Identification of proteins interacting with protein kinase C-δ in hyperthermia-induced apoptosis and thermotolerance of Tca8113 cells

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Abstract. The purpose of the present study was to investigate the differential proteins that interact with protein kinase C-δ (PKC-δ) in hyperthermia-induced apoptosis as well as thermotolerance in Tca8113 cells, and furthermore, to investigate the mechanisms of these processes in tumor cells. Activation of PKC-δ was previously indicated to be involved in the heat sensitivity and thermal resistance of tongue squamous carcinoma cells. Tca8113 cell apoptosis was induced by incubation at 43˚C for 80 min and the thermotolerant Tca8113 cells (TT-Tca8113) were generated through a gradient temperature-elevating method. The apoptotic rate of the cells was determined by flow cytometry, while cleavage and activation of PKC-δ were analyzed by western blot analysis. The proteins that interacted with PKC-δ in the Tca8113 and TT-Tca8113 cells were identified by co-immunoprecipitation coupled with mass spectrometry. Co-immunoprecipitation analysis followed by electrospray ionization mass spectrometric analysis were utilized to identify the pro- and anti-apoptotic proteins that interacted with PKC-δ. Significant cell apoptosis was observed in Tca8113 cells following hyperthermia, and the apoptotic rate was significantly higher than that in the control group ($P<0.05$). Marked PKC-δ cleavage fragmentation was also identified. By contrast, the apoptotic rate of the TT-Tca8113 cells was not significantly increased following hyperthermia and no PKC-δ cleavage fragmentation was observed. Among the proteins interacting with PKC-δ, 39 were found to be involved in the promotion of apoptosis and 16 in the inhibition of apoptosis of Tca8113 cells; these proteins were known to be involved in the regulation of cell proliferation, apoptosis, transcription and intracellular protein transport. The results of the present study provided evidence that PKC-δ is a crucial factor in the heat sensitivity and thermal resistance of tongue squamous carcinoma cells and elucidated the underlying molecular basis, which may aid in the improvement of hyperthermic cancer treatments.

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Introduction

Hyperthermia refers to an increase in body temperature during which the body absorbs more heat than it dissipates. This condition is used in the treatment of cancer with clear efficacy in clinical practice, and has been acknowledged as a therapeutic method for cancer treatment besides surgery, radiotherapy, chemotherapy and biotherapy (1,2). Hyperthermia has direct cytotoxic effects on tumor cells, which may result in the induction of cellular structural damage, allosterism, destabilization of DNA, RNA and proteins, and alterations in cellular metabolism (3). Hyperthermia has additionally been reported to induce cell apoptosis through upregulation of pro-apoptotic genes and/or downregulation of anti-apoptotic genes (4). In addition, previous studies have demonstrated that hyperthermia may enhance the efficacy of radiotherapy and chemotherapy, improve immunity and suppress tumor growth, metastasis and diffusion (5-7).

Apoptosis is the autonomous process of programmed cell death controlled by gene expression, which functions to regulate and maintain a stable internal environment in multicellular organisms. Previous studies have demonstrated that hyperthermia induces apoptosis in tumor cells (4,8,9). Hyperthermia at 40-45˚C was observed to induce apoptosis-mediated cell death in tumor cells, with no significant influence on normal tissues, while hyperthermia at temperatures >45˚C was identified to induce necrosis-mediated cell death with additional damage to normal tissues (10,11). A localized temperature of 43˚C is used in clinical practice for the treatment of oral and

maxillofacial tumors, the therapeutic effects of which are mediated by cellular apoptosis induced by hyperthermia (4). As an external stimulating signal, high temperatures are able to upregulate pro-apoptotic genes and/or downregulate pro-survival genes, activate apoptosis-associated pathways and promote cellular apoptosis. Previous studies have demonstrated that hyperthermia may activate the transcription of p53, B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax), Bcl-2 homologous antagonist killer, c-Myc and the caspase family of genes, thus upregulating the expression of corresponding proteins, inhibiting the expression of the anti-apoptotic Bcl-2 gene, inducing the elevation of the intracellular Ca^{2+} concentration, activating Ca^{2+}/Mg^{2+}-dependent endonucleases, activating and altering the mitochondrial membrane potential, promoting the release of pro-apoptotic factors from the mitochondria and ultimately inducing cellular apoptosis (12-16). Thermotolerance, a phenomenon involving strong resistance of tumor tissues to heat therapy, can be observed subsequent to the first application of hyperthermia (17). The generation of thermotolerance is closely associated with regulators involved in apoptotic pathways and may significantly affect the outcomes of hyperthermia therapy. Previous studies have demonstrated that the heat-shock protein (HSP) family and hypoxia-inducible factor 1α expression are increased, while the mitochondrial apoptosis pathway cascade is suppressed in thermotolerant cells (18,19).

Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine protein kinases and PKC may regulate multiple cell functions, including cell proliferation, diffusion and death. Activation and translocation of PKC-δ, a member of the PKC family, serves important roles in cellular apoptosis (20,21). The translocation pattern and substrates of PKC-δ vary depending on the cell type or stimulation by various apoptosis-inducing factors (22). Full-length PKC-δ and/or its catalytic domain are able to translocate into the majority of subcellular organelles, including the nucleus, mitochondria, Golgi apparatus, endoplasmic reticulum and cell membranes, where they phosphorylate various substrates, resulting in the induction of cell death or survival (23). The translocation of PKC-δ into specific sub-cellular compartments to phosphorylate specific substrates contributes to various biological effects (21). Previous studies have demonstrated that the expression of PKC-δ in tongue squamous carcinoma tissues is low or absent (24). Activation of PKC-δ has been regarded to be a critical molecular event in the apoptosis of human tongue squamous carcinoma Tca8113 cells induced by hyperthermic stress (24,25).

In the present study, apoptosis and thermotolerance models of human tongue squamous carcinoma Tca8113 cells were induced by hyperthermic stress. Co-immunoprecipitation analysis followed by electrospray ionization tandem mass spectrometric (ESI-MS/MS) analysis were used to screen proteins that interacted with PKC-δ under hyperthermia to either induce or inhibit apoptosis. In addition, the molecular mechanisms involved in the apoptosis and thermotolerance induced by hyperthermia were investigated from the perspective of the proteins that interact with PKC-δ.

Materials and methods

Cells and reagents. The human tongue squamous carcinoma Tca8113 cell line was purchased from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). The rabbit anti-human PKC-δ monoclonal antibody (NBP1-30126) was purchased from Novus Biologicals, LLC (Littleton, CO, USA). Rabbit polyclonal immunoglobulin (Ig)G (ab27472) and goat horseradish peroxidase-conjugated anti-rabbit IgG H&L (ab97051) antibodies were purchased from Abcam (Cambridge, UK). Protein A/G PLUS-Agarose was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Propidium iodide (PI), Triton X-100 and RNase A were purchased from Sigma-Aldrich (St. Louis, MO, USA). Radioimmunoprecipitation assay lysis buffer [containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40 and 0.5% sodium deoxycholate] and phenylmethylsulfonyl fluoride (PMSF) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). All other chemicals were of analytical grade unless otherwise stated.

Cell culture and the generation of the thermotolerant TT-Tca8113 cell line. Tca8113 cells were grown in RPMI l640 medium supplemented with 10% heat-inactivated fetal bovine serum, and 1% penicillin and streptomycin. All cells were cultured at 37˚C in a humidified 5% CO_2 atmosphere.

Thermotolerant Tca8113 cells (TT-Tca8113) were induced using the gradient temperature-elevating method. In brief, following incubation of the Tca8113 cells at 39˚C for 60 min, the cells were transferred into RPMI l640 medium and cultured for 48 h. The dead cells were removed and the residual cells were incubated at 41˚C for 60 min, followed by incubation at 37˚C for 48 h in RPMI l640 medium. The dead cells were removed again and the residual cells were incubated at 43˚C for 60 min, followed by incubation at 37˚C for 48 h in RPMI l640 medium, which was repeated five times. The cells were used for the following experiments subsequent to being passaged twice.

Thermal induction of cells and evaluation of the apoptotic rate. For thermal induction, the Tca8113 and TT-Tca8113 cells were directly incubated at 43˚C for 80 min. The cells were harvested 24 h following thermal induction, fixed in 70% ethanol at 4˚C for 2 h and were centrifuged at 35 x g for 5 min. The supernatant was discarded and subsequent to washing with phosphate-buffered saline, 500 μl PI (0.05 mg/ml containing 0.1 mg/ml RNase A and 0.05% Triton X-100) was used to re-suspend the cells for 30 min in the dark. The DNA content of the cells was determined by flow cytometry (Epics xL4; Beckman Coulter, Inc., Brea, CA, USA). A hypodiploid peak at the early G_0/G_1 phase (sub-G1 population) was considered to be an apoptotic peak, and the apoptotic rate of the cells was calculated.

