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Antigen levels of urokinase-type plasminogen activator receptor and its gene polymorphism related to microvessel density in colorectal cancer

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We determined the distribution of genotypes and frequencies of alleles of the $(CA)_n$ repeat polymorphism in intron 3 of the urokinase plasminogen activator receptor (uPAR) gene, uPAR antigen levels and microvessel density (MVD) in tumour and distant mucosa samples from 52 patients with colorectal cancer. The uPAR level was higher for patients with high MVD comparing to patients with lower MVD which may suggest that uPAR can be correlated with progression of colorectal cancer. The significant relationship between the high MVD and uPAR antigen level appeared to be independent of the $(CA)_n$ repeat polymorphism because no differences in the level of uPAR antigen between carriers of alleles were found. The received results, indicate that uPAR might be considered as a target in colorectal cancer patients' therapy.

Keywords: plasminogen activation system, urokinase type plasminogen activator receptor (uPAR), gene polymorphism, colorectal cancer, cancer progression, angiogenesis, microvessel density

INTRODUCTION

The vascularization in human colorectal tumour has been reported to be a significant predictor of an increased risk of haematogenous metastatic disease (Shpitz *et al*., 2003; Pang & Poon, 2006; Rajaganeshan *et al*., 2007). The increase in tumour vessel density raises the chance for cancer cells to enter the circulation (Sieczkiewicz *et al*., 2002). Newly formed vessels or capillaries have leaky and weak basement membranes that can be penetrated by cancer cells more easily than those of mature vessels (Nagy *et al*., 1998). Additionally, escape of tumour cells into the tumour neovasculature is facilitated by the degradative enzymes secreted by the endothelial cells on the tips of growing capillaries, and recent data suggest that the urokinase plasminogen activator (uPA) system and matrix metalloproteinases (MMP) are essential for this process (Baker & Leaper, 2003; Kim *et* al., 2006). The urokinase plasminogen activator receptor (uPAR) is anchored on the cell-surface membrane and specifically recognizes pro- and active uPA (Nielsen *et al*., 1988). The binding of uPA to uPAR strongly enhances uPA-mediated plasminogen activation and localizes the proteolytic activities on the cell surface (Reuning *et al*., 1997). The receptor-bound uPA catalyses the formation of plasmin on the cell surface to generate the proteolytic cascade that contributes to the breakdown of basement membranes and extracellular matrix (Ploug *et al*., 1993; Pepper, 2001). In many types of cancer one can observe increased

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Abbreviations: ECM, extracellular matrix; MMP, matrix metalloproteinases; MVD, microvessel density: PAI-1, plasminogen activator inhibitor type-1; PBS, phosphate-buffered saline; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; VEGF, vascular endothelial growth factor.

levels of uPA, uPAR and PAI-1 in tumour in comparison to normal tissue. Therefore these factors might have an impact on the cancer development (Błasiak *et al*., 2000; Przybyłowska *et al.*, 2001; 2002; Seetoo *et al*., 2003). Because of the essential role of uPA system components in tumour invasion and angiogenesis, it is important to determine if alterations in *uPA, uPAR* and *PAI-1* genes may influence on cancer development and progression. We have recently observed significantly higher levels of uPA antigens in colon cancer samples with the genotype C/C of the C/T polymorphism in exon 6 of the *uPA* gene (Przybyłowska *et al*., 2001) These results suggest that this polymorphism might not be associated with the occurrence of cancer, but the higher level of uPA in tumour samples with the C/C genotype indicates that the C allele may be involved in the development and/or progression of this disease (Przybyłowska *et al*., 2001; 2002). The 4G/5G polymorphism of the *PAI-1* gene has been associated with increased plasma levels of PAI-1 and has been linked with coronary heart disease and/or myocardial infarction (Hamsten *et al*., 1987; Eriksson *et al*., 1995). Our previous study implies that 4G/5G and 1334G/A polymorphism is not directly involved in the development of colorectal and breast cancer (Smolarz *et al*., 2003; Błasiak & Smolarz, 2003). Increased levels of uPAR in tumour and serum are associated with worse prognosis for patients with colon cancer. This may be linked with enhanced tumour angiogenesis dependent on the uPA system (Seetoo *et al*., 2003). Due to the significance of uPAR production in cancer, it is important to check whether it can be affected by genetic variability. Four polymorphisms of the *uPAR* gene have been described: a $(CA)_n$ repeat in intron 3, two restriction fragment length *Pst*I and one *Eco*RI polymorphisms (Borglum *et al*., 1991; 1992; Kohonen-Corish *et al*., 1996). This study was designed to investigate the relationship between the level of uPAR, MVD and frequency of alleles of the $(CA)_n$ repeat polymorphism in colon cancer.

