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Concomitant Inhibition of HSP90, Its Mitochondrial Localized Homologue TRAP1 and HSP27 by Green Tea in Pancreatic Cancer HPAF-II Cells

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Abstract

Pancreatic cancer is a deadly disease characterized by poor prognosis and patient survival. Green tea polyphenols have been shown to exhibit multiple antitumor activities in various cancers, but studies on the pancreatic cancer are very limited. To identify the cellular targets of green tea action, we exposed a green tea extract (GTE) to human pancreatic ductal adenocarcinoma HPAF-II cells and performed two-dimensional gel electrophoresis of the cell lysates. We identified 32 proteins with significantly altered expression levels. These proteins are involved in drug resistance, gene regulation, motility, detoxification and metabolism of cancer cells. In particular, we found GTE inhibited molecular chaperones heat-shock protein 90 (Hsp90), its mitochondrial localized homologue Hsp75 (tumor necrosis factor receptor-associated protein 1, or Trap1) and heat-shock protein 27 (Hsp27) concomitantly. Western blot analysis confirmed the inhibition of Hsp90, Hsp75 and Hsp27 by GTE, but increased phosphorylation of Ser78 of Hsp27. Furthermore, we showed that GTE inhibited Akt activation and the levels of mutant p53 protein, and induced apoptosis and growth suppression of the cells. Our study has identified multiple new molecular targets of GTE and provided further evidence on the anticancer activity of green tea in pancreatic cancer.

Keywords

Green tea; human pancreatic adenocarcinoma HPAF-II cells; Hsp90; Trap1; Hsp27

1 Introduction

Pancreatic cancer was the 4th leading cause of cancer deaths for men and women in the United States in 2010 [1]. The overall 5-year survival rate is approximately 5%, the lowest of all the major cancers. Mutations of *KRAS* (~100% of the pancreatic ductal adenocarcinoma cases), *P53* (>50% cases) and other genes, and the resistance to treatment

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are two of the many factors contributing to the poor prognosis and survival. Gemcitabine (2'-deoxy-2'-difluorodeoxycytidine) is the first-line treatment in patients with locally advanced or metastatic adenocarcinoma of the pancreas. However, it is only moderately effective, producing a response rate of about 12% with a median survival time of 6 months [2–4]. Hence, there is an urgent need to identify new agents or regimens to improve the survival of patients with this disease.

Green tea (*Camellia sinensis* leaves) contains polyphenols that are naturally occurring antioxidants. Tea is generally considered as a safe food item. It is available as dietary supplements, but the concentration of polyphenol in any particular tea beverage depends on the type of tea, the amount used, the brew time, and the temperature [5]. Green tea is a potentially promising chemopreventive agent [6, 7]. Laboratory and animal studies have shown that green tea is protective against many types of cancer, but very limited studies have been conducted on pancreatic cancer. In an attempt to identify non-toxic natural products that could benefit to pancreatic cancer patients, we used a proteomic approach to identify new molecular targets in human pancreatic ductal adenocarcinoma cells HPAF-II in response to GTE exposure. We show that green tea significantly altered the expressions of 32 proteins. Among them, the down-regulation of heat-shock protein 90 (Hsp90), its mitochondria localized homologue Hsp75 (Trap1) and heat-shock protein 27 (Hsp27) were confirmed by western blot analysis. Furthermore, we show GTE down-regulated Hsp90 targets Akt and mutant p53 and induced apoptosis and growth suppression of the cancer cells.

2 Materials and Methods

2.1 Materials

GTE was obtained from Pharmanex (Provo, UT, USA). The purity of the catechins in the GTE was 84% with epigallocatechin gallate (EGCG) being a major component (43.0% by weight) [8]. The GTE contained less than 0.3% caffeine. Sequencing-grade trypsin was purchased from Promega (Madison, WI, USA); TGS (Tris-glycine-SDS buffer) and DTT were purchased from BioRad Laboratories (Hercules, CA, USA).

2.2 Cell culture and GTE stimulation

Human pancreatic adenocarcinoma HPAF-II cells (American Type Culture Collection, Manassas, VA, USA) were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) with 1% penicillin and streptomycin mix solution (Invitrogen), sodium pyruvate 11.0 µg/ml and 10% FBS (Invitrogen). Cultures were maintained at 37°C in 5% CO₂ and 95% air, and the medium was changed two times per week. GTE was dissolved in 10% ethanol to make a stock solution of 20 mg/mL which was diluted with cell medium prior to its use.

