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Analysis of expressions of components in the plasminogen activator system in high- and low-metastatic human lung cancer cells

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Abstract ***Purpose:*** To determine the expressive patterns of the components of the plasminogen activator system in human large-cell lung carcinoma strains and to analyze the effects of the patterns on tumor invasion and metastasis. ***Methods:*** The in vitro and in vivo invasive and metastatic potential of two human large-cell lung carcinoma strains with high (strain 95D) and low (strain 95C) metastatic potential was further confirmed by the Boyden chamber model and nude mice model. After this, the expressions of the components of the plasminogen activator system – including urokinase-type and tissue-type plasminogen activator (uPA and tPA), urokinase receptor (uPAR), and type-1 and type-2 plasminogen activator inhibitor (PAI-1 and PAI-2) in strain 95D and 95C cells – were determined by RT-PCR and immunohistochemical staining. The effects of monoclonal antibodies of uPA, uPAR, and PAI-1 on the invasive potential of strain 95D cell line were also evaluated. ***Results:*** Strain 95D cells were found to have a stronger in vitro and in vivo invasive and metastatic potential than strain 95C cells. In the former, the average number of infiltrating cells in the in vitro model in one field of vision (40055) was 73.75 ± 7.42, while in the latter, it was 56.33 ± 6.28 ($P < 0.001$). Lung metastatic loci were observed in all six nude mice inoculated with 95D cells (6/6), but not in any of the nude mice inoculated with 95C cells (0/6). The high-metastatic strain 95D cells expressed higher uPA and uPAR and lower tPA and PAI-2 than the low-metastatic strain 95C cells. The PAI-1 expressions in both 95D and 95C cells were almost the same. Monoclonal antibodies of uPA and uPAR greatly reduced the invasive potential of strain 95D cells in vitro. ***Conclusions:*** These data suggest that the invasive and metastatic potential of human large-cell lung carcinoma cell lines is associated with differential expressions of the components of the plasminogen activator system and that the determination of these components may be used as a marker for judging clinically the possibility of tumor metastasis as well as the prognoses of patients.

Key words Lung cancer · Plasminogen activator system · Tumor invasion and metastasis

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Introduction

The invasion and metastasis of cancer are the greatest obstacle to successful tumor treatment. The process of cancer metastasis involves several sequential steps in which malignant cells are released from the primary tumor and disseminate to distant sites where they proliferate to form new tumor loci (Woodhouse et al. 1997). Urokinase-type plasminogen activator (uPA) converts inactive plasminogen to active plasmin, which then degrades the extracellular matrix protein directly or indirectly through the activation of other matrix-metalloproteinases. This proteolysis mediated by the uPA/plasmin system could be involved in the dissolution of the connective tissue around cancer cells and the perivascular basement membrane, facilitating the migration and intravasation of cancer cells in the metastatic step (Andreasen et al. 1997). uPA-catalyzed proteolysis is focused by a specific receptor for uPA, urokinase-type plasminogen activator receptor (uPAR), on the cell surface (Behrendt et al. 1998). The activity of receptor-bound uPA is also regulated by two of the specific inhibitors of plasminogen activator (PAI-1 and PAI-2). Recent studies showed that the high expression levels of

uPA, uPAR, and PAI-1 in several malignant tumors were related to the tumor metastasis and the prognosis of the patients (Duffy et al. 1999).

Large-cell lung carcinoma is the most fatal form of lung carcinomas in terms of its invasiveness and metastasis. However, there is still little known about the metastatic mechanism of large-cell lung carcinoma. In this study, we used strain 95D and 95C cell lines with different metastatic potential as models for studying the mechanism of the metastasis of lung carcinomas. We first compared the expressive patterns of the components of the plasminogen activator system between strain 95D and strain 95C cells and then analyzed the effects of monoclonal antibodies of uPA, uPAR, and PAI-1 on the invasive potential of strain 95D cells.

Materials and methods

Cell lines and culture conditions

Strain 95D and strain 95C were provided by the cell culture laboratory of the Pathology Department of the Chinese PLA General Hospital. The cells were maintained in RPMI-1640 medium (Gibco, Gaithersburg, Md., USA) containing 10% fetal bovine serum (Gibco). They grew at 37 °C in humidified air with 5% CO_2.

