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Evaluate the Expression of uPA, PAI-1 in Human Gastric Cancer and its Correlation with the Angiogenesis by the Application of Tissue Microarray

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OBJECTIVE To investigate the expression of urokinase-type plasminogen (uPA), its inhibitor-1 (PAI-1) mRNA and its protein in human gastric cancer and to find out the relationship among the tumor differentiation, angiogenesis, and other clinical pathologic factors.

METHODS In situ hybridization (ISH) was used to get the uPA, PAI-1mRNA in 110 cases with human gastric cancer in 2-tissue microarray (TMA). Immunohistochemical staining (S-P method) for uPA, PAI-1 protein and CD34 were performed in the 110 cases in 2 TMA.

RESULTS The expression of the uPA, PAI-1mRNA and their protein happened in the cytoplasm of gastric cancer cells were induced by the poor differentiation of the GC, and the expression of uPA had an increasing trend while the expression of the PAI-1 had a decreasing trend. The microvessel density (MVD) had a positive correlation with the clinical stages and the significant relationship with the lymph node metastasis ($P < 0.05$). The MVD in uPA positive group was significantly higher than those in uPA negative group ($P < 0.05$). The expression of PAI-1 has no correlation neither with the clinical stages nor the lymph node metastasis.

CONCLUSION The uPA play an important role in invasion and metastasis of GC through promoting angiogenesis. Interdicting the secretion and function of the uPA may allow the target therapy against the tumor invasion. As a new high-throughput technology, the tissue microarray is a valuable way to be used in clinical treatment.

KEY WORDS: stomach neoplasmas, urinary plasminogen activator, plasminogen activator inhibitor 1, angiogenesis, tissue microarray.

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Introduction

Breaking the extracellular matrix and membrane is essential for tumor invasion and metastasis. The generation of the broad specificity protease plasmin stimulated by the urokinase-type plasminogen activator (uPA) is the central effect of these processes, since it is able to degrade most matrix components either by directly breaking it or by activating other latent enzyme systems. This activity is tightly regulated by its specific physiologic inhibitor-PAI-1. The report in the literature showed that the PAI-1 plays an important role in tumor progression[1,2].

Angiogenesis, the formation of new capillaries from existing blood vessels, is essential for the growth and metastasis of a solid tumor.

Endothelial cells use a repertoire of degradation enzymes and receptors to complete the process of the angiogenesis which is similar to what the neoplastic cells do in the extracellular matrix during physiological and pathological angiogenesis. It is generally assumed that microvessel formation around the tumor is stimulated by various angiogenic factors. The MVD may be one of the important predictors for the angiogenesis. A role of the PA system in tumor angiogenesis has been demonstrated in the previous literature[3]. In this study, we examined the expression of uPA and PAI-1 in 110 cases with gastric cancer in 2 TMA, to analyze the relationship among the uPA system, tumor MVD and their clinical pathological features.

Materials and Methods

Tissue samples and groups

The tissue specimens of 185 cases with gastric cancer were respectively obtained from the first affiliated hospital of Anhui Medical University. The results of the pathological grades of the 185 patients (134 males, 51 females, with the median age of 55 years old, ranging from 25-86) were as follows: 20 cases of well differentiated papillary (tubular) adenocarcinoma (grade I), 54 cases of median differentiated tubular adenocarcinoma (grade II), 111 cases of poor differentiated adenocarcinoma, mucinous carcinoma and signet-ring cell carcinoma (grade III). All specimens were fixed in 10% formalin, and then embedded in paraffin followed by the process of 2 "gastric cancer TMA". And finally, the treated specimens were cut in serial 4 sections and then adhered to slides, and after that the slides were treated by Poly-L-Lysine and 0.1% DEPC.

