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# Expression of Protein Kinase Cα and the MZF-1 and Elk-1 Transcription Factors in Human Bladder Transitional Cell Carcinoma Cells

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## Abstract

**In a recent study on hepatocellular carcinoma (HCC), we have shown that the transcription factors Myeloid Zinc Finger-1 (MZF-1) and Ets-like-protein 1 (Elk-1) are significantly related to protein kinase C alpha (PKCα) expression. The purpose of this study was to determine the correlation of the expression of PKCα with the expression of Elk-1 and MZF-1 in various differentiated urinary bladder transitional cell carcinoma (TCC) cell lines: 5637, BFTC905, TSGH8301, HT1376 and HT1197 cells. The malignant potential in the five TCC cell lines was examined by using cell proliferation/migration/invasion assay and the protein and mRNA levels of PKCα, ElK-1 and MZF-1 were examined by Western blot and RT-PCR analysis. The results showed that the rate of cell proliferation in the TSGH8301 cell line was higher than that in other cell lines, while there were obvious signs of cell migration and invasion in 5637, BFTC905 and HT1376 cells, and no sign in TSGH8301 and HT1197 cells. The resulting expression levels of Elk-1 and PKCα were the highest in 5637 cells, but the MZF-1 expression observed in all five cell lines showed no significant difference. To determine whether a correlation exists between PKCα and Elk-1, a shRNA knockout assay was performed and the results showed that the reduction of Elk-1 expression in 5637 cells did not result in the decreased PKCα expression. Therefore, although the findings showed elevated expression of Elk-1 and PKCα in 5637 cells, the regulator of PKCα in bladder cancer cells is yet to be determined.**

**Key Words: PKCα, Elk-1, MZF-1, urinary bladder transitional cell carcinoma**

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## Introduction

Protein kinase C (PKC) is an important family of signaling molecules that regulate proliferation, differentiation, transformation and apoptosis in cells (21). The ten PKC isoforms are divided into conventional (cPKCs: α, βI, βII, and γ), novel (nPKCs: δ, ε, η, and θ) and atypical (aPKCs: ξ and ι/λ) subclasses depending on their requirement for $Ca^{2+}$, phosphatidylserine and diacylglycerol (22). The α-isoform of protein kinase C (PKCα) is widely expressed in tissues regulating apoptosis, proliferation, differentiation, migration and adhesion (20). However, this isoform has been suggested to play an important role in tumorigenesis, invasion and metastasis (2, 10, 11, 14, 17, 19, 26, 27). In fact, overexpression of PKCα has been detected in tissue samples of prostate, breast, high grade urinary bladder and liver cancers by immunohistochemistry (6, 13, 15, 24, 25).

Recently, Finland *et al*. reported that the expression of PKCα increased with increasing grade of urinary bladder transitional cell carcinoma (TCC) (25) and with growth rate of TCC (1). Moreover, PKCα has been demonstrated to play an important role in cell migration and invasion of TCC (11, 19). Therefore, it has been considered that PKCα may represent a target for the development of new therapeutic agents.

Recently, our data showed that the expression of PKCα was the highest in poorly-differentiated hepatocellular carcinoma (HCC) cell lines HA22T/VGH and SK-Hep-1 as compared with well-differentiated cell lines, and PKCα was associated with the expression of Ets-likes-1 (ElK-1) and Myeloid Zinc Finger-1 (MZF-1) transcription factors (7-9, 28, 29). In this study, we investigated whether correlation between PKCα and Elk-1 and MZF-1 is also found in urinary bladder TCC cells. In this study, the expression of PKCα in TCC cells with the potential of cell proliferation, migration and invasion found to be associated with the expression of Elk-1 and MZF-1 is discussed.

## Materials and Methods

*Materials*

Anti-Elk-1, anti-MZF-1 and β-actin polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-labeled anti-mouse secondary antibody was purchased from Promega (Madison, WI, USA). The polyclonal PKCα antibody was obtained from the rabbits on day 42 after immunization as described in the previous study (24).