PATIENTS AND METHODS

Patients. Tumour tissues and distant mucosa samples were obtained from 52 patients with colorectal cancer treated at the 2nd Department of Surgery, Military Academy of Medicine, Łódź, Poland since 2000. There were 34 males and 18 females and their median age was 69 years (quartiles: 52, 74 years). All patients had histologically-confirmed advanced adenocarcinoma. Pathologic staging was obtained by grading according to WHO classification: G1 (n = 16), G2 (n = 27), G3 (n = 9); and the Dukes' criteria: A (n = 12), B (n = 23), C (n = 11) and D (n = 6). Control samples consisted of DNA extracted from blood collected from sex and age matched 60 individuals without cancer.

Determination of the *uPAR* genotype. DNA from tumour tissue, distant mucosa and blood samples was isolated by proteinase K digestion and phenol/chloroform extraction. The genotype was determined by short tandem repeat PCR (STR-PCR), using primers of flanking region that contains $(CA)_n$ repeat in intron 3 of the *uPAR* gene. PCR was carried out in an MJ Research, INC thermal cycler, model PTC-100 (Waltham, MA, USA), in a total volume of 25 µl, containing 50 ng genomic DNA, 10 pmol each primer: (GT strand) 5′ CTT ACA TGA TGC TGG GAA CAC 3′ and ^{32}P-labelled (CA strand) 5′ TGG TGA TGG ATG GGT CTG TC 3′ (Eurogentec, Seraing, Belgium), 200 µM each dATP, dCTP, dGTP and dTTP, 20 mM Tris/HCl, (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, and 1 unit *Taq* polymerase (Qiagen GmbH, Hilden, Germany) (Kohonen-Corish *et al*., 1996). The thermal cycling conditions were: 3 min at 94°C, followed by 35 cycles of 30 s at 95°C, 1 min at 60°C and 1 min at 72°C. To determine allele size the PCR products were subjected to electrophoresis in 6% polyacrylamid formamide sequencing gels. Gels were then dried and exposed to X-ray film (Fig. 1).

The uPAR antigen levels. Samples of tumour tissue and distant mucosa of 50 mg wet mass were pulverised at 4°C in TBS buffer (20 mM Tris/HCl pH 8.5, 125 mM $NaCl_2$, 1% Triton X-100). The suspension was ultracentrifuged (105 000 × ***g***) at 4°C for 45 min and the resulting cytosol fractions were collected and immediately used. uPAR antigen level was quantified by sandwich enzyme linked immunosorbent assay (ELISA) using commercially available IMBIND Total uPAR ELISA kit (American Diagnostica Inc., Greenwich, CO, USA). Absorbance was measured at 450 nm and the antigen levels in ng/ml were obtained from standard curves. The uPAR antigen content was expressed as nanograms of analysed

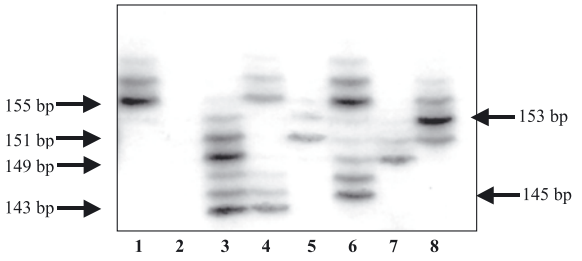

Figure 1. Autoradiogram of SRT-PCR products containing fragments of intron 3 of *uPAR* gene with highly polymorphic $(CA)_n$ region analysed by 6% polyacrylamid formamide sequencing gel electrophoresis.
Lanes display bands for: 1 – 155bp/155bp, 3 – 149/143, 4 – 155bp/143bp, 5 – 151bp/151bp, 6 – 155bp/145bp, 7 – 149bp/149bp, 8 –153/bp153bp.

per milligram of tissue protein. Protein was assayed by the Bradford method (Bradford, 1976).

Microvessel staining and evaluation. All tissue samples of primary colorectal cancer were fixed in 10% buffered formalin and routinely processed for light microscopy. Sections (4-mm thick) were dewaxed in xylene and dehydrated in ethanol. The sections were predigested with protease for 20 min at 37°C and then immersed in 3% H_2O_2 for 30 min to inhibit endogenous peroxidase. After washing with PBS, they were incubated in normal rabbit serum for 30 min, followed by incubation overnight with anti-factor VIII polyclonal antibody (DAKO, Corp.) at a 1 : 50 dilution. The sections were then incubated with biotinylated rabbit antimouse IgG for 15 min. Peroxidase-conjugated with streptavidin was used at a dilution of 1 : 200. After washing in PBS, the slides were developed by immersing into 0.01% H_2O_2 and 0.05% diaminobenzidine tetrahydrochloride for 2 min. Normal mouse IgG was substituted for the primary antibody in the negative control. The sections were counterstained with hematoxylin. After staining, blood vessels appeared intensely brown in colour, which facilitated identification and quantification.

