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QUARTERLY

Plasminogen activator inhibitor 1 (PAI-1) 1334G/A genetic polymorphism in colorectal cancer✪

Beata Smolarz✉, Hanna Romanowicz-Makowska and Andrzej Kulig

Laboratory of Molecular Genetics, Department of Pathology, Institute of Polish Mother's Memorial Hospital, Łódź, Poland

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Plasminogen activator inhibitor 1 (PAI-1) content in colorectal cancer tissue extracts may be of strong prognostic value: high levels of PAI-1 in tumours predict poor prognosis. The gene encoding PAI-1 is highly polymorphic and *PAI-1* gene variability could contribute to the level of PAI-1 biosynthesis. In the present work the distribution of genotypes and frequency of alleles of the 1334G/A polymorphism in 92 subjects with colorectal cancer in samples of cancer tissue and distant mucosa samples as well as in blood were investigated. Blood samples age matched healthy individuals (n = 110) served as control. The 1334G/A polymorphism was determined by PCR amplification using allele specific primers. No differences in the genotype distributions and allele frequencies between blood, distant mucosa samples and cancer tissue were detected. However, the distribution of the genotypes of the 1334G/A polymorphism in patients differed significantly ($P < 0.05$) from those predicted by the Hardy-Weinberg equilibrium. There were significant differences in the frequencies of alleles between the colorectal cancer subjects and controls ($P < 0.05$). The results support the hypothesis that the 1334G/A polymorphism may be associated with the incidence of colorectal cancer.

Invasion and metastasis of malignant tumours require proteolytic degradation of the extracellular matrix (ECM) and the basement membrane and infiltration of tumour cells into the surrounding tissue, the blood stream, and/or the lymphatic vessels (Meyer & Hart, 1998). At least four different types of tumour-associated proteases may be responsi-

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✉Please send all correspondence to Beata Smolarz, Laboratory of Molecular Genetics, Department of Pathology, Institute of Polish Mother's Memorial Hospital, Rzgowska 281/289, 93-338 Łódź, Poland; phone: (48 42) 271 1280; fax: (48 42) 271 1421, e-mail: smolbea@wp.pl

Abbreviations: PAI-1, plasminogen activator inhibitor 1; ECM, extracellular matrix; uPA, urokinase type plasminogen activator; uPAR, urokinase receptor.

ble for degradation of the tumour stroma and tumour cell invasion: cysteine proteases, aspartate proteases, matrix metalloproteases and serine proteases (Dano *et al.*, 1999). The components of the plasminogen activation system: urokinase type plasminogen activator (uPA), the specific plasminogen activator inhibitors PAI-1 and PAI-2 and the urokinase receptor (uPAR), released by cancer cells, stimulate tumour invasion (Carroll & Binder, 1999). High levels of uPA, uPAR and PAI-1 are associated with poor prognosis in a variety of cancers (Lijnen, 2001; Schmitt *et al.*, 1997).

Patients with colorectal cancer can be cured by surgical treatment only if the cancer is detected at an early stage of the disease (Weiss & Itzkowitz, 1995). It is therefore important to identify high-risk and low-risk patients by suitable markers.

Many studies have shown that PAI-1 may be a useful prognostic marker in colorectal cancer (Abe *et al.*, 1999; Fuji *et al.*, 1999; Herszenyi *et al.*, 1999; Sattar & McMillan, 1998; Nielsen *et al.*, 1998). An elevated level of PAI-1 can be associated with shorter recurrence-free survival and shorter overall survival. Changes in PAI-1 biosynthesis are usually preceded by changes in its gene transcription and mRNA level (Henry *et al.*, 1998). Gene variability could contribute to the level of PAI-1 biosynthesis. Ten different polymorphisms within the *PAI-1* gene have been described: an A → G substitution in position +1334 in the propeptide coding region, two $(CA)_n$ repeat polymorphisms, one in the promoter and one in intron 4; a *Hin*dIII restriction fragment length polymorphism; an insertion (5G)/deletion (4G) polymorphism at position –675 of the promoter; two G → A substitutions at positions –844, and +9785; three polymorphisms in the 3′ untranslated region: a T → G substitution at position +11 053 and a 9-nucleotide insertion/deletion located between nucleotides +11 320 and +11 345 in a thrice repeated sequence, and a G → A substitution in position +12078 (Henry *et al.*, 1997; Mansfield *et al.*, 1995; Falk *et al.*, 1999).

In light of the substantial evidence that the progression of colorectal cancer can be associated with elevated level of PAI-1, it seems reasonable to check the possible correlation between these polymorphisms and/or progression of this cancer.

Among the polymorphic variants of the *PAI-1* gene an insertion (5G)/deletion (4G) polymorphism (4G/5G polymorphism) was most frequently studied. It is associated with high plasma PAI-1 levels in patients with coronary artery disease (Grancha *et al.*, 1999; Stegnar *et al.*, 1998), myocardial infarction (Eriksson *et al.*, 1995; Ye *et al.*, 1995) and diabetes (Mansfield *et al.*, 1995; Burzotta *et al.*, 1998). The role of the 4G/5G polymorphism was investigated also in subjects with breast (Smolarz & Błasiak, 1999; 2000), endometrial (Błasiak *et al.*, 2000) and colorectal cancer (Smolarz *et al.*, 2001; Błasiak *et al.*, 2000) but a lack of association between this sequence variation and cancer progression was observed.

