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Original Contribution

# (−)-Epigallocatechin-3-gallate suppresses growth of AZ521 human gastric cancer cells by targeting the DEAD-box RNA helicase p68

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ABSTRACT

(−)-Epigallocatechin-3-gallate (EGCG), the most abundant and biologically active polyphenol in green tea, induces apoptosis and suppresses proliferation of cancer cells by modulating multiple signal transduction pathways. However, the fundamental mechanisms responsible for these cancer-preventive effects have not been clearly elucidated. Recently, we found that EGCG can covalently bind to cysteine residues in proteins through autoxidation and subsequently modulate protein function. In this study, we demonstrate the direct binding of EGCG to cellular proteins in AZ521 human gastric cancer cells by redox-cycle staining. We comprehensively explored the binding targets of EGCG from EGCG-treated AZ521 cells by proteomics techniques combined with the boronate-affinity pull-down method. The DEAD-box RNA helicase p68, which is overexpressed in a variety of tumor cells and plays an important role in cancer development and progression, was identified as a novel EGCG-binding target. Exposure of AZ521 cells to EGCG lowered the p68 level dose dependently. The present findings show that EGCG inhibits AZ521 cell proliferation by preventing β-catenin oncogenic signaling through proteasomal degradation of p68 and provide a new perspective on the molecular mechanism of EGCG action.



Green tea (*Camellia sinensis*) is a widely consumed beverage known to have extensive beneficial health effects, including prevention of cancer and heart disease [1,2]. Extracts of green tea and its polyphenolic components, catechins (Fig. 1), have been shown to inhibit the formation and development of both spontaneous and chemically induced tumors at various organ sites in animal models [1,3,4]. Furthermore, epidemiological studies have revealed that green tea consumption may protect against various cancer types, including prostate, stomach, and breast cancers [1,4]. (−)-Epigallocatechin-3-gallate (EGCG)[2] is the most abundant and biologically active polyphenol in green tea. In various cancer cell lines, there is considerable evidence that EGCG inhibits enzyme activities and modulates multiple signal transduction pathways, resulting in the suppression of cell proliferation and enhancement of apoptosis, as well as cell invasion, angiogenesis, and metastasis [5–8]. Recent accumulating experimental data suggest that EGCG must interact directly with cellular proteins to act on various key proteins involved in cellular proliferation and apoptosis [9,10]. Therefore, the identification of target proteins interacting directly with EGCG is a key step in elucidating the molecular and biochemical mechanisms underlying the anticancer effects of EGCG.

The cell surface catechin receptor 67-kDa laminin receptor (67LR) mediates the EGCG induced-inhibition of cancer cell proliferation [11,12]. Vimentin [13], insulin-like growth factor 1 receptor [14], FYN [15], glucose-regulated protein 78-kDa (GRP78) [16], Ras-GTPase-activating protein SH3 domain-binding protein (G3BP) [17], and ZAP-70 [18] have been shown to be EGCG-binding targets from cultured cell lysates by EGCG–Sepharose 4B affinity chromatography. These proteins are important for the suppression of cell proliferation and enhancement of apoptosis mediated by EGCG in cancer cell lines. Nevertheless, the precise molecular and

*Abbreviations:* CBB, Coomassie Brilliant Blue G-250; Chaps, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; CHX, cycloheximide; DAPI, 4′,6-diamino-2-phenylindole; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EC, (−)-epicatechin; ECG, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin-3-gallate; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; FBS, fetal bovine serum; G3BP, Ras-GTPase-activating protein SH3 domain-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRP78, glucose-regulated protein 78-kDa; HRP, horseradish peroxidase; Keap1, Kelch-like ECH-associated protein 1;MS, mass spectrometry; 67LR, 67-kDa laminin receptor; MALDI, matrix-assisted laser desorption ionization; NBT, nitroblue tetrazolium; Nrf2, nuclear factor-erythroid 2-related factor 2; PAGE, polyacrylamide gel electrophoresis; PBA, *m*-aminophenylboronic acid–agarose; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RIPA, radioimmunoprecipitation assay; SDS, sodium dodecyl sulfate; TBS-T, Tris-buffered saline containing Tween 20; TCF/LEF, T cell factor/lymphoid enhancing factor; TFA, trifluoroacetic acid; TOF, time-of-flight.