Comparison of the invasive potential of strain 95D and strain 95C in vitro

The invasiveness of 95D and 95C cells was determined using the modified Boyden chamber. Polycarbonate filters, 8-μm pore size (Costar, Cambridge, Mass., USA.), were coated with 25.6 μg artificial reconstructed Matrigel (kindly provided by the Department of Biochemistry and Molecular Biology, Beijing Medical University) and dried (2 h, 37 °C). The 100 μl of 95D and 95C cell suspensions (1×10^6 cells/ml) were placed in the upper compartment of a chamber, and the lower compartment was filled with 800 μl of RPMI1640/0.1% BSA. After incubation for 20 h at 37 °C, the cells and the artificial Matrigel on the upper surface of the filter were carefully removed with a cotton swab. The filters were then fixed by formalin, and stained by the conventional HE method. The invasive cells adhering to the lower surface of the filter were quantified under a light microscope (×400). In this assay system, using 95D and 95C cells, some cells came off the lower part of the filter. The data are expressed as the number of cells attached there at a randomly chosen area (each sample was assayed using triplicate filters, and filters were counted at three areas) (Kobayashi et al. 1994).

Comparison of metastasis in vivo of strain 95D and strain 95C in nude mice

BALB/cA male nude mice (Shanghai Institute of Materia Medica, Chinese Academy of Sciences, China) 4–6 weeks old were used in this study. Twelve of the nude mice were separated randomly into two groups; six mice were injected s.c. with 1×10^6 of strain 95D cells and the rest with an equal portion of strain 95C cells. Both groups were kept in laminar-flow cabinets under the specific-pathogen-free condition and killed after 10-weeks cultivation. The growth of primary tumor and the metastatic loci in the lungs, kidneys, spleen, and liver were observed under a light microscope.

RT-PCR

Total RNA was extracted from approximately 2×10^6 cells of strain 95D and 95C using Trizol reagent (Promega). The cDNA was synthesized with 5 μg total RNAs of each cell, and 200 ng oligo (dT15) primers (Promega, Madison, Wis., USA) in a 50 μl solution containing 25 U Avian Myeloblastosis Virus reverse transcriptase (RT; Promega), 20 U RNasin, and 60 nmol dNTP. Subsequently, tPA, uPA, uPAR, PAI-1, PAI-2, and β-actin specific sequences were amplified by 30 cycles of PCR with 4 μl RT products, 50 pmol sense and antisense primers (the sequences are listed in Table 1) and 3 U Taq DNA polymerase (Promega) in a final volume of 50 μl. Each cycle of PCR included 50 s denaturation at 94 °C, 50 s primer annealing at 50–61.50 °C and 50 s extension at 72 °C. Cycles were preceded by incubation at 95 °C for 5 min to ensure full denaturation of the target DNA, and were followed by an extra 5 min of incubation at 72 °C after the final cycle to ensure full extension of the product. The PCR reactions were performed on a DNA thermal cycler (Perkin Elmer/Cetus Instruments, Norwalk, Conn., USA). The amplified PCR products were analyzed by 1.5% agarose gel.

Analysis of tPA, uPA, uPAR, PAI-1, and PAI-2 gene expressions

After the separation by electrophoresis, the PCR products from strain 95D and 95C were separated by 1.5% agarose and the electrophoretic results were printed. The absorbencies (A) and the areas of fragment were read by ImageMaster VDS (Pharmacia Biotech). The expression level of the target gene was calculated as follows: the gene expression (%) = [the target gene fragment area × ($A_{fragment} - A_{background}$)] / [β-actin fragment area × ($A_{fragment} - A_{background}$)].