In the present work the relationship between the 1334G/A polymorphism in the propeptide coding region of the PAI-1 molecule and the appearance and/or invasiveness of colorectal cancer was examined. It was reported earlier that particular genotypes of the 1334G/A polymorphism could be associated with bleeding tendency (Falk *et al.*, 1999), but little is known on possible role of this polymorphism in cancer.

In our study the distribution of genotypes and the frequency of alleles of the 1334G/A polymorphism in subjects with colorectal cancer were investigated.

MATERIALS AND METHODS

Patients. Tumour tissues, distant mucosa samples and blood were obtained from 92 subjects with colorectal cancer treated at the 2nd Department of Surgery, Military Academy of Medicine in Łódź (Poland) between 1998 and 2001. Clinical data for the patients and histo-

logical data were registered. There were 56 males and 36 females and their mean age was 59 years (range: 37–71 years). All tumours were graded according to Dukes's stages. There were 25 tumours of A stage, 40 of B stage and 27 of C stage in total. Blood samples from age matched healthy individuals (n = 110) served as control.

Determination of the 1334G/A polymorphism. DNA was extracted using commercially available QIAmp Kit (Qiagen GmbH, Hilden, Germany) DNA purification kit according to manufacturer's instruction. Genotypes of the 1334G/A polymorphism were determined by polymerase chain reaction amplification of genomic DNA using the following allele specific primers: two 17-mer: 5′–TCA CCA AAG ACA AGG GC–3′, and 5′–TCA CCA AAG ACA AGG GT–3′ in combination with an upstream primer, 5′–TGT TCA CTT ACC ACC TGC TT–3′ (Falk *et al.*, 1999). The amplification results in a DNA fragment of 182 bp and was performed in a total volume of 25 μl. The PCR was carried out in a DNA Thermal Cycler (GeneAmp PCR System 2400; Perkin-Elmer, Norwalk, CT, U.S.A.). The thermal cycling conditions were 30 s at 94°C, 30 s at 60°C, 30 s at 72°C, repeated for 30 cycles. The reaction mixture contained 1 μg of genomic DNA, 0.2 μmol of each appropriate primer (ARK Scientific GmbH Biosystems, Darmstad, Germany), 2.5 mM $MgCl_2$, 1 mM dNTPs (Qiagen GmbH, Hilden, Germany) and 1 unit of Taq Polymerase (Qiagen). PCR products were electrophoresed in a 5% polyacrylamide gel (PAGE) and visualised by ethidium bromide staining. Each subject was classified into one of the three possible genotypes: G/G, G/A or A/A.

Statistical analysis. The allelic frequencies were estimated by gene counting and the genotypes were scored. The observed numbers of each PAI-1 genotype were compared with those expected for a population in Hardy-Weinberg equilibrium by using the χ^2 test. The significance of the differences of the observed allele and genotype frequencies between groups was tested using the χ^2 analysis.

RESULTS

From the PCR analysis, all patients and controls were classified into three genotypes of the 1334G/A polymorphism: G/G, G/A and A/A (Fig. 1). There were no differences in the frequencies of the A (0.28) and G (0.72) alleles between blood, normal and cancer tissue in the patients.

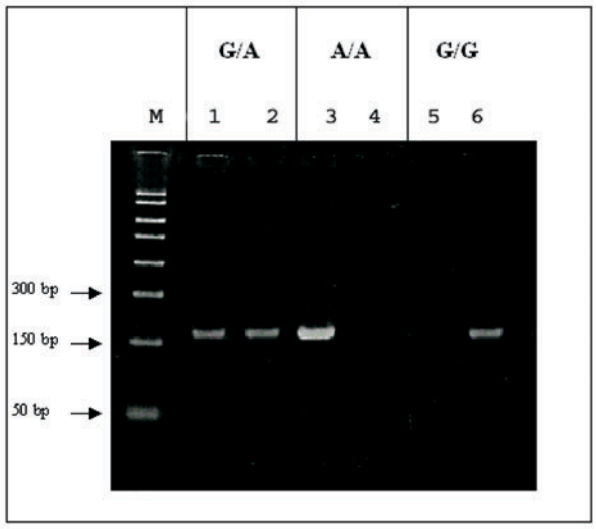

Figure 1. A typical result of allele specific polymerase chain reaction performed with a fragment of the promoter of *PAI-1* gene and analysed by 5% polyacrylamide gel electrophoresis, staining with ethidium bromide and viewed under ultraviolet light.

Lanes 1, 3 and 5 display the product of amplification with the primer pair specific for the A allele; lanes 2, 4 and 6, the G allele; M, molecular mass markers, 50–2000 bp (Sigma, St. Louis, U.S.A.).