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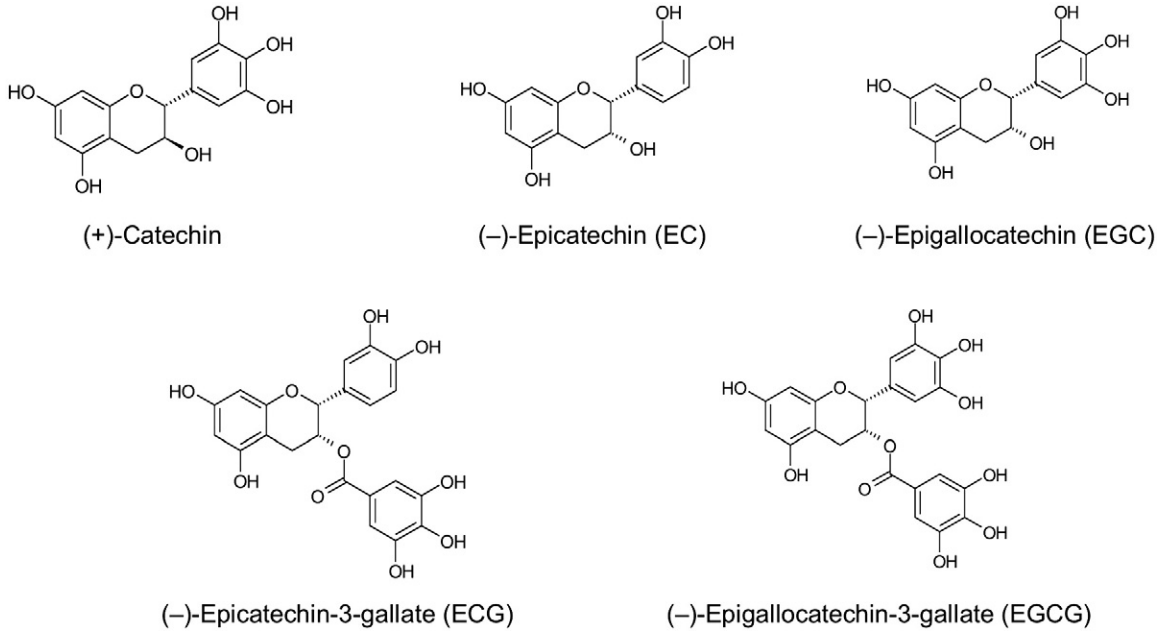


**Fig. 1.** Structures of major green tea catechins.

biochemical mechanism by which the binding of EGCG to target proteins regulates the protein function remains unknown. The EGCG-binding proteins from EGCG-treated cells have not been analyzed comprehensively by proteomics.

We found that EGCG can covalently bind to cysteinyl thiol residues in proteins through autoxidation (Fig. 2) [19]. EGCG is readily autoxidized to form a semiquinone radical that rearranges to an *o*-quinone at the B ring (gallyl) [20–22]. Thus, the electron-deficient *o*-quinone reacts with the nucleophilic thiol group of cysteine to form an *S*-cysteinyl–EGCG adduct. EGCG irreversibly inhibited glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity by specifically binding to the reactive cysteinyl thiol in the active center. Based on these findings, we proposed a novel signaling pathway by which the proteins are covalently modified by EGCG. Reactive cysteines with a low-p$K_a$ thiol group serve as catalytic intermediates at the active site of many enzymes. Furthermore, reactive cysteinyl thiols present in various transcription factors, such as Kelch-like ECH-associated protein 1/nuclear factor-erythroid 2-related factor 2 (Keap1/Nrf2), nuclear factor-κB, and p53, have been suggested to act as redox sensors for the transcriptional regulation of many genes essential for maintaining cellular homeostasis [23–25]. Therefore, the covalent modification of such thiol groups could critically alter protein function, with potential effects on cell signaling. These events may subsequently lead to inhibition of tumor cell growth, induction of apoptosis, or inhibition of angiogenesis.

In this study, we examined the direct binding of EGCG to cellular proteins in EGCG-treated cells by redox-cycling staining. Furthermore, we used the affinity pull-down assay and proteomics approach to identify EGCG-bound proteins in the EGCG-treated cells. Among the identified proteins, we focused on the ATP-dependent RNA helicase DDX5 (referred to as p68) as a novel EGCG-binding target. Recently, p68 was reported to act as a transcriptional coactivator for a number of highly regulated transcription factors (e.g., β-catenin, estrogen receptor α, and androgen receptor) and to be overexpressed in some cancer cells, including intestinal, breast, and prostate tumor, suggesting that it may play an important role in cancer development and/or progression [26–29]. We investigated the effects of EGCG on the p68-dependent transcriptional regulation.

## Materials and methods

### *Materials*

EGCG, (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), (−)-epicatechin (EC), and (+)-catechin were kindly provided by Mitsui Norin Co. Ltd (Shizuoka, Japan). Hybond-P polyvinylidene difluoride (PVDF) membrane was obtained from GE Healthcare UK Ltd. The protease inhibitor cocktail was obtained from Roche Applied Science (Mannheim, Germany). *m*-Aminophenylboronic acid–agarose (PBA) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Fetal bovine