Reagent

The uPA and PAI-1 detection kits were purchased from Boster Biological Technology Ltd. The probe labeled by digoxin is made of two sequences of oligonucleotide of uPA: (1) 5'-CTA GGC CTG GGG AAA CAC AA T TAC TGG AGG-3'; (2) 5'-TGT CTA CAC GAG GGT CTC ACA CTT CCT GGA-3'. The PAI-1 probe is made of 3 sequences of oligonucleotide: (1) 5'-GAT GTC TCC AGC CCT CAC CTG CCT AAC CCT-3'; (2) 5'-GCT GAC ACG GCT GGT GCT GGT GAA TGC CCT-3' ; (3) 5'-AAC AGT CCT CTT CAT GGG CCA AGT GAT GGA-3. The polyclonal antibodies against uPA and PAI-1 extracted from rabbits were purchased from Boster Biological Technology Ltd. The immunohistochemistory kit of the monoclonal mouse antibody against CD34 and S-P were purchased from Beijing Zhongshan Biological Technology Ltd.

In situ hybridization

The specimens were deparaffinized and rehydrated through a graded series of ethanol. The 0.3% hydrogen peroxide was used to block the endogenous peroxidase in the specimens for 10 min. After washed with distilled water treated by 0.1% DEPC three times at 5 min each, the slides were digested by the pepsin added with 3% citric acid at 37°C for 15-20 min. Twenty microliter of prehybridization reagent was added to each slide and kept at 37°C for 2 h, then 20 μl of probe was hybridized to each slide and kept at 40-42°C for 16-18 h. After hybridization, each slide was washed using the SSC at different ways: 2 × SSC at 37°C once for 15 min, 0.5 × SSC at 39°C twice for 5 min and 0.2 × SSC at 39°C twice for 5 min each. Blocking reagent was dropped to slides and incubated for 30 min, and then the mouse anti-digoxin antibody labeled by biotin was added at 37°C for 60 min. After washed with 0.5 M PBS thrice for 3 min each, the slides were incubated with streptavidin-biotin complex (SABC) at 37°C for 20 min, then washed with 0.5 M PBS thrice for 5 min each. At last, the chromogen DAB was added to visualize the reaction products of the peroxidase, then the slides were counterstained by the haematoxylin stain for the visualization of the nuclei. A negative control slides were prepared using the same steps described above but the probe was processed by 2 × SSC. The positive control showed positive result always in the repeated experiments. The positive result of uPAmRNA and PAI-1mRNA expression was demonstrated by the brown-yellow colored ytoplasm.

Immunohistochemistry

Immunohistochemical staining was performed using S-P method. The anti-uPA antibody and anti-PAI-1 was diluted at 1:50, and 1:100, respectively. The anti-CD34 was the reagent ready to use. The negative control slides were dyed according the above method with the primary antibody substituted by animal serum. The positive standard of uPA and PAI-1 protein expression was that the color of the plasma in the cells was turning into brownyellow. The uPA and PAI-1 expressions were classified into three groups. When strong immunostaining was seen in more than 50% of cancer cells, the tumors were determined to be positive (+). When weak immunostaining was seen in less than 50% of cancer cells, the tumors were determined to be negative (-). Weak immunostaining in more than 50% of cells, or strong immunostaining in less than 50% of cells was designated as +/- [4]. The MVD was determined by immunohistochemical staining with CD34 and its positive result was observed in vascular endothelial cells. The MVD was evaluated by counting the number of endothelial deposits/fields under the light microscopy at 400 magnification. The professional who did the counting was not acknowledged of the patients' details. The mean of the four times of counting for each specimen was calculated and statistically analyzed[5].

Statistical analysis

The data were copied down in Excel and analyzed by SPSS version 10.0 for windows. The χ^2 test was used for

statistical analysis, and the Spearman rank correlation was performed for correlation analysis.

Results

Of the specimens collected from 185 cases with gastric cancer, which were processed through the certain procedures, 110 were eligible for analysis. The pathological grades of the 110 cases were as follows: 9 cases of grade I, 39 cases of grade II, 62 cases of grade III.

Expression of uPA, PAI-1 mRNA and protein

The positive mark of uPA, PAI-1 mRNA and their protein was the brownish-yellow cytoplasm of the cells (Figs.1-4). In adjacent cancerous tissues, there was little uPA expression while partly PAI-1 expression. There was a significant positive correlation among the expressions of uPA, PAI-1 mRNA and their protein (Table 1).

