The Expression of Protein Kinase C α and Transcription Factors in Human Bladder Transitional Cell Carcinoma Cells

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Running Head: PKCα and MZF-1 and Elk-1 in Human TCC cells

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Abstract

The purpose of this study was to determine the correlation of the expression of PKCα with the expressions of Elk-1 and MZF-1 in the various differentiated urinary bladder transitional cell carcinoma (TCC) cells: 5637, BFTC905, TSGH8301, HT1376 and HT1197 cells. The malignant potential in the five TCC cells were examined by using cell proliferation/migration/invasion assay and the protein and mRNA levels of PKCα, ElK-1 and MZF-1 were examined by using Western blot and RT-PCR analysis. The results showed that the rate of cell proliferation in TSGH8301 cells was higher than that in other cells, while the potential of cell migration and invasion in 5637, BFTC905 and HT1376cells were higher than those in TSGH8301 and HT1197 cells. The resulting expressions of Elk-1 and PKCα were highest in 5637 cells, but the MZF-1 expression observed in all five cells showed no significant difference. To determine whether a correlation exists between PKCα and Elk-1, a shRNA knockout assay was performed and the result showed that the reduction of Elk-1 expression in the 5637 cell line does not decrease PKCα expression. Therefore, although a correlation between Elk-1 and PKCα expression exists in liver cancer, and the finding showed elevated expressions 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.

Introduction

Protein kinase C (PKC) is an important family of signaling molecules that regulate the 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 cancer and hepatocellular cancers by immunohistochemistry (6, 13, 15, 24, 25).

Recently, Finland's and Hungary's scientists reported that the expression of PKCα increased with increasing the grade of urinary bladder transitional cell carcinoma (TCC) (25) and with accelerated 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 represents an interesting and challenging target for the development of new therapeutic agents.

Recently, our data showed that the expression of protein kinase C alpha (PKCα) was found to be highest in poorly differentiated hepatocellular carcinoma (HCC) cell lines (HA22T/VGH and SK-Hep-1) as compared with the well differentiated ones, and it was associated with the expressions of Ets-likes-1 (ElK-1) and Myeloid Zinc Finger-1 (MZF-1) transcription factors (7, 8, 9, 28, 29). Therefore, we would next like to know whether the correlation of PKCα with Elk-1 and MZF-1 is also found in urinary bladder transitional cell carcinoma (TCC) cells. In this study, the expression of PKCα in TCC cells with the potential of cell proliferation and migration and invasion associated with the expression of the transcription factors is discussed.

Methods and Materials

Materials

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

Cell culture

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

Cell proliferation assay

Cell proliferation is determined by the yellow tetrazolium MTT assay. The cells are seeded in 24-well plates at 1×10^4 cells/well and cultured in DMEM containing 10% FCS at 37°C overnight. These cells are treated with and without various plasmids and incubated for 24 or 48 hours. After incubation, the medium is replaced with fresh medium and the cells are incubated with 5 mg/mL MTT for 4 hours before dissolving in 1 ml isopropanol for 10 minutes. The optical density at 570 nm is then measured using a spectrophotometer.

Migration assay

Migration assay is performed using the 48-well Boyden chamber (Neuro Probe, Gaithersburg, MD) plated with the 8-μm pore size polycarbonate membrane filters (Neuro Probe). The lower compartment is filled with DMEM containing 20 % FCS. Cells are placed in the upper part of the Boyden chamber and incubated for 12 hours, respectively. After incubation, the cells are fixed with methanol and stained with 0.05% Giemsa for 1 hour. The cells on the upper surface of the filter are removed with a cotton swab. The filters are then rinsed in distilled water until no additional stain leached. The cells are then air-dried for 20 minutes. The migratory phenotypes are determined by counting the cells that migrated to the lower side of the filter with microscopy at 200× magnification.

Invasion assay

Invasion assay is performed using a 48-well Boyden chamber with polycarbonate filters. The upper side is precoated with 10 μg/mL Matrigel (Collaborative Biomedical Products, Bedford, MA). Cells are placed in the upper part of the Boyden chamber and incubated at 37°C for 24 hours, respectively. The experimental procedures are the same as that in migration assay.

