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Originals

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PCR Analysis of Matrix Metalloproteinase 3 (MMP-3) Gene Promoter Polymorphism in Ovarian Cancer

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Matrix metalloproteinases (MMPs) are proteolytic enzymes capable of degrading extracellular matrix. Their role has been emphasized in tumor invasion, metastasis and tumor-induced angiogenesis. The gene encoding MMP-3 is polymorphic and an insertion (6A)/deletion (5A) polymorphism (5A/6A polymorphism) in the MMP-3 gene may have functional significance in the regulation of its expression. In the present work the distribution of genotypes and frequency of alleles of the 5A/6A polymorphism in subjects with ovarian cancer were investigated. Paraffin embedded tumor tissues were obtained from 118 postmenopausal women with node-negative and node-positive ovarian cancer. The 5A/6A polymorphism was determined by PCR amplification using the allele specific primers. The distribution of the genotypes of the 5A/6A polymorphism in both control and study patients did not differ significantly ($p>0.05$) from those predicted by the Hardy-Weinberg distribution. There were no significant differences ($p>0.05$) in genotype distributions and allele frequencies between subgroups assigned to histological stage. The results suggest that the 5A/6A polymorphism of MMP-3 gene may not be linked with appearance and/or progression of ovarian cancer.

Introduction

Cancer progression, leading to 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 lymph nodes followed by extravasation at distant sites [21]. A critical step of the progression is crossing tissue boundaries by the malignant cells, which distinguishes proliferative disorders and carcinoma *in situ* from true malignancy [3]. Two main boundaries are basement membrane and extracellular matrix (ECM) and their breakdown facilitates cancer cells invasion into the surrounding normal tissues [14, 33]. This process is mediated by serine proteinases and metalloproteinases [15, 23, 24]. MMPs comprise a family of at least 17 proteolytic enzymes (collagenases, gelatinases, stromelysins) that degrade the extracellular matrix. Matrix metalloproteinase family members are involved in the physiological remodeling of tissues and embryonic development as well as pathological destruction of ECM components [3, 18]. Several of these enzymes have the unique ability to degrade the interstitial collagens (types I, II and III), the body's most abundant proteins [13].

Ovarian cancer, one of the most common gynecological malignancies, has an aggressive phenotype and a relatively poor prognosis; peritoneal dissemination and/or retroperitoneal lymph node metastases are found in two-thirds of patients at the time of diagnosis [5]. The ability of ovarian cancer cells to metastasize to different body sites is the major cause of morbidity and mortality among ovarian cancer patients. It is therefore important to identify high-risk or low-risk patients by suitable markers.

Overexpression of MMP proteins, including MMP-1, MMP-2, MMP-3 and MMP-9 has been demonstrated in various types of cancers and cell lines, and patients whose tumors express a high level of MMP have particularly poor prognoses [1, 6 - 9, 20]. Several reports demonstrated a correlation between the expression of matrix metalloproteinase 3 (MMP-3 or stromelysin 1) and the level of *ras* expressed in cells and with the cell ability to form tumors and with malignant potential [2, 19].

Changes in MMP-3 biosynthesis are usually proceeded by changes in its gene transcription and mRNA level [10, 16]. Gene variability could contribute to the level of the MMP-3 biosynthesis. *MMP-3* gene has 5A/6A polymorphism in the promoter region [34]. Its location at the promoter of the gene indicated its possible role in regulation of *MMP-3* gene transcription. In view of the potential significant role of MMP-3 for tumor spreading, it is important to know, whether 5A/6A polymorphism can account for the development and/or progression of ovarian cancer.

It was shown that particular genotypes of this polymorphism could be associated with rapid progression of