Logarithmically growing HPAF-II cells were harvested and seeded at an initial density of 3×10^6 cells in 20 mL of fresh medium in 100 mm petri dishes. After overnight proliferation, the adherent cells were cultured in RPMI 1640 medium without FBS for 4 h, and then incubated with GTE at final concentrations of 0, 20, and 40 µg/mL for 24 h.

2.3 Protein extraction

HPAF-II cells were washed twice with ice-cold PBS containing protease inhibitors and were scraped from petri dish by rehydration buffer (containing 7 M urea, 2 M thiourea, 20 mM DTT, 1.2% CHAPS, 5% glycerol, 10% isopropanol, 0.4 ASB-14, 0.25% ampholytes, protease inhibitor cocktail set III). Cells were then shaken overnight. The sample was clarified by centrifugation at 20 000×g for 15 min at 15°C, and the supernatants stored at –80°C until use for 2DE. Protein concentrations were quantified using the 2D Quant kit (Amersham Biosciences, Uppsala, Sweden).

2.4 2DE, gel staining and image analysis

A sample volume of 350 μ L containing 100 μ g protein was applied to 17-cm pH 3–10 immobilized pH gradient strips (Bio-Rad) which were allowed to rehydrate for 12 hr at 50 V (22°C). Subsequently, isoelectric focusing using a Protean IEF Cell (Bio-Rad) was performed at 23°C for 1 hr with a linear ramp to 500 V, followed by 3 hr at 500 V, and a 3 hr linear ramp to 10,000 V, and holding at 10,000 V until the V-hrs reached to 99,999. The strips were then equilibrated at room temperature for 15 min in SDS-equilibration buffer (0.375 mM Tris/HCl pH 8.8, 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS, 60 mM DTT) and for another 15 min with SDS-equilibration buffer supplemented with 135 mM iodoacetamide. After equilibration, the IEF strips were applied to 8–16% 17 cm Protean II Ready Gels (Jule, Milford, CT, USA). Molecular weight standards (Mark 12™, Invitrogen) were applied to filter paper beside the strip. Electrophoresis was carried out at 10 mA per gel during the first 30 min followed by 18 mA per gel until complete. Gels were stained using Sypro Ruby (Bio-Rad). For gel-image analysis, gels were scanned at high resolution with Molecular Imager FX (Bio-Rad), and Progenesis SameSpots 2-D Gel Analysis software (Nonlinear Dynamics, Durham, NC) version 3.0 was used for detection of qualitative and quantitative alterations in the protein spots. The statistically ranked top spots were selected based on *p*-value of ANOVA (*p* < 0.05). Selected protein spots were manually checked. Gels were run in triplicate for each sample group in each experiment. Experiments were performed in duplicate.

2.5 Protein identification by LC-MS/MS

Spots of interest were excised from the gels by a ProPic II Spot Cutter (DIGILAB, Holliston, MA). The in-gel reduction, alkylation, trypsin digestion, and peptide extraction were accomplished manually using standard protocols [8, 9]. Data for protein identifications was acquired with an LC-quadrupole time-of-flight (QTOF) system (Dionex/LC Packings nano-LC, Sunnyvale, CA, USA) and Applied Biosystems/Sciex QSTAR XL mass spectrometer (Foster City, CA, USA). The reversed-phase LC pre-column (75 μ m $\times$ 10 mm) and column (75 μ m $\times$ 150 mm) were packed with Jupiter 4 μ m Proteo 90 Å C12 resin (Phenomenex, Torrance, CA, USA). The eluent for the LC binary gradient was comprised of water containing 0.1% formic acid (A) and 95% acetonitrile, 0.1% formic acid (B). The flow rate was 200 nL/min and the gradient profile was 3–21% B in 36 min, 21–35% B in 14 min, 35–80% B in 6 min and 80% B constant for 8 min. Electrospray ionization was performed using a 30 μ m internal diameter nano-bore stainless steel online emitter (Proxeon Biosystems, Odense, Denmark) and a voltage of 1900 V.

