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Transcriptional Regulation of Urokinase Receptor in High- (95D) and Low-metastatic (95C) Human Lung Cancer Cells

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Abstract To study the transcriptional regulation of urokinase receptor (uPAR) in high- (95D) and low-metastatic (95C) human lung cancer cells, we performed PCR to amplify 2238 bp uPAR promoter from 95C and 95D cells. According to the results of sequencing, five different bases are found in uPAR promoter between 95C and 95D cells. The results of luciferase activity assay showed that these differences have no significant effect on the uPAR promoter activity. Based on a normal uPAR promoter, progressive truncated mutants were constructed. The transient transfection/reporter assay showed that the promoter region from –136 to +9 may interact with relevant nuclear factors, which result in different levels of uPAR expression between 95C and 95D cells.

Key words uPAR; promoter; transcriptional regulation

The malignant neoplasm is characterized by progressive growth and the ability to disseminate tumor cells beyond the boundaries of the parent tumor. Tumor invasion and metastasis are important aspects of tumor progression and the formation of tumor and metastasis is a principal contributing factor to cancer morbidity and mortality. During this process urokinase (urokinase-type plasminogen activator, uPA) and its receptor (uPAR) play an important role.

uPAR is a 55–60 kD glycoprotein which is anchored on the cell-surface membrane by a glycosyl-phosphatidylinositol linkage and specifically recognizes pro-uPA and active uPA by their epidermal growth factor terminal domains [1,2]. As 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, uPAR is believed to be of vital importance in cancer cell invasion and metastasis. The increasing data showed that uPAR was over-expressed in a variety of cancer cell lines [3–5]. The uPAR level is correlated strongly with metastatic potential in human cancer cell lines and the high levels of uPAR may predict a high risk of cancer metastasis [3,6–8]. Yang *et al*. [9] reported that higher expression of the uPAR protein in primary tumor tissues was positively correlated with distant metastasis of colorectal cancers and negatively correlated with both patient overall survival and cancer specific survival. The regulation of expression of the *uPAR* gene is therefore important for understanding normal cell migration and tumour metastasis and may be critical in the control of cancer invasion and metastasis.

In this report, we used high- (95D) and low-metastatic (95C) human lung cancer cells to study the transcriptional regulation of uPAR expression. Our data presented here would provide the important insights into the transcription regulation of the *uPAR* gene in particular and enhance our understanding of the general molecular genetic mechanisms applied to a wider range of genes.

Materials and Methods

Materials

Pfu DNA polymerase and dNTP were purchased from

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Shanghai Sangon Corp. Restriction endonuclease, Klenow fragment and T4 DNA ligase were purchased from NEB Corp. Lipofectamine was obtained from Gibco Corp. Dual-luciferase reporter assay system was obtained from Promega Corp. A 1562 bp human uPAR promoter designated as D1553, containing the region from –1553 to +9 relative to the transcription initiation site of the human *uPAR* gene, was a gift from Prof. Peter Jones (University of Nottingham, United Kingdom).

Cell culture

The high- (strain 95D) and low-metastatic potential (strain 95C) sublines of human giant-cell lung carcinoma cell line were grown at 37 °C in atmosphere with 5% CO_2 in RPMI 1640 medium supplemented with 10% calf bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin.

Matrigel invasion assays

Matrigel invasion assays were performed by using 6.5 mm Transwell chamber (pore size: 8 μm, Costar). 50 μl RPMI 1640 medium containing 25.6 μg Matrigel was added to the upper chamber of Transwell and dried in a sterile hood for two hours at 37 °C before the addition of cells. Cells were detached from culture plates using trypsin-EDTA, and washed twice with serum-free medium containing 0.1% BSA. After being washed the cells were resuspended in the same buffer to a concentration of 1×10^6 cells/ml. 100 μl of diluted cell suspensions were added to each of the upper chambers, and then 800 μl conditioned RPMI 1640 media containing 0.1% BSA was added to each of the lower chambers. After 24 h at 37 °C, the cells that had not invaded to the lower surface of the filters were removed from the upper face of the filters by cotton swabs. The cells that had invaded were fixed in formaldehyde solution and then stained with a 0.2% solution of crystal violet in 2% ethanol. Invasion was quantitated through counting cells in 1 mm^2 square using a gridded reticle under bright-field optics. The mean of five randomly chosen 1 mm^2 field in the center of the filter (where invasion was the highest) was obtained for each well.