*Cell Culture*

Five bladder cancer cells HT1376 (BCRC No. 60058), 5637(BCRC No. 60061), BFTC905 (BCRC No. 60068) TSGH8301(BCRC No. 60145) and HT1376 were purchased from the Bioresources Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan). These cell lines were cultured with their respective media (Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G, and 100 μg/ml streptomycin (Sigma Chemicals Co., St. Louis, MO, USA) in a humidified atmosphere containing 5% $CO_2$ at 37°C.

*Cell Proliferation Assay*

Cell proliferation was determined by the yellow tetrazolium MTT assay. The cells were seeded in 24-well plates at $1 \times 10^4$ cells/well and cultured in DMEM containing 10% FBS at 37°C overnight. These cells were incubated for 24 or 48 h. After incubation, the medium was replaced with fresh medium and the cells were incubated with 5 mg/mL MTT for 4 h before dissolving in 1 ml of isopropanol for 10 min. The optical density at 570 nm was then measured using a spectrophotometer.

*Migration Assay*

Migration assay was performed using the 48-well Boyden chamber (Neuro Probe, Gaithersburg, MD, USA) plated with the 8-μm pore size polycarbonate membrane filters (Neuro Probe, Gaithersburg, MD, USA). The lower compartment was filled with DMEM containing 20% FBS. Cells at $2 \times 10^5$ cells/well in serum-free DMEM were placed in the upper part of the Boyden chamber and incubated for 12 h. After the incubation, the cells were fixed with methanol and stained with 0.05% Giemsa for 1 h. The cells on the upper surface of the filter were removed with a cotton swab. The filters were then rinsed in distilled water until no additional stain leached. The cells were then air-dried for 20 min. The migratory phenotypes were determined by counting the cells that migrated to the lower side of the filter with microscopy at 200× magnification.

*Invasion Assay*

Invasion assay was performed using a 48-well Boyden chamber with polycarbonate filters. The upper side was pre-coated with 10 μg/mL Matrigel (Collaborative Biomedical Products, Bedford, MA, USA). Cells at $2 \times 10^5$ cells/well in serum-free DMEM were placed in the upper part of the Boyden chamber and incubated at 37°C for 24 h. The rest of the experimental procedures were the same as that in the migration assay.

Invasive phenotypes were determined by counting the cells that invaded the lower side of the filter with microscopy at 200× magnification.

*Western Blot*

The cultured cells were washed twice with PBS and then lysed with a lysing buffer containing 50 mM Tris/HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 150 mM NaCl, 1 mM PMSF, 1 mM NaF, 1 mM sodium orthovanadate, 1% (v/v) 2-mercaptoethanol, 1% (v/v) Nonidet P40, and 0.3% sodium deoxycholate. The cell lysates were centrifuged at 12000 × g and 4°C for 15 min. The supernatant was collected and the protein concentration was determined by the Bradford method. Equal amounts of protein extracts (50 μg) is subjected to 12.5% SDS-PAGE and blotted onto a polyvinylidene fluoride membrane (Millipore, Belford, MA, USA). After blocking, the membrane was incubated with the specific anti-PKCα antibody (1:500), anti-Elk-1 (1:500), anti-MZF-1 (1:500) or β-actin antibody (1:2000). The blots were then incubated with HRP-conjugated anti-mouse or anti-rabbit antibody (1:3000) at room temperature for 2 h. Proteins were detected by the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

*RNA Isolation and RT-PCR Analysis*

Total RNA was isolated from cell specimens by the guanidinium thiocyanate-phenol method. The RNA integrity was assessed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The total amount of RNA was determined spectrophotometrically. RT-PCR assay was performed according to De Petro *et al*. (5) with slight modifications. An aliquot of total RNA (1 μg) was reverse transcribed. The RT product (2 μl) was diluted with the PCR buffer (50 mM KCl, 10 mM Tris-HCl, and 2 mM $MgCl_2$) to a final volume of 50 μl, containing 0.5 μM dNTPs (final concentration, 0.8 mM) and 0.5 unit of Super-Therm Taq DNA polymerase (Southern Cross Biotechnology, Cape Town, South Africa). PCR was performed on a GeneAmp PCR system 2400 (Applied Biosystems, Foster City, CA, USA). The oligonucleotide primers used in RT-PCR were as described previously (9). The PCR products were analyzed by 1.5% agarose gel electrophoresis and direct visualization after SYBR Green I (Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) staining. The agarose gels were scanned and analyzed using the Kodak Scientific 1D Imaging System (Eastman Kodak Company, New Haven, CT, USA).