Peptide product ion tandem mass spectra were recorded during LC-MS/MS by information-dependent analysis (IDA) on the mass spectrometer. Argon was employed as the collision gas. Collision energies for maximum fragmentation were automatically calculated using empirical parameters based on the charge and mass-to-charge ratio of the precursor peptide. The MS/MS spectra were searched against the IPI protein sequence database (release date 01-09-2007) using the Mascot search program (Matrix Science, London, UK). For searching the sequence database, the following variable modifications were set: carbamidomethylation of cysteines, oxidization of methionines, conversion of N-terminal glutamate and aspartate to pyro-Glu, and cyclization of N-terminal cysteine. One missed tryptic cleavage was tolerated and the peptide and MS/MS mass tolerance was set as ± 0.3 Da. Positive protein identification was based on standard Mascot criteria for statistical analysis of the MS/MS data. A $-10\log(P)$ score, where *P* is the probability that the observed match is a random event, of 72 was regarded as significant.

2.6 Western blotting

Approximately 10 µg of cell protein was electrophoresed on 10% SDS polyacrylamide gels before transfer to nitrocellulose membranes. Horseradish peroxidase-conjugated secondary antibodies (GE Healthcare/Amersham Biosciences, Uppsala, Sweden) were used followed by ECL reaction to develop the blots according to the manufacturer's instructions. Primary antibodies were used to detect the expression of the following proteins: Hsp90 (Enzo, Plymouth Meeting, PA, USA), Hsp-75 (BD Biosciences, Pasadena, CA, USA), Hsp27 (Santa Cruz, Santa Cruz, CA, USA), Hsp27 (Phospho-Ser82) (Enzo), Hsp27 (Phospho-Ser15) (Cell Signaling, Danvers, MA, USA), and Hsp27 (Phospho-Ser78) (GenScript, Piscataway, NJ, USA). Protein expressions were visualized and analyzed using a ChemiDoc XRS (Bio-Rad) chemiluminescent detection and imaging system. After stripping the membrane, monoclonal antibody to GADPH or α -tubulin (GenScript) was applied as loading control. Band intensities were analyzed by IMAGEQUANT 5.2 software (Molecular Dynamics, Sunnyvale, CA, USA).

2.7 Immunofluorescence assay

For immunofluorescence analysis, HPAF-II cells (5×10^4) were seeded in 8 well chambers and treated with GTE at 0, 10, 20 and 40 µg/ml doses. After 24 h, cells were fixed in 4% paraformaldehyde/PBS and permeabilized in 0.1% Triton X-100/PBS and blocked with 3% BSA/PBS for 30 min. Cells were then incubated with primary antibody Hsp90 (GenScript, 1:200 dilution), phospho-Akt (Ser473, Cell Signaling, 1:100), p53 (Santa Cruz, 1:50 dilution) or cleaved caspase-3 (Asp175, Cell Signaling) at 37°C for 1 h, then washed with PBS three times and incubated with donkey anti-mouse or rabbit IgG (H + L) conjugated Alex 488 (Biogenex, San Ramon, CA, USA) at room temperature for 30 min. Cells were sealed after applying *SlowFade*® Gold antifade reagent with DAPI (invitrogen). Images were taken using a Nikon Eclipse 90i fluorescence microscope (Melville, NY, USA).

Cell Viability was determined using the Cell Proliferation Assay kit (Chemicon, Temecula, CA, USA) according to the manufacturer's instructions. Briefly HPAF-II cells were plated in 96-well plates (0.5×10^4 cells/well). GTE at 0, 10, 20, 40, 80, and 160 µg/mL concentrations were added to cell culture media for 24 and 48 hrs. All treatments were performed in triplicate.

2.8 Statistical analysis

Data were expressed as the mean $\pm$ SD and analyzed by student's *t*-test. All differences of $p < 0.05$ were considered significant.

