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Cloning and Expression Analysis of Protein Kinase C Alpha Isolated from MCF7 Cell Line

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Abstract: ***Protein kinase C (PKC) is a group of phospholipid-dependent serine/threonine kinases, which is often additional divided into three PKC isozymes subfamilies: conventional, novel, and atypical. PKC isozymes are recognized to participate in cell proliferation, living expenses, invasion, migration, apoptosis, angiogenesis, as well as drug resistance. PKC isozymes are essential signal transducers associated with natural physiology and disease and have been dramatically implicated in cancers development. Enhanced PKC activity in malignant breast tissue and real correlations between PKC activity and expression of a much more excessive phenotype in breast cancer cell lines propose an activity on this signal transduction pathway in the pathogenesis and/or development of breast cancer. Resulting from their fundamental functions in cell signaling, PKC isozymes include the possibilities to be potential therapeutic objectives for several diseases. The current investigation aimed to monitor the impact of fever-range hyperthermia on human breast cancer cell line MCF7 considering cell viability and proliferation.MCF7 breast adenocarcinoma cell line were evaluated after subjected to 37°C and 40°C, cell viability, cell proliferation, and apoptosis was determined. MCF7 showed reduction in the proliferation activity combined with increasing in PKCα expression after exposing to fever range hyperthermia. Point mutation were determined using the PKCα gene sequence analysis and revealed a missense mutation in the kinase domain converted a conserved hydrophobic amino acid isoleucine into polar threonine that proved its effect on function alternation from suppression to promotion for the proliferation of the MCF7 breast adenocarcinoma cell line.***

Keywords: Protein kinase C, hyperthermia, viability, MCF7.

1. Introduction

Protein kinase C (PKC) is a family group of serine- and threonine-specific protein kinases that could be activated by calcium and second messenger diacylglycerol. PKC members of the family phosphorylate a wide selection of protein targets as they are considered to be associated with wide-ranging cellular signaling pathways. PKC members of the family in addition function as main receptors for phorbol esters, a class of tumor promoters. Every member of the PKC family includes a particular expression profile which is assumed to have a specific function in cells. The protein encoded with this gene is among the PKC family members. It really is a calcium-independent and also phospholipids-dependent protein kinase. It truly is essentially expressed in epithelial cells and also has been demonstrated to exist predominantly in the cell nucleus [11]. Calcium-independent, phospholipid- and also diacylglycerol (DAG)-dependent serine/threonine-protein kinase which is associated with the specifications of cell differentiation in keratinocytes with pre-B cell receptor, mediates specifications of epithelial restricted junction stability in addition to foam cell development, which is necessary for glioblastoma proliferation and even apoptosis protection in MCF-7 cells [15].

PKC family is divided into three subgroups depending on the structure of the regulatory domain: classical (PKC-α, bI, bII, and g), novel (PKC δ, ε, and θ) and a typical (PKC ζ and ι/l) isoforms. Classical and novel PKCs contain a diacylglycerol (DAG)-binding C1 domain and are therefore regulated by activation of pathways that lead to DAG generation. A typical PKCs are DAG-insensitive and regulated in a different manner [7].

The α-isozyme of PKC, PKC-α, is widely expressed in various tissues, it is characterized by their ability to add a phosphate group to other proteins, thus changing their function. PKC-α plays a major role in the balance of proliferation and apoptosis and its protein is widely expressed in various tissues. PKC-α expression was associated with changes in the invasion capacity of cancer cells [16]. Its expression is regulated by Elk-1 and MZF-1 transcription factors; it regulates cell migration and invasion via the activation of p38 MAPK, and regulates cell proliferation through unknown pathway [3].