Western blot analysis. Cells were harvested 4 h following thermal induction. PMSF-containing lysis buffer was added to lyse the cells for 15 min, the lysed cells were centrifuged at 4˚C and 4,800 x g for 15 min, and the supernatant was collected. The protein concentration was determined using the bicinchoninic acid assay (BCA) method (Beyotime Institute of Biotechnology). Subsequent to adjusting the protein concentration to 3.0 mg/ml, the samples were mixed with 5X loading buffer and SDS-PAGE was performed on the samples for 1.5 h. Following SDS-PAGE, the proteins were transferred to a polyvinylidene difluoride membrane for 80 min. The membrane was washed and then blocked with 5.0% skimmed milk powder for 1 h. Subsequently,

the membranes were washed three times with Tris-buffered saline containing Tween 20 (TBST) for 15 min each, and incubated at 4˚C overnight with PKC-δ monoclonal antibody (1:1,000). Following three additional washes with TBST, the membranes were incubated for 1 h at room temperature with the goat anti-rabbit secondary antibody (1:2,000) followed by another wash and imaging. Immunoreactivity was detected using an enhanced chemiluminescence kit (EZ-ECL kit for Horseradish Peroxidase; Beyotime Institute of Biotechnology) and images of the gels were captured on X-ray film (Kodak, Rochester, NY, USA). The sample expression was normalized against the expression of β-actin.

Co-immunoprecipitation coupled with ESI mass spectrometry. Cells were harvested 6 h subsequent to thermal induction. PMSF lysis buffer was used to lyse the cells for 15 min on ice, and the supernatant was collected following centrifugation at 4,800 x g for 15 min at 4˚C. The protein concentration was determined using the BCA method, following which the protein concentration was adjusted to 3.0 mg/ml, then 1.0 μg rabbit IgG and 20 μl Protein A/G PLUS-Agarose were added. The mixture was incubated at 4.0˚C for 30 min and the supernatant was collected. The anti-PKC-δ antibody (5 μg) was added to the mixture, which was then incubated overnight at 4˚C, followed by incubation for 3 h at 4˚C with 50 μl Protein A/G PLUS-Agarose. Co-immunoprecipitation-pellets were collected by centrifugation at 2,000 x g for 5 min. Subsequent to washing three times with 1.0 ml lysis buffer, 30 μl 2X SDS loading buffer was added and the mixture was boiled for 10 min. The supernatant was collected following centrifugation at 20,000 x g for 15 min at 4˚C, then SDS-PAGE and silver staining were performed. Four divided gel sections were subjected to in-gel digestion with trypsin (Sigma-Aldrich) for 20 h followed by liquid chromatography-ESI-MS/MS analysis (LTQ VELOS; Thermo Fisher Scientific, San Jose, CA, USA). The peak list files were used to query the ipi.MOUSE.v3.53 database using Mascot Daemon v.2.1 (Matrix Science, Inc., Boston, MA, USA).

Analysis of differential proteins interacting with PKC-δ. Proteins which interacted with PKC-δ in Tca8113 cells following hyperthermia but not in TT-Tca8113 cells and hyperthermia-treated TT-Tca8113 cells (therefore inducing apoptosis) were identified. In addition, proteins which interacted with PKC-δ in TT-Tca8113 and hyperthermia-treated TT-Tca8113 cells but not with PKC-δ in hyperthermia-induced Tca8113 cells (therefore having an apoptosis-inhibiting effect) were also identified.

Statistical analyses. The apoptotic rates are expressed as the mean ± standard deviation of three independent experiments. Statistical analyses were performed using SPSS software, version 16.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used to compare the differences between the two groups. $P<0.05$ was considered to indicate a statistically significant difference.