3 Results

3.1 Proteins altered in their steady-state levels by treatment of HPAF-II cells with GTE

For the comprehensive analysis of effects of green tea extract on the proteome of HPAF-II cells, cells were exposed to the GTE at doses of 0 (control), 20 and 40 µg/mL for 24 hr, and whole cell lysates were separated by 2DE. Cell proteins were detected and visualized by Sypro Ruby stain. Significant changes in protein expression were defined as ANOVA analysis ($p < 0.05$) among control, 20 and 40 µg/ml GTE-treated groups from the staining intensity of each spot. More than 600 protein spots were resolved on each of the gels. Forty spots exhibited significant changes in expression level responding to GTE treatment. Among them, 32 were identified by LC-MS/MS analysis (Fig. 1A). A variety of proteins involved in drug resistance, metabolism, detoxification, gene regulation, motility and heat-shock proteins displayed significant changes in expression level. Among them expression of 17 proteins were down-regulated and 12 of them showed a dose-responsive decrease. Thirteen proteins were up-regulated and 9 of them showed a dose-responsive increase. Two proteins

showed decreasing then increasing levels (Table 1). In particular, we identified 2 Hsp90s, i.e., Hsp90 α and Hsp75 (Trap1), both with reduced expressions. There were 2 heat-shock protein beta-1 (Hsp27) proteins, one with increased (spot #12) and another reduced (spot #13) expression. Detail of the 2DE images of these Hsps in response to GTE treatment as compared to the untreated control is shown in Fig. 1B.

Human Hsp27 contains 7 serine residues and can be mono-, bi- and tri-phosphorylated, but phosphorylation has been reported at residues Ser15, Ser78 and Ser82 [10, 11]. Tandem mass spectrometry of the tryptic peptides derived from both spots #12 (apparent pI = 5.4) and #13 (apparent pI = 5.7) showed both phosphorylated and unmodified versions of peptide Q80LSSGVSEIR₈₉. The unmodified Hsp27 is expected to have a pI of 5.98 (Table 1). From the MS/MS data, the modified peptide is phosphorylated at Ser82 (Fig. 1C). Another phosphorylation site in both proteins (spots #12 and #13) is suggested (to ensure co-migration of the different forms), but examination of the MS/MS data did not reveal the additional phosphorylation site(s). To summarize, both spots #12 and #13 are phosphorylated at Ser82. Spot #13 is likely to contain at least two phosphorylation sites, and the more acidic spot #12 contains one additional phosphorylation site.

3.2 GTE reduced the expression of molecular chaperones

To verify the expression change of these Hsp proteins, we performed western blot analysis. Both Hsp90 and Hsp75 showed dose-responsive reduction in expression (Fig. 2), consistent with our proteomics findings. Overall Hsp27 expression decreased markedly in our repeated WB analysis.

PhosphoSer78 Hsp27 has been reported recently to have higher immunohistochemical staining intensity in human pancreatic ductal adenocarcinoma tissues compared with adjacent normal tissues (n=10) [11]. We, therefore, tested pSer78-Hsp27, as well as pSer82-Hsp27 and pSer15-Hsp27 by western blot analysis. Our results showed pSer78-Hsp27 expression increased substantially with increasing GTE concentrations (Fig. 2). Western blot analysis of pSer82-Hsp27 and pSer15-Hsp27 showed only a very minor dose-response increase in abundance. Thus, the increase in pSer78-Hsp27 abundance best matches our 2DE measurement for spot #12 (Fig. 1B).

3.3 GTE inhibited Akt activation and mutant p53 protein level and induced apoptosis and growth inhibition of HPAF-II cells

Hsp90 is required for the refolding of proteins in cells exposed to various environmental stressors and for the conformational maturation and stability of a subset of key regulatory proteins including Akt, Her2 and Raf1 [12]. Hsp90 has been reported to modulate tumor cell apoptosis mediated through effects on protein kinase Akt [13]. Another important role of Hsp90 in cancer is the stabilization of mutant proteins such as the mutant forms of p53 [14]. To investigate these targets of Hsp90, we examined levels of Akt and p53 in the GTE treated HPAF-II cell using immunohistochemistry. As shown in Figure 3A, GTE at a dose of 20 μ g/ml significantly inhibited Akt phosphorylation with an intensity of 8.9 ± 0.79 vs. 5.1 ± 1.90 (untreated control, $p < 0.05$, Fig. 3A middle panel) and reduced p53 expression by more than 50% (21.1 ± 5.31 vs. 9.7 ± 4.05 , $p < 0.05$, Fig. 3A bottom panel).

