行政院國家科學委員會專題研究計畫 成果報告

以化學性蛋白質體學方法分析 EGCG 結合蛋白並評估其在化學癌症預防之重要性

研究成果報告(精簡版)

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附件一

行政院國家科學委員會補助專題研究計畫 ☑ 成 果 報 告 □期中進度報告

以化學性蛋白質體學方法分析 EGCG 結合蛋白並評估其在化學癌症預防之重要性

Identification of EGCG-binding proteins by chemical proteomics and their significances in cancer chemoprevention

計畫類別：☑個別型計畫　□ 整合型計畫
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執行單位：中山醫學大學生物醫學科學學系

中　華　民　國　97 年 10 月 31 日

1. 中文摘要

(-)-表沒食子酸兒茶素-3-沒食子酸酯（(-)-Epigallocatechin 3-gallate (EGCG)）為綠茶中含量最豐且生物活性最強之兒茶素（catechins）。近來研究發現，EGCG 會抑制多種腫瘤細胞株生長並誘發其走向細胞凋亡（apoptosis），顯示 EGCG 具有癌症化學預防（cancer chemoprevention）的能力；然而，EGCG 抑制腫瘤細胞生長之分子機轉仍未完全明瞭。推測其中原因之一，可能是 EGCG 與細胞中某種與調節細胞生長或凋亡有關的蛋白結合後，進而影響此蛋白的生化活性，導致最終癌細胞的細胞週期停止或進入細胞凋亡。為了證明此推論，我們利用化學蛋白質體學 (chemical proteomics) 的方法，先將 EGCG 耦合在 CNBr-activated Sepharose 4B beads 上，作為親合性色層分析之配體（ligand），再通入腫瘤細胞萃取液，分離出可與 EGCG 結合的蛋白，續以二維電泳及質譜儀鑑定其身份。基於此，多種 EGCG 結合蛋白被質譜儀鑑定出，其中部份蛋白如 Hsp90，Hsp70 及 PGK1，亦以 *in vitro* pull-down assay 證明可直接與 EGCG 結合。但活性分析顯示 EGCG 對 Hsp90 及 Hsp70 之內生性 ATPase 活性，與 PGK1 激酶活性並無顯著影響。我們認為 EGCG 可能不會改變 Hsp90 和 Hsp70 的 ATPase 活性，但有可能會干擾或阻礙 Hsp90 和 Hsp70 與其他蛋白間的交互作用，因而擾亂細胞內 chaperoning 系統，改變癌細胞正常之生理功能。為證明此，我們將 EGCG 先行處理之癌細胞萃取液，利用免疫沉澱法將其中 Hsp90 或 Hsp70 蛋白純化出，再以梯度電泳將共同沉澱出來的蛋白分離開來，繼以質譜儀鑑定其身份。另一方面，我們也構築 GST-Hsp90 融合蛋白，利用 GST-pull down 分析以辨別 Hsp90/70 client 蛋白與 Hsp90/70 間之交互作用，是否有受到 EGCG 的處理而改變，並進一步評估此交互作用的變化，對癌細胞本身的生理現象有何影響。另外，tritium 標定的 EGCG 將加入活細胞中，用以追蹤 EGCG 和質譜儀鑑定出的 EGCG 結合蛋白是否可在活細胞中結合。我們也將繼續檢驗 EGCG 對其它由質譜儀鑑定出的 EGCG 結合蛋白之生化特性的影響，並評估此影響是否能改變癌細胞的生理活性，以及這種改變是否有足夠能力讓癌細胞生長停止或誘導癌細胞走向細胞凋亡。我們希望能藉由化學蛋白質體學方法所鑑定到的 EGCG 結合蛋白圖譜，幫助我們進一步探索 EGCG 癌症預防的分子機制，以確立 EGCG 在癌症化學預防或治療上之重要地位。

關鍵字：(-)-表沒食子酸兒茶素-3-沒食子酸酯；親合性色層管柱分析；化學蛋白質體學；質譜儀；熱休克蛋白

2. 英文摘要

(-)-Epigallocatechin-3-gallate (EGCG) is the main polyphenol from green tea and it seems to be the most potent compound in tea with respect to inhibiting cell proliferation and inducing apoptosis in cell culture systems. However, the molecular mechanisms of cancer preventive activity by EGCG are not fully elucidated. In order to understand the anti-cancer mechanism of EGCG, we employed a chemical proteomics method to find out the molecular targets for action of EGCG. We suggest that protein interaction with EGCG may be one of the ways by which EGCG exerts its anti-tumor activity. To this end, EGCG was firstly immobilized on CNBr-activated Sepharose 4B beads as specific ligands for the affinity purification of cellular protein target(s) which were then identified by gradient-gel electrophoresis and MALDI-TOF MS. By this way, several EGCG-binding proteins shown in our data were identified and subsequently validated by *in vitro*-pull down assay and immunoblotting. We found that each EGCG-binding protein we identified processes distinct cellular function, suggesting the multi-effects of EGCG on cancer cell biology. Now we make an attempt to evaluate whether the interaction of EGCG and its high affinity proteins can interrupt cell growth or induce apoptosis in cancer cells. The effects of EGCG on the biochemical functions of EGCG-targeted proteins such as Hsp90 and Hsp70 are being examined. The result pointed out no significant influence of EGCG on the ATPase activity of Hsp90 and Hsp70, respectively. We suggest that EGCG may not change Hsp90's and Hsp70's ATPase activity but interrupt Hsp90/Hsp70-based chaperone machinery which maintains homeostasis in cancer cells. To examine this hypothesis, the interaction of Hsp90/70 and client proteins in EGCG-treated A431 cells/cell lysates is being determined by co-immunoprecipitation and GST-pull down assay followed by proteomics identification. Meanwhile, *in vitro* functional study will be performed to determine the biological effects of EGCG on the interaction between Hsp90/Hsp70 and client proteins or co-chaperones. Furthermore, we will use tritium-labeled EGCG to verify whether EGCG would directly interact with EGCG-targeted proteins derived from chemical proteomics findings in intact cells and continuously evaluate the biological alterations of protein interaction with EGCG and their significance in cancer prevention. We expect that using the chemical proteomics method for the identification of EGCG-targeted proteins can help us to determine the molecular actions of EGCG on cancer prevention.

Keywords: EGCG; affinity chromatography; chemical proteomics; mass spectrometry; heat shock proteins

3. **Introduction**

(-)-Epigallocatechin gallate (EGCG, Fig.1), the most abundant and biologically active catechin derived from green tea, is one of the most extensively investigated chemopreventive phytochemicals. Studies in animal models have demonstrated that green tea and EGCG can block each stage of carcinogenesis (*1*) and inhibits tumor angiogenesis, metastasis and invasion in animal models (*2-4*). These cancer preventive properties of EGCG have been attributed to its inhibition of tumor cell proliferation and molecular pathways involved in the cell cycle, angiogenesis, invasion, and growth factor-related proliferation (*5, 6*). The possible mechanisms responsible for the cancer preventive effects of EGCG are known to inhibit a wide variety of enzymatic activities associated with cell proliferation and tumor progression. The enzymes involve mitotic protein kinases, telomerase, DNA methyltransferase, matrix metalloproteinase (MMP), and 26S proteasome, etc (*6-11*). Several studies have focused on the direct interaction of EGCG with molecular targets in cancer. A few EGCG high-affinity binding proteins have been identified, including fibronectin (*12*), Fas (*13*), laminin, 67-kDa laminin receptor(*14, 15*), and intermediate filament vimentin (*16*). In all of these studies, the binding of EGCG to these target proteins consequently results in the inhibition of cancer cell growth or inducing apoptosis. Whether these well-established mechanisms can explain the cases observed in animal models or cell culture system needs to be determined.

