The Expression of Protein Kinase C α and the MZF-1 and Elk-1 Transcription Factors in Human Breast Cancer Cells

Chia-Herng Yue [1], Yung-Wei Chiu[2,3], Jai-Nien Tung [1,3], Bor-Show Tzang[4,5], Jiuan-Jen Shiu[6], Wen-Hung Huang[6], Jer-Yuh Liu[5, 6, 7,*], and Jin-Ming Hwang[8,*]

[1]Department of Surgery, Tungs' Taichung MetroHarbor Hospital, Taichung County

[2]Hyperbaric Oxygen Therapy Center, Tungs' Taichung MetroHarbor Hospital, Taichung County

[3]Institute of Medicine, College of Medicine, Chung Shan Medical University, Taichung County

[4]Clinical Laboratory, Chung Shan Medical University Hospital, Taichung

[5]Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung

[6]Center for Molecular Medicine and [7]Graduate Institute of Cancer Biology, China Medical University and Hospital, Taichung, and

[8]School of Applied Chemistry, Chung-Shan Medical University, Taichung, Taiwan, Republic of China

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*These authors contributed equally to this paper.

Corresponding author: Jer-Yuh Liu PhD., Graduate Institute of Cancer Biology, College of Medical, China Medical University, No 6, Hsueh-Shih Road, Taichung 404, Taiwan. Tel: +886-4-22052121 ext 7801; Fax: +886-4-22347028. E-mail address: jyl@mail.cmu.edu.tw; Jin-Ming Hwang PhD, School of Applied Chemistry, Chung Shan Medical University, No. 110, Sec. 1, Jianguo N. Road, Taichung 40201, Taiwan. Tel: +886-4-24730022 ext. 11300; Fax: +886-4-23248180. E-mail address: hjm@csmu.edu.tw.

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 breast cancer cells: MDA-MB-231, Hs57BT, SKBR3, MDA-MB-468 and MCF-7 cells. The malignant potential in the five breast cancer 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 Western blot and RT-PCR analysis, respectively. The results showed that there were obvious signs of migration and invasion of cells in MDA-MB-231 and Hs57BT cells, few signs of cell migration and invasion in MDA-MB-468 cells, and no sign in SKBR3 and MCF-7 cells. Moreover, the highest expression of PKCα, Elk-1, and MZF-1 were also observed in MDA-MB-231 and Hs57BT cells when compared to the other breast cancer cell lines. These findings confirm that the elevated expression of PKCα in breast cancer cells may be correlated with the potential of cell migration and invasion, and suggest an association between the expression of PKCα and the expression of the transcription factors Elk-1 and MZF-1.

Key words: PKCα; Elk-1; MZF-1; Human breast cancer cells.

Introduction

Protein kinase C (PKC) is an important family of signaling molecules that regulate the proliferation, differentiation, transformation, and apoptosis in cells (26). 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 (29). The α-isoform of protein kinase C (PKCα) is widely expressed in tissues regulating apoptosis, proliferation, differentiation, migration, and adhesion (25). However, this isoform has been suggested to play an important role in tumorigenesis, invasion, and metastasis (1, 12, 14, 16, 19, 22, 34, 35). In fact, overexpression of PKCα has been detected in tissue samples of prostate, breast, high grade urinary bladder, and liver cancer by immunohistochemistry (7, 15, 17, 32, 33). Activation of PKCα has also been observed in breast cancer cells as well as in breast tumor samples (3, 27), while other researchers have instead found down-regulation of the PKCα protein in breast cancer (2, 13). Overexpression of PKCα is associated with decreased levels of the estrogen receptors in breast cancer cells (35, 38), and causes human breast cancer cells to show a more aggressive and metastatic phenotype, anchorage-independent growth in soft-agar and tumorgenicity in nude mice(31). Moreover, patients with breast cancer showing a $PKC\delta^{+}/PKC\alpha^{-}$ phenotype respond to anti-estrogen therapy six-times longer

than patients with the PKCδ^{+}/PKCα^{+} phenotype (4). Therefore, it has been considered that PKCα represents an interesting and challenging research target into the development of new therapeutic agents.