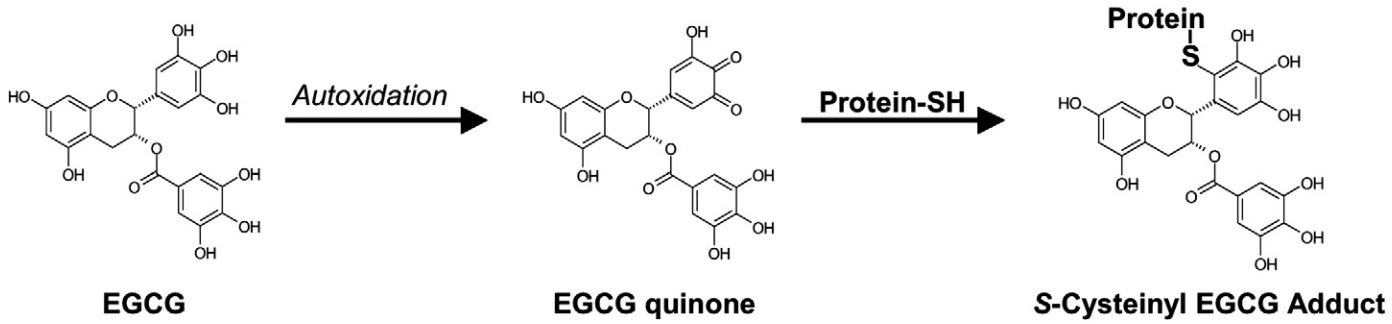


**Fig. 2.** Putative mechanism for EGCG binding to a protein cysteinyl thiol group through autoxidation. EGCG is oxidized to form EGCG quinone at the B ring by autoxidation. The EGCG quinone can react with the nucleophilic thiol group of a cysteine residue to form EGCG–protein adducts.

serum (FBS) was obtained from Invitrogen (Carlsbad, CA, USA). The EZ-ECL kit for chemiluminescence detection was from Biological Industries (Kibbutz Beit Haemek, Israel). Ethylenediamine tetraacetic acid (EDTA), iodoacetamide, skim milk powder, trifluoroacetic acid (TFA), Tween 20, 4′,6-diamino-2-phenylindole (DAPI), and dithiothreitol (DTT) were purchased from Wako Pure Chemical (Osaka, Japan). RPMI 1640 medium, penicillin, streptomycin, Hepes, nitroblue tetrazolium (NBT), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps), phenylmethylsulfonyl fluoride (PMSF), ethylene glycol tetraacetic acid (EGTA), and Triton X-100 were purchased from Nacalai Tesque (Kyoto, Japan).

*Cell culture*

The human gastric cancer cell line AZ521 was obtained from the Cell Resource Center for Biomedical Research, Tohoku University, Japan, and maintained in a 5% $CO_2$ humidified atmosphere at 37 °C in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were seeded into 10-cm petri dishes and cultured until they reached 50 to 60% confluence. The cells were starved in serum-free RPMI 1640 medium for 24 h before they were treated with catechins in the serum-free medium. After treatment, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with lysis buffers. The protein concentration of each lysate was measured with the BCA protein assay reagent (Thermo Fisher Scientific, Waltham, MA, USA) or Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA).

*Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and redox-cycling staining*

AZ521 cells were lysed in buffer containing 8 M urea, 4% Chaps, and 40 mM Tris by sonication. The reaction between catechin and proteins was terminated by the addition of DTT (50 mM final), because thiol compounds can quench the autoxidation products such as superoxide ($O_2^{\cdot-}$), semiquinone radical, and quinone [19]. We confirmed that the addition of 50 mM DTT completely prevented the modification of proteins by catechins. After the protein solutions were subjected to SDS–PAGE, the proteins were stained with Coomassie Brilliant Blue G-250 (CBB). For redox-cycle staining, the gel was transblotted onto a PVDF membrane, and then catechin-modified proteins were detected with NBT and glycinate as previously described [19,30]. After being washed with ultrapure water, the membrane was stained with 0.24 mM NBT in 2 M potassium glycinate (pH 10) for 15–20 min in the dark and then washed thoroughly with ultrapure water.

*Preparation of subcellular extracts*

The cytosolic fraction, organelle/membrane fraction, nuclear fraction, and cytoskeletal fraction were extracted stepwise with the ProteoExtract subcellular proteome extraction kit (Merck, Darmstadt, Germany), in accordance with the manufacturer's instructions. Alternatively, cytoplasmic and nuclear extracts for immunoblotting were prepared as described by Schreiber et al. [31]. Briefly, cells were washed with cold PBS and scraped into PBS. The cells were collected by centrifugation at 1500 *g* for 5 min in microfuge tubes and resuspended in 10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 1× protease inhibitor cocktail (buffer A). After 15 min on ice, Nonidet P-40 (Nacalai Tesque) was added to a final concentration of 10%, and the tubes were vortexed for 10 s. Lysates were centrifuged at 4 °C in a microfuge set at maximum speed to obtain the soluble cytoplasmic fraction (supernatant) and the nuclear pellet, which was resuspended in ice-cold buffer B (20 mM Hepes at pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1× protease inhibitor cocktail) and agitated at 4 °C for 15 min. The nuclear lysate was centrifuged for 5 min at 4 °C to obtain the soluble nuclear fraction (supernatant).