The distributions of the G/A genotypes as well as the frequencies of the A and G alleles for colorectal cancer patients and control are shown in Table 1. It can be seen from the Table that there were significant differences ($P < 0.05$) between the two investigated groups. The frequencies of the A and G alleles were

Table 1. Distribution of G/G, G/A and A/A genotypes and frequencies of the G and A alleles in patients with colorectal cancer and controls

	Patients (n = 92)		Controls (n = 110)	
	Number	Frequency	Number	Frequency
A/A genotype	16	0.17	26	0.24
G/A genotype	19	0.21	52	0.47
G/G genotype	57	0.62	32	0.29
χ^2	23.644[a]		0.309[a]	
A allele	51	0.28[b]	104	0.47
G allele	133	0.72[b]	116	0.53

[a]$P < 0.05$ as compared with Hardy-Weinberg distribution; [b]$P < 0.05$ as compared with the controls.

0.28/0.72 in patients and 0.47/0.53 in controls. In patients the observed frequencies of the G/G, G/A and A/A genotypes differed significantly ($P < 0.05$) from the distribution expected from the Hardy-Weinberg equilibrium.

The dependencies of the distribution of genotypes and frequencies of alleles on the tumour stage evaluated according to Dukes criteria of patients with colorectal cancer are displayed in Table 2. There were no significant differences between the distributions of genotypes in the subgroups assigned to the histological stage and the distribution predicted by Hardy-Weinberg equilibrium ($P > 0.05$). There were no differences in the frequencies of the A and G alleles between the subgroups either ($P > 0.05$).

DISCUSSION

Genetic factors have been shown to influence protein level for several haemostatic factors (Błasiak *et al.*, 2000). The 1334G/A polymorphism of the *PAI-1* gene has been associated with interindividual differences in the

Table 2. Dependency of A/A, G/A and G/G genotypes and frequencies of the A and G alleles on tumour stage in patients with colorectal cancer[a]

Stage[b]	A (n = 25)		B (n = 40)		C (n = 27)	
	Number	Frequency	Number	Frequency	Number	Frequency
A/A genotype	7	0.28	7	0.18	7	0.26
G/A genotype	11	0.44	20	0.50	11	0.41
G/G genotype	7	0.28	13	0.32	9	0.33
χ^2	0.360[c]		0.028[c]		0.886[c]	
A allele	25	0.50	34	0.43	25	0.46
G allele	25	0.50	46	0.57	29	0.54

[a]n = 92; [b]according to Dukes criteria; [c]$P > 0.05$ as compared with Hardy-Weinberg distribution.

basal steady state level of its protein (Falk *et al.*, 1999). In addition, responses to environmental factors have been shown to differ by genotype (Green & Humphries, 1994).

Little is known about the possible role of the *PAI-1* gene polymorphisms in cancer. In our earlier studies we showed that the 4G/5G polymorphism and G → A substitutions at position –844 (G/A polymorphism) of *PAI-1* are not linked with the appearance of colorectal cancer (Smolarz *et al.*, 2001; Błasiak *et al.*, 2000). In the present study we show that there is association between the genotypes of the 1334G/A polymorphism in the propeptide coding region of *PAI-1* and the incidence of colorectal cancer in a group of 92 patients.

In an analysis of the exons of the *PAI-1* gene only one base change was identified at base pair number 1334 (numbering according to Bosma *et al.*, 1988) where both G and A were present. The base change (G1334 → A) causes a change of alanine at residue –9 in the propeptide of the PAI-1 molecule to threonine.

The 1334G/A polymorphism was found to be associated with severe bleeding problems, similar to those seen in patients with haemophilia (Falk *et al.*, 1999), but no data are available on the association or lack of such association in colorectal cancer. In view of the potentially significant role of PAI-1 for tumour spreading, it is important to know whether this polymorphism can account for the appearance and/or development of colorectal cancer.

It should be also taken into account that in addition to the genotype also environmental factors affect plasma PAI-1 levels. PAI-1 synthesis has been related to high blood levels of glucose, insulin and triglycerides (Eriksson *et al.*, 1995), sex hormones (Yang *et al.*, 1993) and angiotensin IV (Kerins *et al.*, 1995). Increased level of PAI-1 can be also linked with smoking habits (Eliasson *et al.*, 1994), alcohol consumption (Hendriks *et al.*, 1994) and acute infections (Pralong *et al.*, 1989).

In the present work a PCR method was used to screen 92 colon cancer patients for the 1334G/A polymorphism. We detected a significant difference in distribution frequency of alleles between patients and control ($P < 0.05$). The distribution of the genotypes in the patients differed from one expected from the Hardy-Weinberg equilibrium, with an overrepresentation of G/G homozygotes. It is possible that the presence of the G allele is in linkage disequilibrium with another, so far unknown, mutation located outside the coding region in the *PAI-1* gene, which may be of importance for the PAI-1 concentration in plasma.

On the other hand we did not detect any significant difference between the genotypes in subgroups assigned to histological stages, which suggests a lack of association between the polymorphism and colorectal cancer invasiveness. Moreover, we did not detect any significant difference between the genotypes in blood, distant mucosa samples and cancer tissue.

Our study implies that the 1334G/A polymorphism of *PAI-1* gene may be associated with the occurrence of colorectal cancer. Further studies, conducted on a larger group, are required to clarify this point.

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