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QUARTERLY

Plasminogen activator inhibitor-1 (PAI-1) gene 4G/5G promoter polymorphism is not associated with breast cancer✪

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The antigen content of plasminogen activator inhibitor-1 (PAI-1) in primary breast cancer tissue extracts may be of strong prognostic value: high levels of PAI-1 in tumors predict poor prognosis for patients. The gene encoding PAI-1 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 the distribution of genotypes and frequency of alleles of the 4G/5G polymorphism in subjects with breast cancer were investigated. Tumor tissues were obtained from 100 postmenopausal women with node-negative and node-positive ductal breast carcinoma with uniform tumor size. Blood samples from age matched healthy women served as control. The 4G/5G polymorphism was determined by PCR amplification using the allele specific primers. The distribution of the genotypes of the 4G/5G polymorphism in both control and patients did not differ significantly ($P > 0.05$) from those predicted by the Hardy-Weinberg distribution. There were no differences in the genotype distributions and allele frequencies between node-positive and node-negative patients. The 4G/5G polymorphism may not be linked with elevated level of PAI-1 observed in breast cancer and therefore may not be associated with appearance and/or progression of breast cancer.

Cancer progression, leading to its invasion and eventually to metastasis, is a multifactorial process that includes adherence to the basement membrane, secretion of proteolytic enzymes and cancer cell migration into vessels and lymphatic nodes followed by extravasation at distant sites [1]. A critical step of the progression is crossing tissue boundaries by the malignant cells which distinguishes proliferative disorders and carci-

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

noma *in situ* from true malignancy [2]. Two main boundaries are basement membrane and extracellular matrix (ECM) and their breakdown facilitates cancer cells invasion into the surrounding normal tissues [3]. This process is mediated by serine proteinases and metalloproteinases [4]. The plasminogen activation system contains proteolytic factors that, released by cancer cells, can degrade ECM and promote tumor invasion and metastasis [5]. 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 kDa glycoprotein belonging to the serine proteinase inhibitor superfamily, is the major physiological inhibitor of the system.

About two third of all breast cancer patients is node-positive and in general receives the adjuvant therapy, the other one third, which is node-negative, may receive or not such therapy depending on the chance of disease recurrence [6]. It is therefore important to identify high-risk node-negative breast cancer patients in order to fight the disease and avoid unnecessary chemotherapy in low-risk patients. Prognostic factors commonly used to such identification are tumor size, nuclear grade, steroid hormone receptor status [7].

PAI-1 was shown first to be a prognostic marker in both node-negative and node-positive breast cancers [8–11]. The elevated level of PAI-1 can be associated with shorter recurrence-free survival and shorter overall survival. Changes in PAI-1 biosynthesis are usually proceeded by changes in its gene transcription and mRNA level [12, 13]. Gene variability could contribute to the level of the PAI-1 biosynthesis [14]. Nine different polymorphisms of the *PAI-1* gene have been described: two $(CA)_n$ repeat polymorphisms, one in the promoter and one in the intron 4 [15, 16]; an *Hin*dIII restriction fragment length polymorphism [17]; an insertion (5G)/deletion (4G) polymorphism at position –675 of the *PAI-1* gene promoter [18]; two G → A substitutions at positions –844 and +9 785; 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 [14] and G → A substitution in position +12 078 [19]. In view of the potential significant role of PAI-1 for tumor spreading, it is important to know, whether these polymorphisms can account for the development and/or progression of breast cancer.

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 transcription of the *PAI-1* gene. It was shown that particular genotypes of this polymorphism could be associated with cerebral sinus thrombosis [20], coronary arterial disease [21, 22] and other vascular disturbances but little is known on possible role of the 4G/5G polymorphism in cancer. In our preliminary study we showed that this polymorphism might have been linked with an appearance of various types of cancer [23].

In the present work the distribution of genotypes and frequency of alleles of the 4G/5G polymorphism in women with node-negative and node-positive breast cancer was investigated.

MATERIALS AND METHODS

Breast cancer samples. Tumor tissues were obtained from 100 postmenopausal women with node-negative (n = 39) and node-positive (n = 61) ductal breast carcinoma treated at the Department of Gynaecological Oncology, Medical University of Łódź, between 1993 and 1997. No distant metastases were found in patients at the time of treatment. The patients ranged in age from 40 to 82 years (median age 58 years). Median fol-

low-up of patients still alive at the time of analysis was 39 months (range: 2–71 months). The average tumor size was 20 mm (range 17–32 mm). All tumors were graded by a method based on the criteria of Scarff-Bloom-Richardson [24, 25]. There were 20 tumors of I grade, 45 of II grade and 35 of III grade in total. In node-negative patients these numbers were: I — 9, II — 16, III — 14; in node-positive: I — 11, II — 29, III — 21. Steroid receptors status was not determined in the investigated group. Blood samples from age matched healthy women (n = 106) served as control.

