guzach pierwotnych. Polimorfizm insercyjno/delecyjny 4G/5G w obszarze promotorowym genu PAI-1 może być związany z osoczym poziomem PAI-1. W pracy badano ten polimorfizm we krwi 53 pacjentów cierpiących na różne rodzaje nowotworów (16 przypadków raka sutka, 12 jelita grubego, 9 żołądka, 9 czerniaków i 7 nowotworów głowy i szyi) oraz 53 odpowiednio dobranych osobników zdrowych. Częstości alleli 4G i 5G u chorych były odpowiednio: 0,53 i 0,47, podczas gdy w grupie kontrolnej wartości te wynosiły 0,43 i 0,57. Rozkłady genotypów w badanych grupach były znacząco różne – u chorych genotyp 4G/5G występował ze zwiększoną częstością niż w grupie kontrolnej. Konieczne są dalsze badania dla oceny częstości występowania danego genotypu polimorfizmu 4G/5G, jak również innych polimorfizmów, genu PAI-1 i stopniem ryzyka zapadnięcia na chorobę nowotworową.