Microvessels within the tumour were counted in immunohistochemically stained sections under light microscopy as an indicator of tumour angiogenesis (factor VIII) (Kumar *et al*., 1998). After screening the areas with intense neovascularization at low magnification (× 40 or × 100), microvessels were counted in a × 200 field. In all samples, the mean value of microvessel number was calculated from four different fields (Fig. 2).

Statistical analysis. The observed numbers of genotypes were compared with those expected for a population in Hardy-Weinberg equilibrium using the χ^2 test. The significance of the observed alleles and genotype differences between groups was tested using χ^2 analyses. To determine normal distribution, the Shapiro-Wilk test was used. Student's *t*-test (for normal distribution) or Mann-Whitney test (for non-normal distribution) was used to compare each parameter between two groups. An ANOVA test was first used to identify parameters that would cause significant differences between more than two groups; Scheffe's test was then used to investigate the significance of difference in each identified parameter between any two groups. Analyses were performed using STATISTICA 6.0 (Statsoft, Tulsa, OK, USA).

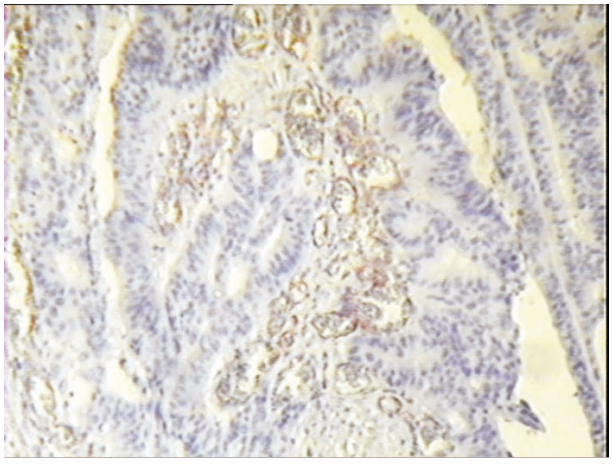

Figure 2. Immunohistochemical staining for factor VIII of primary colorectal carcinoma.
Microvessels are represented by brown capillaries or clusters.

Table 1. Clinicopathologic parameters and microvessel density of colorectal cancer samples

Microvessel count per field (mean ± S.D.)	
Total	94.13 ± 34.19
Sex	
Male	93.76 ± 31.41
Female	91.18 ± 24.85
Grading (WHO)	
G1	95.75 ± 29.41
G2	88.57 ± 31.12
G3	102.61 ± 40.51
Dukes' stage	
A	90.57 ± 34.78
B	96.33 ± 24.04
C/D	118.31 ± 31.51

RESULTS

Table 1 shows the clinicopathologic parameters and MVD of colorectal cancer samples. No differences ($P > 0.05$) in the MVD of colorectal cancer samples between tumour variables were observed.

Table 2. The $(CA)_n$ repeat polymorphism in intron 3 of the *uPAR* gene. Frequencies of alleles by patients with colorectal cancer and in blood samples of controls

Allele $(CA)_n$ repeat	Frequency of alleles		
	Colorectal cancer patients (n = 52)		Controls (n = 60)
Length (bp)	Tumour tissue	Distant mucosa	Blood
139	0.016	0.016	0.033
141	0.081	0.081	0
143	0.016	0.016	0.050
145	0.032	0.032	0.033
147	0.452	0.452	0.483
149	0.161	0.161	0.183
151	0.112	0.112	0.133
153	0.016	0.016	0
155	0.048	0.048	0.050
159	0.045	0.045	0.033

Table 3. The $(CA)_n$ repeat polymorphism in intron 3 of the *uPAR* gene.

Frequencies of alleles by patients with colorectal cancer and tumour variables.