The relationship between the expression of uPA, PAI-1 and the MVD

The microvessel distributed in the stomach of the GC patients was not equal, and the MVD was 25.6 ± 4.2. The mean of the MVD in Grade I, II, III was 20.3 ± 1.5, 24.7 ± 3.1, 26.9 ± 4.4, respectively, and the differences of the mean among one another was significant (P = 0.006). In the result analyzed by the Spearman rank correlation, we found that the expression of the uPA and protein had significantly positive correlation mRNA, and the MVD, while no significant correlation between the PAI-1 expression and MVD was found (Table 2).

The relationship between the expression of uPA, PAI-1, MVD and clinicopathological parameters

We grouped the 110 GC patients whose data of clinical staging and lymph node metastasis were collected, and found the following results. *i*) The MVD of the patients in stage III, IV was significantly higher than that of the patients in stage I, II. *ii*) The positive percentage of the uPA mRNA and protein in the patients in stage III, IV were significantly higher than those in the patients in stage I, II. *iii*) There was no relationship between the expression of PAI-1 and the clinicopathological parameters mentioned above (Table 3).

Table 1. The expression of uPA, PAI-1 mRNA and protein in histologic differentiation of GC.

Group	*n*	uPAmRNA (protein)[a]				PAI-1 mRNA (protein)[b]			
		-	±	+	Positive %	-	±	+	Positive %
I	9	0 (0)	5 (3)	4 (6)	44.4*(66.7)*	0 (0)	4 (2)	5 (7)	54.6*(77.8)*
II	39	5 (3)	13 (13)	21 (23)	53.8 (60.0)	3 (1)	13 (12)	23 (26)	60.0 (66.7)
III	62	15 (10)	10 (7)	37 (45)	59.7 (72.6)	16 (13)	13 (9)	33 (40)	53.2 (64.5)

Comparison among the groups of GC, *P<0.05. a: The relationship between the expressions of uPA mRNA and protein was significantly positive, r = 0.686, P < 0.001. b: The relationship between the expressions of PAI-1 mRNA and protein was significantly positive, r = 0.812, P < 0.001.

Table 2. Relationship between the expression of uPA, PAI-1 and the MVD.

Group	*n*	MVD ($\bar{x} \pm s$)	*P*
uPA mRNA (protein)			0.000 (0.003)
-	20 (13)	22.8 ± 3.1 (22.7 ± 3.8)	
±	28 (23)	23.4 ± 2.6 (24.4 ± 2.6)	
+	62 (74)	27.5 ± 4.1 (26.5 ± 4.4)	
PAI mRNA (protein)			0.560 (0.672)
-	18 (14)	24.7 ± 5.8 (26.5 ± 6.2)	
±	28 (23)	25.2 ± 4.0 (25.6 ± 3.6)	
+	64 (73)	25.9 ± 4.0 (25.4 ± 4.0)	

Table 3. Relationship between the expression of uPA, PAI-1, MVD and clinicopathological parameters in GC.

Parameters	*n*	MVD	uPA positive		PAI-1 positive	
			mRNA (%)	Protein (%)	mRNA (%)	Protein (%)
Clinical stage						
I-II	21	22.9 ± 3.1*	7 (33.3)	9 (42.9)*	7 (33.3)	14 (66.7)
III-IV	89	26.2 ± 4.2	55 (61.8)	65 (73.1)	57 (64.0)	59 (66.3)
Lymph node metastasis						
Absent	37	24.9 ± 3.8	16 (43.2)	23 (62.2)	16 (43.2)	26 (70.3)
Present	73	25.9 ± 4.4	46 (63.0)	51 (69.9)	48 (65.8)	47 (64.4)

*P < 0.05.