The breast cancer tissue samples were fixed routinely in formalin and embedded in paraffin. Archival paraffin-embedded tumor sections on slides were deparaffinized in xylene and rehydrated in ethanol and distilled water. DNA was extracted using commercially available QIAmp Kit (Qiagen GmbH, Hilden, Germany) DNA purification kit according to manufacturer's instruction. Blood was mixed with equal volume of a 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. Following 2 h incubation at 55°C proteinase K was added to a final concentration of 125 μg/ml and incubation continued for additional 2 h, then DNA was extracted one 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

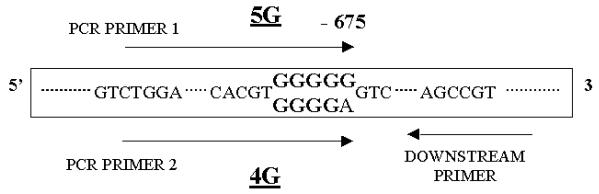

Figure 1. PAI-1 promoter sequences comprising the region of the 4G/5G polymorphism.

(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 [26]. The PCR was carried out in a MJ Research, INC thermal cycler, model PTC-150-162-25 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 of 10 × Taq buffer. The PCR cycle conditions were 94°C for 60 s, 54°C for 30 s then 72°C for 40 s, repeated for 35 cycles. The amplified DNA fragments were separated by a 5% polyacrylamide gel electrophoresis and, after staining with ethidium bromide, viewed under ultraviolet light. Each subject was classified into one of the three possible genotypes: 4G/4G, 4G/5G or 5G/5G.

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 in Hardy-Weinberg equilibrium by using a χ^2 test. The significance of the differences of observed alleles and genotypes between groups was tested using the χ^2 analysis.

RESULTS

From the PCR analysis, all the patients and controls 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 breast cancer patients and controls. Both distributions did not differ significantly ($P > 0.05$) from those predicted by the Hardy-Weinberg distribution. Additionally, there were no differences in the frequencies of the 4G and 5G alleles between patients and controls.

Distributions of the 4G/5G genotypes as well as the frequencies of the 4G and 5G alleles for node-positive and node-negative breast cancer patients are displayed in Table 2. It can be seen from the Table that there were no significant differences between these two groups in both genotype distribution and allele frequencies ($P > 0.05$).

Dependencies of the distribution of genotypes and frequencies of alleles on the tumor grade evaluated according to Scarf-Bloom-Richardson criteria in patients with node-positive and node-negative breast cancer are displayed in Table 3 and 4, respectively. There were no significant differences between distributions of genotypes in subgroups assigned to histological grades and the distribution predicted by Hardy-Weinberg equilibrium ($P > 0.05$). The were no differences in frequencies of the 4G and 5G alleles between subgroups either ($P > 0.05$).

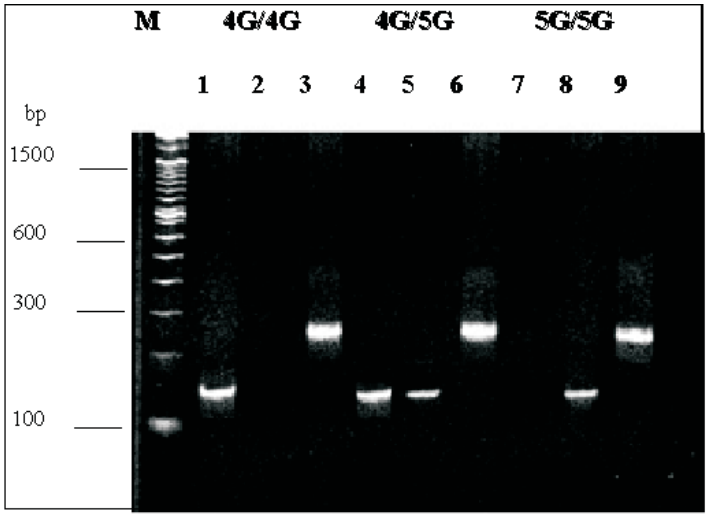

Figure 2. A typical result of allele specific polymerase chain reaction performed with a fragment of the *PAI-1* gene promoter and analysed by a 5% polyacrylamide gel electrophoresis, staining with ethidium bromide and viewed under ultraviolet light.

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. DNA of each genotype was prepared from the same donor. M denotes 100 bp DNA ladder marker.