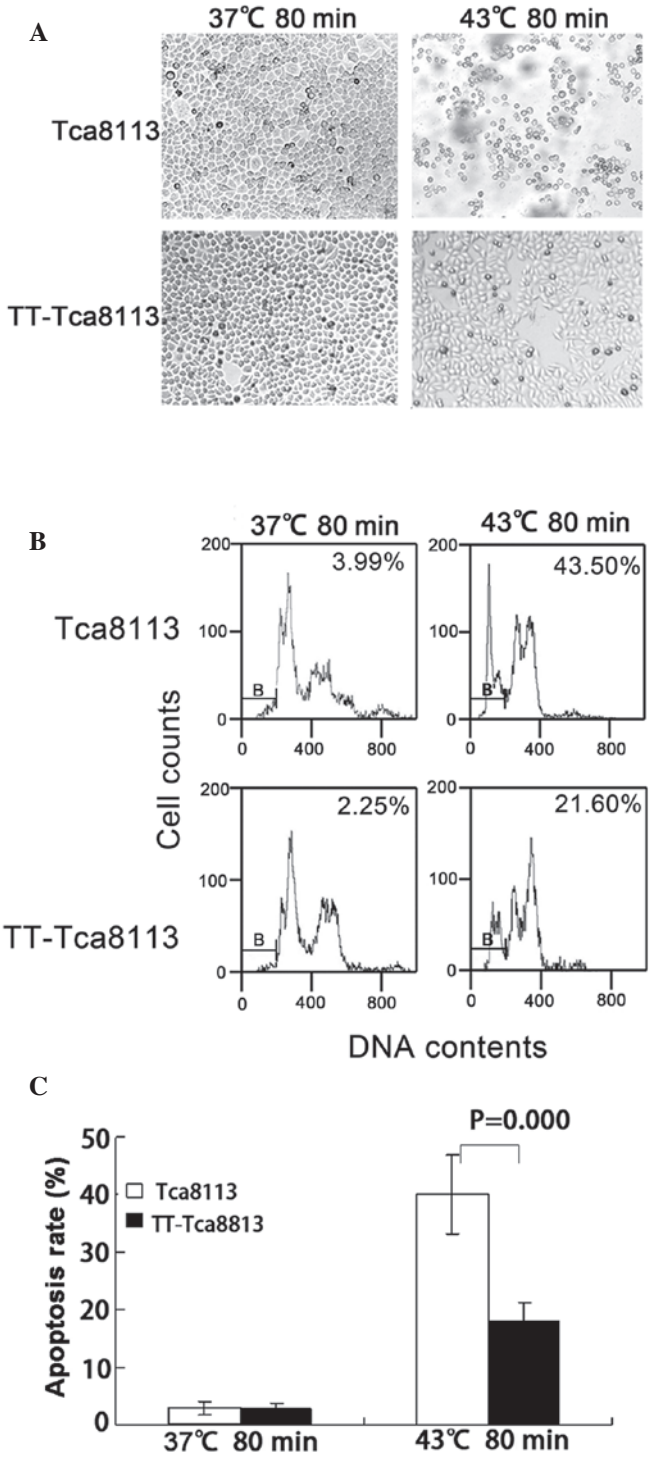

Figure 1. Establishment and characterization of TT-Tca8113 cells. (A) Tca8113 cells and TT-Tca8113 cells were heated at 43˚C for 80 min and the morphology of the cells was observed under an inverted microscope (magnification, x100). (B) Tca8113 cells and TT-Tca8113 cells were heated at 43˚C for 80 min and harvested for apoptosis assays using flow cytometry subsequent to incubation at 37˚C for 24 h. The apoptotic population was determined by propidium iodide staining. The sub-G_1 population was marked and used to represent the apoptotic population. (C) Quantified apoptotic rate of the cells. Values are expressed as the mean ± standard deviation (n=3). TT-Tca8113, thermotolerant Tca8113.

Results

Characterization of TT-Tca8113 cells. The morphology of Tca8113 cells was observed using an inverted microscope. The cells were observed to adhere to the culture plate, with a fusiform or polygonal shape and varied cell sizes. Subsequent to exposure to 43˚C for 80 min, the cells were observed to have shrunk, become rounded and floated. Flow cytometric evaluations were

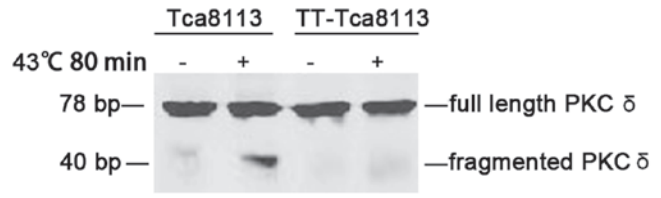

Figure 2. Analysis of heat-induced PKC-δ activation in Tca8113 and TT-Tca8113 cells. Tca8113 cells and TT-Tca8113 cells were heated at 43˚C for 80 min and then cultured at 37˚C for 4 h. Whole cell lysates were extracted and analyzed by western blot analysis using antibodies against PKC-δ. Full-length PKC-δ was expressed in the Tca8113 cells and an obvious fractured fragment of PKC-δ was present in the heat-induced Tca8113 cells, while no fractured fragment of PKC-δ was observed in TT-Tca8113 or heated-induced TT-Tca8113 cells. PKC-δ, protein kinase C-δ; TT-Tca8113, thermotolerant Tca8113; bp, base pairs.

