PLASMINOGEN ACTIVATOR INHIBITOR–1 (PAI–1) LEVEL AND 4G/5G GENETIC POLYMORPHISM IN PATIENTS WITH COLORECTAL CANCER

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СОДЕРЖАНИЕ ИНГИБИТОРА–1 АКТИВАТОРА ПЛАЗМИНОГЕНА (ИАП–1) И ГЕННЫЙ ПОЛИМОРФИЗМ 4G/5G У БОЛЬНЫХ КОЛОРЕКТАЛЬНЫМ РАКОМ

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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 tumors predict poor prognosis. *PAI-1* gene is highly polymorphic, and an insertion (5G)/deletion (4G) polymorphism in the *PAI-1* gene promoter (the 4G/5G polymorphism), may have functional significance in *PAI-1* expression. In the present work PAI-1 level and distribution of genotypes and frequency of alleles of the 4G/5G polymorphism in 25 subjects with colorectal cancer in samples of cancer tissue and distant mucosa samples as well as in blood were studied. PAI-1 level was measured by ELISA, and the 4G/5G polymorphism was determined by PCR using allele specific primers. PAI-1 level in cancer tissue was significantly ($P < 0.05$) higher than in distant mucosa. No differences between genotypes of the 4G/5G polymorphism in distant mucosa, cancer tissue, and blood of each patient were found. The distribution of the genotypes in the population under study did not differ significantly ($P > 0.05$) from those predicted by Hardy-Weinberg distribution. The results support a hypothesis, that higher PAI-1 level is associated with colorectal cancer. Meanwhile 4G/5G polymorphism is not directly involved in the development of colorectal cancer.

Key Words: **plasminogen activator inhibitor-1 (PAI-1), *PAI-1* gene, gene polymorphism, colorectal cancer, prognostic marker.**

Содержание ингибитора-1 активатора плазминогена (ИАП-1) в экстрактах ткани колоректального рака имеет существенное прогностическое значение: высокое его содержание свидетельствует о неблагоприятном прогнозе. Ген, кодирующий ИАП-1, является в высокой степени полиморфным. Полиморфизм вставка (5G)/делеция (4G) в промоторе гена *ИАП-1* (полиморфизм 4G/5G) может иметь функциональное значение для экспрессии ИАП-1. В работе проанализированы содержание ИАП-1, распределение генотипов и частота аллелей полиморфизма 4G/5G в опухолевой ткани, в слизистой оболочке кишечника и в крови 25 больных колоректальным раком. Содержание ИАП-1 определяли методом ELISA, полиморфизм 4G/5G – методом ПЦР с праймерами, специфичными для каждого из анализируемых аллелей. Содержание ИАП-1 в ткани опухоли было значительно выше ($p < 0,05$), чем в слизистой оболочке участка кишки, взятого от опухоли. Не обнаружено различий между генотипами по полиморфизму 4G/5G у каждого конкретного больного в ткани опухоли, в нормальной слизистой оболочке и в крови. Распределение генотипов исследуемой популяции не отличалось от такового, предсказанного распределением Харди–Вайнберга. Полученные данные подтверждают гипотезу о том, что высокое содержание ИАП-1 связано с колоректальным раком. Однако полиморфизм 4G/5G может не иметь отношения к повышенному содержанию ИАП-1, наблюдаемому при колоректальном раке, и, таким образом, не ассоциирован с возникновением и/или прогрессированием заболевания.

Ключевые слова: **ингибитор-1 активатора плазминогена (ИАП-1), ген *ИАП-1*, генный полиморфизм, колоректальный рак, прогностический маркер.**

Breakdown of tissue boundaries by malignant cells represents a critical step in cancer progression facilitating invasion of surrounding normal tissues [1–2]. This process is mediated by serine proteinases and metalloproteinases [3]. The plasminogen activation system contains proteolytic factors that, released by cancer cells, can degrade extracellular matrix and promote tumor invasion and metastasis [4]. The system includes the urokinase type plasminogen activator (uPA), the tissue type plasminogen activator (tPA), the specific plasminogen activator inhibitors PAI–1 and PAI–2 and the urokinase receptor (uPAR). PAI–1, an approximately 50 kD glycoprotein belonging to the serine proteinase

Received: November 11, 1999.

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Abbreviations used: ELISA — enzyme linked immunosorbent assay; PAI–1 — plasminogen activator inhibitor–1; tPA — tissue type plasminogen activator; uPA — urokinase type plasminogen activator; uPAR — urokinase receptor.

inhibitor superfamily, is the major physiological inhibitor of the system.