*Cell growth assay*

AZ521 cells were seeded in 96-well plates (5000 cells/well). After 24 h of culture in 10% FBS–RPMI 1640 medium, the medium in the 96-well plate was exchanged with 100 μl of serum-free RPMI 1640 medium. Twenty-four hours later, the cells were treated with EGCG (0–10 μM) in serum-free medium (100 μl) for 24 h. After the treatment, the medium was discarded and replaced by 100 μl of RPMI 1640 medium containing 10% FBS. The cells were further incubated for 48 h, and then cell growth was determined by WST-8 reduction assay using a Cell Count Reagent SF kit (Nacalai Tesque) according to the manufacturer's instructions.

*PBA isolation of EGCG-bound proteins*

We isolated EGCG-bound proteins by a specific interaction between catechols and boronic acid using PBA according to the previous method [19,32]. AZ521 cells were washed twice with ice-cold PBS and lysed with lysis buffer (100 mM Tris, pH 8.6, 1% Triton X-100, 50 mM DTT, 1 mM PMSF, and 1× protease inhibitor cocktail). After protein quantification, equal amounts of protein (total protein, 1.0–1.5 mg) were mixed with 50 μl of PBA in 200 μl of 100 mM Tris (pH 8.6) containing 0.1% Triton X-100. The mixture was incubated overnight at 4 °C with rotary shaking. Then, the resin was washed for 10 min each in ~40 bed volumes of 1 mM Tris–HCl (pH 8.6), 2×250 mM Tris (pH 6.5), and 1 mM Tris (pH 6.5). Proteins were released from the boronate resin by elution with 50 μl of 50 mM glycine (pH 2.0).

*Protein separation by two-dimensional (2D) gel electrophoresis*

The total protein extract was desalted and put into a solution composed of 8 M urea and 4% Chaps using MicroSpin G-25 columns (GE Healthcare) as previously reported [33]. Then, 150 μl of total protein extract (50 μg protein) was mixed with 6 μl of 0.5 M DTT, 1 μl of pH 3–10 Zoom carrier ampholyte (Invitrogen), and 3 μl of 0.1% bromophenol blue. Isoelectric focusing (IEF) was carried out using a Zoom IPGRunner system (Invitrogen) and an immobilized pH gradient strip (Zoom strip), pH 3–10NL (Invitrogen), according to the manufacturer's instructions. A Zoom strip was hydrated overnight with each sample at room temperature. IEF was performed at 200 V for 20 min, 450 V for 15 min, 750 V for 15 min, and 2000 V for 30 min. After IEF, the strips were equilibrated in 1× NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) containing 50 mM DTT at room temperature for 15 min with shaking and then for 15 min in 1× NuPAGE LDS sample buffer containing 250 mM iodoacetamide. The equilibrated strip was applied to a NuPAGE 4 to 16% polyacrylamide gel (Invitrogen) and electrophoresed. After electrophoresis, the gels were stained with CBB R-250 or an EzStain silver staining kit (Atto, Osaka, Japan).

*Protein identification*

Gel pieces were washed in ultrapure water containing 10 mM ammonium bicarbonate buffer (pH 8.0) and 50% methanol for 1 h, dehydrated in acetonitrile, and dried in a Speed Vac for 30 min. Samples were proteolyzed with 100–500 ng of sequence-grade modified trypsin (Promega, Madison, WI, USA) in 50 mM Tris–HCl buffer (pH 8.8) overnight at 37 °C. The supernatant was collected, and peptides were further extracted with water containing 0.1% TFA, 50% acetonitrile containing 0.1% TFA, and acetonitrile. Peptide extracts were vacuum-dried and resuspended in water containing 0.1% TFA.

Peptide mass fingerprints (PMF) were generated with an UltraFLEX matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS; Bruker Daltonics Japan, Tokyo, Japan). After being desalted and concentrated using a Zip Tip μ-C18 (Millipore, Billerica, MA, USA), a few microliters of the sample was mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid (Bruker Daltonics) in 50% acetonitrile containing 0.1% TFA, and 1 μl of the mixture was deposited on the MALDI-TOF MS target. Proteins were identified using MASCOT (Matrix Science, UK), searching algorithms using the nonredundant database. Probability-based MOWSE scores were estimated by comparison of search results against the estimated random-match population and were reported as $\sim 10 \times \log 10\ (p)$, where $p$ is the absolute probability. Scores greater than 65 were considered significant, meaning that for scores higher than 65 the probability that the match was a random event was lower than 0.05. All analyses were carried out in the positive ion mode, and the instrument was calibrated immediately before each analysis.