Semi-quantitative analysis by immunohistochemical staining

Both strain 95D and 95C cells were cultured on 96-well plates, which then were stained for the presence of tPA, uPA, uPAR, PAI-1, and PAI-2 proteins using monoclonal antibodies (kindly provided by

Table 1 The primer sequences and annealing temperature of RT-PCR. The abbreviations used are: tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; PAI-1, plasminogen activator inhibitor type-1; PAI-2, plasminogen activator inhibitor type-2; tPA, uPA, uPAR, PAI-1, PAI-2, β-Actin appeared in the Table 1 mean those cDNAs will be amplified by PCR with corresponding primer

cDNA to be amplified	Primer sequences	Length of amplified fragments (bp)	Annealing temperature (°C)
tPA	Sense: 5′-GCGGCCGCCTCTGAGGAAACAGT-3′ Antisense: 5′-TCACGGTCGCATGTTGTCACGAAT-3′	979	61.5
uPA	Sense: 5′-TTCTCTGCGTCCTGGTCGTG-3′ Antisense: 5′-AGGCTGCCTCCACACACGTA-3′	612	61.5
uPAR	Sense: 5′-AAGCTTAGAGAAGACGTGCAGGGA-3′ Antisense: 5′-TTCACCTTCCTGGATCCAGT-3′	513	60.0
PAI-1	Sense: 5′-GCCATGGTCCAGCTGACAACA-3′ Antisense: 5′-GTCTGATTTGTGGAAGAGGGG-3′	450	60.0
PAI-2	Sense: 5′-AATGAAGTGGGAGCCAAT-3′ Antisense: 5′-GAGGATCCTTAGGGTGAGCAAAA-3′	1067	50.0
β-Actin	Sense: 5′-CATGGGTCAGAAGGATTCCTAT-3′ Antisense: 5′-GCGCTCGGTGAGGATCTTCAT-3′	450	50.0–61.5

Professor Hou-yan Song, Shanghai Medical University, Shanghai, China). The avidin-biotin-peroxidase method was applied with a commercial kit. The adopted antibody concentrations provided maximal staining intensity with minimal background staining. The negative controls consisted of the samples preincubated with non-immune mouse serum, but the other steps remained unchanged. Immunocytochemical staining of different components of the plasminogen activator system was scored in a semi-quantitative fashion, which incorporates in the analysis both the intensity and the percentage of distribution of specific staining, as described by McCarthy et al. (Bacus et al. 1988). In brief, the evaluations were recorded as percentages of positively stained target cells in each of four intensity categories, which were denoted as 0 (no staining), 1 (weak staining, but detectable above control), 2 (distinct), and 3 (intense). For each well, the score was derived by adding the percentages of cell staining at each intensity multiplied by the weighted intensity of staining according to the following formula: score: $\Sigma Pi \times i$ ($i = 0,1,2,3$ and Pi varies from 0 to 1, 1 = 100% cells staining positively).

Effects of the monoclonal antibodies of uPA, uPAR, and PAI-1 on the invasiveness of strain 95D cells in vitro

The effects of these mAbs were assayed in a modified Boyden chamber with an 8-μm pore size polycarbonate filter. The mAbs (40 μg/ml final concentration) were added to the artificial Matrigel, coating the filter, which was then dried. One hundred μl of 95D cell suspension (1×10^6 cells/ml) were pretreated with the mAbs at the final concentrations of 40 μg/ml for 2 h at 37 °C in serum-free medium, with occasional shakings, and then the mixtures were placed in the upper compartment of the chamber. The following procedures were the same as those of the invasion assay. Meanwhile, 0.1% bovine serum albumin (BSA) served as a negative control.

Statistical analysis

The statistical analysis of the results was performed by the *t*-test and *q*-test. Differences were considered statistically significant at value of $P < 0.05$.

Results

Comparison of the invasive potential of strain 95D and strain 95C cells in vitro

The high metastatic strain 95D cells strongly penetrated the filter (73.75 ± 7.42 infiltrating cells), whereas low-metastatic strain 95C cells had a weak penetration (only 56.33 ± 6.28 penetrating cells, Fig. 1). Thus, there was a significant difference between these two groups as shown by the *t*-test ($P < 0.01$).