Chemical proteomics is a strategy for characterization of chemical and protein interaction. This approach employs immobilized chemicals as specific ligands for affinity purification of cellular protein targets, which are then identified by mass spectrometry. Based on this chemical proteomic method, the relationship of anti-cancer chemical drugs such as protein kinase inhibitors, and their cellular targeted proteins can be profiled, and the selectivity of these chemical drugs can be rapidly systemically tested (*17-21*). Significantly, the potential of chemical proteomics provide rationales for the development of potent anti-cancer chemical drugs, which combine rather unexpected biological modes of action by simultaneously targeting defined sets of proteins involved in cancer progression.

In order to understand the anticancer mechanism of EGCG, we developed a chemical proteomics method for the characterization of EGCG-binding proteins in cancer cells. EGCG will be immobilized as specific ligands for the affinity purification of cellular protein target(s) which are then identified by sensitive techniques such as one-dimension or two-dimension electrophoresis, mass spectrometry and Western blot analysis. By this way, we identified several cellular targets (such as heat shock protein 90, heate shock protein 70 and phosphoglycerate kinase 1, etc) that directly bind to EGCG. Now, we are constructing glutathione-S transferase (GST) recombinant protein that fuses with EGCG-targeting proteins identified by chemical proteomics. We want to use GST-pull down assay to analyze the effects of EGCG on protein-protein interaction of EGCG-targeting proteins and further evaluate whether the interrupt of protein-protein interaction by EGCG can affect cancer cell physiological function such as inhibition of cell growth or induction of apoptosis. We expect that using the chemical proteomic method for the identification of EGCG-targeted proteins can help us to determine the molecular basis that is responsible for cancer preventive activity of EGCG.

4. **Materials and methods**

4.1 Materials

EGCG, cycloheximide, actinomycin D, the selective tyrosine kinase inhibitor genistein, EGFR inhibitor PD153035 and the kinase inhibitors LY294002, wortmannin, SB203580, PD98059 Propidium iodide (PI), cycloheximide, and phosphoglycerate kinase1 (PGK1) were obtained from Sigma (St. Louis, MO). TF1 (theaflavin), TF2a (theaflavin-3-gallate), TF2b (theaflavin-3'-gallate), and TF3 (theaflavin-3,3'-gallate) were isolated from black tea as described previously (*22*). The authentic compounds of these theaflavins were kindly provided by Prof. C.T. Ho, Rutgers University, NJ, USA. Anti-Hsp70, anti-PGK1, anti-Bax, anti-β-actin antibodies, protein A/G plus-agarose and glutathione-agarose were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-p21 and anti-cytochrome C antibodies were from PharMingen (San Diego, CA); anti-p27 antibody was from Transduction Laboratory (Lexington, KY); anti-phospho-p27 (Thr187) antibody was from Upstate Biotechnology (Lake Placid, NY); anti-phospho-Rb (Ser807/811), anti-phospho-Akt (Ser473), anti-Akt antibodies were from Cell signaling (Beverly, MA); anti-Rb antibody and HER2 inhibitor AG825were purchased from Calbiochem (La Jolla, CA). Antibody against FAS was obtained from BD Biosciences (Los Angeles, CA, USA). RT-PCR reagents were from Promega (Madison, WI, USA). Anti-Hsp90 antibody was generous gifts from Jau-Song Yu (Medical College of Chang Gung University, Tao-Yuan, Taiwan). Purified Hsp90 and recombinant Hsp70 proteins were from Stressgen (Victoria, B. C., Canada).

4.2 Cell culture and cell extract preparation

Human epidermoid carcinoma A431 cells and human breast cancer MCF7 cells were grown in Dulbecco's minimal essential medium (DMEM). All of the cells were supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, NY), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and kept at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were harvested, washed with cold-phosphate buffered saline twice, and homogenized in a lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% (v/v) Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) for 30 min at 4 °C. Afterward, the lysates were centrifuged at 12,000*g* for 30 min, and then the supernatants were collected as whole cell extracts. Protein determination was by the method of Bradford (kit available from BioRad, Richmond, CA, U.S.A.) with BSA as standard.

4.3 Affinity chromatography

Firstly, EGCG was coupled to the CNBr-activated Sepharose 4B beads according to the instruction provided by the manufacturer. The cell lysates from A431 cells cultured in 10-cm dishes were homogenized in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% (v/v) Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol). The lysate was sonicated and centrifuged at 15,000 x *g* for 30 min, and the supernatant fraction was applied to the polyphenol-coupled Sepharose 4B column at 4°C. The mobile phase was buffer A (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM 6-aminohexanoic acid, 1 mM PMSF, 1 mM benzamidine hydrochloride, and 1 mM EDTA) running at a flow rate of 0.5 ml/min. The bound proteins were then eluted with buffer B with three different ionic strength (0.15 M NaCl; 1 M

NaCl; and 1 M NaCl, 4 M urea in buffer A, respectively). Protein in eluted fractions was monitored using the Bio-Rad protein assay kit.

4.4 Two-dimensional gel electrophoresis (2DE)

Cells were solubilized in appropriate volume of 2DE lysis buffer (8 M urea, 2% CHAPS, 40 mM Tris-base, 1 mM benzamidine, and 1mM phenylmethylsulfonyl fluoride). One hundred microliters of cell extracts (200 μg protein) were mixed with 150 μL of rehydration buffer (8 M urea, 2% CHAPS, 20 mM dithiothreitol, 0.5% IPG buffer, and 0.01% bromophenyl blue) and separated in the first-dimension by isoelectric point in 13-cm IPG strips (pH 4-7) using Ettan IPGphor IEF system (Amersham Biosciences) at 20°C under the following condition: 50 V for 12 h, 100 V for 0.5 h, 150 V for 0.5 h, 250 V for 0.5 h, 500 V for 0.5 h, 1,000 V for 0.5 h, 4,000 V for 0.5 h, then 4,000-8,000 V for about 12 h with a total of 45,000 V-h. After IEF, the strips were incubated in SDS equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% dithiothreitol and 0.01% bromophenyl blue) at room temperature for 15 min, and then in 2.5% iodoacetamide (in SDS equilibration buffer) for another 15 min. The strips were then subjected to second-dimension separation by molecular weight in 10% SDS-gels. After gel electrophoresis, proteins were silver-stained for protein identification or transferred to PVDF membrane for immunoblot.

4.5 Mass spectrometric analysis of secreted proteins

Silver-stained bands were excised and in-gel digested with trypsin according to procedures described previously (*23*). Briefly, the gels were destained by 1% potassium ferricyanide and 1.6% sodium thiosulfate (Sigma, St. Louis, MO, USA). Then the proteins were reduced with 25 mM NH_4HCO_3 containing 10 mM DTT (Biosynth, Switzerland) at 60°C for 30 min and alkylated with 55mM iodoacetamide (Amersham Biosciences, UK) at room temperature for 30 min. After reduction and alkylation, the proteins were digested with trypsin (Promega, Madison, WI, USA) (20 mg/mL) at 37°C overnight. After digestion, the tryptic peptides were acidified with 0.5% TCA and loaded onto an MTP AnchorChip^{TM}. 600/ 384 TF (Bruker-Daltonik). MALDI-TOF MS analysis was performed on an Ultraflex^{TM}. MALDI-TOF mass spectrometer (Bruker-Daltonik). Monoisotopic peptide masses were assigned and used for database searches with the MASCOT search engine (http://www.matrixscience.com) (Matrix Science, London, UK).

4.6 In Vitro pull-down assay

2 μg of recombinant Hsp90, Hsp70, PGK1, or 400 μg of total cellular proteins from A431 cells was incubated with the EGCG-Sepharose 4B (or Sepharose 4B as control) beads (100 μL, 50% slurry) in buffer A. After incubation with gentle rocking overnight at 4 °C, the beads were washed five times with buffer C (1 M NaCl, 4 M urea in buffer A), and proteins bound to the beads were analyzed by Western blotting.