*Immunoblot analysis*

The proteins were boiled in Laemmli sample buffer for 5 min at 100 °C. The samples were run on 12.5% SDS–polyacrylamide gels, transferred to a PVDF membrane, incubated with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) at room temperature for blocking, washed three times in TBS-T for 10 min, and treated overnight with primary antibody at 4 °C. After being washed three times in TBS-T, the blots were further incubated for 1 h at room temperature with IgG antibody coupled to HRP in TBS-T. The blots were then washed three times in TBS-T before visualization. An ECL kit and LAS-4000 system (Fujifilm, Tokyo, Japan) were used for chemiluminescence detection. Anti-p68 RNA helicase goat polyclonal IgG (dilution 1:3000), anti-β-actin mouse monoclonal IgG (dilution 1:4000), anti-histone H2B rabbit polyclonal IgG (dilution 1:2000), anti-ubiquitin mouse monoclonal IgG (dilution 1:1000), donkey anti-goat IgG–HRP (dilution 1:4000), goat anti-mouse IgG–HRP (dilution 1:4000), and goat anti-rabbit IgG–HRP (dilution 1:4000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-cyclin D1/Bcl-1 Ab-1 mouse monoclonal IgG (dilution 1:2000) and anti-c-Myc Ab-5 mouse monoclonal IgG (dilution 1:2000) were purchased from Thermo Fisher Scientific. Anti-β-catenin rabbit polyclonal IgG was purchased from Sigma–Aldrich.

*Immunoprecipitation assays*

AZ521 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris–HCl, 50 mM NaCl, 0.5% Nonidet P-40, 0.25% sodium deoxycholate, 0.05% SDS, 0.5 mM EDTA, pH 7.4) containing 1 mM PMSF and 1× protease inhibitor cocktail by sonication. After removal of debris and preclearing, 2 μl of antibodies was added and, 2 h later, 20 μl of PureProteome protein G magnetic beads (Millipore) was also added. After 30 min of rotation, beads were collected using a magnetic separation rack and then washed four times with RIPA buffer. Bound proteins were resolved by SDS–PAGE and then subjected to immunoblot analysis.

*Immunofluorescence microscopy*

AZ521 cells grown on coverslips were washed twice with PBS, fixed in 4% formaldehyde in PBS for 30 min, and extracted in 0.1% Triton X-100 in PBS for 30 min at room temperature to allow subsequent antibody penetration. After being washed three times in PBS containing 0.1% Tween 20 (PBS-T), the cultures were blocked with a 1% bovine serum albumin/PBS solution for 60 min at room temperature, rinsed with PBS-T three times, and then incubated with anti-p68 RNA helicase goat polyclonal IgG at a dilution of 1:400 for 2 h at room temperature. The cells were rinsed three times with PBS-T and incubated with Alexa Fluor 488 rabbit anti-goat IgG (Molecular Probes) at a dilution of 1:500 and DAPI (2 μg/ml) for 2 h at room temperature. After being rinsed three times with PBS-T, the cells were mounted onto slides using PermaFluoro aqueous mounting medium (Thermo Fisher Scientific). Fluorescence microscopy was performed using BZ-9000 (Keyence, Osaka, Japan).

## Results

*Detection of cellular protein modification by EGCG*

We exposed cultured cells of a human gastric cancer cell line, AZ521 cells, to major green tea catechins for 3 h and then detected the catechin-bound proteins in the cells by SDS–PAGE/electroblotting followed by redox-cycle staining with NBT/glycinate [19,30]. The mechanism of redox cycling is proposed as follows [34,35]: at an alkaline pH, the catechol moiety is easily oxidized to the corresponding quinone concomitant with the evolution of $O_2^-$ via autoxidation. Glycine covalently binds to the quinone to form quinone ketimine followed by the *trans*-Schiff-base reaction, giving phenolaldimine. Then, the adduct progressively releases aldehyde through hydrolysis, thereby yielding aminophenol. The $O_2$-dependent oxidation of aminophenol can regenerate quinone accompanying the release of $O_2^-$ and $NH_3$, and then the regenerated quinone undergoes the subsequent redox reactions. The generated $O_2^-$ reduces NBT to the blue-purple insoluble formazan on the PVDF membrane, allowing the detection of catechin-bound protein. As shown in Fig. 3A, the EGCG-treated cell lysate generated positive bands with a wide range of molecular weights when assayed by redox-cycle staining, but the other catechins produced no such effect, indicating that EGCG was bound to a wide range of cellular proteins. Modified proteins increased with the dose of EGCG (Fig. 3B). Furthermore, as shown in Fig. 3C, the positive bands were detected after exposure of AZ521 cells to 20 μM EGCG for 15 min by redox-cycle staining. The exposure of AZ521 cells to EGCG resulted in a time-dependent increase in redox-cycle staining after treatment for 3 h.

We examined the subcellular distribution of EGCG-bound proteins. After exposure to EGCG, AZ521 cells were sequentially fractionated into cytosol, membrane/organella, nucleus, and cytoskeleton by stepwise extraction. As shown in Fig. 3D, EGCG-modified proteins were observed in cytosol, membrane/organella, and cytoskeleton fractions by redox-cycle assay but not in the nucleus fraction, suggesting that EGCG was incorporated into the cytoplasm and bound to a wide range of target proteins.