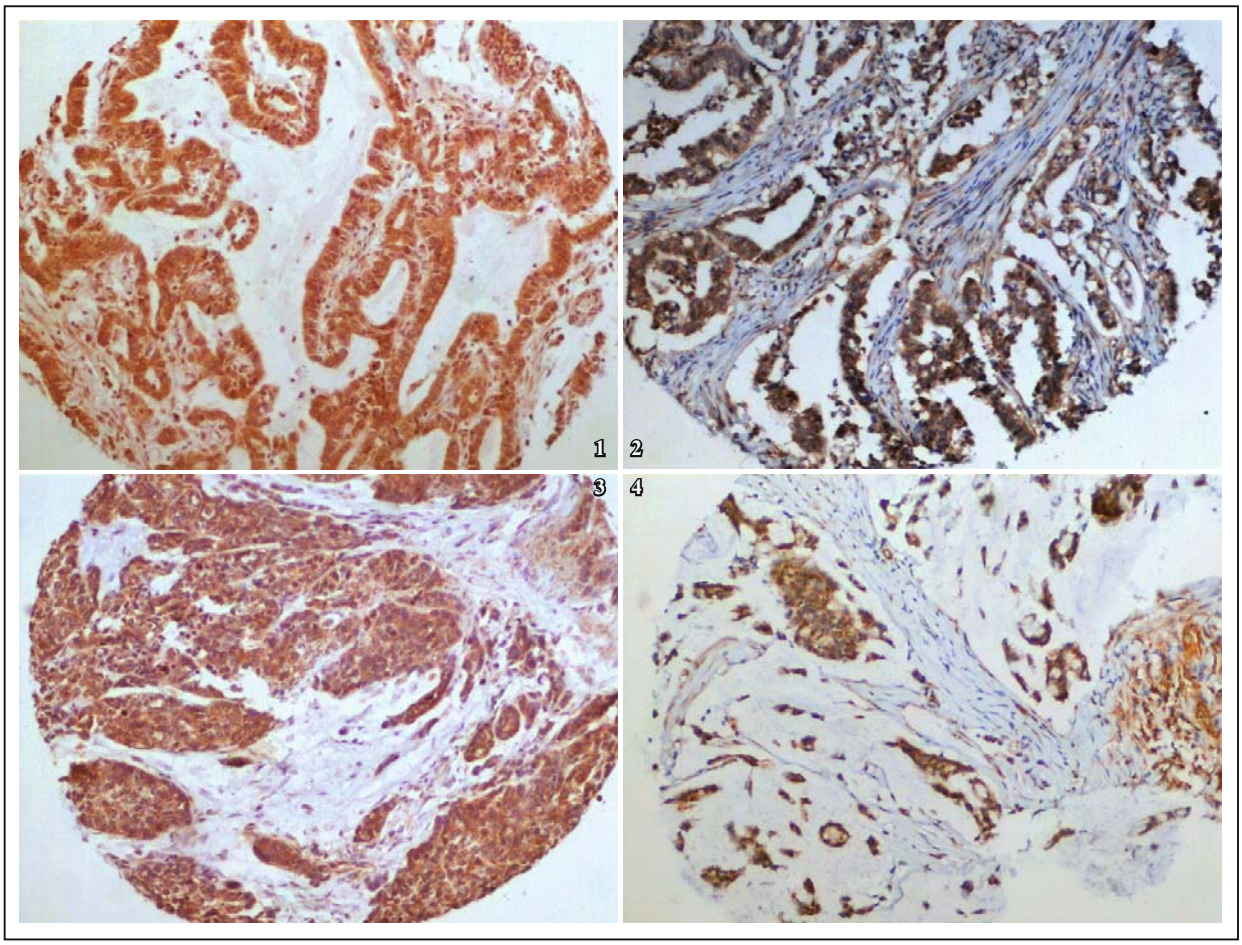

Fig.1. The expression of uPAmRNA is moderate positive in papillary adenocarcinoma × 100.
Fig.2. The expression of uPA protein is strong positive in tubular adenocarcinoma × 100.
Fig.3. The expression of PAI-1mRNA is moderate positive in poor differenciated adenocarcinoma × 100.
Fig.4. The expression of PAI-1 protein is strong positive in mucoid adenocarcinoma × 100.