Western blot analysis

The cells were lysed in 1×SDS, and protein concentration was measured using BCA protein assay (Pierce, Rockford, IL). Protein extracts were subjected to 12% SDS-PAGE, and electrophoretically transferred to nitrocellulose membranes, which were then blocked for 1 h with 5% fat-free milk in wash buffer containing 0.6% NaCl (*W*/*V*), 10 mM Tris-HCl, pH 7.4, 0.025% Tween-20. The membranes were incubated with anti-uPAR (1:500, Santa Cruz), mouse anti-tubulin alpha monoclonal antibody (1:500, Newmarker), and subsequently incubated with corresponding secondary antibody conjugated with horseradish peroxidase (HRP). The resulting chemiluminescence was recorded on Kodak film.

PCR to obtain a 2238 bp uPAR promoter

The genomic DNA was prepared respectively from cultured 95C and 95D strain cells. Three pairs of primer, which were designed by Primer3 software, were used to amplify uPAR promoters from part to part. The sizes of PCR product were 807 bp (sense primer 5′-CCC CAC TGT CAA CAC AAC AA-3′, and antisense primer 5′-CGC TTG AAC CTG GGA ACT G-3′), 939 bp (sense primer 5′-GAG TGC AGT GGT GCA ATC ATA G-3′, and antisense primer 5′-TTG TCA GGA GGG ATA CTG GTA TTT-3′), and 648 bp (sense primer 5′-AAG CAA AGC AAG GGT TAA GTG T-3′, and antisense primer 5′-CTC CAA CTC ATC CTC TGA CAA C-3′), respectively. Purified PCR products were digested with *Kpn*I and *Hin*dIII, and subcloned into pGEM-3zf (+) vector which was digested with the same restriction endonucleases and then sequenced. The whole procedure including genomic DNA extraction, PCR and sequencing was repeated for three times. Full-length *uPAR* products were obtained by digesting and ligating with the convenient restriction sites which lie in the overlapping sequences and then subcloned into promoterless pGL_3-Basic vector to generate two new plasmids C2050 and D2050.

Construction of deletion mutants

The luciferase reporter construct D1553 was kindly provided by Professor Peter Jones (University of Nottingham, Nottingham, United Kingdom). Based on this plasmid, a further 5′-deletion was generated by progressive deletion of the D1553 by digesting convenient restriction sites or using PCR. D1226, D823, D401 and D136 were constructed by digesting the plasmid D1553 with *Pst*I+*Hin*dIII, *Eco*RV+*Hin*dIII, *Xba*I+*Hin*dIII and *Sma*I+*Hin*dIII respectively and then religation. D260 and D202 were generated by insertion of PCR products (5′ primers: D260 5′-CGG GGT ACC GGG TCC CAC GTT AGG AAG AGA-3′ with *Kpn*I site and D202 5′-CGG GGT ACC AGC TGT GAT CAC AAC TCC ATG A-3′ with *Kpn*I site; 3′ forward/reverse primer: 5′-TAC CGG AAT GCC AAG CTT TCT C-3′) into *Kpn*I and *Hin*dIII sites in the plasmid pGL_3-Basic. All deletion mutants were verified by sequencing.

Transient transfections and luciferase activity assay

Cultured cells were seeded in a 12-well plate (1×10^5 cells/well) in 1 ml of complete growth medium and grown to 70% –80% confluence prior to transfection. Transfections were performed by using Lipofectamine reagent according to the manufacturer's instructions. 1 μg of the promoter-reporter constructs and 0.1 μg pRL-CMV (Promega, USA) were used in each well. After 48 h incubation at 37 °C, cells were harvested in 1×passive lysis buffer (Promega, USA), and both firefly and renilla luciferase activities were measured with a single-tube assay (Dual-luciferase reporter assay system, Promega, USA), in a Luminoskan TL plus [Thermo Life Sciences (HK) Ltd.]. All the experiments were carried out in duplicate. The promoter potencies of the tested fragments were presented as the mean and standard deviation of the ratio of the firefly luciferase over the renilla luciferase activity of the duplicates and plotted against the tested constructs. All the experiments were carried out three times, and the result from one representative experiment was presented.

Results

95D cells have higher invasiveness than 95C cells *in vitro*

We used a Matrigel assay to study the invasion of lung cancer cells, one of the hallmark behaviors of this cancer. As shown in Fig. 1, the average number of infiltrating cells in one field of vision under 400× magnification was 39 ± 14 in 95C strain, and 139 ± 38 in 95D strain. Compared with 95D, the number of 95C cells that invaded through the Transwell inserts pre-coated with reconstituted basement membrane Matrigel was significantly fewer ($P<0.01$), which indicated that the invasive ability of 95D cells was stronger than that of 95C cells.

