Tumour invasion and metastasis require proteolytic degradation of the extracellular matrix (ECM) and the basement membrane to allow for the infiltration of tumour cells into the surrounding tissue, the bloodstream, and/or into the lymphatic vessels [1]. At least four different types of tumour-associated proteases may be responsible for tumour stroma degradation and tumour cell invasion. They are: cysteine proteases, aspartate proteases, matrix metalloproteases and serine proteases [2]. The components of the plasminogen activation system (urokinase type plasminogen activator uPA, specific plasminogen activator inhibitors PAI-1 and PAI-2 and the urokinase receptor uPAR) released by cancer cells stimulate tumour invasion and metastasis.

Genetic polymorphism of plasminogen activator inhibitor 1 (PAI-1) 1334G/A in patients with colorectal cancer

Hanna Romanowicz-Makowska¹, Beata Smolarz¹, Elżbieta Kozłowska¹, Karolina Przybyłowska², Andrzej Kulig¹

Introduction. High concentrations of plasminogen activator inhibitor 1 (PAI-1) are a poor predictive factor for colorectal cancer patients. The PAI-1 gene is highly polymorphic and it is supposed that its variability could contribute to the level of the PAI-1 biosynthesis.

Material and methods. We investigated the distribution of genotypes and frequency of alleles of the 1334G/A polymorphism in samples of cancer tissue, distant mucosa and in the blood of 100 patients with colorectal cancer. 1334G/A polymorphism was determined by PCR amplification using allele-specific primers.

Results. The distribution of the genotypes of 1334G/A polymorphism did not differ significantly (p > 0.05) from that predicted by the Hardy-Weinberg equilibrium. There were no differences in genotype distribution between blood, normal mucosa samples and cancer tissue. Additionally, we found no significant differences in frequencies alleles between colorectal cancer subjects and controls (p > 0.05).

Conclusion. Our results suggest that 1334G/A polymorphism of PAI-1 gene does not correlate with the development of colorectal cancer.

Key words: Plasminogen activator inhibitor 1 (PAI-1), 1334G/A polymorphism, colorectal cancer, PCR

Słowa kluczowe: Inhibitor aktywatorów plazminogenu typu 1 (PAI-1), polimorfizm 1334G/A, rak jelita grubego, PCR
invasion [3]. High levels of uPA, uPAR and PAI-1 are associated with poor prognosis in a number of malignancies [4, 5].

Patients with colorectal cancer can be cured by surgical treatment only if the cancer is detected at an early stage [6]. It is therefore important to identify high-risk or low-risk patients with the aid of suitable markers.

Many studies have shown that PAI-1 may be a useful prognostic marker in colorectal cancer [7-11]. The elevated level of PAI-1 can be associated with shorter recurrence-free survivors and shorter overall survivals. Changes in PAI-1 biosynthesis are usually preceeded by changes in its gene transcription and mRNA level [12]. Gene variability could contribute to the level of PAI-1 biosynthesis. Ten different polymorphisms within the PAI-1 gene have been described: A→G substitution in position +1334 in propeptide coding region, two (CA)n repeat polymorphisms, one in the promoter and one in the intron 4; the HindIII restriction fragment length polymorphism; an insertion (5G)/deletion (4G) polymorphism at position +11 053 and 9-nucleotide insertion/deletion located between nucleotides +11 345 and +11 345 in a three-fold repeated sequence, G→A substitution in position +12 078 [13-15].

In view of the potentially significant role of PAI-1 for tumour spread, it is important to determine whether this polymorphism can account for the appearance and/or development of colorectal cancer.

Among the polymorphic variants of the PAI-1 gene an insertion (5G)/deletion (4G) polymorphism was studied most frequently. It is associated with high plasma PAI-1 levels in patients with coronary artery disease [16, 17], myocardial infarction [18, 19] and diabetes [20, 21]. The role of 4G/5G polymorphism was also investigated in subjects with breast [22, 23], endometrial [24] and colorectal cancer [25, 26] but no association was observed between this sequence variation and cancer progression.