PKCα role differs in different types of cancer cells. Kang (2014) reported that PKCα shows proliferative functions in several types but has antiproliferative functions in colon cancer cells. It's also mentioned that there is a relationship between PKCα and MDR phenotype. Since overexpression of P-glycoprotein (P-gp) is typical in cancer cells, P-gp-mediated MDR is one of the most serious problems facing cancer treatment. He stated that PKC isozymes, especially PKCα and ε, are involved in the Pgp-mediated MDR in several types of cancer, such as colon cancer, pancreatic cancer, gastric cancer, breast cancer, leukemia, and prostate cancer. However, he suspected that these combination therapy trials will most likely be the only way to overcome the problem of MDR. As, PKCα-specific inhibitors have been used in combination with anticancer drugs in clinical trials, but satisfactory results have not yet been obtained [5].

In breast cancer cells , a number of PKC isozymes , which include PKC α , β , δ , ε , ζ , η , and θ involved in cell proliferation , differentiation , survival , and even apoptosis . However PKC α is essential for regulating the proliferation of breast cancer cells throughout the stimulation of extracellular signal-regulated kinase (ERK) and then telomerase a number of investigation initiatives emphasis its

actual function in metastasis and drug resistance . PKCα expression leads to the metastasis of breast cancer cells throughout upregulation of some activity of matrix metalloproteinases , urokinase-type plasminogen activator , F-κB and osteopontin receptor αvβ3 in addition to throughout improving the cell surface degrees of C-X-C chemokine receptor type 4 (CXCR4) (also known as CXCL12) , and that is related to lung metastasis of breast cancer cells . Throughout tyrosine kinase receptor ErbB2 mediated breast cancer cell invasion, ErbB2 upregulates PKCα throughout c-Src kinase, causing upregulation of uPA and uPA receptors that enable cell invasion and then migration [5].

As PKCα and other PKCs have been shown to play important roles in cancer development; this kinase has become one of the major targets of therapeutic interventions for various cancers [14]. Many efforts have been made to create molecules that target PKCs for cancer therapy, but attempts to date have been largely unsuccessful. This may owe to the difficulty in creating inhibitors that target specific PKC isozymes in cancer, coupled with the challenges associated with indirectly disrupting the physiological role of PKCs in normal cells [4]. Several mutations existing in the entire coding region of the PKC family exist in a diverse range of cancers. However, it is worth noting that the majority of mutations found in PKCs resulted in a loss of function and none were activating [4].

Antal *et al.* (2015) reported that clinical trials targeting PKC have been based on the wrong assumption; it is not inactivation of PKC but, rather, activation that suppresses tumor growth. Thus, therapies should target mechanisms to restore the PKC signaling output rather than reduce it. Their comprehensive analysis revealed that 61% of the PKC mutations characterized were loss-of-function (LOF) and none were activating. They did not account for nonsense mutations or deletions, so an even higher proportion of PKC mutations are LOF [1].

The aim of this work is studying the PKCα and its relation to cell growth under the effect of fever-range hyperthermia on human breast cancer cell line MCF7 considering cell viability, proliferation and the expression of the gene.

2. Materials and Methods

Gene source: MCF-7 (human breast adenocarcinoma, CLS GmbH, Germany), cell line were generously provided by Dr. Aly Fahmy Mohamed , Head of R&D Sector (VACSERA-Cairo, Egypt), cultured using DMEM basal medium (31885-023, Gibco). Cells were collected and centrifuged and mRNA was isolated using kits GeneJET RNA purification kit, Thermo Scientific (#K0731, #K0732).

PCR: Primers (forward and reverse) were used to isolate the *PKC*α gene. Restriction sites of *Hind*III and *Xho*I were added to primers (Table 1). Two micrograms of the total RNA was transcribed into cDNA used RevertAid first strand cDNA synthesis kit (Thermo Scientific, Cat. No. K1621). cDNA fragment was amplified used the specific primers to isolate the PKCα gene. PCR was done used high fidelity Taq enzyme from Takara. The reaction was carried out in a volume of 25 µl containing 50 ng of cDNA template, 1 µM of each of the forward and reverse primers, 200 µM dNTPs, 0.5 units of high fidelity Taq polymerase enzyme and 1xTaq polymerase buffer. Amplification was carried out in Veriti® 96-Well Thermal Cycler (Applied Biosystems Catalog Number 4479071) programmed with an initial strand separation cycle at 95°C for 5 min, this was followed by 30 cycles including a denaturation step at 95°C for 30 sec., an annealing step at 60°C for 45 sec. and a polymerization step at 72°C for 2 min. The final cycle was a polymerization cycle performed at 72°C for 10 min. PCR products were examined using gel electrophoresis and stained with ethidium bromide.