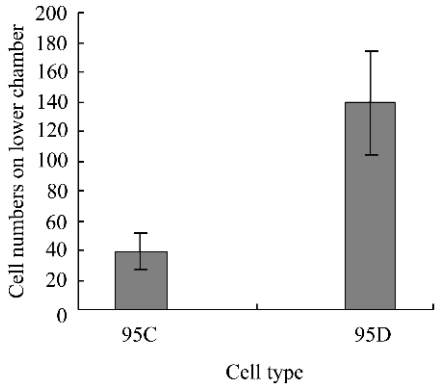

Fig. 1 Comparison of the *in vitro* invasiveness of 95C and 95D cells

95D cells express more uPAR protein than 95C cells

The expression levels of uPAR protein in 95C and 95D cells were studied by Western blot analysis. The result showed that the uPAR protein level in 95D cells was much higher than that in 95C cells (Fig. 2). Densitometry of the uPAR protein bands on the Western blot further proved this observation.

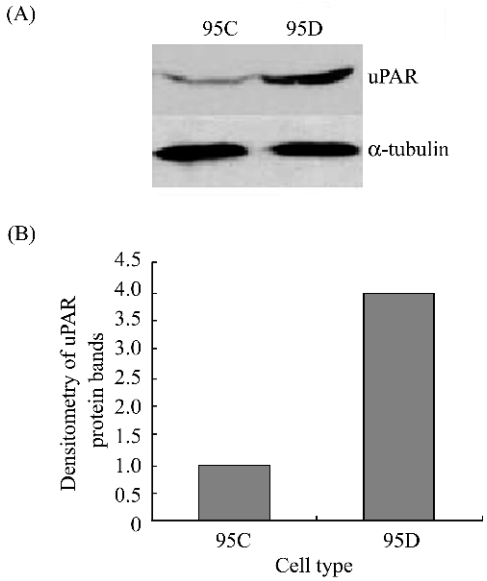

Fig. 2 Comparison of the uPAR expression levels in 95C and 95D cell

(A) The result of Western blot. Equal amounts of total proteins prepared from 95C and 95D cells were analyzed by 12% SDS-PAGE and probed with an anti-uPAR antibody. The filter was then reprobed with an anti-tubulin alpha antibody as a control for protein loading. (B) Densitometry of the uPAR protein bands. The results are shown as the ratio of uPAR and α-tubulin.

Different bases in uPAR promoter have no significant effect on promoter activity

We performed PCR to amplify the 2238 bp uPAR promoter sequences including 2050 bp of 5′-flanking region and 188 bp of downstream sequence from transcriptional initial site. The two promoter sequences from 95C and 95D cells were compared. The result showed that there were five places different in the uPAR promoter sequence between 95C and 95D cells, they were A at –464 in 95C instead of G in 95D, G at –943 in 95C instead of A in 95D, A at –1199 in 95C instead of G in 95D and A at –1272 in 95C instead of G in 95D. In addition, 95D uPAR promoter