In our study we examined the relationship between 1334G/A polymorphism in the propeptide coding region of the PAI-1 molecule and the appearance and/or invasiveness of colorectal cancer. It has been reported that particular genotypes of 1334G/A polymorphism could be associated with a tendency to haemorrhage [15], but little is known about the possible role of this polymorphism in cancer.

We focused on the distribution of genotypes and the frequency of alleles of the 1334G/A polymorphism in patients with colorectal cancer.

**Materials and methods**

**Patients**

Tumour tissue, distant mucosa samples and blood were obtained from 100 patients (56 men and 44 women; mean age: 59 years; range: 37-68 yrs.) with colorectal cancer treated at the 2nd Department of Surgery of the Military Academy of Medicine in Łódź, Poland between 1997 and 2001. The patients' clinical and histopathological data was registered. All tumours were graded according to Dukes' stages: 29 in stage A, 50 in stage B and 21 in stage C. Blood samples from age-matched healthy individuals (n=106) served as control.

**DNA isolation**

DNA from cancer tissue and distant mucosa samples was extracted using the commercially available Qiagen Kit (Qiagen GmbH, Hilden, Germany) DNA purification kit according to manufacturer's instruction. Blood was mixed with an equal volume of buffer containing 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 µg/ml. After 2h incubation at 55°C proteinase K was added to a final concentration of 125 µg/ml and incubation continued for another 2h, then DNA was extracted: once with phenol and twice with chloroform.

**Determination of the 1334G/A polymorphism**

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’ [15]. The amplification resulted 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 step cycles. The reaction mixture contained 1 µg of genomic DNA, 0.2 µmol of each appropriate primer (ARK Scientific GmbH Biosystems, Darmstadt, Germany), 2.5 mM MgCl2, 1 mM dNTPs (Qiagen GmbH, Hilden, Germany) and 1 unit of Taq Polymerase (Qiagen GmbH, Hilden, Germany). PCR products were electrophoresed in a 5% polyacrylamide gel (PAGE) and visualised by ethidium bromide staining (Figure 1). 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 genotypes were scored. The observed numbers of each PAI-1 genotype were compared with that expected for a population acc. to the Hardy-Weinberg equilibrium by using the χ² test. The significance of the differences of observed alleles and genotypes between groups was tested using the χ² 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 (Figure 1).

Table I shows genotype distributions in normal mucosa samples, tumour tissue and blood. The distributions of the genotypes and the frequencies of the A and G alleles did not differ significantly (p > 0.05) from those predicted by the Hardy-Weinberg equilibrium. Additionally, there were no differences in the frequencies of the A (0.51) and G (0.49) alleles between blood, healthy tissue and cancer tissue.

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 II. It can be
seen that there were no significant differences (p>0.05) between the investigated groups. Frequencies of the A and G allele were 0.51/0.49 in cancer patients and 0.42/0.58 in controls. Among cancer patients the observed frequencies of the G/G, G/A and A/A genotype did not differ 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 grade evaluated according to Dukes’ criteria of patients with colorectal cancer are displayed in Table III. There were no significant differences between the distribution of genotypes in subgroups assigned to histological stage and the distribution predicted by the Hardy-Weinberg equilibrium.

Table I. Distribution of G/G, G/A and A/A genotypes and frequencies of the G and A alleles in blood, tumour tissue and in distant mucosa of patients with colorectal cancers

<table>
<thead>
<tr>
<th></th>
<th>Blood</th>
<th>Tumour tissue</th>
<th>Distant mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>number</td>
<td>frequency</td>
<td>number</td>
</tr>
<tr>
<td>A/A genotype</td>
<td>31</td>
<td>0.31</td>
<td>31</td>
</tr>
<tr>
<td>G/A genotype</td>
<td>40</td>
<td>0.40</td>
<td>40</td>
</tr>
<tr>
<td>G/G genotype</td>
<td>29</td>
<td>0.29</td>
<td>29</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td></td>
<td></td>
<td>3.987</td>
</tr>
<tr>
<td>A allele</td>
<td>102$^b$</td>
<td>0.51</td>
<td>102</td>
</tr>
<tr>
<td>G allele</td>
<td>98</td>
<td>0.49</td>
<td>98</td>
</tr>
</tbody>
</table>

$n = 100; ^b p > 0.05$ as compared with Hardy-Weinberg distribution

The dependencies of the distribution of genotypes and frequencies of alleles on the tumour grade evaluated according to Dukes’ criteria of patients with colorectal cancer are displayed in Table III. There were no significant differences between the distribution of genotypes in subgroups assigned to histological stage and the distribution predicted by the Hardy-Weinberg equilibrium.