*Isolation of EGCG-bound proteins from EGCG-treated cells by the boronate-affinity method*

We isolated the EGCG-modified proteins from EGCG-treated cells by a pH-dependent interaction between catechols and boronic acids with PBA resin. At alkaline pH the EGCG-bound proteins and PBA can form a covalent complex between catechols and boronic acids, which can subsequently withstand washing. At a strongly acidic pH, the hydroxyl groups can become protonated and release the EGCG-bound proteins from the PBA resin, allowing the isolation of the EGCG-modified proteins [19]. After exposure of AZ521 cells to EGCG for 3 h, we incubated the whole-cell lysates with PBA resin at an alkaline pH, washed the resin under stringent conditions, and determined the EGCG-binding proteins by SDS–PAGE and redox-cycle staining. As shown in Fig. 4A (left), SDS–PAGE analysis of PBA eluates confirmed that the binding of cellular proteins to PBA was specific and dependent on the dose of EGCG. The proteins bound to PBA were visualized by redox-cycle staining (Fig. 4A, right), suggesting that PBA-bound proteins are modified by EGCG. These results support the suitability of the boronate-affinity method for the isolation of EGCG-bound proteins from cells exposed to EGCG.

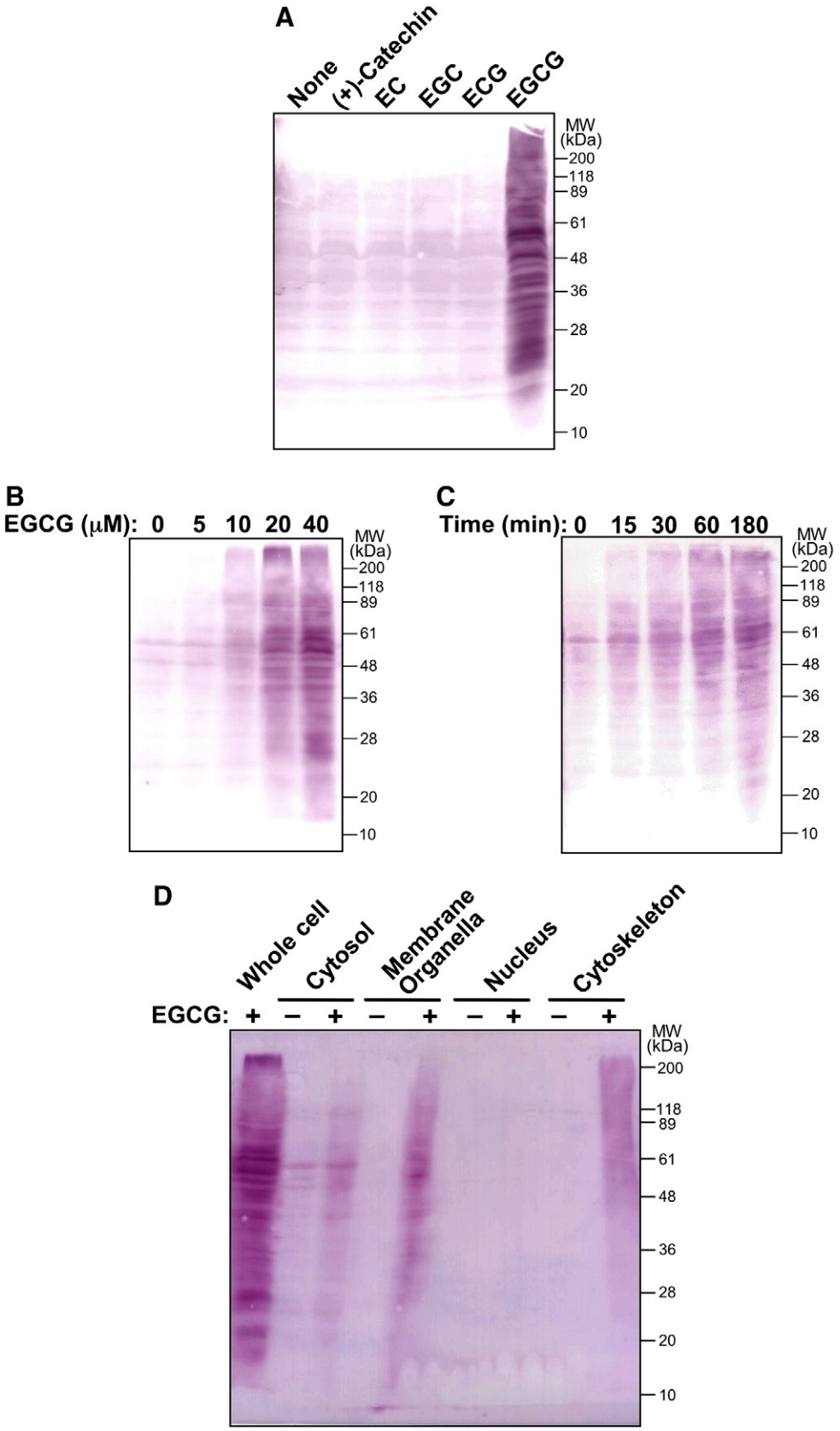


**Fig. 3.** Detection of EGCG binding to cellular proteins in AZ521 cells. (A) Detection of catechin-bound cellular proteins by redox-cycle staining. AZ521 cells were exposed to each green tea catechin (40 μM) for 180 min. After reduction with DTT, catechin-modified proteins were analyzed by SDS–PAGE/electroblotting followed by redox-cycle staining with NBT/glycinate. (B) Concentration-dependent binding of EGCG to cellular proteins. AZ521 cells were incubated with the indicated concentrations of EGCG (0–40 μM) for 180 min, and then EGCG-modified proteins were detected by redox-cycle staining. (C) Time-dependent binding of EGCG to cellular proteins. AZ521 cells were cultured with 20 μM EGCG for 0–180 min, and then EGCG-modified proteins were detected by redox-cycle staining. (D) The subcellular distribution of EGCG-bound proteins in EGCG-treated AZ521 cells. After exposure to 40 μM EGCG for 3 h, AZ521 cells were sequentially fractionated into cytosol, membrane/organella, nucleus, and cytoskeleton by stepwise extraction, and then EGCG-modified proteins were detected by redox-cycle staining.