*shRNA Elk-1 and MZF-1 Plasmid Construction*

We constructed the shRNA Elk-1- and MZF-1-expressing plasmid vector using the pcDNA-HU6 vector (denoted by Dr. J. Tsai Chang, Institute of Toxicology, College of Medicine, Chung Shan Medical University, Taichung, Taiwan, PRC) as the vector backbone according to Chang (4). The sequence of the shRNA Elk-1 and shRNA MZF-1 duplex from the human Elk-1 and MZF genes (GenBank, NCBI) was designed using the BLOCK-iT™ RNAi Design available at http://www.invitrogen.com and corresponded to the coding regions relative to the first nucleotide of the start codon. The sequences were designed to produce hairpin RNAs identical to the oligonucleotide shRNA duplex. To generate shRNA duplex, sense and antisense oligonucleotides (40 μM) were annealed by incubating the mixed oligonucleotides in the PCR thermocycler using the following profile: 37°C for 30 min and 65°C for 15 min. The completed shRNA duplex was then cloned into the pcDNA-HU6 vector in frame of the *BamHI* and *HindIII* sites. The insert was screened by PCR with HU6 primer and confirmed by sequencing with HU6 primer.

*Transfection*

Transfections were performed using lipofectin. Cells seeded at 60-mm dish were cultured in DMEM supplemented with 10% FBS at 37°C for 24 h. After incubation, the cells were rinsed with serum-free MEM before adding 1 ml MEM containing 15 μg/ml Lipofectamine 2000 Transfection Reagent (Invitrogen, USA) and 2 or 5 μg of the indicated plasmid. The cells were then incubated at 37°C for 6 h before adding 1 ml MEM supplemented with 20% FBS to the medium. After incubation at 37°C for 18 h, the medium was replaced with fresh 10% FBS-DMEM and the cells were incubated at 37°C for 24 h. The cells were then lysed for the above assays.

*Statistical Analysis*

Data were expressed as means ± SEM and analyzed using analysis of variance (ANOVA). Student's *t*-test was used in two-group comparisons. The association between the various factors was determined using the Pearson correlation. $P < 0.05$ was considered to be statistically significant.

## Results

*Cell Proliferation, Migration, and Invasion*

The malignant potential in the five TCC cell lines was examined by using a cell proliferation/migration/invasion assay. The results showed that the rate of cell proliferation in TSFH8301 cells was much higher

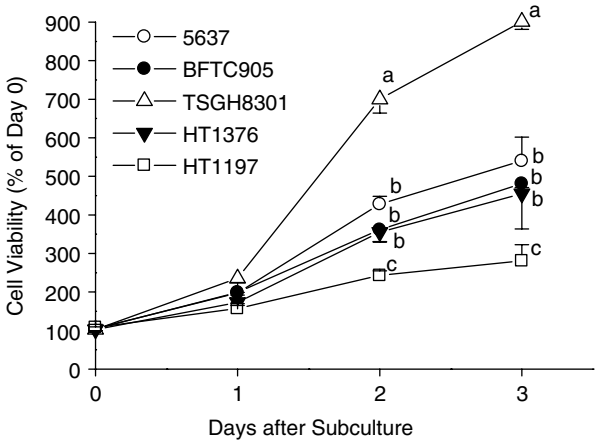


Fig. 1. Cell growth in five bladder cancer cell lines. Cell growth was determined 1-4 days after subculture using the MTT assay as described in Materials and Methods. Absorbance values obtained from cells on day 0 after subculture were taken as 100%. Data are presented as means ± SE of three replicates from three independent experiments. Means at the same day after subculture not sharing the same superscripts differ significantly ($P < 0.05$).