DISCUSSION

The 4G/5G polymorphism may be related to differential binding of proteins that influence its transcription [21]. 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 evi-

Table 1. Distribution of 4G/5G genotypes and frequencies of the 4G and 5G alleles in patients with breast cancer and controls

	Breast cancer patients (n = 100)		Controls (n = 106)	
	Number	Frequency	Number	Frequency
4G/4G genotype	31	0.31	21	0.20
4G/5G genotype	40	0.40	48	0.45
5G/5G genotype	29	0.29	37	0.35
χ^2	3.987[a]		0.591[a]	
4G allele	102	0.51[b]	90	0.42
5G allele	98	0.49[b]	122	0.58

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

dence that the progression of breast cancer can be associated with elevated levels of PAI-1, it seems reasonable to check a possible correlation between the polymorphism and clinical status of breast cancer patients. In this work conducted on 100 ductal breast carcinoma patients we did not find any correlation between 4G/5G genotypes and occurrence of cancer. Moreover, we did not detect any significant difference between genotypes of node-positive and node-negative patient, that suggests a lack of association between 4G/5G genotype and breast cancer invasiveness.

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 [27, 29], sex hormone [30] and angiotensin IV [31]. Increased level of PAI-1 can be also linked with smoking habits [32], alcohol consumption [33] and acute infections [34].

Clinical studies have shown the association between 4G/5G polymorphism and plasma PAI-1 activity, the 4G allele being associated with higher levels of PAI-1, in young healthy controls and young myocardial infarction pa-

Table 2. Distribution of 4G/5G genotypes and frequencies of the 4G and 5G alleles in patients with node-positive and node-negative breast cancer

	Node-positive breast cancer patients (n = 61)		Node-negative breast cancer patients (n = 39)	
	Number	Frequency	Number	Frequency
4G/4G genotype	17	0.28	14	0.36
4G/5G genotype	24	0.39	16	0.41
5G/5G genotype	20	0.33	9	0.23
χ^2	2.732[a]		1.070	
4G allele	58	0.48[a]	44	0.56
5G allele	64	0.52[a]	34	0.44

[a] $P > 0.05$ as compared with node-negative patients

Table 3. Dependency of the distribution of 4G/5G genotypes and frequencies of the 4G and 5G alleles on the tumor grade in patients with node-positive breast cancer[a]

Grade[b]	I (n = 11)		II (n = 29)		III (n = 21)	
	Number	Frequency	Number	Frequency	Number	Frequency
4G/4G genotype	3	0.27	9	0.31	3	0.14
4G/5G genotype	5	0.46	12	0.41	9	0.43
5G/5G genotype	3	0.27	8	0.28	9	0.43
χ^2	0.089[c]		0.852[c]		0.085[c]	
4G allele	11	0.50	30	0.52	15	0.36
5G allele	11	0.50	28	0.48	27	0.64

[a] n = 61; [b] according to Scarf-Bloom-Richardson criteria; [c] $P > 0.05$ as compared with Hardy-Weinberg distribution

tients from Sweden [27] and in the larger French and Irish population of myocardial infarction patients and healthy controls of the ECTIM study [35]. Such association has been recently shown for large Italian [36] and Japanese [22] populations.

Despite many results suggesting an importance of the 4G/5G polymorphism in vascular diseases and prevalence of the 4G/4G genotype in these conditions, some reports suggest that the distributions of the 4G/5G genotypes among men who developed myocardial infarction or venous thromboembolism were virtually identical to those free of vascular diseases, and that relative risk of future thrombosis among those with the 4G/4G genotype compared with those with 4G/5G or 5G/5G genotype was just 1.02 [37]. It is worth noting that this study was conducted in a large cohort (almost 15 000) and there was no modification in the effect as a result of age, smoking, family history of vascular diseases and drug use. It was also shown that the 4G/5G polymorphism did not lead to an increased risk of myocardial infarction in young (not more than 45 years) men [38]. Populations of 241 patients and 179 controls were enrolled in that study.

Since the 4G/5G polymorphism is associated with the *PAI-1* gene promoter activity under interleukin-1 stimulation [22], it may influence the transcription of the gene through the regulation by cytokines released by tumor cells but so far no such effect has been shown.

Table 4. Dependency of the distribution of 4G/5G genotypes and frequencies of the 4G and 5G alleles on the tumor grade in patients with node-negative breast cancer[a]

Grade[b]	I (n = 9)		II (n = 16)		III (n = 14)	
	Number	Frequency	Number	Frequency	Number	Frequency
4G/4G genotype	2	0.22	4	0.25	7	0.50
4G/5G genotype	5	0.55	7	0.43	5	0.35
5G/5G genotype	2	0.22	5	0.32	2	0.15
χ^2	0.104[c]		0.238[c]		0.465[c]	
4G allele	9	0.50	15	0.47	19	0.68
5G allele	9	0.50	17	0.53	9	0.32

[a] n = 39; [b] according to Scarf-Bloom-Richardson criteria; [c] $P > 0.05$ as compared with Hardy-Weinberg distribution

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 [39]. 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 [40].

Our study implies that it is possible that the 4G/5G polymorphism of the *PAI-1* gene may not be directly involved in the development and/or progression of breast cancer but further research, conducted on larger population, are needed to clarify this point.

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