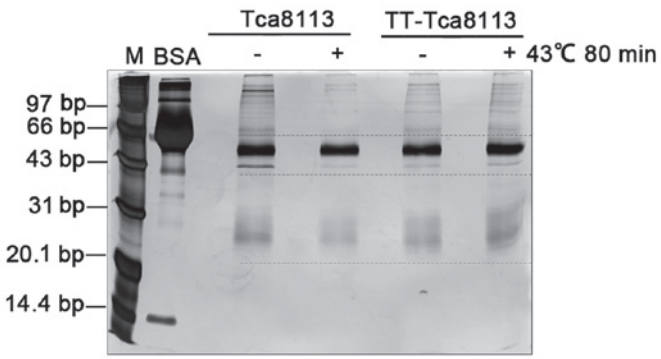

Figure 3. SDS-PAGE of proteins interacting with PKC-δ. Tca8113 cells and TT-Tca8113 cells were heated at 43˚C for 80 min, then cultured at 37˚C for 4 h and whole cell lysates were extracted. Proteins interacting with PKC-δ were immunoprecipitated from whole cell lysates. Subsequent to separation by SDS-PAGE, the proteins interacting with PKC-δ were divided into four groups (indicated by dotted lines) in each lane for mass spectrometric analysis. PKC-δ, protein kinase C-δ; TT-Tca8113, thermotolerant Tca8113; bp, base pairs; BSA, bovine serum albumin; M, marker.

performed following PI staining. The hypodiploid peak in the early G_0/G_1 phase (sub-G1 population) was considered to be the apoptotic peak, and the apoptotic rate was calculated. The apoptotic rate in the Tca8113 cells was 2.92±1.16%, while that of cells exposed to 43˚C for 80 min 24 h subsequent to exposure was increased to 40.10±6.86%, with a clear hypodiploid peak in the early G_0/G_1 phase. The apoptotic rate of the TT-Tca8113 cells was 2.86±0.86%, while following exposure to hypothermia (43˚C) for 80 min, the apoptotic rate 24 h subsequent to exposure had increased to 18.03±3.23% (Fig. 1).

Cleavage and activation of PKC-δ. Subsequent to treatment at 43˚C for 80 min, the Tca8113 and TT-Tca8113 cells were cultured for 4 h and were then harvested. The cell lysate was collected and subjected to western blot analysis for the evaluation of PKC-δ cleavage and activation. The results demonstrated that expression of full-length PKC-δ (78 kDa) was evident, while no expression of 40-kDa cleavage fragments (PKC-δ-CF) was observed; however, thermal induction of the Tca8113 cells substantially increased the amount of PKC-δ-CF. By contrast, no distinct PKC-δ-CF was identified in the TT-Tca8113 cells either prior or subsequent to incubation at 43˚C for 80 min (Fig. 2).

Differential proteins interacting with PKC-δ. Tca8113 and TT-Tca8113 cells were harvested 4 h subsequent to incubation at 43˚C for 80 min, and cell lysates were collected and treated with anti-PKC-δ antibody for co-immunoprecipitation followed by SDS-PAGE and silver staining. As demonstrated in Fig. 3, gel fragments were obtained according to the distribution of the proteins on the SDS-PAGE and subjected to digestion and mass spectrometric identification. Analysis of the proteins using the ipi.MOUSE.v3.53 database identified 103 proteins from the Tca8113 cells and 109 proteins from the hyperthermia-induced Tca8113 cells which interacted with PKC-δ. In addition, 103 proteins from TT-Tca8113 cells and 107 proteins from hyperthermia-induced TT-Tca8113 cells that interacted with PKC-δ were identified. A difference analysis among these four groups of proteins was performed, analyzing whether proteins interacting with PKC-δ were present or absent in the four groups. A total of 39 apoptosis-inducing proteins that were present in hyperthermia-induced Tca8113 cells but not in TT-Tca8113 cells or hyperthermia-induced TT-Tca8113 cells were identified. In addition, 16 apoptosis-inhibiting proteins that existed in TT-Tca8113 and hyperthermia-induced TT-Tca8113 cells but not in hyperthermia-induced Tca8113 cells were identified (Tables I and II).

Discussion

PKC-δ is an important factor in the process of apoptosis, and it has been previously demonstrated that PKC-δ serves a central role in the regulation of cell apoptosis during the responses to various apoptosis-stimulating signals (21). The functions of PKC-δ are associated with the cell type, cellular context and transposition positions (22). Full-length PKC-δ and the catalytic domain may translocate into the majority of sub-cellular compartments, including the nucleus, mitochondria, Golgi apparatus, endoplasmic reticulum and cell membranes, in order to phosphorylate various substrates and thus mediate cell death or survival (26). Translocation of PKC-δ to specific sub-cellular compartments and phosphorylation of specific substrates have been acknowledged as the mediators of cell survival or apoptosis (27).