Recently, our data from research into the cells of other organs has shown that the expression of PKCα was found to be higher in the poorly differentiated hepatocellular carcinoma (HCC) cell lines (HA22T/VGH and SK-Hep-1) as compared with the well differentiated ones, and the expression of PKCα was associated with the expressions of Ets-like-protein 1 (ElK-1) and Myeloid Zinc Finger-1 (MZF-1) transcription factors (9, 10, 11, 36, 37). The focus of this research is whether the correlation of PKCα with Elk-1 and MZF-1 is also found in breast cancer cells. In this study, the expression of PKCα in breast cancer cells with the potential of cell proliferation, 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 (32).

Cell culture

Five breast cancer cells are MDA-MB-231, Hs57BT, SKBR3, MDA-MB-468 and MCF-7 cells 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.(6) 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(11). 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).

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 breast cancer cells were examined by using cell proliferation/migration/invasion assay. In the results, the highest proliferation rate was exhibited by MCF-7 cells at a doubling time of 38.5 hours, and the slowest proliferation rate was exhibited by SKBR3 cells at a doubling time of 65.1 hours.

The results also show that the potentials of cell migration and invasion in MDA-MB-231 and Hs57BT cells were highest amongst the tested cells (Fig. 2 and 3). Furthermore, MDA-MB-468 cells have shown little signs of migration and invasion, while SKBR3 and MCF-7 cells didn't show any signs of migration.

Expressions of PKCα and Elk-1 and MZF-1

The protein levels of PKCα, ElK-1 and MZF-1 were examined by using Western blot analysis. In the results, the PKCα expressions were significantly higher in MDA-MB-231 and Hs57BT cells than those in the other three cells while PKCα expression in MDA-MB-468 and MCF-7 cells were not detected (Fig. 4). In RT-PCR assays, the mRNA expressions of PKCα in MDA-MB-231 and Hs57BT cells were also significantly higher than those in the other three cells (Fig 5). Statistical analysis of the above findings indicates that the expression of PKCa in breast cancer cell lines is significantly correlated with the potential of cell migration and invasion ($P<0.05$), but not with the rate of cell proliferation.

The ElK-1 and MZF-1 expressions were also the highest in MDA-MB-231 and Hs57BT cells (Fig. 4). In RT-PCR assays, the mRNA expressions of ElK-1 and MZF-1 were also the highest in MDA-MB-231 and Hs57BT cells, similar to the

results of PKCα (Fig 5). These results indicate that high expression of PKCα gene has some correlation with the expression of Elk-1 and MZF-1 genes in human breast cancer cells.

Discussion

In this study, we found that PKCα expression, cell migration, and cell invasion were significantly higher in MDA-MB-231 and Hs 578T cells than those in SKBR3, MDA-MB-468 and MCF-7 cells. These results indicate that the elevated expression of PKCα in MDA-MB-231 and Hs 578T cells may be correlated with the potential of cell migration and invasion. These phenomena were similar to a previous report done on Human HCC cells, which in the results also showed increased PKCα expression as well as increased cell migration and invasion in poorly differentiated HA22T/VGH and SK-Hep-1 cells (11, 36). Moreover, many papers about experiments of the same nature have been published which show that PKCα expression is more pronounced in MDA-MB-231 when compared to MCF-7 and SKBR3 (18) and that no PKCα was present in MDA-MB-468 (23). These results confirmed that PKCα level is higher in MDA-MB-231 than that in the other breast cancer cell lines (20, 21)

The significance of PKC expression and activity in breast cancer had been demonstrated in a previous study to be higher than that in normal breast tissue (8, 28). It was also shown that the increase in urokinase plasminogen activator (uPA) secretion level in MDA-MB-231 cells, correlated with cell migration and invasion (5), can be induced by PKCα activation (24, 30) through the AP-1 and NF-kappaB signal pathways and makes the cells estrogen receptor-negative, highly invasive, and chemotherapy-resistant. Moreover, when PKCα genes are transfected to nonmetastatic MCF-7 cells, a more aggressive neoplastic phenotype is produced, and an increase in uPA expression is observed (31, 35). The results of this study reveal that PKCα expression is correlated with cell migration and invasion in breast cancer and is in agreement with the studies mentioned above.