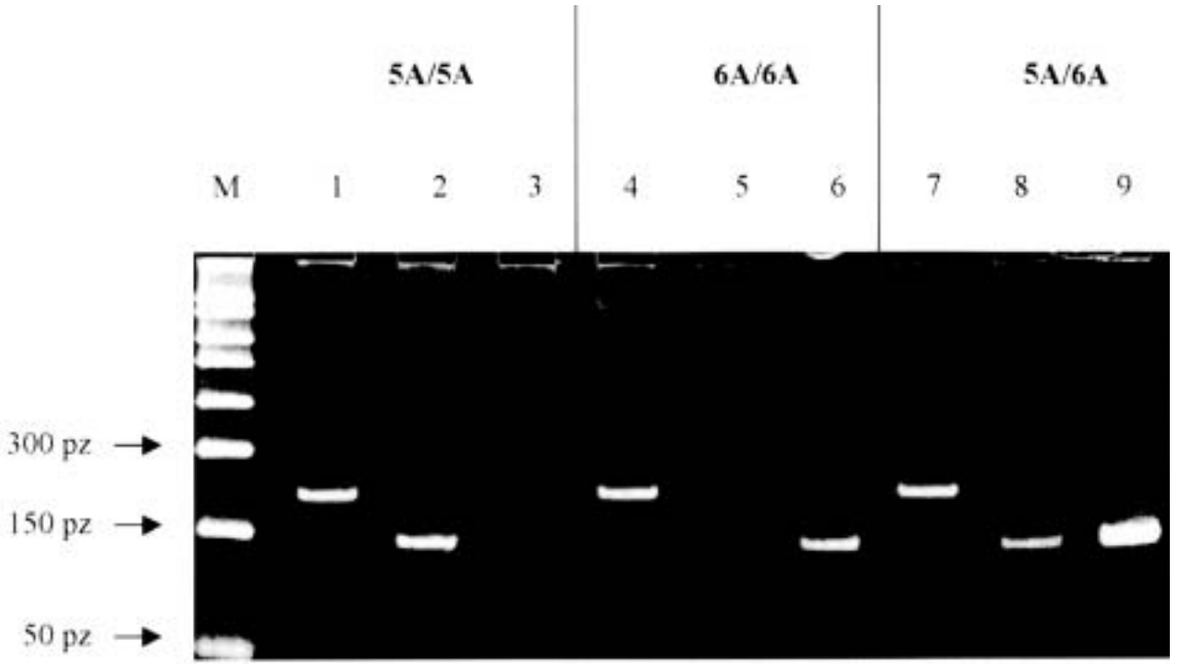

Fig. 1. A typical result of allele specific polymerase chain reaction performed with fragment of the promoter of the *MMP-3* gene and analysed by a 5% polyacrylamide gel electrophoresis, staining with ethidium bromide and viewed under ultraviolet light. Lanes 1, 4 and 7 display the product of amplification with a primers specific to controls; lanes 2, 5 and 8 - the 5A allele and lanes 3, 6 and 9 - the 6A allele. M denotes molecular weight ladder marker 50 - 2000 bp (Sigma, St. Louis, USA).

aneurysm [34], rheumatic diseases [27] and primary sclerosing cholangitis [30] but little is known on possible role of the 5A/6A polymorphism in cancer. In the present work the distribution of genotypes and frequency of alleles of the 5A/6A polymorphism in subjects with ovarian cancer were investigated.

Material and Methods

Ovarian cancer samples

Tumor tissues were obtained from 118 postmenopausal women with node-negative (n=57) and node-positive (n=61) ovarian cancer treated at the Department of Surgical Gynecology of the Institute of Polish Mother's Memorial Hospital between November 1997 and February 2001. No distant metastases were found in patients at the time of treatment. The patients ranged in age from 52 to 82 years (median age 57 years). All tumors were staged according to the criteria of the International Federation of Gynecology and Obstetrics (FIGO). There were 26 tumors of I stage, 51 of II stage and 41 of III stage in total. In node-negative patients these numbers were: I - 15, II - 22, III - 20; in node-positive: I - 11, II - 29, III - 21. Blood samples from age matched healthy women (n=110) served as control.

The 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 OIAmp 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, 0.8M urea, 20mM EDTA, 0.4M NaCl, 200mM Tris, pH 8.0, and RNase A was added to a final concentration of 100μg/ml. Following 2h-incubation at 55˚C proteinase K was added to a final concentration of 125μg/ml and incubation continued for additional 2h, then DNA was extracted once with phenol and twice with chloroform.