A previous study demonstrated that the expression of pro-apoptotic PKC-δ protein in tongue squamous carcinoma tissues is either absent or expressed at low levels, while hyperthermia was shown to induce cell apoptosis in tongue squamous carcinoma Tca8113 cells by activating PKC-δ (24). In the present study, thermotolerant TT-Tca8113 cells were induced using the gradient temperature-elevating method. Flow cytometric examination indicated that the apoptotic rates of Tca8113 and TT-Tca8113 cells were 40.10±6.86 and 18.03±3.23%, respectively, following incubation at 43˚C for 80 min. Cleavage and activation of PKC-δ was further evaluated by western blot analysis, which demonstrated substantial fragmentation of PKC-δ in hyperthermia-induced Tca8113 cells, but not in TT-Tca8113 cells. These observations further suggested that PKC-δ serves a key role in the apoptosis of Tca8113 cells induced by hyperthermia.

To explore the PKC-δ-associated proteins involved in the process of apoptosis induced by hyperthermia, a thermotolerant TT-Tca8113 cell model was induced and then co-immunoprecipitation coupled with mass spectrometry and

Table I. Proteins interacting with protein kinase C-δ to promote hyperthermia-induced apoptosis in Tca8113 cells.

Protein abbreviation	Protein name	Protein localization
TNRC6B	Trinucleotide repeat-containing gene 6B protein	Cytoplasm
HSD17B10	17 β-hydroxysteroid dehydrogenase 10	Mitochondrion
MINA	MYC induced nuclear antigen	Nucleus
PMS1	DNA mismatch repair protein MS1	Nucleus
HLCS	Holocarboxylase synthetase	Nucleus, mitochondrion
ZNF767	Zinc finger family member 767	Cytoplasm
ZNF12	Zinc finger protein 12	Nucleus
PRRT2	Proline rich transmembrane protein 2	Cell membrane
LGALS8	Lectin galactoside binding soluble 8	Cytoplasm
TRAPPC3	Trafficking protein particle complex 3	Golgi apparatus, endoplasmic reticulum
POLR3E	RNA polymerase III polypeptide E	Nucleus
CEP290	Centrosomal protein of 290 kDa	Cytoplasm, cytoskeleton
S100A9	S100 calcium binding protein A9	Cytoplasm
ARFGAP1	ADP ribosylation factor GTPase activating protein 1	Cytoplasm, golgi apparatus
FAM115A	Family with sequence similarity 115, member A	Cytoplasm
FLNC	Filamin-C	Cytoplasm
TNR	Tenascin-R	Secreted, extracellular space
IPPK	Inositol-1,3,4,5,6-pentakisphosphate 2-kinase	Cytoplasm, nucleus
ACTB	Cytoskeletal actin	Cytoplasm, cytoskeleton
TUBB2C	Tubulin β-2C chain	Cytoplasm, cytoskeleton
TTN	Titin	Cytoplasm, nucleus
DNAH2	Dynein heavy chain 2	Cytoplasm, cytoskeleton
ITSN1	Intersectin 1 SH3 domain protein	Endomembrane system
LMNB2	Lamin B2	Nucleus inner membrane
TCEB3	Transcription elongation factor B polypeptide 3	Nucleus
FLJ22374	Hypothetical protein LOC84182	ND
KIAA0776	E3 UFM1-protein ligase 1	Endoplasmic reticulum
LYZ	Lysosyme	Secreted
C3 or f63	Uncharacterized protein	ND
C21 or f88	Putative uncharacterized protein	ND
THAP2	THAP domain-containing protein 2	Nucleus
WDR42A	WD repeat-containing protein 42A	ND
CTNNA1	α E-catenin	Cell membrane, cytoplasm, cytoskeleton
cDNA FLJ59573	ND	ND
RECQL5	RecQ helicase protein-like 5-β	Cytoplasm, nucleus, nucleoplasm
KIAA0467	Uncharacterized protein	ND
PTCHD2	Patched domain-containing protein 2	Membrane
P2RX5	P2X purinoceptor 5	Membrane
MGC3032	Hypothetical protein MGC3032	ND

ND, not determined.

differential protein analysis were performed. This successfully identified 39 proteins interacting with PKC-δ in the Tca8113 cells challenged by hypothermia. Of note, these proteins did not interact with PKC-δ in the TT-Tca8113 or stressed TT-Tca8113 cells, indicating that they are a potential cause of apoptosis. These proteins were predominantly comprised of the following types: i) Cytoskeletal protein, including β-actin (ACTB), lamin B2 (LMNB2) and filamin-C (FLNC). ACTB is prevalent in the cytoplasm and is involved in cell division, growth, mobility and endocytosis. PKC-δ has been reported to be able to directly phosphorylate cytoskeletal actin (ACTB) following binding, in order to mediate cell apoptosis (28). Type B nuclear lamina protein (LMNB2) is predominantly distributed in the nucleus and participates in nuclear stabilization, chromatin structure regulation and genetic expression. It was reported that following translocation into the nucleus, PKC-δ is able to co-localize with LMNB. LMNB phosphorylation by activated PKC-δ has been reported to be able

Table II. Proteins interacting with protein kinase C-δ to inhibit hyperthermia-induced apoptosis in Tca8113 cells.