Since Hsp90 modulates tumor cell apoptosis mediated through effects on Akt [13], and Hsp27 regulates apoptosis by interacting with key components of the apoptotic signaling pathway, particularly those involved in caspase activation [15], we examined levels of caspase-3 activation using immunohistochemistry. Figure 3B showed that GTE induced cleaved caspase-3 activation dose-dependently. Treatment of HPAF-II cells with 20 μ g/ml GTE significantly increased cleaved caspase-3 by nearly 3-fold (20.8 ± 4.4 vs. 7.2 ± 0.47 ,

$p < 0.5$). Meanwhile, our cell viability assay indicated that GTE at concentrations of 20, 40 and 80 $\mu\text{g/mL}$ inhibited HPAF-II cell viability by 19%, 41% and 82%, respectively. The inhibition of cell growth by GTE was dose- and time-dependant (Fig. 3C). The IC_{50} of GTE on HPAF-II cells was 42 $\mu\text{g/mL}$ at 24 hr and 18 $\mu\text{g/mL}$ at 48 hr treatment.

4 Discussion

In this study we demonstrated that GTE regulates a variety of proteins involved in drug resistance, gene regulation, detoxification, metabolism, motility and molecular chaperones in HPAF-II cells. HPAF-II is a human pancreatic ductal adenocarcinoma cell line that displays ductal characteristics such as secretory granules and mucin production with unlimited replicative capability. It is a well-differentiated cell line with high metastatic potential and carries TP53 mutation [16, 17]. We report here that GTE concomitantly inhibited the expression of the Hsp90 family proteins Hsp90 and Hsp75, and Hsp27. Furthermore, we demonstrated that GTE inhibited Hsp90 target Akt activation and mutant p53 levels and induced the cancer cell apoptosis and growth suppression.

Heat-shock or stress proteins are constitutively expressed molecular chaperones that guide the normal folding, intracellular disposition and proteolytic turnover of many of the key regulators of cell growth and survival. Among them, Hsp90 assists the maturation of multiple oncoproteins and mutant proteins to retain functions such as proliferation, survival and metastasis in the pancreatic cancers [12]. The family of Hsp90 molecular chaperones includes the cytosolic Hsp90 α (stress-inducible) and β (constitutive) isoforms, the mitochondrial localized homologue tumor necrosis factor receptor-associated protein 1 (Trap1, or Hsp75), and the endoplasmic reticulum (ER)-restricted glucose-regulated protein 94 (Grp94). Human Hsp90 α shows 85% sequence identity to Hsp90 β . Trap1 protects mitochondria from oxidative stress. Trap1 expression is low in the mitochondria of normal tissues but is increased in tumor mitochondria. Inhibition of Trap1 has been reported to cause the collapse of mitochondrial function and selective tumor cell death in several murine tumor models and tumor cell lines [18, 19]. Targeting Trap1 has been suggested to be a potential novel target of many solid tumors. The mitochondria-directed Hsp90 antagonists may provide a new class of potent anticancer agents [19]. Hsp90 participates in stabilizing and activating more than 200 proteins, referred to as Hsp90 “clients” [12]. Because of the diverse functions of its numerous client proteins, Hsp90 inhibition impacts all of the hallmarks of cancer [20]. As such, developing highly specific inhibitors of Hsp90 is a current research interest. Small molecules as inhibitors of Hsp90, such as geldanamycin (GA) and its modified derivative 17-AGG, have been found to target Akt, Her kinases, Raf, met tyrosine kinase, etc. [21, 22] and are currently on clinical trials [19].

Green tea processes multiple health benefits and chemopreventive activities that have been well documented [7, 23, 24]. Although there are very limited *in vivo* studies on the inhibition of tumorigenesis in the pancreas by green tea, results consistently demonstrated its inhibitory activity on nitrosamine-induced pancreatic cancer in animal models [25–27]. Studies by Shankar et al. showed significant reductions in volume, proliferation, angiogenesis and metastasis and inductions in apoptosis, caspase-3 activity and growth arrest in tumors of mice treated with ECCG at 60mg/kg dose for 6 weeks [27]. In *in vitro* studies, green tea extract and EGCG have been reported to decrease the expression of the K-ras gene [28], inhibit viability, capillary tube formation and migration of HUVEC cells [27, 29]. Of special interest is a recent report that EGCG binding to the C-terminal domain of Hsp90 impairs Hsp90 superchaperone complex for down-regulation of its client proteins Akt, Cdk4, Raf-1, Her2 and pERK in human pancreatic cancer cell line Mia Paca-2 [30]. However, EGCG treatment of cells for 24 hrs at the dose of 80 μM did not show the inhibition of either Hsp90 or Hsp70 by western blot analysis. In our study, we used a whole