Table 1: Sequence of primers used in PCR for isolating PKCα genes

Primer	Sequence (5'-3')	Length	Expected product size
PKCα F	AAGCTTATGGCT GACGTT TTCCCG	18 (24)	2020 bp
PKCα R	CTCGAGTTCATA CTGCACTCTGTA AGATGGG	25 (31)	

Cloning and DNA sequence: The PCR products obtained were cloned into pGEM®-T Easy Vector System (Promega, Cat. No. A1360). Clones were isolated after transformation of *E. coli* (DH10β). Plasmid purification and agarose gel electrophoresis were performed using alkaline lysis protocol according to Birnboim and Doly (1979)[2] and used for sequence analysis.

Double digestion reaction for the construct: The construct was digested using two restriction enzymes, *Hind*III and *Xho*I (TaKaRa), the reaction was incubated overnight in water bath at 37°C. Then a double digestion using one restriction enzyme, *Eco*RI from thermo scientific, the reaction was then incubated in water bath at 37°C for 1, 5 and 24 hours. Gel electrophoresis of digestion products was used as standard method for analyzing the reaction yield.

Sequence analysis and protein prediction: The obtained sequence was analyzed using NCBI blast and then converted into amino acid sequence, compared using multiple sequence alignment http://www.ebi.ac.uk/Tools/msa/muscle/ MUSCLE database then viewed using the SEAVIEW http://doua.prabi.-fr/software/seaview.

Proliferation assay of the effect of hyperthermia on cell culture: MCF-7 (human breast adenocarcinoma, CLS GmbH, Germany), cell line cultured using DMEM basal medium (Gibco, 31885-023,). Cells were maintained at standard conditions, 37°C, 5% CO_2 in humidified atmosphere. Cells were seeded in 6-well plates at a density of 200,000 cells/well, the plates were incubated at 37°C for 3h to facilitate adherence and normal growth and then were placed at 37°C and 40°C, for 24, 48, and 72 hours respectively. Proliferations assays for all the examined cell lines and growth temperatures were performed simultaneously. Cell proliferation measurements were performed in a 24h interval using hemocytometer counting method as described by Rouge, 2002 [12].

mRNA gene expression analysis: Total RNA was isolated using Qiagen's RNeasy mini kit following the manufacturer's instructions. Two micrograms of the total RNA was transcribed into cDNA using RevertAid first strand cDNA synthesis kit (Thermo Scientific, Cat. No. K1621). Quantitative PCR analysis was performed using BioRad SYBER green PCR instructions with Rotor-gene RT-PCR system (Qiagen). Relative quantification was calculated using the $2^{-\Delta\Delta Ct}$ method with β-actin according to Livak & Schmittgen (2001) [8].

3. Results and Discussion

Cloning of the *PKCα* full gene: All PCR products gave the expected band with different concentrations (Figure 1). The PCR products were then cloned into pGEM®-T-easy Vector System. *E. coli* DH10β was transformed with PKCα-pGEM-T-easy construct, white colonies screened with colony PCR technique. Positive cells gave band at 2020 bp which is the expected size of *PKCα* gene (Figure 2).

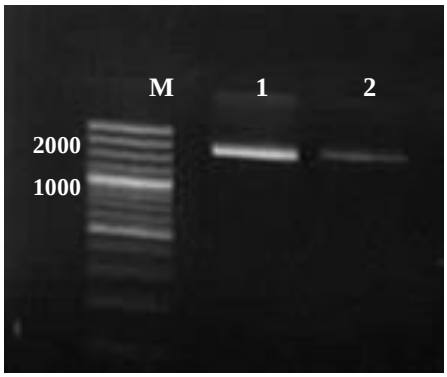

Figure 1: PCR amplification with specific primers for *PKCα* gene, M: Molecular marker 100 bp plus Fermentas, lane 1 and 2 PKCα PCR products.