Determination of MMP-3 genotype

MMP-3 5A/6A promoter genotype was established for each subject by polymerase chain reaction (PCR) amplification of genomic DNA using the allele specific primers: 5'-GTA TGG GCT CGT AAAAAG-3' for the deletion 5A allele and 5'-GTA TGG GCT CGT AAAAAA-3' for the insertion 6A allele, each in a separate reaction together with the common downstream primer 5'-TGC AGC GCT GGT CGT TAG TGT GACT-3'. The fourth primer 5'-GAA CTT ATT CCA GGT TAA GGG TGCTG-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. The PCR was carried out in a DNA Thermal Cycler (GeneAmp

TABLE 1

Distribution of 5A/5A, 5A/6A and 6A/6A genotypes and frequencies of the 5A and 6A alleles in patients with ovarian cancer and controls

	Ovarian cancer patients (n = 118)		Controls (n = 110)	
	Number	Frequency	Number	Frequency
5A/5A genotype	37	0.31	26	0.24
5A/6A genotype	46	0.39	52	0.47
6A/6A genotype	35	0.30	32	0.29
χ^2	3.987[a]		0.309[a]	
5A allele	120	0.51[b]	104	0.47
6A allele	116	0.49[b]	116	0.53

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

TABLE 2

Distribution of 5A/5A, 5A/6A and 6A/6A genotypes and frequencies of the 5A and 6A alleles in patients with node-positive and node-negative ovarian cancer

	Node-positive ovarian cancer patients (n = 61)		Node-negative ovarian cancer patients (n = 57)	
	Number	Frequency	Number	Frequency
5A/5A genotype	17	0.28	20	0.35
5A/6A genotype	24	0.39	22	0.39
6A/6A genotype	20	0.33	15	0.26
χ^2	2.732[a]		1.070[a]	
5A allele	58	0.48[b]	62	0.54
6A allele	64	0.52[b]	52	0.46

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

PCR System 2400; Perkin-Elmer, Norwalk, CT, USA). The thermal cycling conditions were 30s at 94˚C, 30s at 57˚C, 40s at 72˚C, repeated for 35 step cycles. 25µl of PCR reaction contained 30ng genomic DNA, 0.2µmol of each appropriate primer (ARK Scientific GmbH Biosystems, Darmstad, Germany), 2.5mM $MgCl_2$, 1mM 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 visualized by ethidium bromide staining (Fig. 1).

Statistical analysis

The allelic frequencies were estimated by gene counting and genotypes were scored. The observed numbers of each *MMP-3* 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 *MMP-3* gene promoter region: 5A/5A, 5A/6A and 6A/6A (Fig. 1). Table 1 shows genotype distribution between ovarian cancer patients and controls. Both distributions 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 5A and 6A alleles between patients and controls.

Distribution of the 5A/6A genotypes as well as the frequencies of the 5A and 6A alleles for node-positive and node-negative ovarian cancer patients are summarized 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).

Correlation between the distribution of genotypes, frequencies of alleles and the tumor stage evaluated according to FIGO criteria in patients with node-positive and node-negative ovarian cancer are presented in Tables 3 and 4, respectively. There were no significant differences between distributions of genotypes in subgroups assigned to histological stage and the distribution predicted by Hardy-Weinberg equilibrium (p>0.05). There were no differences in frequencies of the 5A and 6A alleles between subgroups either (p>0.05).

TABLE 3

Dependency of the distribution of 5A/5A, 5A/6A and 6A/6A genotypes and frequencies of the 5A and 6A alleles on the tumor stage in patients with node-positive ovarian cancer[a]

Stage[b]	I (n = 11)		II (n = 29)		III (n = 21)	
	Number	Frequency	Number	Frequency	Number	Frequency
5A/5A genotype	3	0.27	9	0.31	3	0.14
5A/6A genotype	5	0.46	12	0.41	9	0.43
6A/6A genotype	3	0.27	8	0.28	9	0.43
χ^2	0.089[c]		0.852[c]		0.085[c]	
5A allele	11	0.50	30	0.52	15	0.36
6A allele	11	0.50	28	0.48	27	0.64

[a]n=61; [b]according to FIGO criteria; [c]p>0.05 as compared with Hardy-Weinberg distribution

TABLE 4

Dependency of the distribution of 5A/5A, 5A/6A and 6A/6A genotypes and frequencies of the 5A and 6A alleles on the tumor stage in patients with node-negative ovarian cancer[a]