Allele $(CA)_n$ repeat	Frequency of alleles				
	Grading		Dukes' stage		
Length (bp)	G1 (n = 16)	G2/ G3 (n = 36)	A (n = 12)	B (n = 23)	C/D (n = 17)
139	0	0.022	0	0	0.05
141	0.05	0.087	0.125	0.076	0.05
143	0	0.022	0.062	0	0
145	0	0.043	0	0.076	0
147	0.7	0.304	0.375	0.269	0.6
149	0.1	0.195	0.25	0.153	0.15
151	0.1	0.131	0	0.269	0.05
153	0.5	0.022	0	0.076	0
155	0	0.065	0.125	0.038	0
159	0	0.065	0.062	0.038	0.05

Table 4. Average uPAR antigen levels in tumour tissue and distant mucosa samples in patients with colorectal cancer

Allele $(CA)_n$ repeat	Distant mucosa	Tumour tissue
Length (bp)	Antigen uPAR levels (ng/mg protein)	
	Median (quartiles)	Median (quartiles)
139	0.239 (–;–)	0.283 (–;–)
141	2.143 (–;–)	3.266 (–;–)
145	0.172 (–;–)	1.118 (–;–)
147	1.018 (0.235; 2.227)	0.734 (0.390; 3.755)
149	1.792 (0.235; 3.127)	3.447 (0.696; 4.881)
151	1.334 (0.736; 1.792)	2.835 (0.364; 4.881)
155	1.173 (–;–)	2.212 (–;–)
157	2.454 (–;–)	2.355 (–;–)
159	1.985 (–;–)	3.092 (–;–)
total	1.792 (0.234; 2.455)	2.356 (0.617; 4.208)

*$P < 0.05$ as compared with distant mucosa samples.

Table 5. Average uPAR antigen level in tumour tissue and distant mucosa samples in patients with colorectal cancer and tumour variables

Average uPAR level (ng/mg protein)		
	Distant mucosa sample Median (quartiles)	Tumour tissue sample Median (quartiles)
Grading (WHO)		
G1	1.79 (0.95; 2.14)	3.27 (0.62; 4.88)
G2	1.03 (0.23; 2.45)	1.46 (0.73; 3.09)
G3	1.27 (0.57; 1.89)	2.41 (0.30; 4.52)
Dukes' stage		
A	2.26 (0.21; 3.13)	2.35 (0.39; 3.45)
B	0.87 (0.23; 2.14)	1.14 (0.69; 3.27)
C/D	1.73 (0.65; 2.05)	2.43 (0.31; 4.32)

Ten alleles of $(CA)_n$ repeat polymorphism in intron 3 of the *uPAR* gene were detected in the control and the patients. Table 2 shows the $(CA)_n$ repeat polymorphism frequencies of alleles in tumour tissue and distant mucosa samples from colorectal cancer patients and blood samples from controls. Both kinds of samples from each patient had the same genotype. There were no differences ($P > 0.05$) between the frequencies of alleles in patients and controls, but two alleles (141 bp, 153 bp) were detected only in patients. A distinct preference of heterozygotes (67% – patients, 64% – controls) was observed. Table 3 shows the frequencies of alleles of $(CA)_n$ repeat polymorphism in intron 3 of the *uPAR* gene by patients and tumour variables. There were no differences ($P > 0.05$) between the distributions of the genotypes in these groups.

The results of the uPAR antigen level measurements in tumour tissues and distant mucosa samples are presented in Table 4. The uPAR antigen level was compared for different groups of homozygotic and heterozygotic patients depending on allele present. A significant ($P < 0.05$) increase of the uPAR antigen level in tumour tissue samples was observed as compared with distant mucosa. No differences in the level of uPAR antigen between the alleles of the $(CA)_n$ repeat polymorphism was found. Table 5 shows relationships between the median levels of uPAR antigen in tumour tissues with tumour characteristics. No differences ($P > 0.05$) in the level of uPAR antigen between tumour variables were detected.

Table 6 shows relationships between allele frequencies and the median levels of uPAR antigen and microvessel density. The level of uPAR antigen in tumour samples with 126.92 ± 27.48 microvessels per field was significantly ($P < 0.05$) higher then the level in samples with 54.14 ± 5.01 or 83.83 ± 9.95 microvessels per field. Additionally, no differences in the MVD between the alleles of the $(CA)_n$ repeat polymorphisms were found.