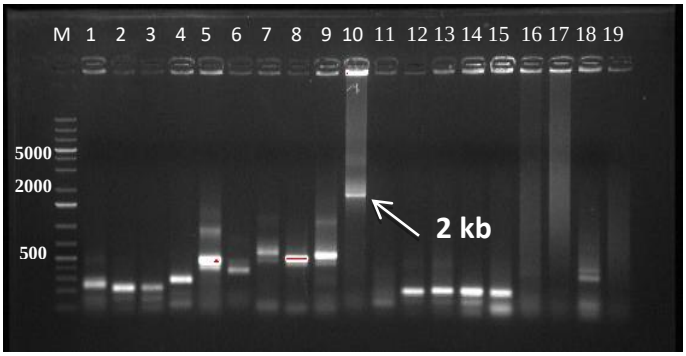

Figure 2: Molecular detection of PKCα gene in pGEM-T-easy vector using PKCα gene specific primers, M: Marker 1 kb plus Fermentas, lane 1 - 19: different colonies, lane 10: positive result for the gene detected in one colony.

Plasmid digestion: Plasmid restriction digestion using *Xho*I and *Hind*III showed a band at 1000 bp that indicates a partial digestion for the insert (Figure 3). To ensure the presence of the full gene sequence (Figure 4), *Eco*RI that presented on both borders of the multicloning site of pGEM-t-easy vector was used for digestion (Figure 5). The partial digestion is as an evidence for an expected point mutation produced a *Hind*III restriction site in the middle of the gene sequence.

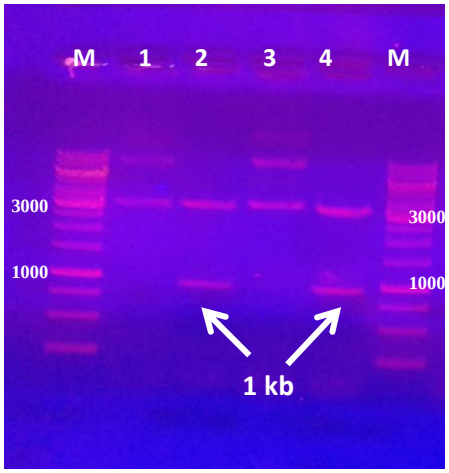

Figure 3: Restriction enzyme digestion of PKCα construct, M: Marker 1 kb Fermentas, lane1: PKCα construct, lane2: PKCα construct digested with *Hind*III, lane3: PKCα construct digested with *Xho*I, lane4: PKCα construct digested with both *Hind*III and *Xho*I.

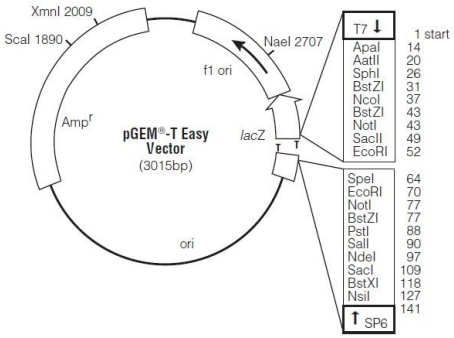

Figure 4: Map of the pGEM-T-Easy Vector, *Eco*RI was found in both sides of the multicloning site, and also T7 and SP6 universal primers that were used in sequencing.

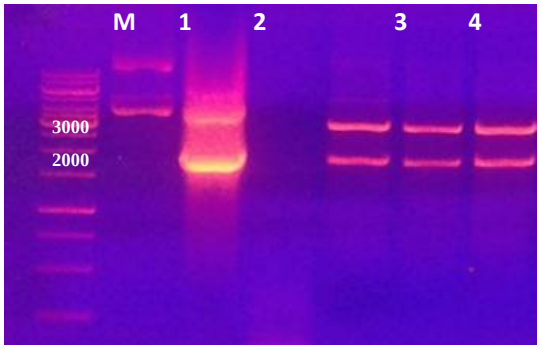

Figure 5: Restriction enzyme digestion of PKCα construct using *Eco*RI, M: Marker 1 kb Fermentas, lane1: PKCα construct, lane2: PCR product for PKCα using its primers, lane3: PKCα construct digested by *Eco*RI for 1 hr, lane4: for 5 hrs, lane5: for 24 hrs.