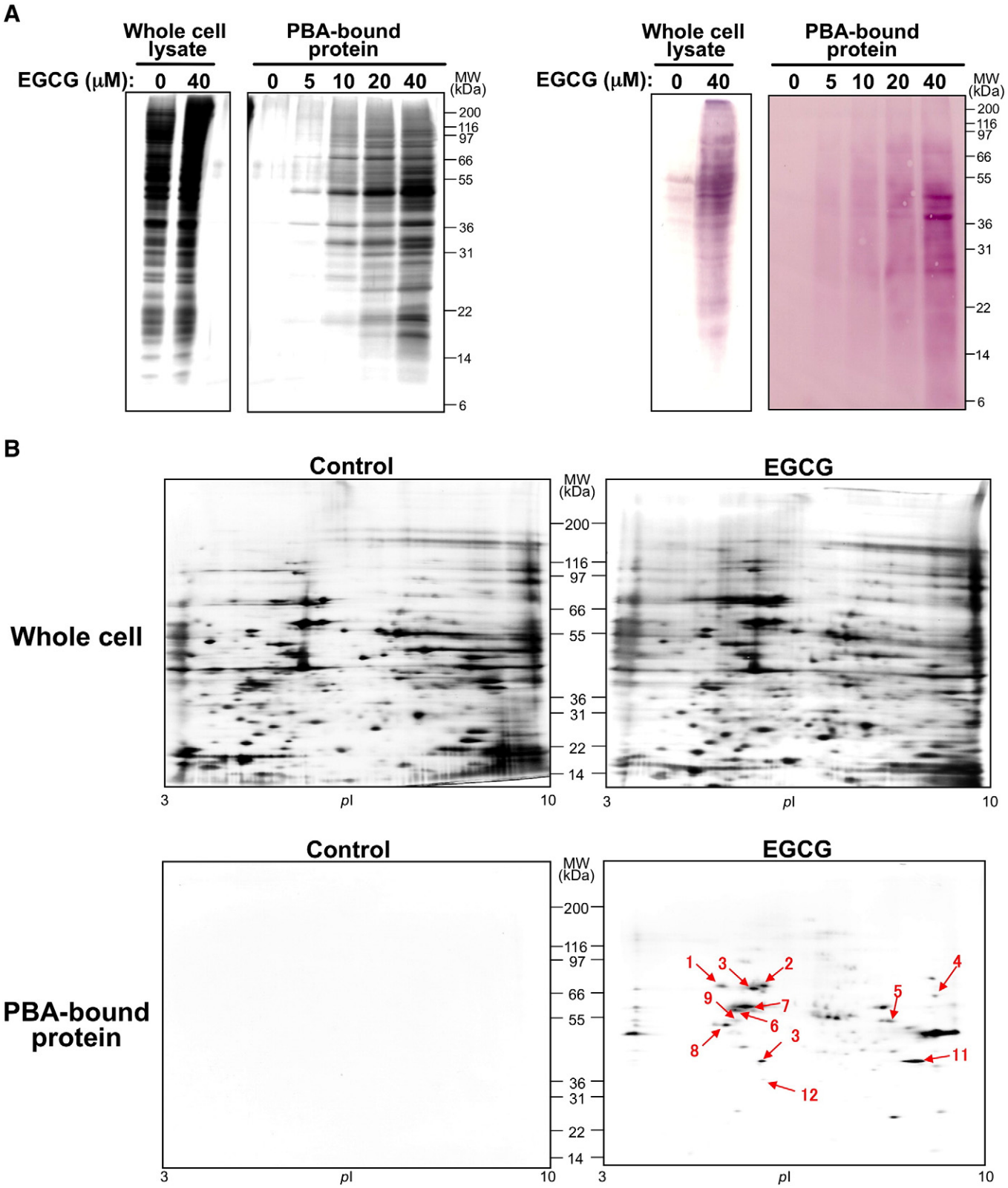


**Fig. 4.** Affinity purification of EGCG-modified proteins from EGCG-treated AZ521 cells using the boronate-affinity method. (A) Selective binding of EGCG-modified proteins to the PBA resin. AZ521 cells were exposed to the indicated concentrations of EGCG (0–40 μM) for 180 min. Then, EGCG-modified proteins were separated using the boronate-affinity method as described under Materials and methods. EGCG-modified proteins in the PBA eluate were separated by SDS–PAGE and then detected by silver staining (left) and redox-cycling staining (right). (B) 2D gel electrophoresis of whole-cell proteins (top) and EGCG-modified proteins (bottom) from EGCG-treated and control AZ521 cells. AZ521 cells were cultured with or without 40 μM EGCG for 180 min. EGCG-modified proteins were separated using the boronate-affinity method as described under Materials and methods. The proteins were first separated according to their isoelectric point on an immobilized pH gradient gel strip (pH 3–10, nonlinear). Second, the separated proteins were further resolved according to their molecular weight on a 4 to 16% polyacrylamide gel. The proteins identified by MALDI-TOF MS analysis are shown by arrow and number.

*Identification of EGCG-bound proteins in cells exposed to EGCG*

We analyzed EGCG-modified proteins using an MS-based proteomics approach. Proteins binding with PBA from EGCG-treated AZ521 cells were separated by 2D gel electrophoresis with a different isoelectric point and molecular weight. Experimental conditions were established to obtain a reproducible pattern of spots by 2D electrophoresis. Fig. 4B shows typical 2D maps of whole cellular protein (top) and PBA-bound protein (bottom) from control and EGCG-treated AZ521 cells. We detected more than 20 spots of proteins in the PBA-bound fraction from EGCG-treated AZ521 cells but not from control cells. The respective spots were excised from the 2D gel and in-gel digested with trypsin, and the resulting peptides were subjected to analysis by MALDI-TOF MS. Consequently, we identified 12 proteins by a database search with the PMF mode of MASCOT. Table 1 shows the p*I*, molecular weight, and gel spot location of each protein.