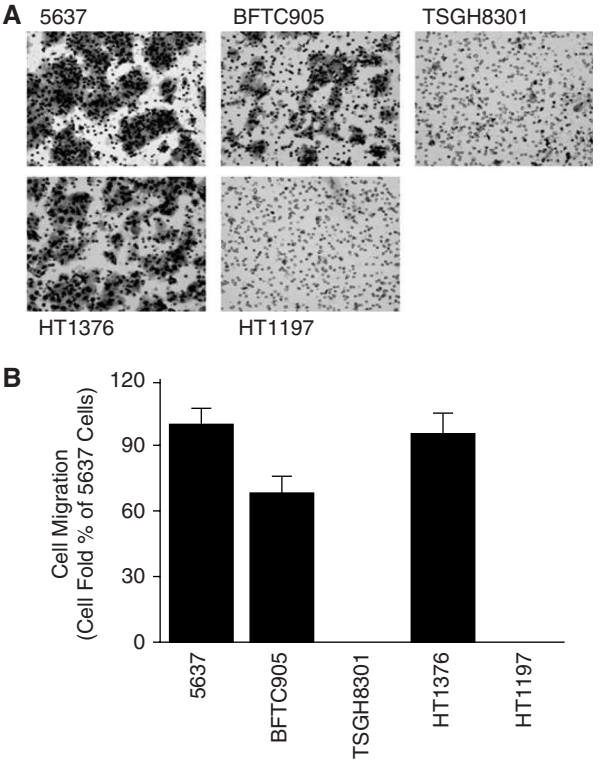


Fig. 2. Cell migration (A) and statistical analysis (B) of cell migration in five bladder cancer cell lines. Migration assays were performed on cell cultures as described in Materials and Methods. Grey dots are pores in the filter, and black areas are actually dots of migrated cells. Cell migration is determined by counting the number of cells which appear as black dots on the filter. Data are presented as means ± SE of three replicates from three independent experiments.

than those in 5637, BFT905, HT1376 and HT1197 cells (Fig. 1). The doubling time of the above cell lines was about 19.4 h for 5637 cells, 21.6 h for BFTC905 cells, 13.6 h for TSFH8301 cells, 23.1 h for HT1376 cells and 40.7 h for HT197 cells. The results showed that there were obvious signs of cell migration and invasion in 5637, BFT905 and HT1376 cells, while TSGH8301 cells and HT1197 cells did not show any sign of migration or invasion (Figs. 2 and 3).

*Expression of PKCα, Elk-1 and MZF-1*

The protein levels of PKCα, Elk-1 and MZF-1 were examined by using Western blot analysis. PKCα expression was significantly higher in 5637 cells than that in the other four cell lines while expression in HT1376 cells was not observed (Fig. 4). Elk-1 expression was also the highest in 5637 cells, similar to the PKCα expression above. Statistical analysis showed that the high expression level of PKCα gene was significantly correlated with the expression of Elk-1 gene in human bladder cancer cells (r = 0.95, $P < 0.05$). However, it was observed that there was no significant difference in MZF-1 expression in all five cell lines.

From the RT-PCR assay, it was also observed that the mRNA expression levels of PKCα and Elk-1 in 5637 cells were significantly higher than those in the other four cell lines (Fig. 5). This result was consistent to the protein assay above. However, the expression levels of PKCα and Elk-1 in the five cell lines were not significantly correlated with the potential of cell migration (r = 0.40, $P > 0.05$ and r = 0.45, $P > 0.05$, respectively) and invasion (r = 0.47, $P > 0.05$ and r = 0.49, $P > 0.05$, respectively), indicating that PKCα may not be a good indicator for predicting the potential of cell migration and invasion in bladder cancer cells.