green tea extract instead of active ingredients and observed inhibition of Hsp90 by proteomics analysis, and confirmed by western blot analysis. In addition, we report, for the first time to our best knowledge, GTE inhibited the expression of mitochondrial chaperone Trap1 in cancer cells. Our previous green tea studies demonstrated that whole extract is more effective compared to the individual components for inducing actin remodeling and suppressing proliferation in various cancer cells [9, 31]. The concomitant inhibition of multiple heat-shock proteins by GTE further demonstrated that a large diversity of structurally related and unrelated constituents present in green tea (in other natural products as well) contribute to its multiple biological activities.

SHB1 (Hsp27) regulates apoptosis by interacting with key components of the apoptotic signaling pathway, particularly those involved in caspase activation [15]. Cancer develops resistance to chemotherapy through the antiapoptotic action of Hsp27 [32]. Intrinsic or acquired resistance of pancreatic cancer to apoptosis is a major cause of treatment failure [3]. One study reported a shorter survival of pancreatic cancer patients correlating with high Hsp27 expression compared with low Hsp27 expression ($p=0.0025$), as measured in pancreatic tumor tissues [33]. When this manuscript was in revision we found a recent publication reporting that EGCG, a major polyphenol present in green tea, down-regulates Hsp27 in human urinary bladder cancer cells [34]. The result is consistent with our observation for green tea-regulated Hsp27 expression. Thus, an agent such as green tea that targets multiple signaling pathways and inhibits Hsp27 of pancreatic cancer cells might enhance the cytotoxic and apoptotic effects of gemcitabine when used in combination.

Hsp27 is a multifunctional protein with several functional phosphorylation sites. The exact function of the different phosphorylated forms of Hsp27 is unclear and needs to be further studied [35]. It is known that phosphorylation of Hsp27 is a reversible event that modulates the oligomerization of the protein. The phosphorylated Hsp27 forms small oligomers that decrease its chaperone properties [36]. The influence on actin stabilization and regulation of cytoskeletal organization have been connected to small oligomers [37]. Specifically, phosphorylated Hsp27 organized in small oligomers may interact directly or indirectly with F-actin, protect the actin filament against breakage, and promote its subsequent reorganization [38]. In our previous studies, we have shown that GTE modulates actin remodeling by increasing actin polymerization in transformed urothelial MC-T11 cells [9], lung cancer A549 cells [39], and in several live metastatic cancer cells including the pancreas but not in normal cells [40]. We have further demonstrated that GTE-induced ANX1 expression mediates actin polymerization, resulting in enhanced cell adhesion and decreased motility. Our WB analysis indicated that GTE induced ANX1 expression in HPAF-II cells (unpublished data), suggesting GTE induced actin polymerization may take place. However, further investigations are needed to establish the association of Hsp27 phosphorylation with cytoskeletal reorganization.

There are several other GTE regulated proteins that are of considerable importance in the chemopreventive application of GTE. Major vault protein mediates drug resistance, possibly via a transport process [41]. This protein is widely distributed in normal tissues, and overexpressed in multidrug-resistant cancer cells including the human pancreatic ductal adenocarcinoma [42]. Its overexpression is a potentially useful marker of clinical drug resistance. Heterogeneous nuclear ribonucleoprotein F protein has been shown to modulate the alternative splicing of the apoptotic mediator Bcl-x [43]. Lactate dehydrogenase (LDH) is a tetrameric enzyme comprising five isozymes that can catalyze the forward and backward conversion of pyruvate to lactate. LDHA favors the conversion of pyruvate to lactate. It has been known that many human cancers have higher LDHA levels than normal tissues [44]. Inhibition of LDHA was reported to reduce cell transformation, tumor formation and to increase cell oxidative stress that is linked to cell death [45, 46]. Alpha-actinin-4, an actin-

binding protein, is associated with cell motility and cancer metastasis [47]. High expression of actinin-4 has been reported to correlate with poor survival or advanced tumor stages of several solid tumors and is considered as a potential prognostic marker in colorectal, ovarian and pancreatic cancers [48–51].