Metastatic potential of strain 95D and strain 95C cells in nude mice

Strain 95D and strain 95C cells exhibited different degrees of metastatic potential, reminiscent of metastatic behaviors in nude mice. At the late stage, numerous visible metastatic loci were found in the lungs of all six nude mice with strain 95D (Fig. 2a), whereas there were none in the lungs of all six nude mice with strain 95C (Fig. 2b), although there were no significant differences in tumor growth in situ. Interestingly enough, metastatic loci in the

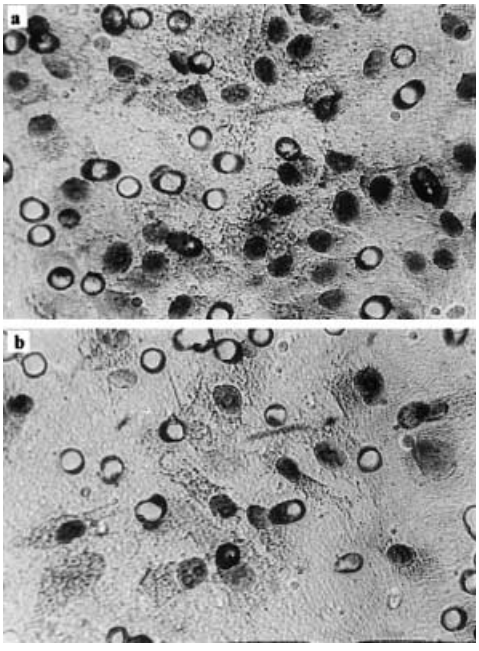

Fig. 1 Comparison of in vitro invasive potential of strain 95D (**a**) and strain 95C (**b**) cells by Boyden Chamber Model

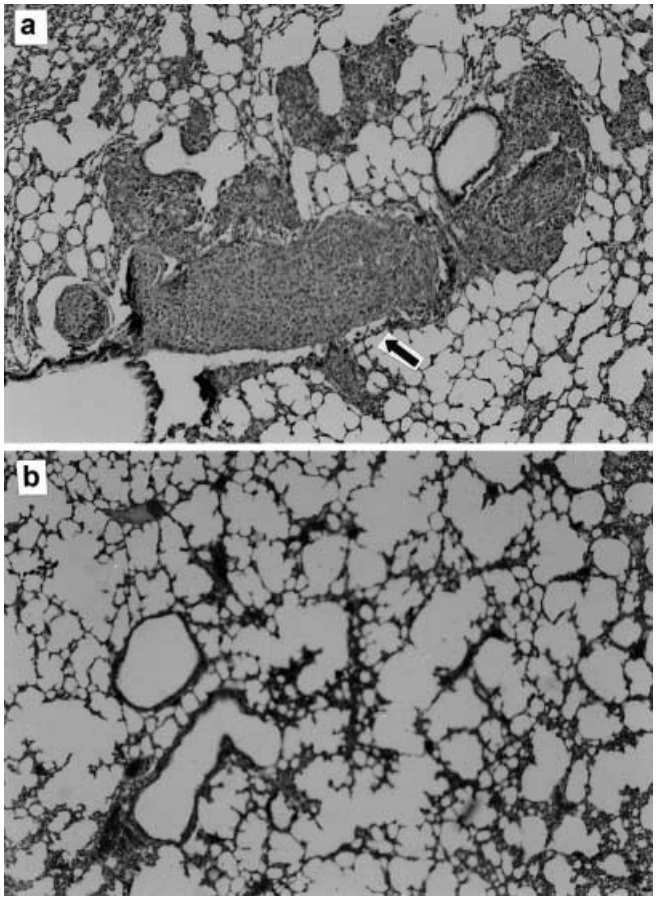

Fig. 2 Comparison of in vivo metastatic potential of strain 95D (**a**) and strain 95C (**b**) cells. The *arrow* indicates the metastatic loci of tumor in the lungs

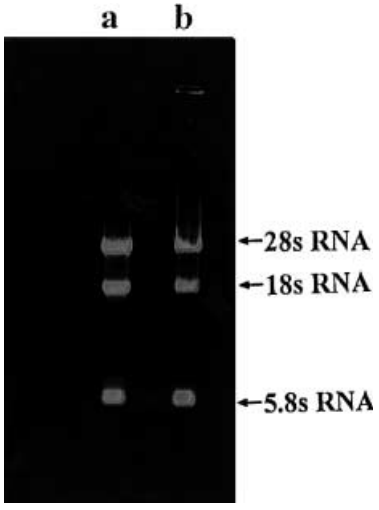

Fig. 3 Total RNA extracted from strain 95D (**a**) and strain 95C (**b**) cells

spleen, liver, and kidneys were not found in any of the twelve nude mice inoculated with 95D cells or 95C cells.