Protein abbreviation	Protein name	Protein localization
HSPA9	Heat shock 70 kDa protein 9	Mitochondria
ALDOA	Fructose-bisphosphate aldolase A	Cytoplasm
PRAP1	Poly (ADP ribose) polymerase 1	Nucleus
BANF1	Barrier-to-autointegration factor 1	Nucleus, cytoplasm, chromosome, significantly enriched at the nuclear inner membrane
CDK2	Cyclin-dependent kinase 2	Nucleus, cytoplasm
HBB	Hemoglobin β	Membrane
SFPQ	Polypyrimidine tract binding protein associated splicing factor	Nuclear matrix
PC	Pyruvate carboxylase	Mitochondrial matrix
PSMD1	26S proteasome non-ATPase regulatory subunit 1	Nucleus, cytoplasm
OBSCN	Obscurin	Cytoplasm
RPL11	60S ribosomal protein L11	Nucleus
hnRNPF	Heterogeneous nuclear ribonucleoprotein F	Nucleus
RPL22	60S ribosomal protein L22	Cytoplasm
PCMT1	L-isoaspartate (D-aspartate)-O-methyltransferase	Cytoplasm
DOCK9	Dedicator of cytokinesis protein 9	Intracytoplasmic membrane
LOC342346	ND	ND

ND, not determined.

to trigger nuclear lamina disassembly and thus induce cell apoptosis (29). Filamin-C (FLNC) is a cytoskeletal protein that is localized in the chromosomes. Filamin A, a member of the FLNC family, has been reported to promote cell apoptosis through the Rac/PAK/p38 signaling pathway (30). The co-immunoprecipitation in the present study demonstrated that these cytoskeletal proteins were able to interact with PKC-δ, suggesting that PKC-δ was capable of targeting these cytoskeletal proteins to initiate hyperthermia-induced cell apoptosis. ii) Proliferation-associated proteins, including MYC-induced nuclear antigen (MINA) and catenin α-1 (CTNNA1). MINA is localized in the nucleolus and participates in ribosome synthesis and cell proliferation. As a target gene of c-Myc that is involved in cell proliferation, the expression of MINA53 has been reported to be increased in tumor cells (31). CTNNA1 predominantly presents in the cytoplasm and it is involved in cell adhesion in addition to the mediation of signal transduction. CTNNA1 gene knockout in cutaneous squamous cell carcinoma was reported to reduce cellular apoptosis via the upregulation of the nuclear factor-κB pathway (32). Due to the fact that CTNNA1 has been demonstrated to be able to combine with ACTB to form a complex to exert biological effects, it was hypothesized in the present study that PKC-δ was able to regulate CTNNA1 indirectly through ACTB and thus promote hyperthermia-induced apoptosis. iii) Apoptosis-associated proteins, including THAP domain-containing protein 2 (THAP2) and S100A9. THAP2 is distributed in the nucleus and participates in gene transcription, cellular apoptosis and cell-cycle regulation. THAP was first identified to function in tumor necrosis factor (TNF)-induced apoptosis of HeLa cells (33). Roussigne *et al* (34) demonstrated that overexpression of THAP resulted in an increase of cellular apoptosis in NIH3T3 cells treated with TNF-α or induced by serum starvation. Although THAP serves an important role in apoptosis, the mechanisms involved have remain elusive. Leite *et al* (35) demonstrated that miR-100 was able to downregulate THAP2 in prostate cancer through the retinoblastoma protein (pRb) signaling pathway and thus increase cellular proliferation. Koike *et al* (36) additionally demonstrated that PKC-δ resulted in the induction of cell-cycle arrest at the G_1 phase in NPA thyroid cancer cells via the pRb signaling pathway. The observations of the present study additionally demonstrated that PKC-δ was able to combine with THAP2 to form a complex, suggesting that these proteins interact with each other to mediate apoptosis; however, the exact mechanisms remain elusive and require further investigation. S100A9 is a member of the calgranulin S100 family that localizes in the cytoplasm. S100A9 predominantly forms a heterodimer with S100A8 to mediate the cleavage of a caspase 3 precursor and thus promotes cell apoptosis (37). In addition, Hiratsuka *et al* (38) demonstrated that S100A8/A9 was able to inhibit the metastasis of tumor cells. Apoptosis-associated proteins regulate the initiation of cell death and thus serve important roles in cellular apoptosis. The results of the present study additionally demonstrated that PKC-δ was able to regulate cellular apoptosis by interacting with apoptosis-associated proteins. iv) Transcription factor protein homolog 1 (PMS1). PMS1 is located in the nucleus and belongs to a mismatch repair (MMR) gene family. Dong *et al* (39) investigated MMR gene single-nucleotide polymorphisms in 154 patients with pancreatic cancer and identified that PMS1 serves an important role in repairing damaged oncogenes. It has been demonstrated that PKC-δ may exert biological effects by phosphorylating transcription factors. For example, PKC-δ may phosphorylate