Discussion

TMA is a new technology first introduced in 1998[6]. It contains hundreds or even thousands of small tissue samples arranged into a grid for rapid, cost effective and high-throughput analysis. TMA has been used widely in gene or protein expression analysis, antibody screening, detection of tissue specific proteins, phenotype versus genotype analysis, and RNA or DNA in situ-hybridization. Based on TMA, we studied the expressions of uPA, PAI-1 mRNA and their protein, MVD and their correlationship with the clinicopathological parameters in GC patients. As many as 185 cylindrical tissue biopsies from individual tumor can be distributed in 2 TMA, and the sections of the microarray provided targets for parallel in situ detection of mRNA and protein targets for each specimen on the array at one time, so the results were more credible than those analyzed using the traditional methods.

Tumor invasion and metastasis are the complex processes and many of these procedures, including angiogenesis, involve the controlled degradation of ECM components by proteinases. The extracellular proteinases involved in ECM degradation are mainly the matrix metalloproteinases (MMPs) and the serine proteinases such as PAs. The urokinase plasminogen activator system consists of the serine protease uPA, its glycolipidanchored receptor, uPAR and its 2 serpin inhibitors, plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2). The plasminogen activator, as its name demonstrates, convert inactive plasminogen into the active serine proteinase-plasmin. The plasmin is involved in ECM degradation directly because it is a broad substrate proteinase that can degrade most proteins within the ECM (for example, fibronectin, laminin, and proteoglycans). The plasmin also acts on the ECM indirectly by activating the latent forms of several MMPs (for example, MMP-1). The function, distribution and regulation of the PAs differ and their differences are as follows. The uPA is important in tissue degradation in both physiological and pathological processes, whereas tPA is important in fibrinolysis. The plasminogen activa-

tors also differ in inhibitory functions. The PAI-1 inhibits both uPA and tPA, however, PAI-2 inhibits only uPA. In vivo, it is balanced between plasminogen activators and their inhibitors that determine matrix degradation by plasmin[7].

The uPA system is causally involved in multiple steps in cancer progression. In particular, uPA has been implicated in remodeling of the extracellular matrix, enhancing both cell proliferation and migration and modulating cell adhesion. The uPAs has been studied extensively in various human cancers. Lately, the relationship between the uPAs and angiogenesis has been reported. The reports showed that the endostatin inhibited the angiogenesis through the down-regulation of the uPAs[8]. Although additional studies are needed to clarify the difference in the angiogenesis among various tumors, Kaneko et al.[4] suggested that uPA was significantly correlated with the clinicopathological factors: depth of tumor invasion, differentiation, vascular invasion. The MVD assessed using the immunohistochemical method was significantly higher in the patients with the expression of uPA and the stepwise analysis identified it as an independent correlated factor with MVD. The uPA is a key factor in the PAs, being associated with a poor outcome of gastric cancer, and contributes not only to tumor invasion, but also the tumor angiogenesis.

In our study, we found that the expressions of the uPA mRNA and protein happened in the cytoplasm of gastric cancer cells and as the differentiation of GC was decreased, the expression of uPA developed an increasing trend. The positive expression rates of the uPA mRNA and protein in GC patients with medium-poor differentiated carcinoma (53.8% and 60.0%, 59.7% and 72.6%, respectively) was significantly higher than those in the GC patients with well-differentiated carcinoma (44.4% and 66.7%). Further more, the MVD in the patients with the uPA mRNA positive (27.5 ± 4.1) was significantly higher than that in the patients with the uPA mRNA negative (22.8 ± 3.1). Meanwhile, there was a positive correlation between the expression of uPA mRNA and the clinical stages. The concurrent result was found in the protein. Our data suggest that the uPA was highly correlated with the angiogenesis, invasion and metastasis of GC. The potential mechanism was that the uPA catalyzed the inactive proenzyme plasminogen to convert into the plasmin and activated the MMPs, which degraded the ECM, as a result, the angiogenesis will be promoted by facilitating endothelial cells migration and tumor cells diffusion. In conclusion, the uPA plays an important role in the ECM remodeling, cell migration, angiogenesis and tumor invasion, being associated with a poor outcome of gastric cancer, and contributing to both tumor invasion and the angiogenesis in tumor.