Stage[b]	I (n = 15)		II (n = 22)		III (n = 20)	
	Number	Frequency	Number	Frequency	Number	Frequency
5A/5A genotype	4	0.27	6	0.27	9	0.45
5A/6A genotype	7	0.47	9	0.41	7	0.35
6A/6A genotype	4	0.26	7	0.32	4	0.20
χ^2	0.104[c]		0.238[c]		0.465[c]	
5A allele	15	0.50	21	0.48	25	0.63
6A allele	15	0.50	23	0.52	15	0.37

[a]n = 57; [b]according to FIGO criteria; [c]p>0.05 as compared with Hardy-Weinberg distribution

Discussion

The expression of *MMP-3* undergoes changes during variety of pathological process, for example cancer invasion and metastasis [7 - 9, 26]. Matrix metalloproteinases facilitate cellular invasion by degrading the extracellular matrix and their regulation is partially dependent on transcription [2]. The 5A/6A polymorphism may be related to differential binding of proteins that influence its transcription [4, 11]. Such connection between genotype and phenotype has been reported in vascular disease, but little is known about possible role of the 5A/6A polymorphism in cancer. In the light of substantial evidence that the progression of ovarian cancer can be associated with elevated levels of MMP, it seems reasonable to check a possible correlation between the polymorphism and clinical status of ovarian cancer patients.

In this work performed on 118 ovarian cancer patients we did not find any correlation between 5A/6A genotypes and occurrence of the cancer. Moreover, we did not detect any significant difference between genotypes in subgroups assigned to histological stages, that suggests a lack of association between 5A/6A genotype and ovarian cancer invasiveness.

It should be taken into account that in addition to genotype, a series of environmental factors affects plasma MMP-3 levels. The consequence of altered expression is usually elevated levels MMP-3 protein observed in cancer patients [1, 7 - 9, 16]. It is supposed that gene variability could contribute to the level of the MMP-3 biosynthesis.

The location 5A/6A polymorphism at the promoter of the *MMP-3* gene indicates their possible role in the regulation of its expression at transcriptional level. The 5A/6A polymorphism may be associated with regulation of the *MMP-3* promoter activity by Ets factors [11, 32]. The Ets transcription factor family comprises more than 30 members and shares a highly conserved DNA binding motif terms ETS domain. Ets recognizes a specific nucleotide sequence with a GGAA/T core [28, 31]. Binding sites for members of the Ets family of transcription factors is present within *MMPs* promoters and is potent positive regulators [29].

Erg and Ets family member differentially regulates *MMP-3* gene expression by physically interacting with the Fos/Jun complex [11]. Fos and Jun transcription factors, products of *jun* and *fos* oncogenes, participate in the formation of AP-1 protein, which functions as homodimers Jun/Jun and heterodimers Jun/Fos. Ets stimulates transcrip-

tion factor AP1 that binds to the area of promoter *MMP-3* [4]. It is supposed that the 5A/6A polymorphism may be associated with the *MMP-3* gene promoter activity under interleukin and tumor necrosis factor α (TNFα) stimulation and it may influence the transcription of the gene through the regulation by cytokines released by tumor cells [22, 25].

The role of 5A/6A polymorphism in abdominal aortic aneurysm (AAAs), rheumatic diseases and in primary sclerosing cholangitis (PSC) was investigated [12, 27, 30, 34]. In these studies 5A allelic frequencies were increased and 6A homozygosity was reduced. These data support hypothesis about the use of 5A/6A polymorphism as a prognostic marker in AAAs, rheumatic and PSC disease [27, 30, 34].

However little is known about possible role of *MMP-3* gene 5A/6A polymorphism in cancer. Because much knowledge has been gained in recent years on the prognostic values of the MMP-3 in cancer progression, it is important to know whether polymorphic variants of the gene encoding this protein can be considered as markers of appearance and/or progression of cancer. Ghiraldi et al. showed that the presence of 5A polymorphism at the *MMP-3* promoter region may represent an unfavorable prognostic feature in breast cancer patients associated with more invasive disease [17].

Our study implies that it is possible that the 5A/6A polymorphism of the *MMP-3* gene may not be directly associated with development of ovarian cancer but further research, including larger population, are needed to clarify this point.

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