In summary, using a proteomic approach we have identified several oncoproteins with reduced expression in pancreatic cancer cells upon green tea treatment. In particular, GTE down-regulates molecular chaperone Hsp90 that modulates function of oncoproteins important to the biology of pancreatic cancer. GTE also reduces the expressions of Trap1 and Hsp27 in a dose-dependent fashion. GTE induces apoptosis and growth suppression of pancreatic cancer HPAF-II cells. Our study provides further evidence that green tea possesses anti-cancer activities by targeting multiple oncogenic pathways.

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Abbreviations

ANX1	Annexin I
2DE	two-dimensional polyacrylamide gel electrophoresis
EGCG	(–)-epigallocatechin gallate
GTE	green tea extract
Hsp	heat-shock protein
Trap1	tumor necrosis factor receptor-associated protein 1
WB	western blotting

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B

Figure 1.

Protein separation by two-dimensional gel electrophoresis. (A) Image of a Sypro Ruby-stained 2DE derived from 20 μ g/ml GTE treated HPAF-II cell lysate. Cell lysate (100 μ g) was isoelectric focused on 17-cm IPG (pH 3–10) strips followed by 8–16% SDS-PAGE. After Sypro Ruby staining, spot intensities were determined by Progenesis SameSpots 2-D Gel Analysis software. Proteins with enhanced or decreased expression levels in the presence of GTE compared to the control were selected, digested and identified by LC-MS/MS. The identities of numbered proteins on the gel are listed in Table 1. (B) Suppressed spots of Hsp90, Trap1 and Hsp27 by GTE treatment as compared to the control. (C) MS/MS spectra of tryptic peptide Q₈₀LSSGVSEIR₈₉ (2+ charge) from spot #12, showing the presence of both unphosphorylated peptide (top) and the phosphorylated peptide (bottom). The MS/MS spectrum indicates that Ser82 is phosphorylated (The peaks are labeled with conventional notation for the product ions. Labels marked with an asterisk denote the loss of 18 Da, or water, from the product ions).

Figure 2.

Confirmation of heat shock proteins by Western blot analysis. HPAF-II cells were treated with 0, 20, and 40 $\mu\text{g/mL}$ of GTE for 24 h. Data represent one of the two or three experiments.

A

B

C

Figure 3.

GTE targeting Hsp90 client proteins and inducing apoptosis and growth suppression in HPAF-II cells. (A) Immunohistochemical analysis of HPAF-II cells treated with 0, 10, 20 and 40 µg/mL of GTE for 24 h. Cells were washed, fixed, and labeled sequentially for Hsp90, Akt or p53 (green), and (B) cleaved caspase-3 (green) and DNA (blue); and (C) effects of GTE on HPAF-II cell viability. Cells were plated at a density of 1×10^4 cells/mL and treated with increasing concentrations of GTE for 24 and 48 h. Cell viability was assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Images were taken using a Nikon Eclipse 90i fluorescence microscope at $\times 20$ objects. All data represent one of the two separate experiments, and three independent microscopic fields were examined for each sample.