DNA Sequencing: The *PKCα* cloned gene was sequenced using universal primers T7 and SP6 presented on the borders of the multicloning site of pGEM-t-easy vector (Figure 4) as Sanger et al., 1977 [13]. The sequenced result of the clone confirmed the existence of PKCα open reading frame. The DNA sequence was aligned to the other PKCα DNA sequences using GenBank database National Center for Biotechnology Information (NCBI). The comparative results was shared 97% identity with expected (E) value equal zero,

indicated that a construct contained the *PKCα* gene. The nucleotide sequence was translated into amino acid sequence using nBLASTp and blast with *PKCα* protein using MUSCLE database, the result revealed a missense mutation in the kinase domain converting a conserved hydrophobic amino acid isoleucine that normally buried inside the protein core to a polar amino acids threonine (Figure 6). That may cause the protein to lose its function, which in return will affect the internal balance between proliferation and apoptosis turning normal cell into cancerous cell.

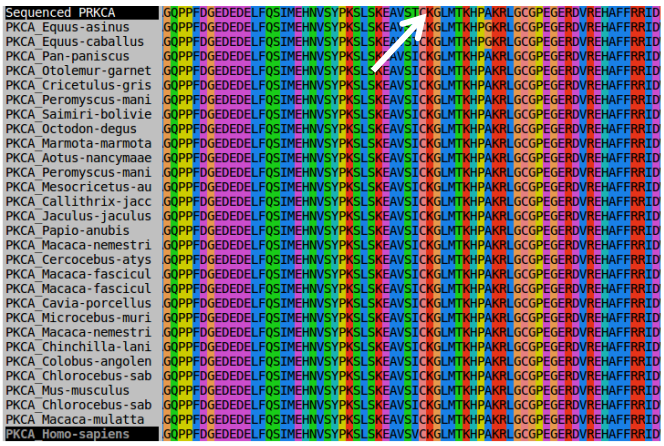

Figure 6: Multiple sequence alignment of PKCα partial sequence with submitted in GenBank database, mutation detection analysis showed a hydrophobic amino acid isoleucine (green) substituted with polar amino acid threonine (blue).

Effect of hyperthermia on MCF7 cell growth: Evaluated cell viability following a 3-day incubation period under fever range hyperthermia, changes of cell proliferation/viability were observed (Figure 7). The results obtained were reduction in cell proliferation by 2.7, 4 and 7 fold respectively, compared to control (37°C) samples, as calculated on the three days incubation. MCF7 cell line changes indicated that a decreased growth rate is linked with hyperthermia. Kalamida et al. (2015) reported that cell lines react differently toward temperature changes, showing extremes of resistance or sensitivity [5]; however MCF7 cell line in the current study showed moderate sensitivity toward hyperthermia, as the reduction was 1-2 folds during 24 hours.

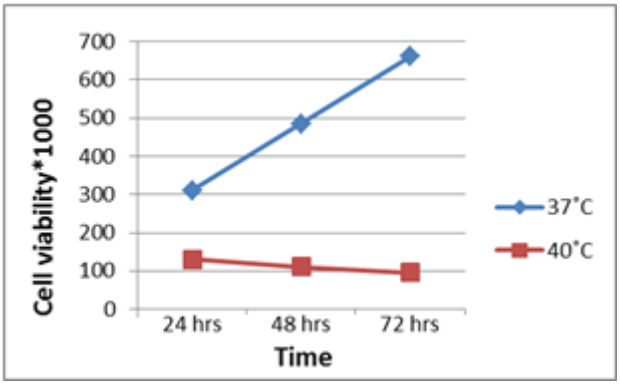

Figure 7: Cell proliferation studies after 3 days incubation at 37˚C and 40˚C.