DISCUSSION

In intratumoural angiogenesis, one of the first mechanisms activated during cancer cell inva-

Table 6. Frequencies of alleles and average uPAR antigen level in tumour tissue and distant mucosa samples of patients with colorectal cancer and microvessel density

	Microvessel count per field (mean ± S.D.)		
	Low MVD	Medium MVD	High MVD
Alleles	0–60 per ×200 field	60–100 per ×200 field	>100 per ×200 field
$(CA)_n$ repeat	(5414 ± 501)	(8383 ± 995)	(12692 ± 2748)
Length (bp)	Frequency of alleles		
139	0.036	0	0
141	0.142	0	0.083
143	0.036	0	0
145	0.036	0	0.083
147	0.250	0.545	0.583
149	0.320	0.136	0
151	0.071	0.227	0.083
153	0.036	0	0
155	0.071	0.045	0
159	0.036	0.045	0
	Antigen uPAR levels (ng/mg protein)		
	Median (quartiles)	Median (quartiles)	Median (quartiles)
Tumour tissue	0.736 (0.212; 0.870)	1.115 (0.364; 3.209)	3.357 (1.774; 4.882)*
Distant mucosa	0.734 (0.390; 1.463)	1.029 (0.283; 2.745)	2.131 (1.792; 2.387)

* $P < 0.05$ as compared with 0–60 and 60–100 microvessel count samples.

sion and metastasis is peritumour proteolysis. The two predominant proteinase systems involved in ECM proteolysis are the plasminogen activators and matrix metalloproteinases which act in the pericellular environment through a series of interacting activation cascades regulated by specific inhibitors. uPA specifically generates plasmin, which has been shown to initiate the autoactivation of many MMPs (Pepper, 2001; Gillette *et al*., 2003). The hypothesis of an uPA system role in angiogenesis is supported by two observations: neovascularization inhibition by an antagonist of uPA and a correlation between microvessel density and uPA levels in cancer (Hildenbrand *et al*., 1995; Min *et al*., 1996). Recently, it has been reported that endostatin inhibits angiogenesis through down-regulation of the PA system in a colorectal cancer cell line (Reijerkerk *et al*., 2003; Dkhissi *et al*., 2003). Thus, the inhibition of uPA and/or uPAR activity may inhibit not only tumour invasion, but also angiogenesis in colorectal cancer, and uPA and/or uPAR can be used as a marker of colorectal cancer biological behaviour.

In the present work a significantly higher level of uPAR antigen was observed in samples with high microvessel density (> 100 per field) than in samples with low (< 60 per field) or medium density (60-100 per field). Additionally, we found the level of uPAR antigen in tumour samples to be higher than the level in distant mucosa samples. These results confirm earlier observations on increased level of uPAR in colorectal cancer and an association between the uPA system and angiogenesis. VEGF has been shown to cause up-regulation of uPA and uPAR in endothelial cells (Mandriota *et al*., 1995). Moreover, a previous study demonstrated that the PA system and VEGF synergistically contributed to liver metastasis of colorectal cancer (Baker *et al*., 2000; Konno *et al*., 2001).

VEGF promotes proliferation of endothelial cells and tube formation after degradation of the ECM by the PA system and/or MMPs (Zheng *et al*., 2006; Kong *et al*., 2007). Interestingly, stepwise analysis demonstrated that uPA mRNA, uPAR mRNA and VEGF protein expression were significantly correlated with MVD. The uPA, uPAR and VEGF produced by colon cancer cells destroy ECM, which may promote migration of both cancer cells and endothelial cells. On the other hand, cancer cells with high invasive ability may have various malignant potentials, including VEGF production. Since we found a positive correlation between high MVD and higher level of uPAR antigen, it is possible that the PA system enhances VEGF-induced tumor angio-

genesis, which is in agreement with a study in gastric cancer (Kaneko *et al*., 2003).

Transcriptional regulation of the *uPAR* gene is important in the control of the malignant behaviour of colon cancer cells (Wang *et al*., 1994). It is not known yet whether the $(CA)_n$ repeat polymorphism of the *uPAR* gene may have an effect on the regulation of the uPAR gene expression. This polymorphism occurs in intron 3, and it might be involved in some events in nuclear splicing. We found here no differences in the level of uPAR antigens in groups with different alleles in intron 3. These results confirm the assumption that this site polymorphism is probably phenotypically silent. However, in previous study three alleles (127 bp, 141 bp, 145 bp) were specific to colon cancer cell lines (Kohonen-Corish *et al*., 1996). We found eight alleles 139 bp, 143 bp, 145 bp, 147 bp, 149 bp, 151 bp, 155 bp, 159 bp in both: patients and control groups. There were no significant differences in the frequencies of alleles between cancer patients and control, but two alleles 141 bp and 153 bp with frequencies 0.081 and 0.032, respectively, were unique to the colon cancer patients group. These results indicate that the $(CA)_n$ repeat polymorphism in intron 3 may not be associated with occurrence of colorectal cancer.

The association of the increased level of uPAR antigen with high microvessel density suggests that uPAR can be correlated with progression of colorectal cancer, but further study, on a larger population, should be performed to verify this hypothesis.

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