Analysis of components of the plasminogen activator system by RT-PCR

About 16 μg of total RNAs were extracted from 2×10^6 of strain 95D and 95C cells, which contained 5.8S, 18S, and 28S of RNA fragments without any contaminants (Fig. 3).

The β-actin functioned as the control. Compared with the expression of β-actin, the expressions of uPA, uPAR, PAI-1, PAI-2, and t-PA in strain 95D cells were 88.1%, 91.5%, 75.9%, 32.5%, and 28.0%, respectively. In strain 95C cells, however, they were 47.8%, 21.7%, 84.4%, 81.6%, and 51.6%, respectively. It is obvious that the expressions of uPA and uPAR in strain 95D cells were much stronger than those in strain 95C cells, but the expressions of PAI-2 and tPA in the former were significantly weaker than those in the latter. The PAI-1 level in strain 95D cells was a little lower than that in strain 95C cells (Figs. 4 and 5). Meanwhile, there was one non-specific fragment (about 450 bp) in the amplified products of uPA cDNA (612 bp), which may have been caused by the primer design.

Immunohistochemical analysis of the expression of the components of the plasminogen activator system

The expressions of uPA and uPAR were shown to be much stronger in strain 95D cells than those in strain 95C cells by the McCarthy method, with the score value ($\Sigma Pi \times i$) 2.1 vs 1.75 and 2.4 vs 1.9, respectively. However, the former presented weaker expressions of PAI-2 and tPA than the latter, the score value being 1.6 vs 1.7 and 1.7 vs 1.9, respectively. The expression of PAI-1 did not show any significant differences between the two groups (2.2 vs 2.15) (Fig. 6).

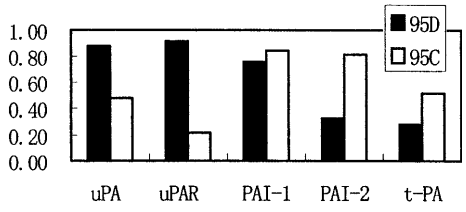

Fig. 5 Semiquantitative analysis of t-PA, uPA, uPAR, PAI-1, and PAI-2 expressed in 95D and 95C cells by RT-PCR

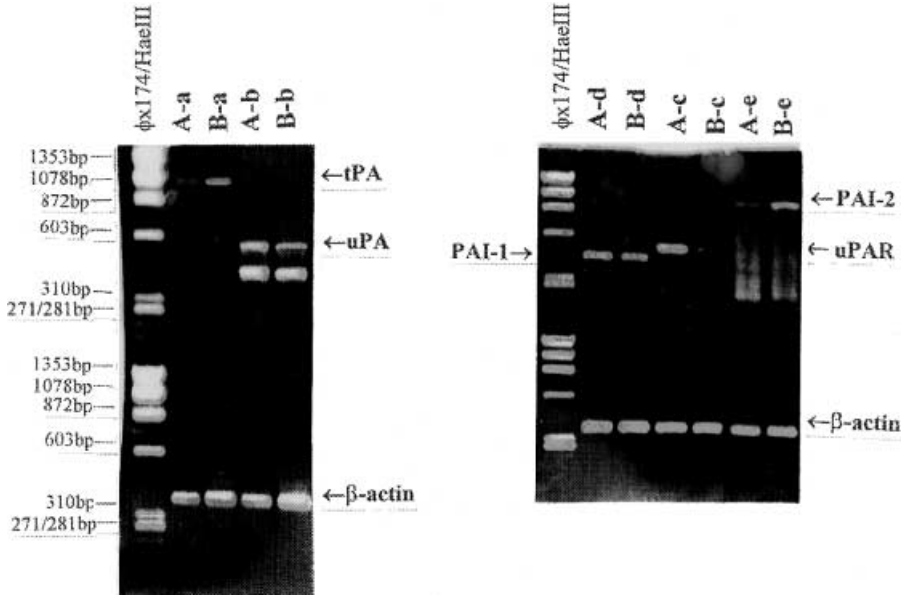

Fig. 4a–e Comparative analysis of t-PA, uPA, uPAR, PAI-1 and PAI-2 expressed in 95D (*A*) and 95C (*B*) cells by RT-PCR. **a** Amplified t-PA cDNA fragment: 979 bp; **b** amplified u-PA cDNA fragment: 612 bp; **c** amplified u-PAR cDNA fragment: 513 bp; **d** amplified PA-1 cDNA fragment: 450 bp; **e** amplified PAI-2 cDNA fragment: 1067 bp