Effect of hyperthermia on *PKCα* gene expression level: Hyperthermia sharply induced PKCα level of expression by 1.3 fold in the first day to 1.8 after 3 days, indicating that PKCα gene expression is directly correlated with sensitivity of MCF7 toward hyperthermia (Figure 8).

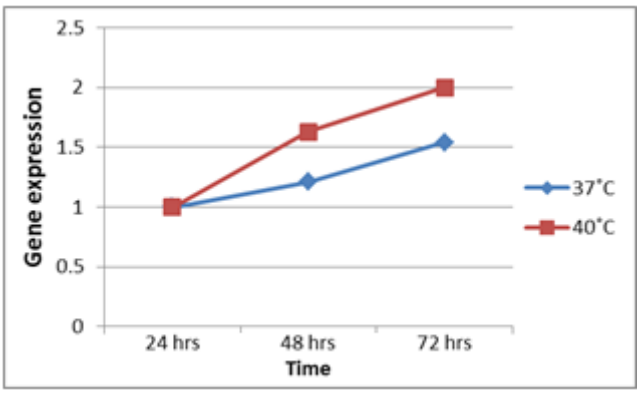

Figure 8: PKCα gene expression level in MCF7 growth at 37˚C and 40˚C

PKCα gene expression increased at 37˚C through time when the cells also showed regular proliferation rate (Figure 9). While, the cells showed a decrease in proliferation rate at 40˚C, the gene was affected positively with hyperthermia showing an increase in expression (Figure 10).

Despite PKCα mutation and expected loss of function, it cannot affect the aggressiveness of cancer cell by increasing cell proliferation.

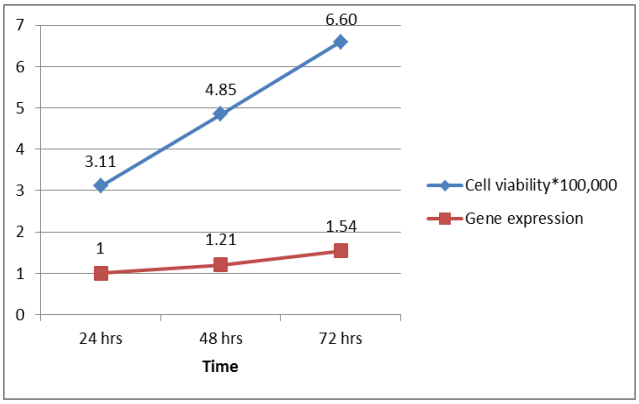

Figure 9: PKCα gene expression level vs viability of MCF7 cells growth at 37˚C.

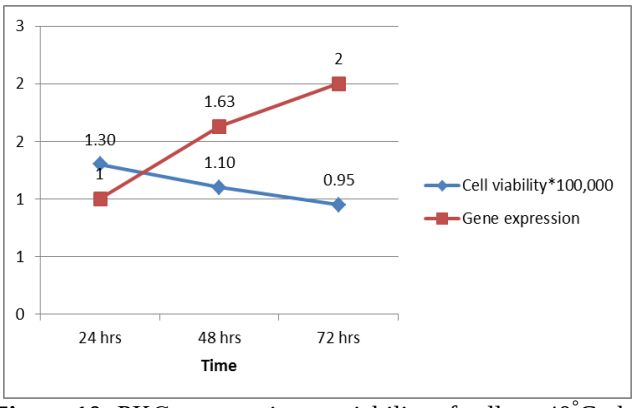

Figure 10: PKCα expression vs viability of cells at 40˚C, the gene expression increased while the inhibition in cell growth.

4. Conclusion

The ideas presented in this article highlight the effect of one of the PKC family that was considered as one of the main targets in cancer treatment; we analyze one of the PKCα mutation presented in the kinase domain that may cause the protein to lose its function, which in return will affect the internal balance between proliferation and apoptosis turning normal cell into cancerous cell. We demonstrate a high level of PKCα gene expression under the effect of heat stress that showed negative correlation to the cell growth.

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