Western blot

The cultured cells are 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 are centrifuged at 12000×*g* and 4°C for 15 minutes. The supernatant is collected and the protein concentration is 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). After blocking, the membrane is 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 are then incubated with HRP-conjugated anti-mouse or anti-rabbit antibody (1:3000) at room temperature for 2 hours. Proteins are detected by the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

RNA isolation and RT-PCR analysis

Total RNA is isolated from cell specimens by the guanidinium thiocyanate-phenol method. The extract integrity is assessed by 1.5% agarose gel electrophoresis and RNA is visualized by ethidium bromide staining. The total amount of RNA is determined spectrophotometrically. RT-PCR assay is performed according to De Petro et al. (5)with slight modifications. An aliquot of total RNA (1 μg) is reverse transcribed. The RT product (2 μL) is 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 is performed on a GeneAmp PCR system 2400 (Applied Biosystems, Foster City, CA). The oligonucleotide primers used in RT-PCR are as described previously(9). The PCR products are analyzed by 1.5% agarose gel electrophoresis and direct visualization after SYBR Green I (Cambrex Bio Science Rockland, Inc., Rockland, ME) staining. The agarose gels are scanned and analyzed using the Kodak Scientific 1D Imaging System (Eastman Kodak Company, New Haven, CT).

shRNA Elk-1 and shRNA MZF-1 Plasmid Construction

We constructed the shRNA Elk-1- and shRNA 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) 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) is 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 designed to produce hairpin RNAs identical to the oligonucleotide shRNA duplex sequences. To generate siRNA duplex, sense and antisense oligonucleotides (40 μM) are 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 is then cloned into the pcDNA-HU6 vector in frame of the *BamHI* and *HindIII* sites. The insert is screened by PCR with HU6 primer and confirmed by sequencing with HU6 primer.

Transfection

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

Statistical Analysis

Data were expressed as mean ± SEM and analyzed using analysis of variance (ANOVA). Student's t-test was used in two-group comparisons. $P<0.05$ was considered to be statistically significant.

Results

Cell Proliferation, Migration, and Invasion

The malignant potential in the five TCC cells were examined by using cell proliferation/migration/invasion assay. The results showed that the rate of cell proliferation in TSFH8301 cells was much higher than those in 5637, BFT905, HT1376 and HT1197 cells (Fig. 1). The doubling time of the above cells is about 19.4 hrs for 5637 cells, 21.6 hrs for BFTC905 cells, 13.6 hours for TSFH8301 cells, 23.1 hours for HT1376 cells, and 40.7 hours for HT197 cells.

The results showed that the potential of cell migration and invasion in 5637, BFT905, and HT1376 cells were highest amongst the tested cells, while TSGH8301 cells and HT1197 cells didn't show any sign of migration or invasion (Fig. 2 and 3).

Expressions of PKCα and Elk-1 and MZF-1

The protein level 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 cells while expression in HT1376 was not observed (Fig. 4). ElK-1 expression was also the highest in 5637 cells, similar to the results of PKCα. It was also observed that there was no significant difference in MZF-1 expression in all five cells.

From the RT-PCR assay, it was also observed that the mRNA expression of PKCα in 5637 cells was significantly higher than those in the other four cells (Fig. 5), similar to the protein assay. Also similar to the results of PKCα is the mRNA expression of Elk-1, which was also the highest in 5637 cells. However, comparing to the above result of the potential of cell migration and invasion, the difference in the five cell lines indicate that PKCα expression 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 the transcription factor Elk-1, the shRNA knockdown assay was performed and it showed that the reduction of Elk-1 expression in the 5637 cell lines did not decrease the expression of PKCα (Fig. 6). This result indicates that the high expression of PKCα gene is not correlated with the expression of Elk-1 gene in human TCC cells.

Discussion

In bladder cancer, PKCα expression increases with increasing tumor grade as assessed by western immunoblot (18), and the ratio of PKCα expression in the membrane to that in cytosol was greater in cancerous 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 had a shorter recurrence-free period than did those with lower a 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 mechanisms 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, is lost in urinary bladder carcinoma 5637 cells, and this can be improved by applying Go6976 (11). These findings reveals that PKCα expression may be involved in cell malignant progression in urinary bladder carcinoma cells, which is consistent with our study, which was to show that both high PKCα expression and high potential of cell migration and invasion are found in 5637 cells, and is also similar to a previous report we have done on human HCC cells, which in the results showed increased PKCα expression as well as increased cell migration and invasion in poorly differentiated HA22T/VGH and SK-Hep-1 cells (9, 28).