*Reduction of Elk-1 not Decrease PKCα Expression*

To determine the connection between PKCα and Elk-1, an shRNA knockdown assay was performed and it showed that the reduction of Elk-1 expression in 5637 cells did not decrease the expression of PKCα (Fig. 6). This result indicates that the high expression level of PKCα gene is not correlated with the expression level of Elk-1 gene in human TCC cells.

## Discussion

In bladder cancer, PKCα expression increases with increasing tumor grade as assessed by immunoblot (18), and the ratio of PKCα expression in the membrane to that in cytosol was greater in cancerous

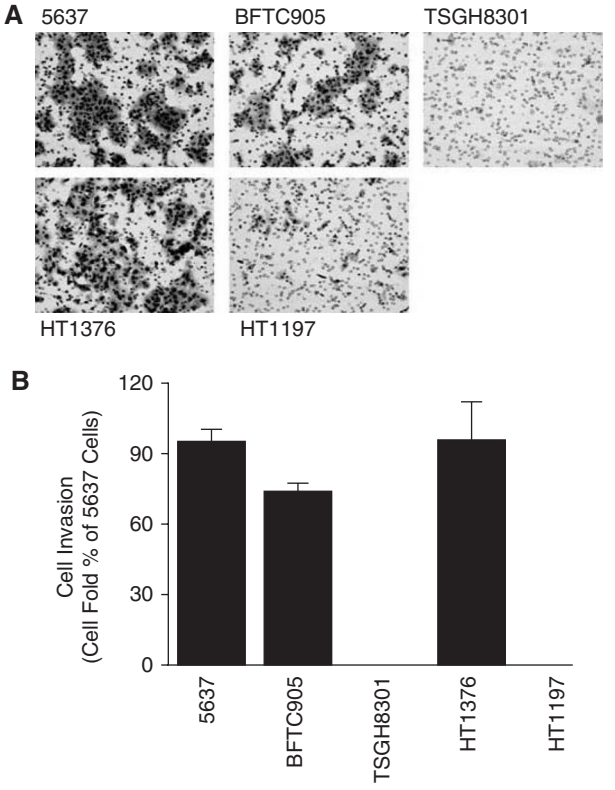


Fig. 3. Cell invasion (A) and statistical analysis (B) in five bladder cancer cell lines. The invasion assays were performed on cell cultures as described in Materials and Methods. Grey dots are pores in the filter, and black areas are actually dots of invaded cells. Cell invasion is determined by counting the number of cells which appear as black dots on the filter. Data are presented as means ± SE of three replicates from three independent experiments.

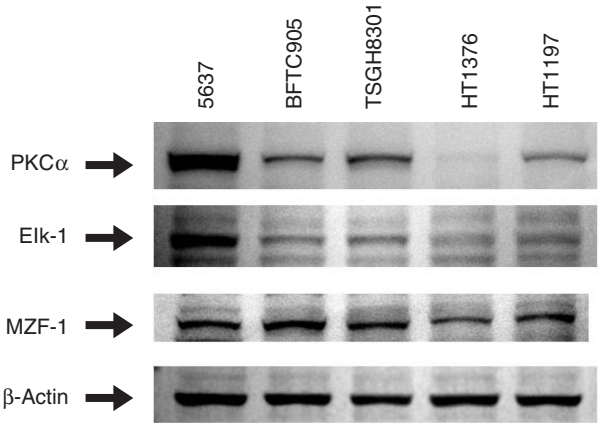


Fig. 4. Expression of PKCα and two transcription factors in five bladder cancer cell lines. The protein levels of PKCα, Elk-1, and MZF-1 were detected by Western blotting. β-Actin was used as an internal control.