4.7 Western blot analysis (Immunoblotting)

Equal amounts of total cellular proteins (50 μg) were resolved by SDS-polyacrylamide gel electrophoresis (10% SDS-PAGE), transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore, Bedford, MA, USA), and then probed with primary

antibody followed by secondary antibody conjugated with horseradish peroxidase. The immunocomplexes were visualized with enhanced chemiluminescence kits (Amersham, UK). The intensity of the band was scanned and quantified by densitometer.

4.8 Immunoprecipitation

300 μg of total cellular proteins were first pre-cleared by incubating with protein A/G-agarose (10 μL, 50% slurry) for 15 min. The clarified supernatants were collected by microfugation, and then incubated with primary antibody for 2h at 4°C. The reaction mixtures were added with 20 μL of protein A/G-agarose to absorb the immunocomplexes at 4°C overnight. Immunoprecipitated proteins were subjected to 10% SDS-PAGE, and then transferred onto PVDF membrane (Millipore). Proteins were visualized by Western blotting.

4.9 GST pull-down assay

Firstly, expressed GST fusion Akt1 was incubated for immobilization with glutathione-agarose beads in binding buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 0.01% Nonidet P-40, 2 μg/ml bovine serum albumin, 0.02 mM PMSF, 1× protease inhibitor mixture) for 2 h at room temperature. Then 1μg of Hsp90 or Hsp70 proteins with 10 μM of EGCG or not was added followed by incubation for another 2 h. The supernatant was collected and concentrated, and the pellets were washed extensively with wash buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 0.01% Nonidet P-40, and 0.02 mM PMSF). The bound and unbound proteins were resolved by 10% SDS-PAGE and visualized by immunoblotting.

4.10 ATPase activity assay

The ATPase activities of Hsp70 and Hsp90 were measured in vitro with a Luminescent ATP detection assay kit (Packard BioScience B.V.). Briefly, 1 μg of Hsp70 or Hsp90 protein was incubated in 100 μL of assay buffer (100 mM Tris, 20 mM KCl, 6 mM $MgCl_2$, pH 7.4) containing 1 mM ATP with 50 μM EGCG or DMSO (vehicle control) for 90 min at 37°C. The decrease in the production of luminescent light caused by the reaction of ATP with added luciferase and D-luciferin was measured on a Top Counter microplate scintillation and luminescence counter (Packard 9912V1, Meriden, CT) in single photon counting mode for 0.1 min/well, following a 2-min adaptation in the dark.

4.11 Assay for phosphoglycerate kinase activity

The activity of phosphoglycerate kinase was determined in vitro with a Luminescent ATP detection assay kit (Packard BioScience B.V.). Briefly, 1 μg of PGK protein and 1 μg of 3-phosphoglycerate (as the substrate)was incubated with 50 μM EGCG or DMSO (vehicle control) in 100 μL of assay buffer (100 mM Tris, 20 mM KCl, 6 mM $MgCl_2$, 1 mM ATP, pH 7.4) for 90 min at 37°C. The decrease in the production of luminescent light caused by the reaction of ATP with added luciferase and D-luciferin was measured on a Top Counter microplate scintillation and luminescence counter (Packard 9912V1, Meriden, CT) in single photon counting mode for 0.1 min/well, following a 2-min adaptation in the dark.

4.12 In vitro translation assay

In vitro translation reaction was based on a rabbit reticulocyte lysate system (Promega, Madison, WI, USA). Briefly, the standard reaction was carried out at 37°C for 90 min in a 50 μL solution containing 35 μL of rabbit reticulocyte lysate, 1 mM amino acid mixture, 40 units/μL of RNasin® ribonuclease inhibitor (Promega) and 1 g/L luciferase mRNA (Promega). After reaction, 2.5 μL of the 50 μL luciferase control translation reaction was added to 50 μL of luciferase assay reagent (Promega) to measure the synthesis of functional luciferase. The luciferase activity was determined on a Top Counter microplate scintillation and luminescence counter (Packard 9912V1, Meriden, CT).

4.13 Construction of expression vector

The PCR products were separated in 1 % agarose gel, and the band containing Hsp70 or Hsp90 cDNA was cut off and purified using the QIAquick spin column. The expression vector pQE30 (Qiagen, Chatsworth, CA, U.S.A.) and Hsp70 or Hsp90 cDNA were digested by restrictive enzymes *Bam*H I, *Sph*I or *Xma*I. Then the inserted cDNA was ligated with the linearized pQE30 downstream the His-tag sequence (pQE30HisDD) by T4 ligase at 18 °C overnight. The recombinant was transformed into *E. coli* DH5α by $CaCl_2$ method and selected by agar plate containing ampicillin and confirmed by restriction enzyme mapping. The positive recombinant was transformed into *E. coli*. M15 as the host strain. The sequence of inserted fragment was confirmed by DNA sequencing.

4.14 The expression of Hsp fusion protein

The ampicillin-resistant colony of *E. coli* cells transformed with plasmid were cultured in LB cultural medium containing 100 mg/L ampicillin and 25 mg/L kanamycin, and induced by 1 mM IPTG. The cultured cells were harvested at 4~6 hr after culture. The optimum time of maximum expression of proteins was analyzed through SDS-PAGE. The expressed Hsp70 and Hsp90 proteins were tested through Western blot with specific antiserum.

4.15 Purification of the His-tagged protein

One hundred milliliters of *E. coli* expressing recombinant Sxh56 induced by IPTG was centrifuged for10 min at 8,000 rpm, and the pellets were resuspended in 10 ml of 10 mM Tris-HCl (pH 7.0) containing 1 mM EDTA (buffer A). Disruptions of cells were performed with an Ultrasonic Processor at 250 W for 60 min (30 s of sonication and a 30-s pause each time with cooling on ice). The disrupted cell extracts were centrifuged at 8,000 rpm for 30 min. The pellets were vortexed to a homogeneous suspension with buffer A (10 mM Tris-HCl [pH 7.0], 1 mM EDTA) containing 1% (vol/vol) Triton X-100 and shaken at room temperature for an additional 20 min. The suspension was centrifuged for 15 min at 7,000 rpm. The pellets were suspended in buffer A containing 2 M urea, and then the suspension was treated as described above. Finally, the pellets were dissolved in 10 ml of 10 mM Tris-HCl (pH 7.0) containing 100 mM NaH_2PO_4 and 8 M urea (buffer B) and applied to a Ni-nitrilotriacetic acid (Ni-NTA) affinity chromatography column (Qiagen GmbH). The proteins were washed with 10 mM Tris-HCl (pH 6.3) containing 100 mM $NaH_2PO_4$and 8 M urea (buffer C) and eluted with 10 mM Tris-HCl (pH 4.5) containing 100 mM NaH_2PO_4 and 8 M urea (buffer D). The purification was done as described in the

instructions to the Ni-NTA affinity chromatography purification kit (Qiagen GmbH).

4.16 Statistical analysis

Mean values between the groups were compared using the Student's unpaired two-tailed *t*-test. All statistical tests were two-sided, and differences were considered significant when $p < 0.05$.

5. Results

Characterization of EGCG-interacting proteins by chemical proteomics

To elucidate the anti-cancer action mechanism of EGCG, we used mass spectrometry-based proteomics analysis to identify proteins binding with EGCG. Firstly, EGCG was coupled to the CNBr-activated Sepharose 4B beads and the bindings between EGCG and total proteins in cancer cell lysates were examined by affinity chromatography. The binding protein elution was monitored in fractions by Bradford's method (Fig. 2). Then the fractions containing proteins binding with EGCG were separated by gradient-gel electrophoresis (Fig. 3) and the resultant bands were identified by MALDI-TOF mass spectrometer and NCBInr database interrogation (Tab. 1). The proteins identified in our study can be classified into eight functional groups, namely, chaperoning, cell signaling, transcription, translation, metabolism, cytoskeleton, DNA-binding proteins and uncharacterized proteins (Tab. 2).