transcription factor signal transducer and activator of transcription 3 to induce apoptosis (40-42); however, it remains elusive whether PKC-δ activates apoptosis through the phosphorylation of PMS1.

A total of 16 proteins that interact with PKC-δ in unchallenged TT-Tca8113 cells or those challenged by hypothermia were identified. Due to the fact that these proteins did not interact with PKC-δ in the challenged Tca8113 cells, they may be involved in the suppression of apoptosis and induction of thermal tolerance. The proteins were as follows: i) Heat shock 70 kDa protein 9 (HSPA9), a heat-shock protein located in the mitochondria, endoplasmic reticulum, cell membrane and cytoplasm vacuoles. HSPA9 is a key protein involved in the process of thermoresistance, and may interact with various intracellular proteins to promote cellular differentiation and the hypothermic stress response (43). HSPA9 interference may additionally increase senescence and apoptosis of tumor cells (44). Previous studies have demonstrated that HSP27 was able to inhibit PKC-δ-mediated phosphorylation of plastin 3 and thus suppress apoptosis of TT-Tca8113 cells following the induction of hyperthermia (24,25). The observations of the present study suggested that PKC-δ may combine with HSPA9 in TT-Tca8113 cells, thus further indicating that HSPs may mediate thermotolerance through the inhibition of PKC-δ proteases, including cyclin-dependent kinase 2 (CDK2) and L-isoaspartate-O-methyltransferase (PCMT1). CDK2 is predominantly located in the nucleus, regulates progression of cells from G_1 to S phase and has been reported to be a protein closely associated with cell apoptosis. CDK2 is able to alter the permeability of the mitochondrial membrane and thus activate caspase 3 and Bax, in addition to suppressing Bcl-2 to increase cellular apoptosis (45-49). Sun *et al* (50) demonstrated that PKC-δ may mediate cellular apoptosis by regulating cyclins. Sinha *et al* (51) additionally demonstrated that inhibition of the PKC family resulted in the promotion of cellular apoptosis via downregulation of CDK2 activation. The observations of the present study further confirmed that CDK2 is likely to be an important target protein of PKC-δ, which may regulate heat sensitivity and thermotolerance of tumor cells. Human PCMT1 is predominantly distributed in the cytoplasm and serves a critical role in repairing and degrading damaged proteins. In a study by Sambri *et al* (52), downregulation of PCMT1 expression by microRNA interference resulted in a significant increase in the isomerization of Bcl extra large protein and thus enhanced cellular apoptosis. Yan *et al* (53) additionally demonstrated that PCMT1 was able to combine with macrophage stimulating 1 (hepatocyte growth factor-like) (MST1) to form a complex, thus reducing MST1 activity, which, in turn, reduces the levels of apoptosis in myocardial cells. ii) Other proteins, including obscurin (OBSCN) and dedicator of cytokinesis protein 9 (DOCK9). OBSCN is mainly distributed in the cytoplasm and is involved in the synthesis of myosin. Expression of OBSCN has been reported to be increased in tumor cells (54), suggesting that OBSCN is closely associated with tumors. DOCK9 is a guanine nucleotide exchange factor that is predominantly distributed on the inner cytoplasmic membrane and may activate CDC42 by promoting the conversion of guanosine diphosphate to guanosine triphosphate (55). As a critical factor involved in cancer-cell metastasis, CDC42 may facilitate the migration of cancer cells to other tissues through the blood circulation (56).

In conclusion, PKC-δ was suggested to serve a critical role in the apoptosis of tongue squamous cancer cells induced by hyperthermia. The functions of PKC-δ are associated with the interactions with various proteins in different subcellular compartments. Significant differences in the proteins that interact with PKC-δ were observed between the tumor cells with or without hyperthermia induction, were indicative of the underlying molecular basis of heat sensitivity and thermotolerance of tumor cells; however, further studies are required to fully elucidate these mechanisms.

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