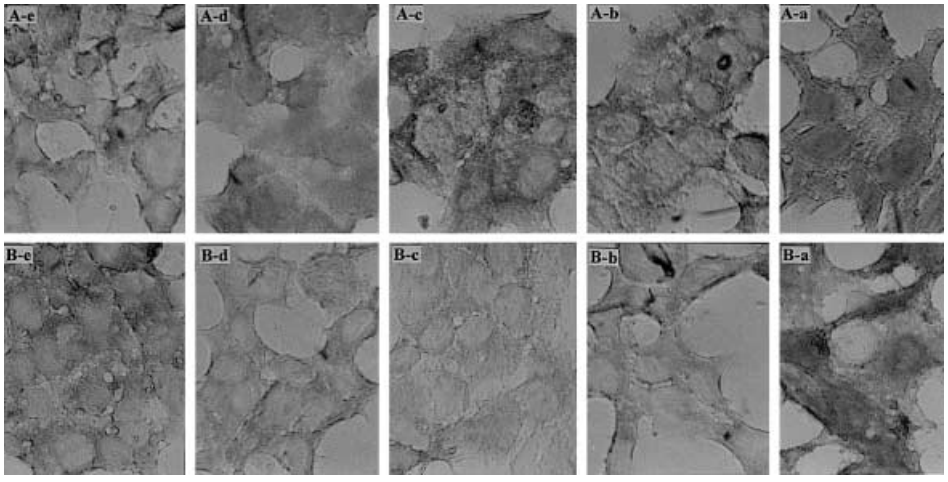

Fig. 6 Typical examples of immunocytochemical staining for **a** t-PA, **b** uPA, **c** uPAR, **d** PAI-1, and **e** PAI-2, expressed in 95D (*A*) and 95C (*B*) cells

The effects of anti-uPA, anti-uPAR, and anti-PAI-1 monoclonal antibodies on the invasive potential of strain 95D cells in vitro

The invasive potential of strain 95D cells treated with anti-uPA, anti-uPAR, and anti-PAI-1 monoclonal antibodies (40 μg/ml) was reduced to 23.62%, 48.4%, and 86.9%, respectively, compared with the control group (Table 2).

Discussion

Strain 95D and 95C cells, isolated from low-different human large-cell lung carcinoma cell line PLA-801, were of different degrees of metastatic potential and tumorigenesis (Lu et al. 1989). They came from the same cell line and have a similar genetic background, so they are ideal models for studying the mechanism of tumor metastasis. In this study, we further confirmed that the invasive and metastatic potential of strain 95D cells was stronger than that of strain 95C cells ($P < 0.001$). It indicates that the metastatic potential of 95D and 95C cells has not changed since they were cloned in 1989.

The endogenous expressions of components of the plasminogen activator system in the two groups of cells were compared and analyzed using RT-PCR and immunohistochemical staining. We found that the expression levels of uPA and uPAR in strain 95D cells were higher than those in strain 95C cells, while PAI-2 and t-PA were weaker in expression. PAI-1 showed almost the same strong expression in both cell lines. These results suggest that the high-expression of uPA/uPAR and the low-expression of PAI-2/tPA are associated with the high invasive/metastatic potential of tumor cells. Previous reports maintained that the high-expression of uPA and uPAR was positively related to metastatic potential in the primary and metastatic locus of breast, colon, liver, prostate, and lung cancer and many other cancers (Miyake et al. 1999; Hudson et al. 1997; Park et al. 1997; Pappot et al. 1999). uPA could bind to uPAR on the surface of tumor cells which not only mediated the fibrolytic activities to enhance the degradation of the extracellular matrix and the migration of tumor cells, but enhanced the differentiation of tumor cells by signal transduction (Dear et al. 1998). In these processes, PAI-1 can bind to uPAR-bound uPA and results in internalization of the PAI-1:uPA:uPAR complex, leading to degradation of PAI-1 and uPA and recycle of uPAR on the cell surface (Nykjaer et al. 1997). Many clinical and experimental studies have proved that the high expression of uPA, uPAR, and PAI-1 was closely related to the unfavorable prognosis of tumors (Harbeck et al. 1999; Abe et al. 1999; Knoop et al. 1998). uPA, uPAR, and PAI-1 levels were considered as independent and significant prognostic markers. In this study, the high expression of PAI-1 in strain 95D and 95C cells could be associated with their low-differentiated characters.