Validation of EGCG-binding proteins from Mass analysis

To validate the chemical proteomics findings, the binding between EGCG and its targeted proteins (such as Hsp90, Hsp70 and PGK1) was assessed *in vitro* by pull-down assay and immunoblotting. Fig. 3A displays that EGCG directly binds with purified Hsp90, Hsp70 and PGK1, respectively, and Fig. 3B also shows EGCG can also target to Hsp90, Hsp70 or PGK1 in A431 cell lysates. These results strongly indicate that Hsp90, Hsp70 or PGK1 directly binds with EGCG.

Functional analysis of EGCG-binding proteins after they interact with EGCG

To clarify whether EGCG affects the intrinsic ATPase activity of Hsp90 or Hsp70 and kinase activity of PGK1, *in vitro* ATPase and kinase activity assay were performed under EGCG incubation. As shown in Fig. 4, 50 μM of EGCG slightly reduced ATPase activity of Hsp90 and Hsp70 and somewhat enhanced PGK1 activity. We suggest that EGCG may not change Hsp90's and Hsp70's ATPase activity but interrupt Hsp90/Hsp70-based chaperone machinery. Gasiewicz *et al.* have reported that EGCG directly binds with Hsp90 and inhibits aryl hydrocarbon receptor (AhR) gene transactivity through stabilizing AhR-Hsp90 complex in nucleus (*24*). It raises the possibility that EGCG may interrupt the interaction between Hsp90/Hsp70 and client proteins or co-chaperones which maintains homeostasis in cancer cells. To examine this hypothesis, the interaction of Hsp90 and client proteins in EGCG-treated A431 cells was determined by co-immunoprecipitation (Fig. 5). The Hsp90-client proteome in EGCG-treated A431 cells will be analyzed by MALDI-TOF mass spectrometer.

By the other way, GST was fused at N-terminal of Hsp90 to generate GST-Hsp90 fusion

protein, and the GST-Hsp90 protein will be applied to GST-pull down assay to determine the effects of EGCG on the interaction of Hsp90 and its clinet proteins identified by proteomic analysis.

6. Discussion

The basic principle of chemotherapeutic or chemopreventive agents used to treat neoplastic disease is to interfere with tumor cell metabolism and the mitotic process by blocking protein and DNA synthesis. Understanding the molecular mechanisms of the anti-cancer effects of cancer preventive agents is necessary for drug design and further clinical trials. Recently, worldwide interest in tea as a cancer preventive agent for human has increased, because it is non-toxic and effective in a wide range of organs. Epidemiologic studies suggest that consumption of tea is linked to a decreased incidence of various cancers. Animal and clinical studies have also demonstrated the anti-tumor activity and cancer preventive effects of tea consumption. Therefore, it is essential for understanding the biochemical and pharmacological mechanisms by which tea achieves its preventive action. Among tea constituents, EGCG, the major and most active catechin from green tea, has been shown to inhibit cell proliferation and induce cell cycle arrest and apoptosis in cancer cells. However, the molecular mechanisms responsible for cancer preventive effects of EGCG are not well understood.

To this end, we developed a chemical proteomics method for the characterization of EGCG-binding proteins in cancer cells. EGCG was immobilized as specific ligands for the affinity purification of cellular protein target(s) which was (were) then identified by sensitive techniques such as one-dimensional or two-dimensional electrophoresis, mass spectrometry and Western blot analysis. By this way, several EGCG-targeting proteins are characterized as shown in Tab. 1. Among them, Hsp90, Hsp70, and PGK1 were further validated by *in vitro* binding assay, suggesting that EGCG may directly bind to them and then affect their physiological function.

Hsp90 assists the folding and activation of a range of client proteins involved in cell cycle regulation, steroid hormone responsiveness, and signal transduction. X-ray crystallographic studeies reveal that Hsp90 comprises two ATP-binding sites within N-terminal and C-terminal regions, respectively (*25-27*). Among them, the site within N-terminus can be recognized by the selective Hsp90 inhibitor geldanamycin (GA) that leads to not the interaction of incompletely folded client polypeptides with native Hsp90, indicating that binding and hydrolysis of ATP plays a essential role in the protein folding function of Hsp90 (*25, 28, 29*). Like Hsp90, Hsp70 is able to interact with unfolded proteins to prevent irreversible aggregation and catalyze the refolding of their substrates in an ATP and co-chaperone dependent manner (*30*). Hsp70 often functions together with Hsp90 to form a multi-chaperone machinery, which also requires a minimal set of co-chaperones: Hsp40, Hop and p23 (*31, 32*). The co-chaperones either regulate the intrinsic ATPase activity of Hsp70 and hsp90 or recruit them to specific target proteins or subcellular localizations (*33*). To determine whether The binding of EGCG to Hsp90 or Hsp70 would affect the ATPase activity of Hsp90 or Hsp70, respectively, the Hsp90's and Hsp70's ATPase activities

were assessed by ATP-luciferase kit in the presence of EGCG as shown in Fig. 4. No signifigant difference of ATPase activity can be observed between the control and EGCG-treated experiments either in Hsp90's ATPase activity assay or in Hsp70's one. We suggest that EGCG does not influence the ATPase activity of Hsp90 or Hsp70 but blocks the interaction of Hsp90 or Hsp70 with their client proteins. To this end, we constructed GST-Hsp90 fusion protein to further study the effect of EGCG on the interaction between Hsp90 and its client proteins. We hope that we can define a new mechanism by which EGCG exerts its anti-cancer activity.

Phosphoglycerate kinase 1 (PGK1), a EGCG-binding protein we identified, not only catalyzes the transfer of the high-energy phosphate group of 1,3-bisphosphoglycerate to ADP, forming ATP and 3-phosphoglycerate but also acts as a disulphide reductase in tumor angiogenesis (*34*). Whether PGK1 could be a molecular target for EGCG was investigated. Firstly, we found that EGCG does not interrupt tha ATPase activity of PGK1 in Fig. 4. Next, we will check the effect of EGCG on the disulphide reductase of PGK1 and the role of EGCG in tumor angiogenesis.

On the basis of our findings, we characterize several EGCG-binding proteins such as Hsp90, Hsp70, and PGK1 and suggest that the binding of EGCG to these proteins would change the physiological function of these proteins. Our works are in progress and have been finished about 60~70%. The following experiments have been or will be done and we hope this study can be organized and published in a SCI journal in the furtur.