However, we found that while cell migration and invasion were significantly increased in BFT905 and HT1376 cells, less PKCα expression was observed in BFT905 cells and no expression in HT1376 cells was observed. These results indicate that there are no significant correlations between PKCα expression and cell migration and invasion in the bladder cancer cells other than that in the 5637 cells. It is unsure how other bladder cancer cells are different from 5637 cells, however, a novel finding is the observation that PKCε, an isoform of the

PKC family, also regulates human cancer cell invasion(3), 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 aggressiveness of bladder cancer.

According to the results, the expression of PKCα and Elk-1 in 5637 cells displayed the highest expressions among the tested cells. Other papers have also shown that Elk-1 expression was higher in 5637 cells than that in HT1376 cells (23), which is consistent with our results. In an attempt to determine the connection between PKCα and the transcription factor Elk-1 through a shRNA knockdown assay, however, it was found that the reduction of Elk-1 expression in the 5637 cell lines does not decrease the expression of PKCα (Fig. 6). Moreover, the MZF-1 expressions in our experiment also showed no significant differences amongst the tested cells with 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 in the HCC study where both the Elk-1 and the MZF-1 were regulators (9). There could be other regulators involved in PKCα expression for TCC cells, and these results provided information for the search of those other regulators in the future.

Acknowledgements

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Legends

Fig. 1. Cell growth in five bladder cancer cells. 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.

Fig. 2. Cell migration (A) and Statistical analysis (B) of cell migration in five bladder cancer cells. The migration assays were performed on cell cultures as described in Materials and Methods. Data are presented as means±SE of three replicates from three independent experiments.

Fig. 3. Cell invasion (A) and statistical analysis (B) in five bladder cancer cells. The invasion assays were performed on cell cultures as described in Materials and Methods. Data are presented as means±SE of three replicates from three independent experiments.

Fig. 4. The expressions of PKCα and two transcription factors in five bladder cancer cells. The protein levels of PKCα, Elk-1, and MZF-1 were detected by Western blotting as described in Materials and Methods. β-actin was used as an internal control.

Fig. 5. The mRNA of PKCα and two transcription factors in five bladder cancer cells. The mRNA levels of PKCα, Elk-1, and MZF-1 were detected by RT-PCR as described in Materials and Methods. B2-MG was used as an internal control.

Fig. 6. The expressions of PKCα and two transcription factors in 5637 bladder cancer cells transfected with Elk-1 shRNA. The protein levels of PKCα, Elk-1, and MZF-1 were detected by Western blotting as described in Materials and Methods. β-actin was used as an internal control.

Fig. 1.

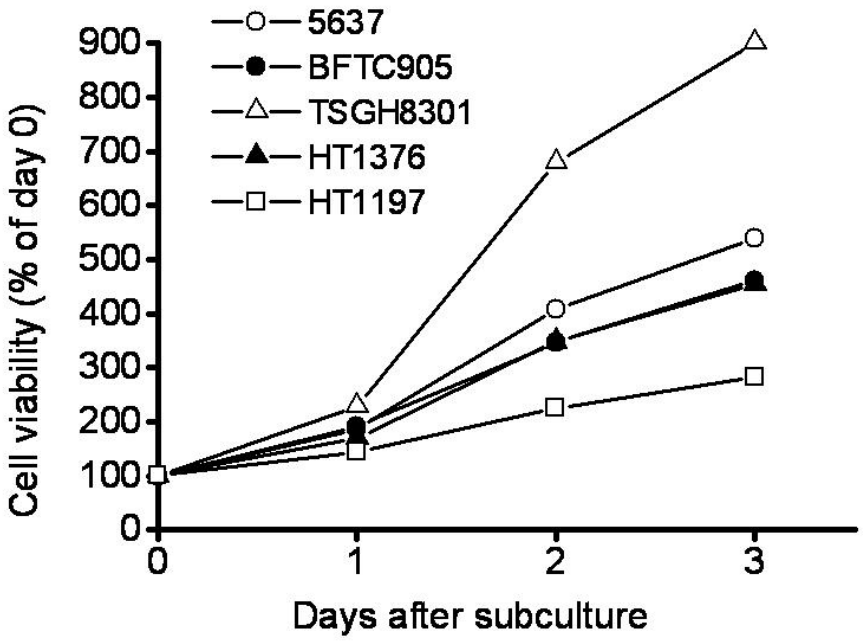

5637
BFTC905
TSGH8301
HT1376
HT1197
Cell viability (% of day 0)
900
800
700
600
500
400
300
200
100
0
0
1
2
3
Days after subculture

Fig. 2.