Our previous study showed that ElK-1 and MZF-1 expression were increased in poorly differentiated HCC cells and demonstrated that they were able to regulate PKCα expression (11). Similarly, the present data also shows that the expression of PKCα may be associated with the expression of the transcription factors Elk-1 and MZF-1, as both protein and mRNA expressions of PKCα, Elk-1 and MZF-1 in MDA-MB-231 and Hs57BT cells were significantly higher than those in the other three cells (Fig.4 and 5). These phenomena were confirmed by real-time RT-PCR analysis (data dot shown). Moreover, to confirm the connection between PKCα and the two transcription factors Elk-1 and MZF-1, a shRNA knockdown assay was performed and showed that the reduction of Elk-1 or MZF-1 expression in the MDA-MB-231 cell lines decreased the expression of PKCα (data not shown, in preparation for publication). These results indicate a significant relationship between the expression of the two transcription factors and PKCα in MDA-MB-231 cells and that high expression of PKCα gene in human breast cancer cells may be regulated by the Elk-1 and MZF-1 genes.

Acknowledgements

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Legends

Fig. 1. Cell growth in five breast 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 untreated cells on day 0 after subculture were taken as 100%. Data are presented as means±SE of three replicates from three independent experiments.

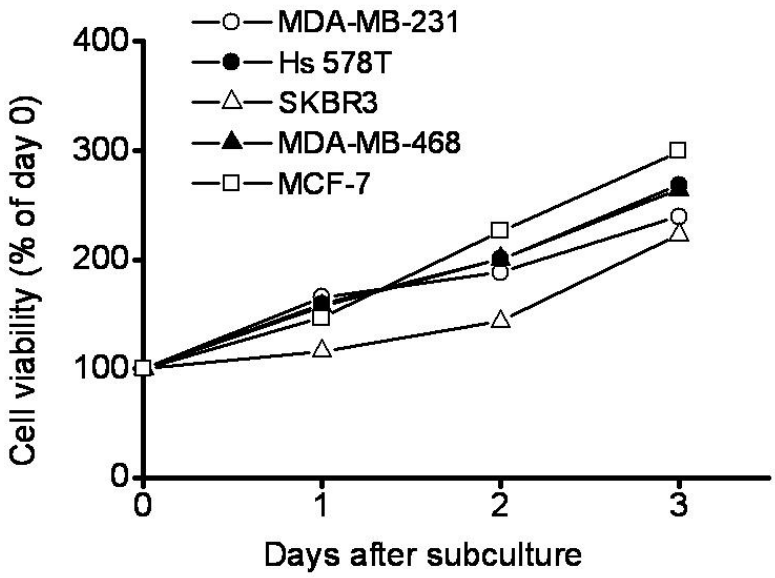
Fig. 2. Cell migration (A) and Statistical analysis (B) of cell migration in five breast cancer cells. The migration assays were performed on cell cultures as described in Materials and Methods. Data are presented as means±SE of three replicates from three independent experiments.

Fig. 3. Cell invasion (A) and statistical analysis (B) in five breast 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 breast 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 breast 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. 1.


MDA-MB-231
Hs 578T
SKBR3
MDA-MB-468
MCF-7
Cell viability (% of day 0)
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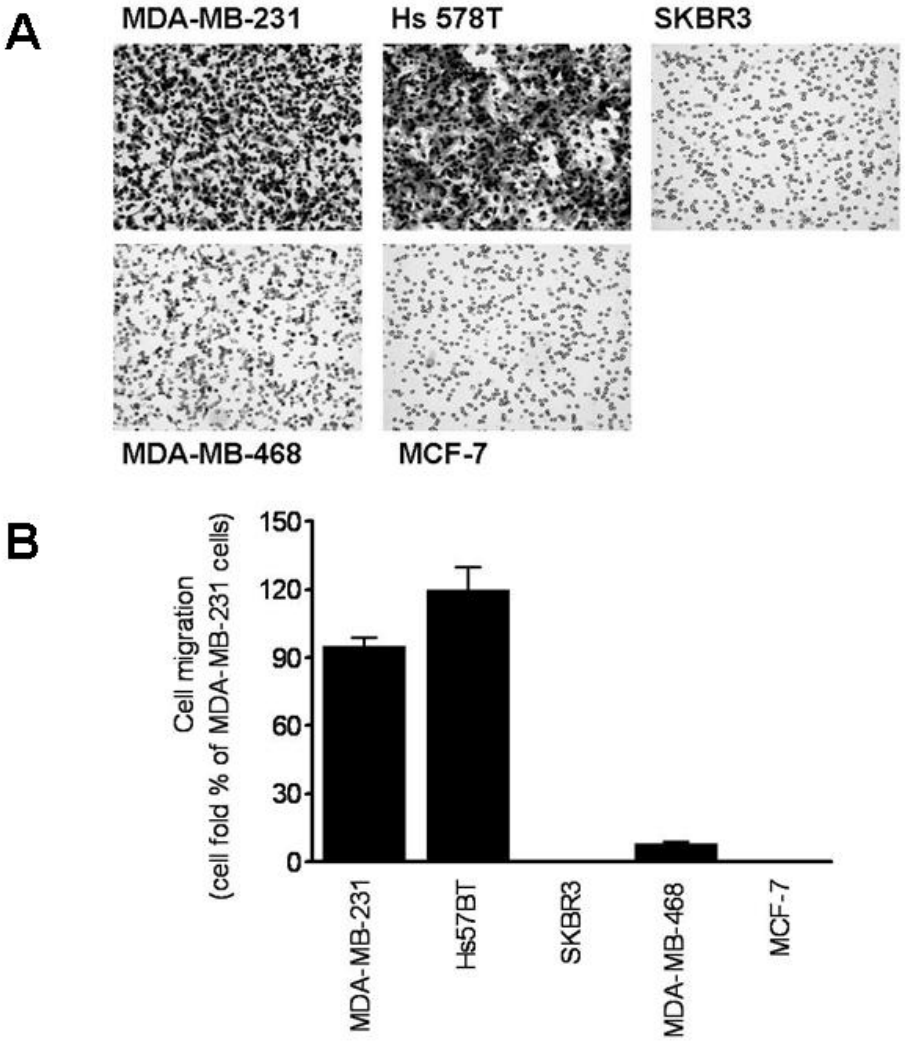
300
200
100
0
0
1
2
3
Days after subculture