7. Acknowledgments

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9. Figures and Tables

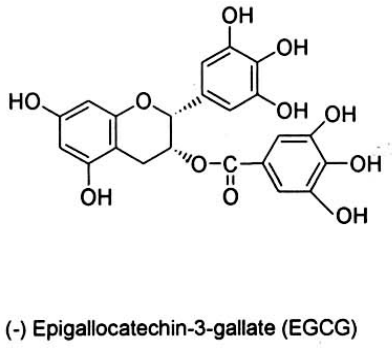
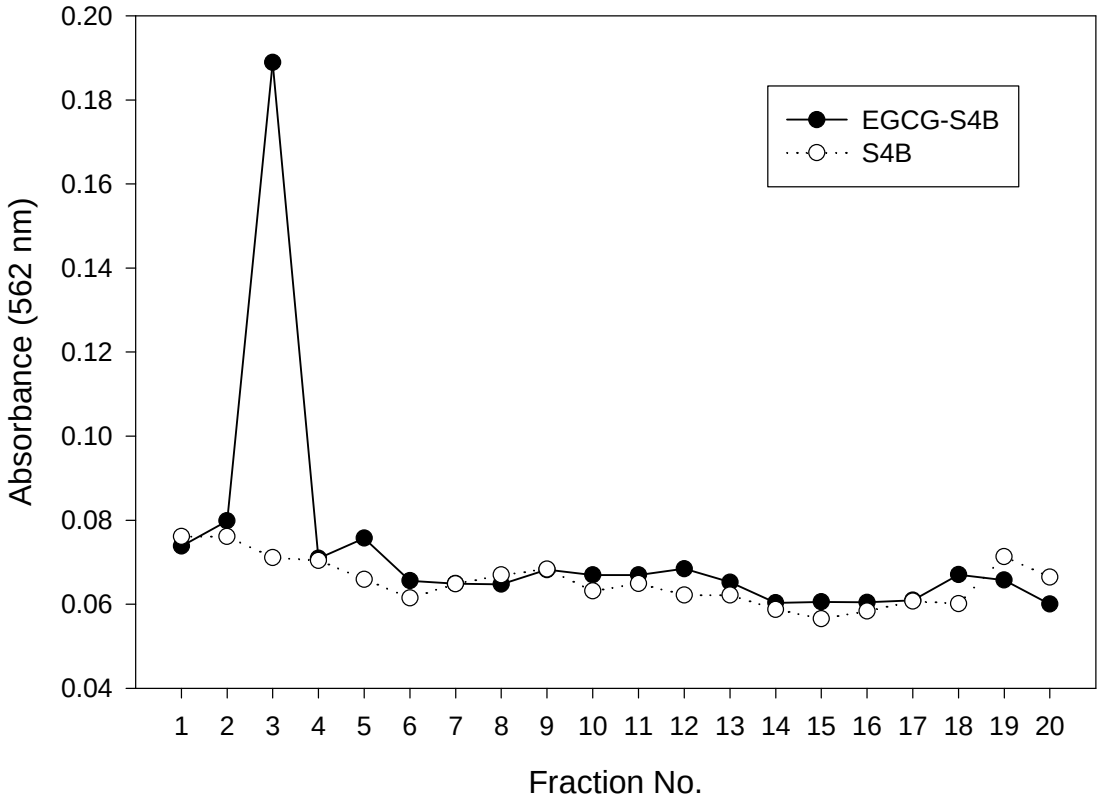


Fig. 1. Chemical structures of (-)-epigallo-catechin 3-gallate (EGCG).

elute in 0.15M NaCl



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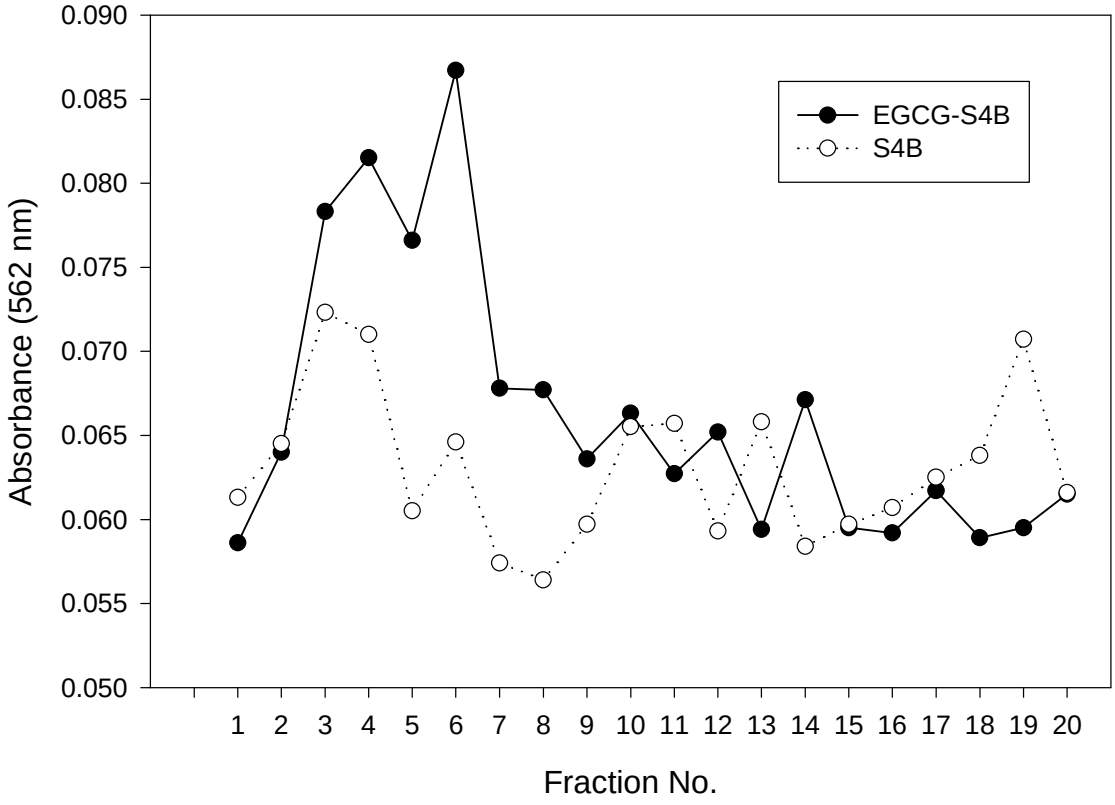


elute in 1 M NaCl and 4 M urea

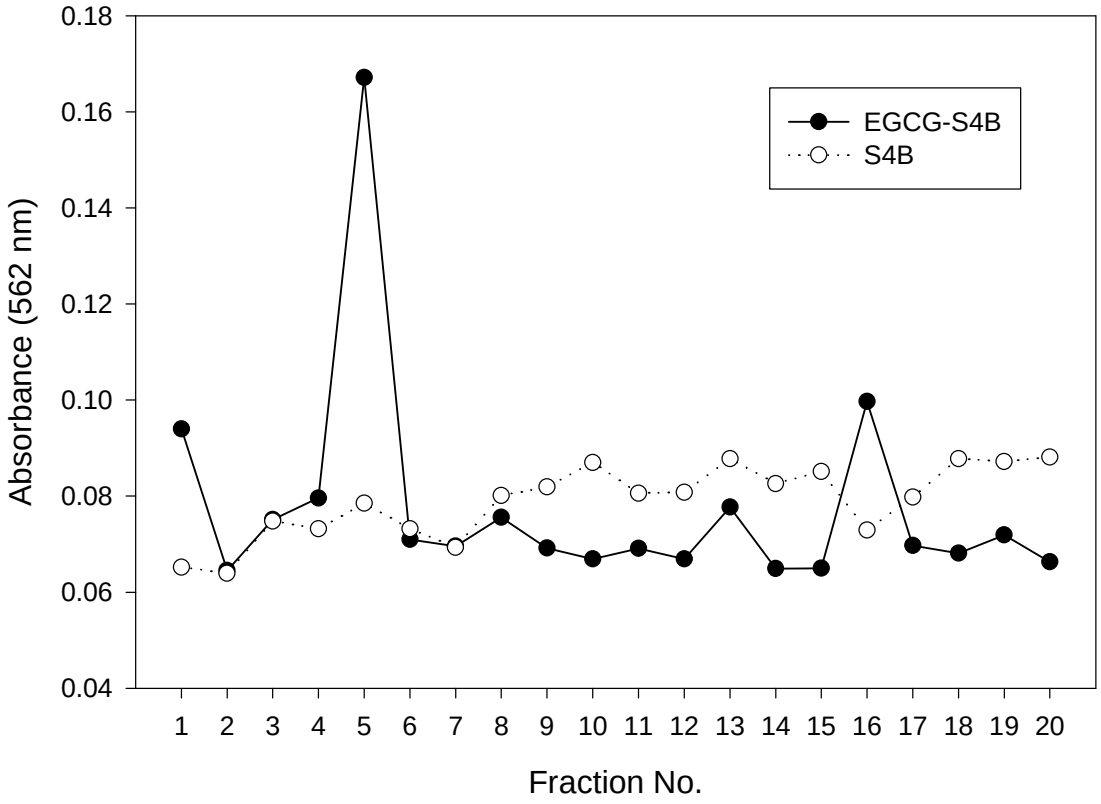


Fig. 2. Affinity chromatography of A431 cell lysates on EGCG-immobilized Sepharose 4B resin. Lysates of A431 cells were applied to an EGCG- (EGCG-S4B), or Sepharose 4B (S4B) affinity column equilibrated in buffer A. The unbound proteins were washed with buffer A, and the proteins bound to EGCG were eluted with buffer B containing three kinds of ionic strength (A) 0.15 M NaCl, (B) 1 M NaCl, and (C) 1 M NaCl and 4 M urea. Fractions eluted with buffer B were monitored protein assay kit, and then collected and concentrated by Centricon. The purified proteins were analyzed by SDS-PAGE later.