Table II. 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

<table>
<thead>
<tr>
<th></th>
<th>Patients (n = 100)</th>
<th>Controls (n = 106)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>number</td>
<td>frequency</td>
</tr>
<tr>
<td>A/A genotype</td>
<td>31</td>
<td>0.31</td>
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<tr>
<td>G/A genotype</td>
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<td>0.40</td>
</tr>
<tr>
<td>G/G genotype</td>
<td>29</td>
<td>0.29</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>3.987$^a$</td>
<td></td>
</tr>
<tr>
<td>A allele</td>
<td>102$^b$</td>
<td>0.51$^b$</td>
</tr>
<tr>
<td>G allele</td>
<td>98</td>
<td>0.49$^b$</td>
</tr>
</tbody>
</table>

$^a p > 0.05$ as compared with Hardy-Weinberg distribution; $^b p > 0.05$ as compared with the controls
(p > 0.05). Neither were there any differences in the frequencies of the A and G alleles between the subgroups (p > 0.05).

**Discussion**

Genetic factors have been shown to influence the protein level of several haemostatic factors. The 1334G/A polymorphism of the PAI-1 gene has been associated with interindividual differences in the basal steady state level of its protein [27]. In addition, responses to environmental factors have been shown to differ by genotype [28].

In this study we have shown on a group of 100 patients that there is no association between the genotypes of the 1334G/A polymorphism of PAI-1 gene and the appearance of colorectal cancer. Moreover, we did not detect any significant difference between genotypes in subgroups assigned to histological stages. This suggests a lack of association between gene polymorphism and colorectal cancer invasiveness. Previously we have shown a similar relationship for 4G/5G and -844G/A polymorphism of PAI-1 gene for breast and colorectal cancers [23, 25, 26].

The 1334G/A polymorphism was found to be associated with severe bleeding problems, similar to those seen in patients with haemophilia [15], but no data is available on the association, or lack of such association, in colorectal cancer. Because the recent years have brought a development in the understanding of the prognostic values of the components of the plasminogen activation system in cancer progression, it is important to know whether polymorphic variants of the genes encoding the components can be considered as markers of appearance and/or progression of cancer.

It should also be considered that, in addition to genotype, a series of environmental factors affect plasma PAI-1 levels. PAI-1 synthesis has been related to high serum concentrations of glucose, insulin and triglycerides [29], sex hormone [30] and angiotensin [31]. An increased level of PAI-1 can be also linked with smoking habits [32], alcohol consumption [33] and acute infections [34].

The expression of PAI-1 undergoes changes during a variety of pathological processes, including cancer invasion and metastasis. The consequence of altered gene expression is the usually elevated level of PAI-1 protein observed in cancer. It is probable that gene variability could contribute to the level of PAI-1 biosynthesis [35].

In the analysis of the exons of the PAI-1 gene only one base change was identified at base pair number 1334 (number according to Bosma et al.) [35], where both G and A were present. The base change (G1334→A) causes a change of alanine at residue -9 in the propeptide of PAI-1 molecule to threonine. It is known that 1334G/A polymorphism is associated with the PAI-1 gene activity under interleukin-1 stimulation, that may influence the transcription of the gene [36]. Such influence is regulated by cytokines which are released by tumour cells.

Our study implies that the 1334G/A polymorphism of the PAI-1 gene may not be directly involved in the development of colorectal cancer. However, further research conducted on a larger population is needed to clarify this tentative conclusion.

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**Hanna Romanowicz-Makowska**

Laboratory of Molecular Genetics
Department of Pathology, Institute of Polish Mother’s Memorial Hospital
Rzgowska 281/289, 93-338 Lodz, Poland

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