Table 1

List of proteins showing differential expression upon GTE treatment

Protein assigned	Spot no	Accession number ^a	Mr(kDa)/pI theoretical	Mr(kDa)/pI observed	Score ^c	Ave ratio 20/40 GTE ^d	Peptides matched	Possible function	
Major vault protein	1	IP100000105	99.1/5.34	125.8/5.4	17	321	0.76/0.48	17	Drug resistant
Protein disulfide-isomerase precursor	2	IP100010796	57.1/4.76	63.5/3.9	11	82	2.13/1.00	6	Protein folding
Tubulin beta-2C chain	3	IP100007752	49.8/4.79	55.8/3.9	15	227	0.68/0.41	7	Microtubules
Heat shock protein 75 kDa, mitochondrial precursor	4	IP100030275	80.0/8.30	82.1/6.4	9	152	0.44/0.24	7	Mitochondrial HSP90
Enolase 1	5	IP100465248	47.1/7.01	47.6/6.6	8	77	0.71/0.60	5	Glycolysis
Adenyl cyclase-associated protein 1	6	IP100008274	51.7/8.29	52.3/8.6	6	96	0.43/0.30	4	Metabolism
Isoform B of mannose-6-phosphate receptor-binding protein 1	7	IP100303882	47.0/5.30	46.7/5.3	19	309	1.24/1.58	7	Protein binding
Heterogeneous nuclear ribonucleoprotein F	8	IP100003881	45.6/5.38	47.3/5.4	14	100	2.17/2.43	6	hnRNP
Eukaryotic translation initiation factor 3 subunit 5	9	IP100654777	39.1/5.24	46.5/5.4	5	55	0.92/0.88	2	Translation
Isoform 1 of actin-like protein 6A	10	IP100003627	47.4/5.39	46.9/5.5	7	80	0.79/0.57	3	Transcription
14-3-3 protein epsilon	11	IP100000816	29.2/4.63	27.8/4.2	29	200	0.64/1.48	7	Cell cycle regulation
Heat-shock protein beta-1	12	IP100025512	22.8/5.98	25.1/5.4	26	220	1.60/2.58	9	Stress resistance
Heat-shock protein beta-1	13	IP100025512	22.8/5.98	24.9/5.7	35	260	0.72/0.78	11	Stress resistance
Peroxiredoxin-4	14	IP100011937	30.5/5.86	24.8/5.8	15	89	1.11/1.53	4	Antioxidant enzyme
Peroxiredoxin-6	15	IP100220301	24.9/6.02	24.6/6.6	19	83	0.59/0.77	6	Antioxidant enzyme
Triosephosphate isomerase 1 variant	16	IP100465028	30.8/5.65	24.6/6.7	16	113	3.75/7.00	4	Glycolysis
Protein-L-isoaspartate (D-aspartate) O-methyltransferase	17	IP100024989	24.7/6.05	23.2/7.4	4	51	0.98/1.20	1	Metabolism
Cytochrome b5 outer mitochondrial membrane precursor	18	IP100303954	16.7/4.88	19.6/4.3	8	86	2.18/3.10	1	Electron carrier
Alpha-actinin-4	19	IP100013808	104.8/5.27	125.6/5.4	8	173	0.54/0.35	7	Cytoskeletal protein
Heat shock protein HSP 90-alpha 2	20	IP100382470	98.1/5.07	108.2/5.3	16	364	0.60/0.36	16	Molecular chaperone
Leukotriene A-4 hydrolase	21	IP100219077	69.2/5.80	63.2/6.1	20	247	0.59/0.26	13	Metabolism
Lactate dehydrogenase A	22	IP100217966	36.7/8.44	33.6/8.5	24	171	0.11/0.18	9	Glycolysis
Peroxiredoxin-1	23	IP100000874	22.1/8.27	24.3/8.5	46	179	0.17/0.02	10	Antioxidant enzyme
UDP-glucose 6-dehydrogenase	24	IP100031420	55.6/6.70	56.2/7.4	37	386	1.70/1.56	7	Metabolism
Protein disulfide-isomerase A3	25	IP100025512	52.8/5.49	57.2/5.8	29	356	1.35/1.46	11	Protein folding
Leucine aminopeptidase 3	26	IP100419237	63.8/6.43	53.8/6.7	3	70	1.26/1.36	10	Proteolysis
Transaldolase	27	IP100744692	37.5/6.36	35.9/6.3	10	58	0.37/0.37	4	Metabolism
S-methyl-5-thioadenosine phosphorylase	28	IP100011876	31.2/6.75	28.6/7.4	6	66	1.44/1.17	2	Metabolism

Protein assigned	Spot no	Accession number ^a	Mr(kDa)/pI theoretical	Mr(kDa)/pI observed	SC ^b	Score ^c	Ave ratio 20/40 GTE ^d	Peptides matched	Possible function
Isorform 1 of growth factor receptor-bound protein 2	29	IP100021327	25.2/5.52	24.7/6.1	20	67	1.61/1.41	2	Adapter protein
Septin-11	30	IP100019376	49.2/6.38	53.6/6.7	5	50	1.53/1.61	2	Cytoskeletal GTPase
Elongation factor 2	31	IP100186290	95.1/6.42	118.6/7.2	8	105	0.53/0.37	8	Translation
Isoform 1 of neutral alpha-glucosidase AB precursor	32	IP100383581	106.8/5.74	122.3/6.7	6	88	0.33/0.39	6	Metabolism

^a International Protein Index database (IPI) protein accession number.

^b SC: sequence coverage of the protein in percentage.

^c Mowse scoring algorithm.

^d Average ratio was calculated between two treated groups vs untreated control.