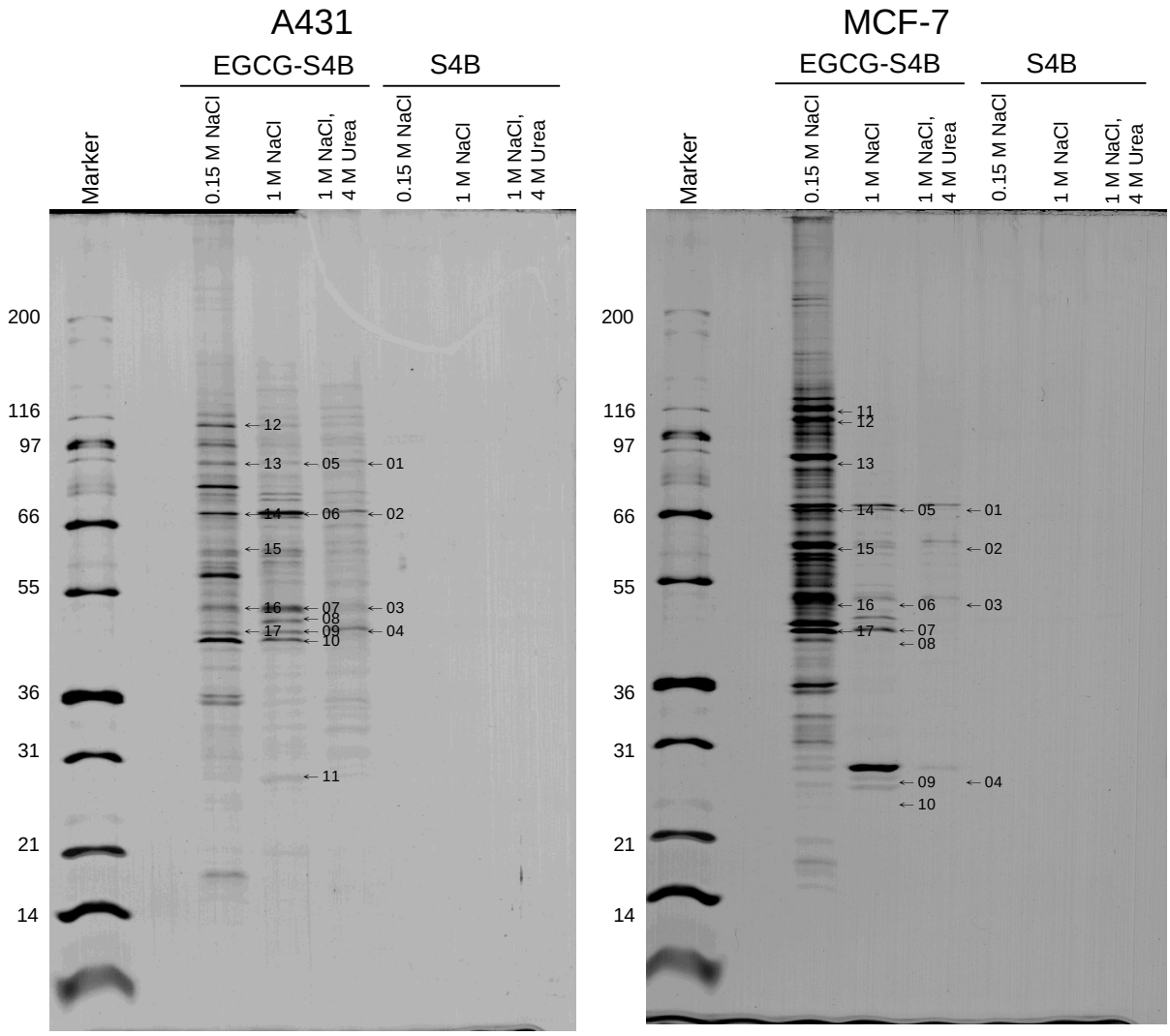


Fig. 3. Separation of proteins captured from EGCG-Sepharose 4B beads. Cell lysates from A431 or MCF7 cells were applied to EGCG-Sepharose 4B column or Sepharose 4B, and ECGG-binding proteins were used for analysis by gradient gel electrophoresis (8% to 14% SDS-PAGE) and stained by silver nitrate. The “number-marked” arrows indicate the protein slots which were picked up, in gel-digested and then analyzed by mass spectrometry (Table 1).

Table 1. EGCG-binding proteins identified by MALDI-TOF MS

Protein identified (Accession no.)	Band no.[a] (Score[b]/Seq Cov[c])					
	A431			MCF-7		
	0.15M NaCl	1M NaCl	1M NaCl, 4M Urea	0.15M NaCl	1M NaCl	1M NaCl, 4M Urea
90kDa heat shock protein (gi|306891)			1 (41/21%)			
Heat shock 70kDa protein 8, isoform 1 (gi|16741727)	14 (116/48%)	6 (192/54%)	2 (74/36%)	14 (112/49%)	5 (122/44%)	1 (145/48%)
epidermal growth factor receptor pathway substrate 15 (gi|55961757)			3 (76/31%)			
ACTB protein (gi|15277503)			4 (88/51%)			
heat shock 90kDa protein 1, beta (gi|20149594)	13 (116/45%)	5 (93/28%)				
eukaryotic translation elongation factor 1 alpha-like 3 (gi|55665593)		7 (72/40%)		16 (69/45%)		
erbB3 binding protein EBP1 (gi|4099506)		8 (111/61%)				
citrate synthase (gi|33337556)		9 (75/37%)		17 (114/42%)		
PGK1 (gi|48145549)	17 (131/62%)	10 (97/57%)				
unnamed protein product (gi|21753501)	11 (65/25%)					
100 kDa coactivator (gi|799177)	12 (129/42%)			12 (164/47%)		
unnamed protein product (gi|34536294)	15 (51/63%)					
SPG7 protein (gi|34783526)	16 (62/27%)					
Chaperonin (gi|49522865)						2 (62/29%)
RAB37, member RAS oncogene family isoform 3 (gi|28376635)						3 (62/50%)
PREDICTED: hypothetical protein XP_936973 (gi|88995874)						4 (50/24%)
AK5 (gi|49456735)					6 (55/51%)	
hepatoma-derived growth factor (high-mobility group protein 1-like) (gi|55960781)					7 (58/51%)	
migration-inducing gene 10 protein (gi|41350401)					8 (111/61%)	
high-mobility group box 1 (gi|55958714)					9 (88/80%)	
HMGB3 protein (gi|47124341)					10 (64/48%)	
ACLY variant protein (gi|68533125)				11 (240/46%)		
Heat shock protein HSP 90-alpha 4 (gi|61656605)				13 (168/57%)		
Pyruvate kinase 3, isoform 1 (gi|31416989)				15 (64/37%)		