The PAI-1 protein is a multifaceted proteolytic factor. It not only functions as an inhibitor of the protease uPA, but also plays an important role in signal transduction, cell adherence, and cell migration. Thus, an apparent paradox for its name and its functions exists. Although it inhibits uPA during the process of the blood coagulation and other aspects, it actually promotes the invasion and metastasis. PAI-1 is considered as one of the key regulators of tumor invasion, metastasis, as well as cancer-related angiogenesis[9]. In different kinds of cancer, the PAI-1 plays a totally reverse role. On the one hand, it inhibits the activity of the uPA and interdicts many steps of tumor metastasis. So, the high expression of PAI-1 has been found in several kinds of tumors such as lung cancer, ovarian caner, prostate cancer, endometrial cancer, etc. and also found in tumors of which the patients had better prognosis[10,11]. PAI-1 and uPAR are prognostic factors for overall survival of the non-small cell lung cancer (NSCLC) patients. Moreover, they add independent prognostic information with regard to established clinical and histomorphological factors in NSCLC[12].

On the other hand, a possible function of PAI-1, which promotes the growth of tumor, is demonstrated by its potential to modify cell adhesion capacity, which is independent of uPA inhibitory activity. PAI-1 expression has been demonstrated in fibroblast contacting endothelial cells, which can only occur during angiogenesis when the basement membrane has been degraded and endothelial cells are in the process of migrating. The higher levels of PAI-1 correlated with the metastasis and poor prognosis in patients with breast cancer, hepatocellular carcinoma (HCC), colorectal cancer, etc. Zhen et al.[13] reported that the expression of PAI-1 mRNA and protein were higher in HCC cells in comparison with those in normal liver cells. PAI-1 plays an important role in signal transduction, cell adherence, and cell migration, and it actually promotes the invasion and metastasis. Fox et al.[14] found that there was significant relationship between the PAI-1 and nodal states, tumor grade, but no association between PAI-1 and vascularity. Sakakibara et al.[15] suggested that PAI-1 might serve as a new parameter for the prediction of prognoses in colorectal cancer (CRC), that is, a significantly increased scores of the PAI-1 expression was observed in the CRC patients whose lymph node had metastasized to the other parts of the body compared with those in the patients who had negative lymph nodes ($P = 0.0037$). The higher scores of the PAI-1 also occurred in the CRC patients who had distant metastasis compared with those in the patients who had negative lymph nodes. The PAI-1 expression score was markedly increased with the tumor stage ($P = 0.0063$). Moreover, multivariate analysis revealed that the PAI-1 expression score was a strong and independent prognostic factor for the CRC patients ($P = 0.0432$).

In our study, the positive signals of PAI-1 mRNA and protein were brownish-yellow stains located in cytoplasm in GC cells. There were some cells of which showed positive signals of the PAI-1 mRNA and protein surrounding the cancerous tissues; and as the differentiation of the GC was decreased, the expression of PAI-1 had a decreased trend. No significant associa-

tion was seen between PAI-1 and lymph node metastasis, clinical stage. Furthermore, no significant association was found between PAI-1 and MVD. These results were consisted with those reported by Kaneko et al.[4] In addition, the Spearman correlation analysis demonstrated that the uPA and PAI-1 has no significant correlation too.

In summary, these findings suggest that the uPA contributes to the angiogenesis and invasion of GC. PAI-1 may be an inhibitor of uPA in GC. But, as PAI-1 also has a strong expression in GC cells and has a positive correlation with tumor differentiation, it may not be a simplex inhibitor of GC, but be a regulator or guardian of tumor cells. The increased knowledge about the plasminogen activation system may allow us to utilize these factors as targets for anti-invasive therapy.

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