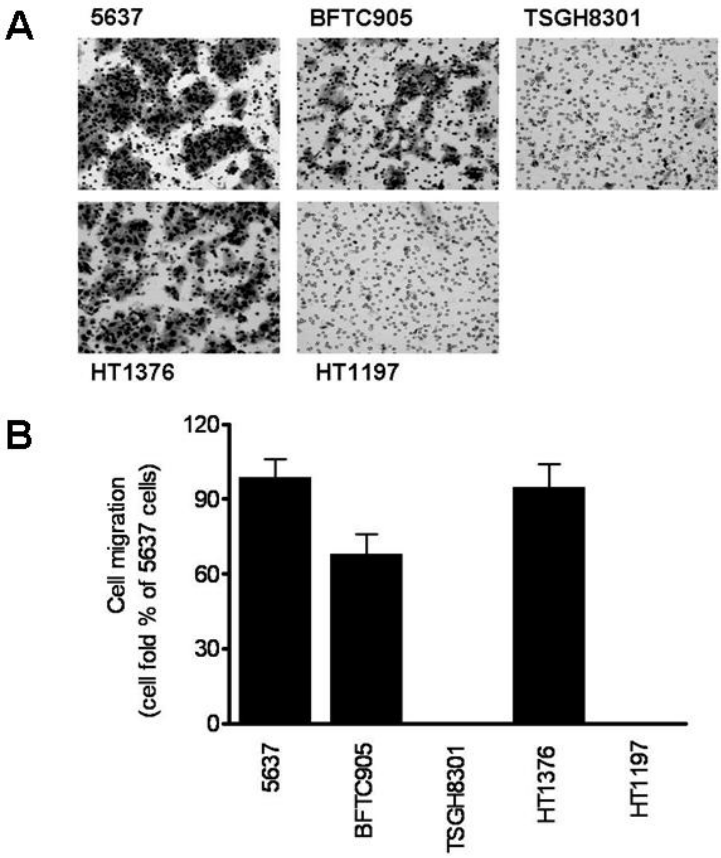
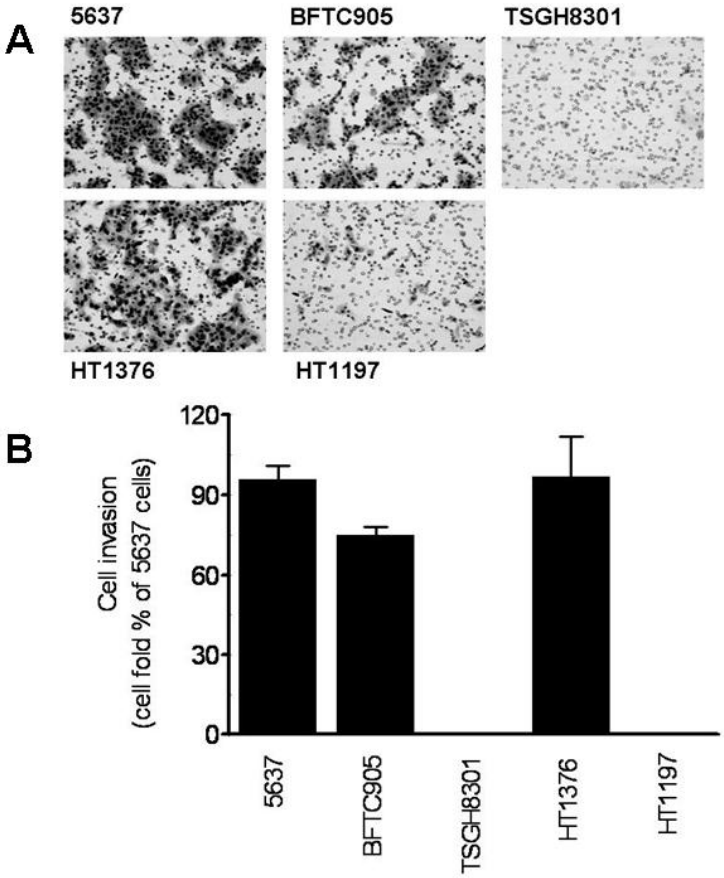

A
5637
BFTC905
TSGH8301
HT1376
HT1197
B
Cell migration
(cell fold % of 5637 cells)
120
90
60
30
0
5637
BFTC905
TSGH8301
HT1376
HT1197

Fig. 3.