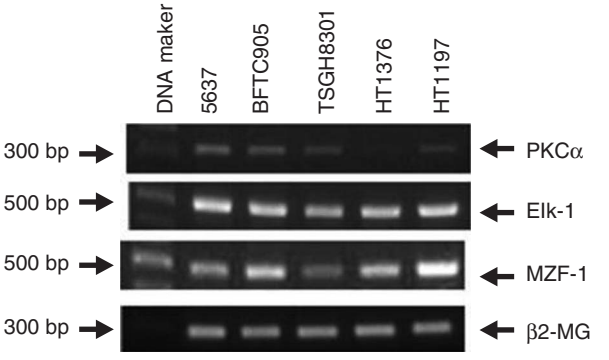


Fig. 5. mRNA levels of PKCα, Elk-1 and MZF-1 in five bladder cancer cell lines detected by RT-PCR. β2-MG was used as an internal control.

tissues than in normal tissues (12). Furthermore, with each progressive tumor grade, PKCα expression in the membrane increased and in cytosol decreased. When a survey was performed on superficial bladder carcinoma patients 2 years after their standard ADM treatment, those with a greater membrane/cytosol ratio of PKCα expression had a shorter recurrence-free period than did those with a lower ratio (12). It is suggested that PKCα may be a potential prognosis marker in malignant bladder cancer.

In a study by Leinonen *et al*. of PKCα involvement in bladder tumor development (16), when treated with PKCα-specific inhibitor Go6976, migration and invasion of 5637 cells were effectively inhibited. Moreover, gap junctions, which play a major role in intercellular calcium signaling in urothelial cells, are lost in urinary bladder carcinoma 5637 cells, and this can be improved by applying Go6976 (11). These findings revealed that PKCα expression may be involved in cell malignant progression in urinary bladder

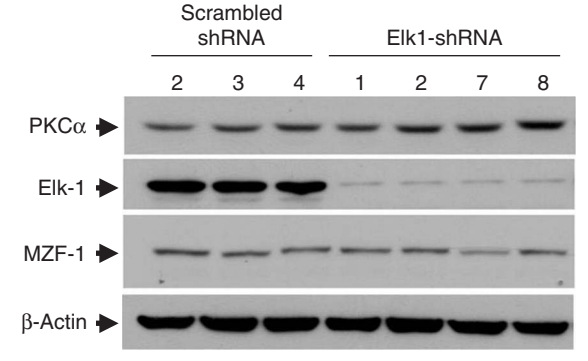


Fig. 6. Expression of PKCα, Elk-1 and MZF-1 in 5637 bladder cancer cells transfected with Elk-1 shRNA detected by Western blotting. β-Actin was used as an internal control.

carcinoma cells. Our previous study also showed that PKCα activation can promote cell migration and invasion in human HCC cells (9, 28).

However, we found in this study that while cell migration and invasion were significantly increased in BFT905, HT1376, and 5637 cells, significant level of PKCα expression in 5637 cells, low level of PKCα expression in BFT905 cells and no expression in HT1376 cells were observed. Although Leinonen *et al*. observed that in 5637 cells there was PKCα involvement in cell migration and invasion (16), the results of this study indicate that there were no significant correlations between PKCα expression and cell migration and invasion in bladder cancer cells. It is unsure how other bladder cancer cells are different from 5637 cells, however, a novel observation made by Aziz *et al*. is that PKCε, another isoform of the PKC family, also regulates human cancer cell invasion (3) and not only in the skin and the prostate but also in several cancer cell lines including the ones in the bladder (RT-4 and UM-UC-3). PKCε becomes activated in human cancers (3) and directs its partner Stat3 to maintain the invasive cancer. Therefore, it is suggested that other genes may also be involved in the malignant progression of bladder cancer.

According to the results, the expression levels of PKCα and Elk-1 in 5637 cells were the highest among the tested cells. Other papers have also shown that Elk-1 expression level was higher in 5637 cells than in HT1376 cells (23), which is consistent with our results. In an attempt to determine the connection between PKCα and Elk-1 through an shRNA knockdown assay, however, it was found that the reduction of Elk-1 expression level in the 5637 cell line did not decrease the expression level of PKCα (Fig. 6). Moreover, MZF-1 expression levels in our experiment also showed no significant difference amongst the tested cell lines which had different levels of PKCα expression. It could be that the major regulator of PKCα expression in TCC cells is not Elk-1 or MZF-1 as slhown in the HCC study that both were regulators (9). There could be other regulators involved in PKCα expression for TCC cells, and these results provided information for the search of these other regulators in the future.

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