As to the importance of PAI-2 and t-PA in the invasion and metastasis of cancer, research data indicated

Table 2 The effects of monoclonal antibodies of uPA, uPAR, and PAI-1 on the in vitro invasive potential of strain 95D cells

Number	Groups	No. of infiltrated cells (X ± SD)[a]	Comparison between groups	*q* value	*P*
1	Strain 95D + BSA	73.75 ± 7.75			
2	Strain 95D + PAI-1mAb	64.08 ± 4.75	1–2	5.12	< 0.01
3	Strain 95D + uPARmAb	35.70 ± 8.03	1–3	19.22	< 0.01
4	Strain 95D + uPAmAb	17.42 ± 8.03	1–4	29.80	< 0.01

[a] The number of infiltrating cells in one vision field of 400×

that the high expression of PAI-2 could weaken the potential of invasion and metastasis of cancer, which could result in a favorable prognosis (Yoshino et al. 1998; Robert et al. 1999). However, the expression level of t-PA has no significant relationship with the invasion and metastasis of cancer. Recently, some researchers found that when the expressions of uPA and PAI-1 were low and t-PA was high, the recurrence rates of breast cancer obviously decreased (Kim et al. 1998). In our study, strain 95C cells produced low uPA and uPAR, and high PAI-2 and tPA, which may account for its low metastasis.

Regulating the activities of the plasminogen activator system in the anti-metastasis and anti-invasion of cancer therapy is an important strategy. An anti-sense RNA expression vector of uPA or uPAR was constructed and transferred to tumor cells to block the expression of uPA or uPAR (Go et al. 1997; Mohanam et al. 1997). On the other hand, the antagonists or antibodies of uPA or uPAR were used to block the interaction of uPA and uPAR (Abaza et al. 1998; Ignar et al. 1998). These strategies can decrease the invasion and metastasis of cancer in vivo and in vitro. In our study, the treatments of strain 95D cells with 40 μg/ml of monoclonal antibodies of uPA or uPAR also greatly decreased the invasive potential of 95D cells in vitro, the number of invasive cells being 23.62% and 48.40%, respectively, as compared with the controls. However the anti-PAI-1 antibody had little effect on the invasion of strain 95D cells. This phenomenon can be explained in terms of the complicated effects of PAI-1. On the one hand, PAI-1 suppressed the activity of uPA and t-PA, resulting in suppression of the degeneration of ECM and cancer invasion. On the other hand, PAI-1 can bind to vitronectin in matrix, which interferes with the binding of integrin and uPAR on the cell surface to vitronectin (Stefansson et al. 1996; Carriero et al. 1997; Loskutoff et al. 1999) and suppresses cell adhesion. Moreover, the endocytosis of the uPA/uPAR complex mediated by PAI-1 and recycling uPAR enhances the migration and invasion of cancer cells. The effect of anti-PAI-1 antibody on the invasion potential of strain 95D cells depends on the total action of PAI-1 (Blasi et al. 1997)

In conclusion, we compared the differences of components of the plasminogen activator system between high-metastasis and low-metastasis human lung large cancer cells from the same origin. We verified that the high expressions of uPA and uPAR were related to tumorigenesis and high-metastasis/invasion of cancer cells, and that the monoclonal antibodies of uPA or/and uPAR could effectively decrease the invasion potential of strain 95D cells in vitro. These results provide a theoretical basis for studying the molecular mechanisms of invasion and metastasis of cancers, and for finding a new anti-metastasis therapy.

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