A
5637
BFTC905
TSGH8301
HT1376
HT1197
B
Cell invasion
(cell fold % of 5637 cells)
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90
60
30
0
5637
BFTC905
TSGH8301
HT1376
HT1197

Fig. 4.

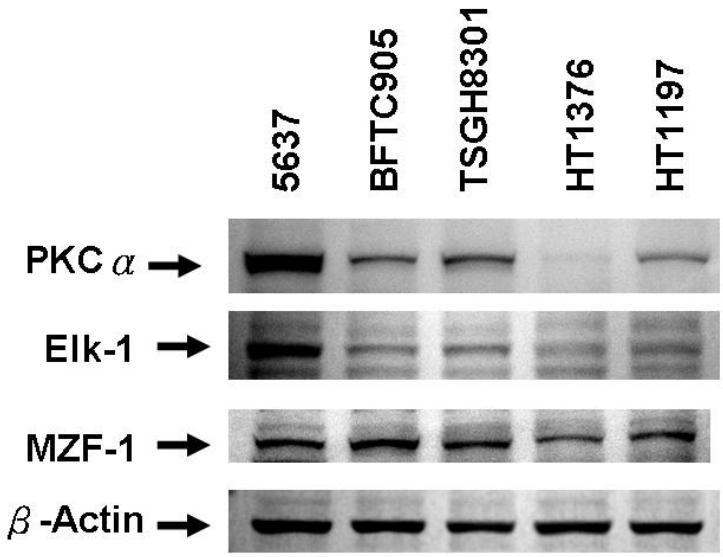

Fig. 5.

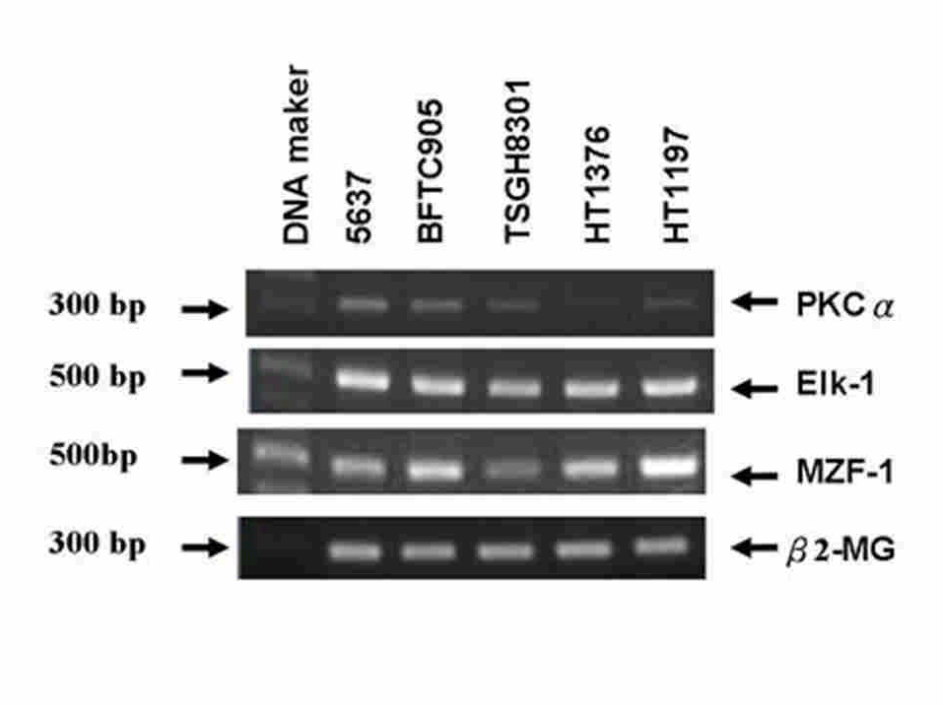
DNA maker
5637
BFTC905
TSGH8301
HT1376
HT1197
300 bp
PKC α
500 bp
Elk-1
500bp
MZF-1
300 bp
β 2-MG

Fig. 6.