Fig. 2.


A
MDA-MB-231
Hs 578T
SKBR3
MDA-MB-468
MCF-7
B
Cell migration
(cell fold % of MDA-MB-231 cells)
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60
30
0
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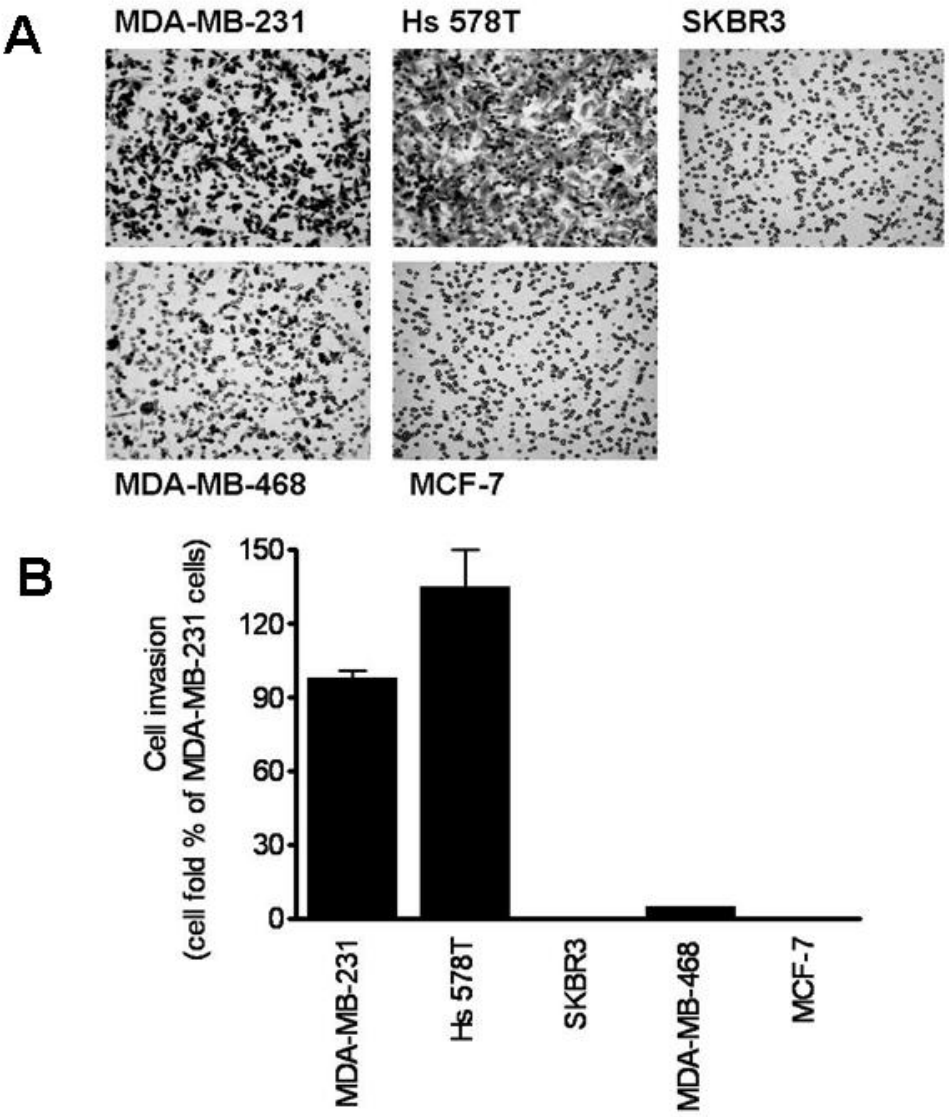
Hs57BT
SKBR3
MDA-MB-468
MCF-7