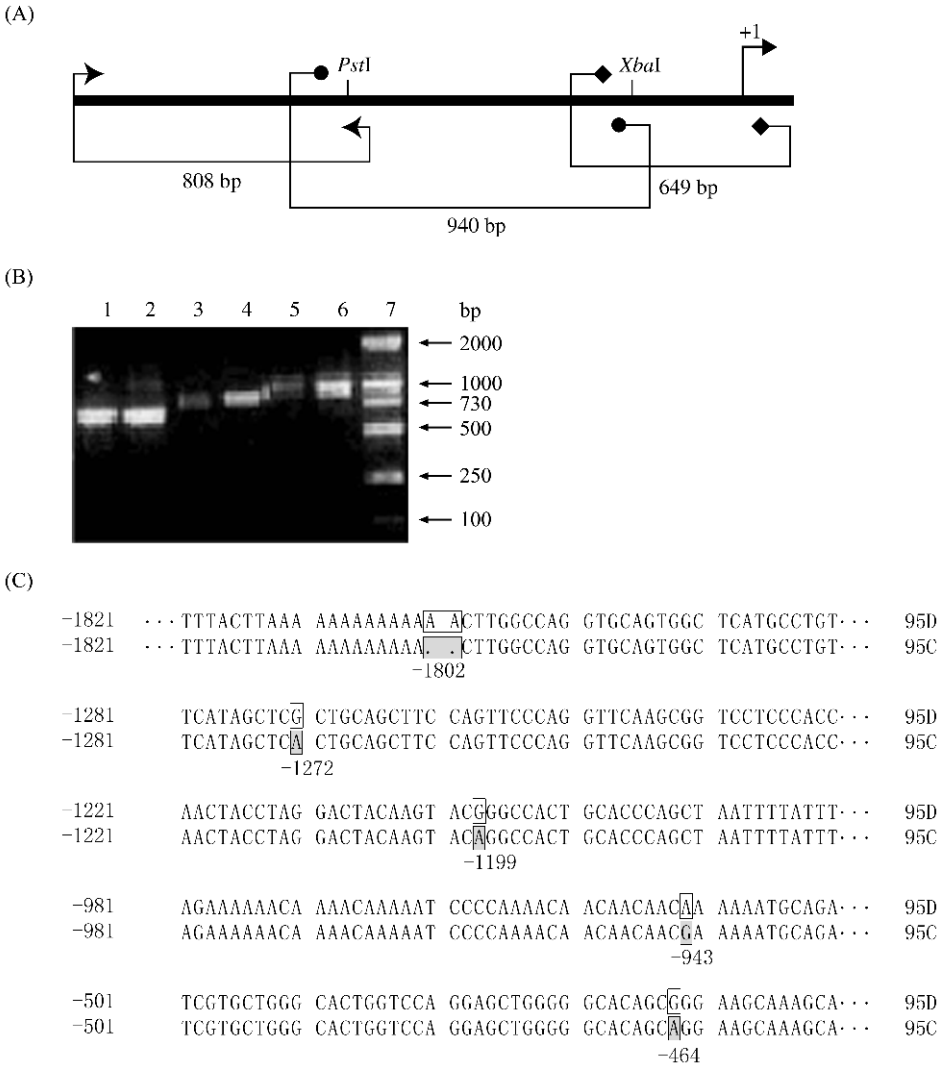

Fig.3 Electrophoresis analysis of PCR products of *uPAR* gene promoter from 95C and 95D cell strains

(A) Three pairs of primers used to amplify uPAR promoter and the size of PCR products. (B) Analyze the PCR products with 1% agarose gel. 1 and 2, 649 bp fragment amplified from 95C and 95D cell strains; 3 and 4, 808 bp fragment amplified from 95C and 95D cell strains; 5 and 6, 940 bp fragment amplified from 95C and 95D cell strains; 7, DL2000 marker from TaKaRa. (C) The different bases in *uPAR* promoter sequence copied from 95C and 95D cell strains.

sequence had two A at –1802 but 95C had none (Fig. 3).

Two 2238 bp uPAR promoter sequences respectively from 95C and 95D cell strains were subcloned into promoterless pGL_3-Basic vector to generate two new plasmids C2050 and D2050. Following the cross-transfection of 95C and 95D cells with C2050 and D2050, luciferase activities in both cell strains were assayed. The results showed that the five-base difference in uPAR promoter between 95C and 95D cells had no significant effect on promoter activity. However, both C2050 and D2050 showed stronger activity in 95D cells than that in 95C cells. This result indicated that the different levels of uPAR expression between 95C and 95D cells were not caused by the different bases but the different transcription factors (Table 1, Fig. 4).

Deletion analysis of the 1553 bp 5′-flanking region of the *uPAR* gene

To identify the region of the uPAR promoter responsible for the difference of gene expression, plasmid D2050 and constructs containing deletions of the *uPAR* gene fragments were tested respectively in 95C and 95D cells. The deletion from 5′ end to as much as –1553 in D1553 (–1553/+9) construct did not significantly affect the potency of the promoter activity in the transfected 95C and 95D cells. A remove of 287 base pairs or more from 5′ end

Table 1 Analysis of relative luciferase activity of C2050 and D2050 in 95C and 95D cells

Plasmid DNA	Relative luciferase activity (95C)	Relative luciferase activity (95D)	95D/95C	*P* value
C2050	0.010 ± 0.002	0.027 ± 0.004	2.78	0.002
D2050	0.013 ± 0.002	0.033 ± 0.005	2.62	0.002
P value	0.125	0.146	–	–