PAI–1 was shown to be a prognostic marker in many cancers, including colorectal cancer [5]. The elevated level of PAI–1 can be associated with shorter recurrence–free and overall survival. Changes in PAI–1 biosynthesis are usually proceeded by changes in its gene transcription and mRNA level [6]. Gene variability could contribute to the level of the PAI–1 biosynthesis [7]. Nine different polymorphism patterns of the *PAI–1* gene have been described: two $(CA)_n$ repeat polymorphisms, one in the promoter and one in the intron 4; an *Hin*dIII restriction fragment length polymorphism; an insertion (5G)/deletion (4G) polymorphism at position –675 of the *PAI–1* gene promoter; two G → A substitutions at positions –844 and +9785; three 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 and G → A substitution in position +12 078. Among the variants of the *PAI–1* gene an insertion (5G)/deletion (4G) polymorphism (the 4G/5G polymorphism) was most frequently studied. Its location at the promoter of the gene indicated its possible role in the regulation of the *PAI–1* gene transcription. It was shown that particular genotypes of this polymorphism could be associated with cerebral sinus thrombosis [8], coronary arterial disease [9] and other vascular disturbances, but little is known on possible role of the 4G/5G polymorphism in cancer. In the present work the PAI–1 level and the distribution of genotypes and frequency of alleles of the 4G/5G polymorphism in subjects with colorectal cancer was investigated.

MATERIALS AND METHODS

Blood, tumor tissues and distant mucosa samples were obtained from 25 patients ranged in age from 37 to 69 years (mean age 56 years). All tumors were graded according to Dukes's stages. The cytosol fraction from tumor and mucosa samples was obtained by pulverisation with a detergent and ultracentrifugation. Protein was determined by Bradford method. PAI–1 antigen level was quantified by a sandwich enzyme linked immunosorbent assay (ELISA) using commercially available kit Imulyse PAI–1 (Biopol, Umea, Sweden). The assay was performed in triplicate and results were expressed in nanograms per milligram of total protein.

DNA was isolated by proteinase K digestion and phenol/chloroform extraction. Genotypes of the 4G/5G polymorphism were determined by polymerase chain reaction amplification of genomic DNA using the following allele specific primers: 5'–GTC TGG ACA CGT GGGGA–3' for the deletion 4G allele (Primer 1) and 5'–GTC TGG ACA CGT GGGGG–3' for the insertion 5G allele (Primer 2), each in a separate reaction together with the common downstream primer 5'–TGC AGC CAG CCA CGT GAT TGT CTAG–3' (Fig. 1). A fourth primer 5'–AAG CTT TTA CCA TGG TAA CCC CTGGT–3' located upstream of the polymorphic region was used as a positive control in the PCR reaction to verify the occurrence of DNA amplification in the absence of the allele in the genomic DNA [10]. The PCR was carried out in a MJ Research, INC thermal cycler, model PTC–150–16α–25 (Waltham, MA, USA). The thermal cycling conditions were 30 s at 94°C, 30 s at 54°C, 40 s at 72°C, repeated for 35 step cycles. 25 μl of PCR reaction contained 10 ng genomic DNA, 10 pmol of each appropriate primer (ARK Scientific GmbH Biosystems, Darmstad, Germany), 2.5 mM $MgCl_2$ 1 mM dNTPs (Boehringer, Mannheim, Germany) and 1 unit of Taq Polymerase (Promega Corporation, Madison, USA). PCR products were electrophoresed in a 5% polyacrylamide gel (PAGE) and visualised by ethidium bromide staining (Fig. 2). 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 in Hardy–Weinberg equilibrium using a χ^2 test. The significance of the differences of observed alleles and genotypes between groups was tested using the χ^2 analysis. The difference between PAI–1 levels in tumor tissue and distant mucosa samples was assayed by the *t*–Student test.

RESULTS AND DISCUSSION

According to the data of PCR analysis, all the patients were divided into three genotypes of the *PAI–1* gene promoter region: 4G/4G, 4G/5G and 5G/5G (Fig. 2). Table 1 shows genotype distribution between blood, tumor tissue and distant mucosa samples. For each patient the distributions were identical in all kind of sample and they did not differ significantly *(P* > 0.05) from those predicted by the Hardy–Weinberg distribution.

The results of the PAI–1 level measurements in tumor tissue and distant mucosa samples were displayed in Table 2. It can be seen that the level of PAI–1 in samples of tumor tissue was significantly *(P* < 0.05) higher than the level in distant mucosa samples. These