Fig. 3.


A
MDA-MB-231
Hs 578T
SKBR3
MDA-MB-468
MCF-7
B
Cell invasion
(cell fold % of MDA-MB-231 cells)
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90
60
30
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MCF-7

Fig. 4.

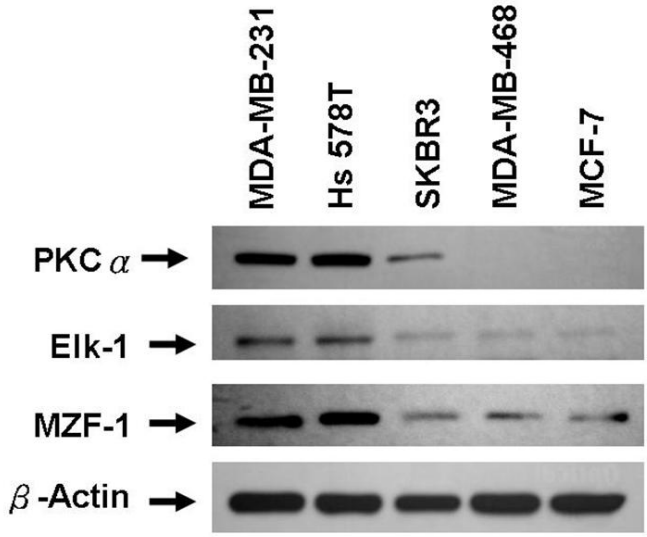
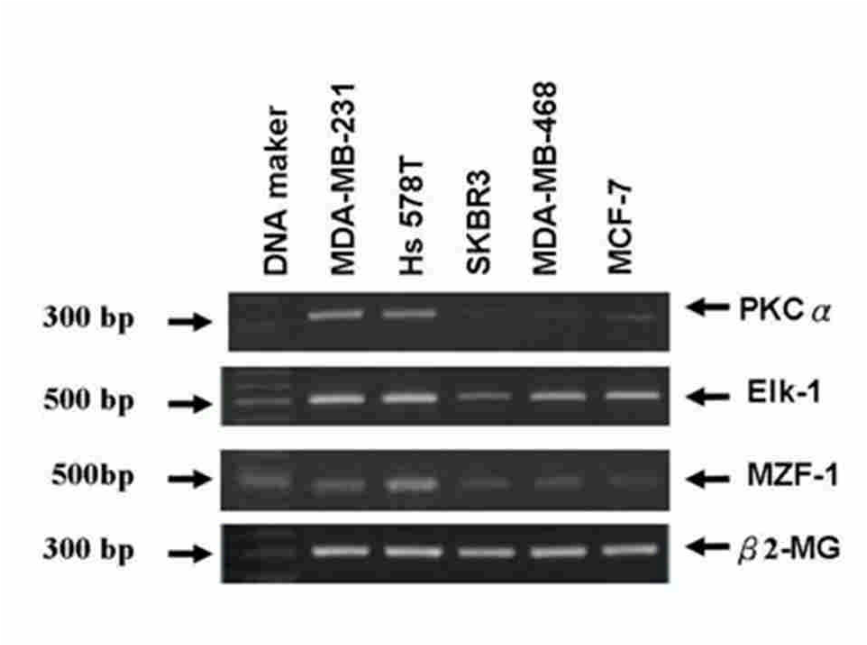


Fig. 5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PKC α
500 bp
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300 bp
β2-MG