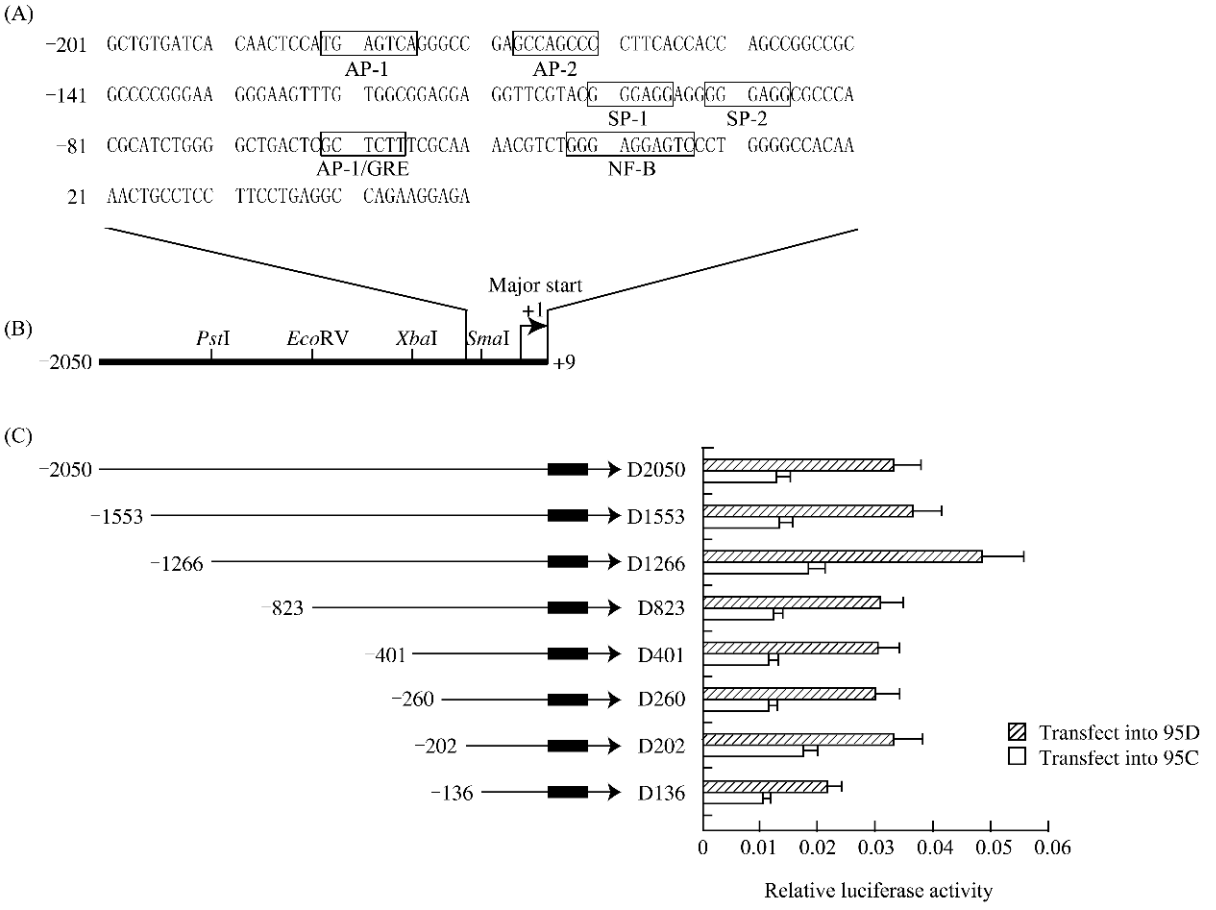

Fig. 4 Deletion mapping of the uPAR promoter

(A) Nucleotide sequence (from –201 to +9) of the genomic region surrounding the transcription starts (the major start +1 is indicated by a curved arrow). Potential *cis*-acting elements are indicated by box. (B) A partial restriction map of the uPAR promoter from –2050 to +9. The bent arrow indicates the major start site. (C) Schematic illustration of the uPAR reporter constructs used in transient transfection analysis of promoter activity in 95C and 95D cell strain. Positions relative to the transcription initiation site were indicated. The promoter-reporter constructs were co-transfected with a control plasmid (PRL-cmv) and assayed 48 h later. The results were shown as the ratio of firefly luciferase activity and renilla luciferase activity, which were expressed in mean ± SD.

of promoter segment represented by D1266 (–1266/+9) resulted in approximately 35.77% increase of the promoter activity compared with D1553. A further remove of 443 bp, represented in D823 (–823/+9), made the promoter activity recovered as much as D1553. Compared with D202, a deletion of 66 bp, represented by D136 (–136/+9) resulted in approximately 37.65% reduction of the promoter activity.