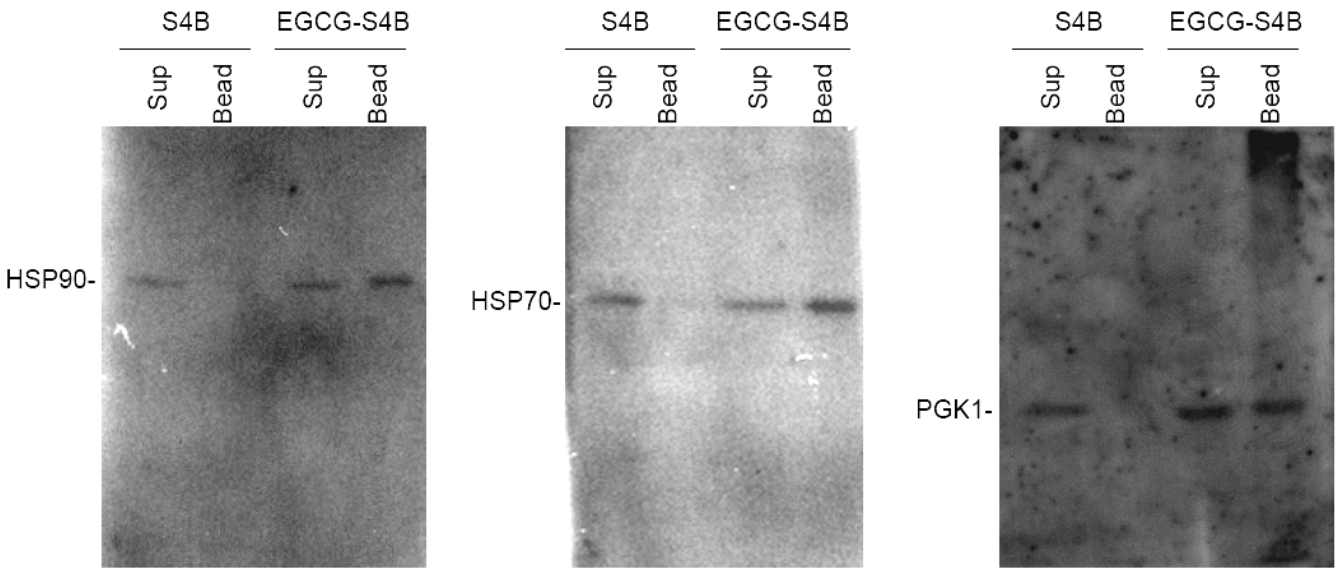
[a]Numbering of the protein bands detected in Fig. 1
[b]MASCOT search score of identified proteins
[c]Sequence coverage (Seq Cov) of the matched peptides in protein

Table 2.

Functional groups of the identified proteins

Functional groups	EGCG-binding Proteins (Band no./A: A431; M: MCF-7)	Biological function
Chaperoning	Hsp90 (A/1)	Chaperoning, stress response
	Hsp90β (A5, A13)	Chaperoning, stress response
	Hsp90α (M13)	Chaperoning, stress response
	Hsp70 protein 8 (isoform 1)(A2, A6, A14; M1, M5, M14)	Chaperoning, stress response
	Chaperonin (M2)	Chaperoning, stress response
Cell signaling	EGFR substrate 15 (A3)	a protein that is part of the EGFR pathway
	RAB37 (M3)	low molecular mass GTPase, regulator of vesicle trafficking
Transcription	100 kDa coactivator	coactivator for STAT6 transcriptosome
Translation	Erb3 binding protein EBP1 (A8)	dsRNA-binding protein, protein translation control
Metabolism	Citrate synthase (A8, M17)	Glycolysis
	PGK1 (A10, A17)	Glycolysis
	Pyruvate kinase 3, isoform 1 (M15)	Pyruvate oxidation
	AK5	nucleotide metabolism
	ACLY variant protein (M11)	the synthesis of cytosolic acetyl-CoA
	MIG10 (M8)	also named PGK1
Cytoskeleton	ACTB protein (A4)	cell motility, structure and integrity
DNA-binding proteins	HMGB1 (M9)	transcriptional regulation
	HMGB3 (M10)	transcriptional regulation
Uncharacterized proteins	EF-1 α-like 3 (A7, M16)	Uncharacterized
	Unnamed protein (gi|21753501, A11)	Uncharacterized
	Unnamed protein (gi|34536294, A15)	Uncharacterized
	SPG7 protein (A16) metalloproteases	Uncharacterized
	hypothetical protein xp_936973 (M4)	Uncharacterized
	hepatoma-derived growth factor (M7)	Uncharacterized

(A)



(B)

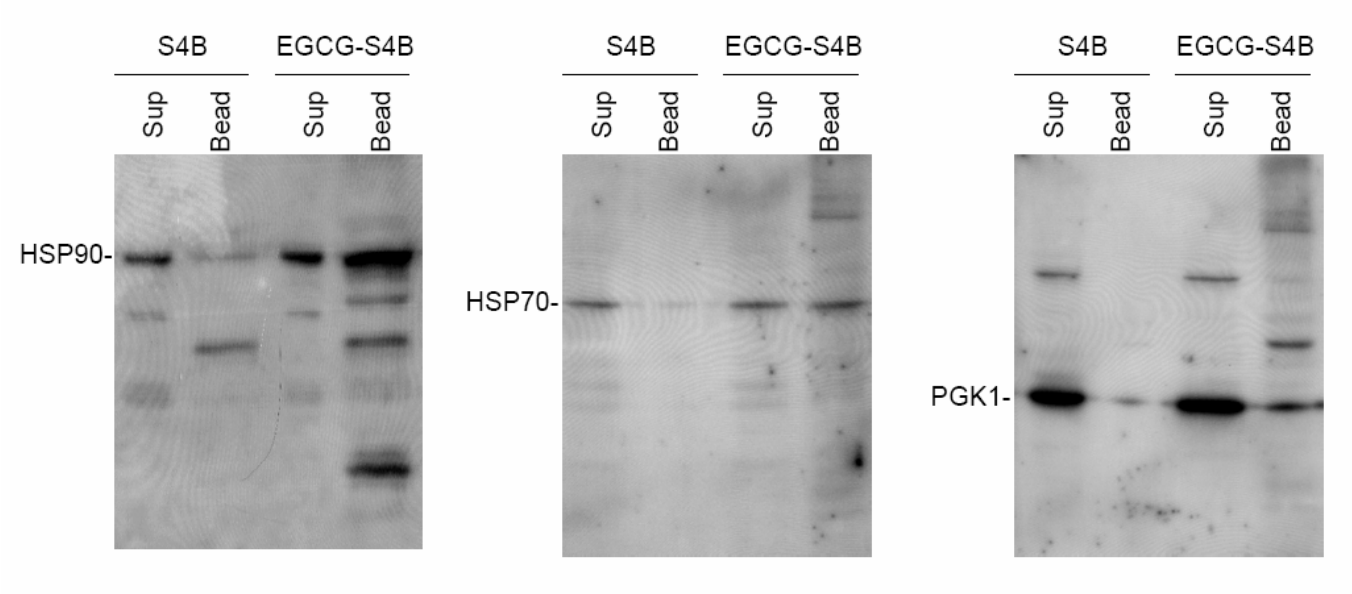


Fig. 3. Validation of the chemical proteomics findings by *in vitro* pull-down assay. (A) 2 μg of purified or recombinant Hsp90, Hsp70, or PGK1 proteins were incubated with EGCG-S4B or S4B at 4°C overnight. (B) 500 μg of total proteins in A431 cell lysates were incubated with EGCG-S4B or S4B at 4°C overnight. After microfugation, the supernatant (Sup) was concentrated, and the pellet (Bead) was clarified. The unbound (in Sup) and bound proteins (on bead) were applied to 10% SDS-PAGE and then visualized by immunoblotting.