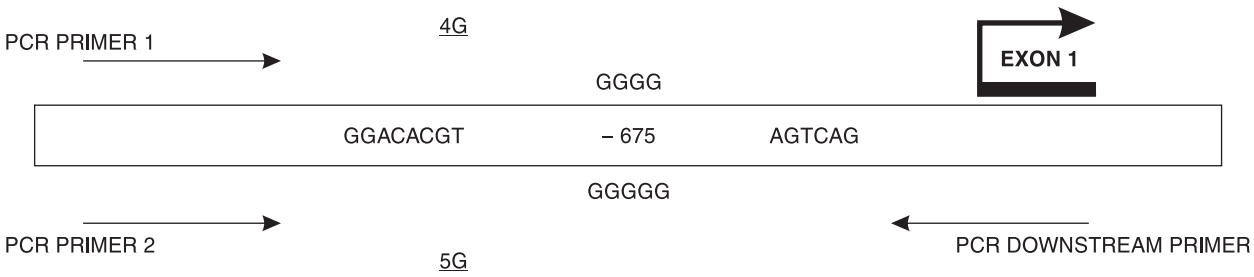

Fig. 1. PAI–1 promoter sequences comprising the region of the 4G/5G polymorphism

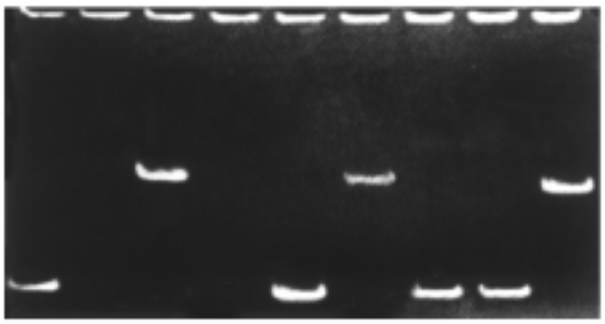

Fig. 2. A typical result of allele specific polymerase chain reaction performed with genomic DNA isolated from tumor tissues and analysed by a 5% polyacrylamide gel electrophoresis, stained with ethidium bromide and viewed under ultraviolet light.
Lanes *1, 4* and *7* display the product of amplification with a primer specific to the 5G allele; lanes *2, 5* and *8* — the 4G allele and lanes *3, 6* and *9* — controls

It is known that 4G/5G polymorphism is associated with the *PAI–1* gene promoter activity under interleukin–1 stimulation [9] that may influence the transcription of the gene. Such influence is regulated by cytokines which are released by tumor cells. Nevertheless no such effect has been shown so far. As mentioned above, the data on possible correlation between the polymorphism and occurrence or progression of cancer are scarce. There were no significant differences in the 4G/5G genotype distributions and allele frequencies between a small population of the advanced ovarian cancer cell lines and peripheral blood lymphocytes of healthy control [18]. It should be noted that in a separate study elevated levels of PAI–1 was found in tumor tissues obtained from patients with advanced ovarian cancer (FIGO IIIc) when compared to those in normal ovarian tissues [19].

Table 1. Distribution of 4G/5G genotypes and frequencies of the 4G and 5G alleles in blood, tumor tissue and distant mucosa samples of patients with colorectal cancer

	Blood		Tumor tissue sample		Distant mucosa sample	
	Number	Frequency	Number	Frequency	Number	Frequency
4G/4G genotype	10	0.40	10	0.40	10	0.40
4G/5G genotype	9	0.36	9	0.36	9	0.36
5G/5G genotype	6	0.24	6	0.24	6	0.24
χ^2				2.264[a]		
4G allele	29	0.58	29	0.58	29	0.58
5G allele	21	0.42	21	0.42	21	0.42

[a] $P > 0.05$ as compared with Hardy–Weinberg distribution.

Table 2. Average PAI–1 level in tumor tissue and distant mucosa samples in subjects with colorectal cancer[a]

	Average PAI–1 level[b]	
Genotype	Tumor tissue samples	Distant mucosa samples
4G/4G genotype	4.71	4.17
4G/5G genotype	4.65	3.77
5G/5G genotype	4.81	3.78
total	4.68*	3.93

[a]n = 25; [b](ng/mg protein); *$P < 0.05$ as compared with distant mucosa samples.

results confirm earlier observations that the level of PAI–1 is increased in colorectal cancer and support hypothesis about the use of PAI–1 level as a prognostic marker in this disease [5].

The 4G/5G polymorphism may be related to differential binding of proteins that influence its transcription [11]. Such connection between genotype and phenotype has been reported in vascular disease, but little is known about possible role of the 4G/5G polymorphism in cancer. In light of substantial evidence that the progression of colorectal cancer can be associated with elevated level of PAI–1, it seems reasonable to check a possible correlation between the polymorphism and clinical status of cancer patients. We did not find any correlation between 4G/5G genotypes and occurrence of cancer. It should be taken into account that in addition to genotype, a series of environmental factors affects plasma PAI–1 levels. PAI–1 synthesis has been related to high blood levels of glucose, insulin and triglycerides [12], sex hormone [13] and angiotensin IV [14]. Increased level of PAI–1 can be also linked with smoking habits [15], alcohol consumption [16] and acute infections [17].

Our study implies that 4G/5G polymorphism is not directly involved in the development of colorectal cancer but further research, conducted on larger population, are needed to clarify this point.

ACKNOWLEDGEMENTS

This work has been supported by grant 4 P05B 013 17 16 from the State Committee of Scientific Research (KBN).

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