This result suggested that there might exist a negative regulatory element from –1266 to –1553 and some positive regulatory elements from –136 to –202 and –823 to –1266. In addition, all deletion mutants showed much higher activity in 95D cells than that in 95C cells, which suggested that the sequence covering from –136 to +9 may

play an important role in interacting with relevant transcription factors so as to result in different levels of uPAR expression between 95C and 95D cells.

Discussion

There is a strong correlation between uPAR expression and the cancers' invasive phenotype [10,11]. uPAR may play a critical role in the process of cancer invasion and metastasis, as antisense uPAR mRNA can inhibit cancer spread *in vitro* and *in vivo* [12]. However, at present, the regulation of its expression is yet not understood thoroughly. In this paper, we have demonstrated that the region between –136 and +9 is clearly required for elevated expression of this gene.

High-metastatic human giant-cell lung carcinoma strain 95D and low-metastatic human giant-cell lung carcinoma strain 95C were established by the pathological department of General Hospital of People's Liberation Army in 1989 [13,14]. Because both 95C and 95D were derived from the same metrocyte, they shared the similar genetic background, but owned different metastatic potential respectively. Our previous study showed that 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) [15]. For their high comparability, they were used to study the transcriptional regulation of uPAR expression.

Two 2238 bp uPAR promoter sequences (from –2050 to +9) were copied respectively from 95C and 95D genomic DNA and subcloned into the promoterless pGL3-Basic vector to generate two new plasmids C2050 and D2050. Sequencing and BLAST analysis showed that there are five different bases in uPAR promoter between 95C and 95D cell strains. Cross-transfection and luciferase assay showed that these five different bases had no significant effect on promoter activity. However, both D2050 and C2050 showed higher activity in 95D cells than that in 95C cells, which suggested that some *trans*-acting factors between two strains might be different that regulate the uPAR expression on the transcriptional level.

Deletion and transient transfection analyses suggested that there may exist a negative regulatory element from –1266 to –1553 region and some positive regulatory elements from –136 to –202 and –823 to –1266. Bioinformatic analysis of the 5′-flanking region of the human *uPAR* gene revealed an AP-1 motif at –184 position. Lengyel *et al*. [16] found that the constitutive expression of u-PAR required an AP-1 motif located 184 bp upstream of the transcriptional start site in colon cancer cell lines. Our results further proved their view. The negative regulatory element in the region from –1266 to –1553 may correlate with Alu repeats [16] which are identified as a silencer that can suppress uPAR promoter activity in an orientation- and promoter-independent fashion in both PC3 prostate and HCT116 colon cancer cells. A full-length conserved Alu sequence is necessary for maximal silencer activity. According to Dang *et al.* [17], the deletion of sequences from –1266 to –401 had no effect on promoter activity, which was different from our results. The discrepancy may reflect different mechanisms of regulation of *uPAR* gene expression in different cell lines.

All mutants showed higher promoter activity in 95D cells than that in 95C cells by more than 2-fold, which indicated that the region covering from –136 to +9 may play a central role for elevated expression of uPAR in 95D cells. According to Dang *et al.* [17], a strong promoter was located in a 188 bp element between –141 and +47 relative to the transcription-start site. From our results, the elevated expression of uPAR in high-metastatic 95D cells was also because the elements located in this region interacted with relevant transcription factors.

Bioinformatic analysis of the 5′-flanking region of the human *uPAR* gene showed that there existed several *cis*-elements in the uPAR promoter region from –136 to +9 including an AP-1, two SP-1 and an NF-κB. Zannetti *et al*. [18] reported that Sp1-binding activity and uPAR expression were coordinately up-regulated in breast cancer. NF-κB motif at –45 was also required for constitutive expression of the *uPAR* gene [19]. The presence of multiple *cis*-regulatory elements in the 5′-flanking region of uPAR promoter suggested a complex mechanism controlling *uPAR* gene expression. In addition to the AP-1, Sp-1 and NF-κB elements, the 5′-flanking region of the human *uPAR* gene may also contain potential binding sites for transcription factors AP-2 and DPE, which play roles in governing transcription of the gene.

In conclusion, our results showed that the different expression of uPAR between the two cell strains was not due to the 5-base differences in promoter sequence but the interaction between the promoter region from –136 to +9 and relevant nuclear factors, which results in different levels of uPAR expression between 95C and 95D cells.

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