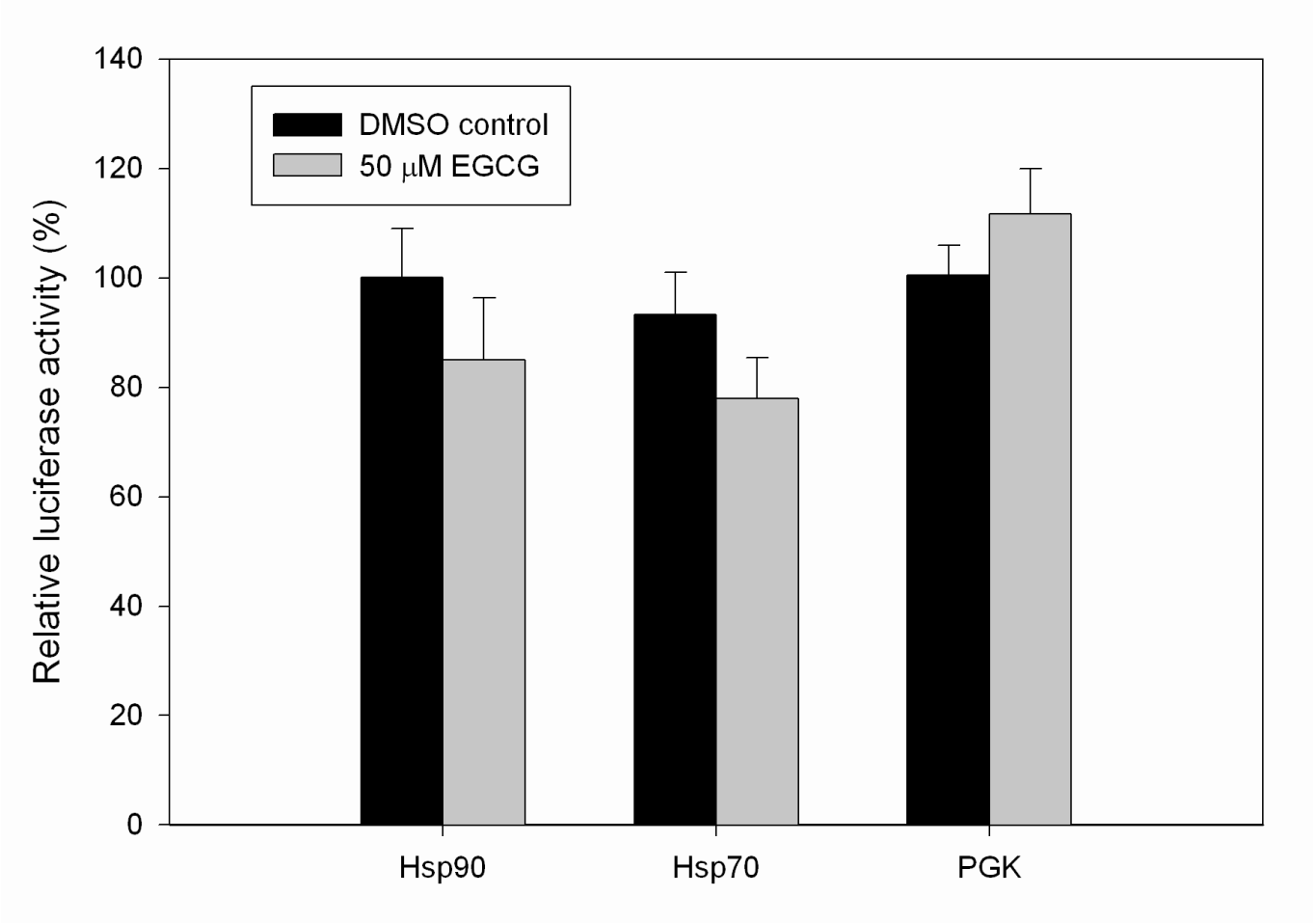


Fig. 4. The effects of EGCG on Hsp90 and Hsp70 ATPase activities and PGK kinase activity. 1 μg of purified Hsp90, Hsp70 or PGK1 was incubated with 50 μM EGCG for ATPase or PGK kinase activity assay (performed as described under "Materials and methods") in assay buffer for 90 min at 37°C. After the end of incubation, the consumption of ATP was determined by ATPLite-M assay. Results were obtained from three independent experiments as mean ± S.D..

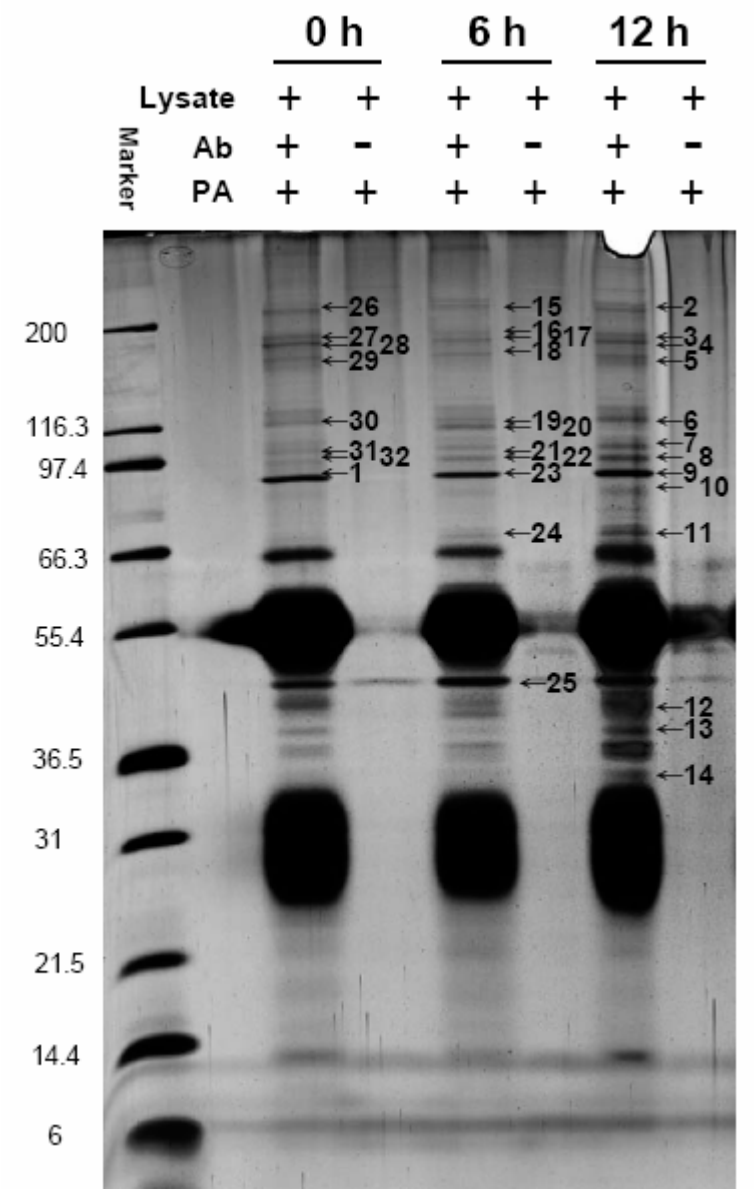


Fig. 5. Silver-stained gradient-PAGE profiles of Hsp90 co-immunoprecipitated proteins from EGCG-treated or untreated A431 cells. The exponentially growing A431 cells were treated with 40 μM of EGCG for indicated times. Afterward, EGCG-treated or untreated A431 cell lysates were used to immunoprecipitated by anti-Hsp90 antibody and then the proteins in the immunocomplexes were resolved by 8%-14% SDS-PAGE followed by sequential silver-staining. The "number-marked" arrows indicate the protein slots which were picked up, in gel-digested and then analyzed by mass spectrometry

9. 計畫成果自評

(1) 本研究證明綠茶中之兒茶素 EGCG 能結合細胞內特定蛋白如 Hsp90，Hsp70，與 PGK1 等，是否 EGCG 會影響這些結合蛋白的活性，並進一步改變癌細胞的生理特性，則仍在評估中。

(2) 本計劃目前已達預定之成效。

(3) 評估本研究已完成約三分之二的工作，目前研究成果與資料持續彙整中，未來將積極投稿至 SCI 排名期刊。