We focused on the ATP-dependent RNA helicase DDX5 (p68) as a novel EGCG-binding target. p68 is a prototypical member of the DEAD-box family of RNA helicases and plays a very important role in cell proliferation and early organ development and maturation [36–38]. Related to the role of p68 in cell proliferation, the expression of the protein was shown to correlate with tumor progression and transformation [39,40]. Recently, p68 has been demonstrated to interact with and thereby activate various transcription factors and is implicated to be involved in cancer development and/or progression [26–29,37]. We studied the potential influence of the interaction between EGCG and p68 on anti-cancer activity.

**Table 1**
Identification of EGCG-binding proteins by MALDI-TOF MS of tryptic peptides.

| Spot | Protein name [taxonomy] | Nominal mass | Calculated p*I* | Score | Sequence coverage (%) | Gi No. |
|---|---|---|---|---|---|---|
| 1 | Heat shock 70-kDa protein 9B precursor (mtHSP75) [*Homo sapiens*] | 73,920 | 5.87 | 117 | 41 | 24234688 |
| 2 | Heat shock 70-kDa protein 5 (GRP78) [*H. sapiens*] | 72,402 | 5.07 | 92 | 23 | 16507287 |
| 3 | Heat shock 70-kDa protein 8 isoform 1 variant [*H. sapiens*] | 71,083 | 5.28 | 154 | 41 | 62897129 |
| 4 | ATP-dependent RNA helicase DDX5 (p68) [*H. sapiens*] | 69,078 | 9.06 | 96 | 23 | 197692465 |
| 5 | Chain A, structure of human muscle pyruvate kinase (Pkm) [*H. sapiens*] | 60,277 | 8.22 | 71 | 27 | 67464392 |
| 6 | Vimentin [*H. sapiens*] | 53,676 | 5.06 | 158 | 56 | 62414289 |
| 7 | Ras-GTPase-activating protein SH3-domain-binding protein (G3BP) [*H. sapiens*] | 52,189 | 5.36 | 100 | 33 | 5031703 |
| 8 | Mitochondrial ATP synthase, $H^+$ transporting F1 complex β subunit [*H. sapiens*] | 48,083 | 4.95 | 154 | 48 | 89574029 |
| 9 | Cardiac muscle α-actin 1 proprotein [*H. sapiens*] | 42,334 | 4.88 | 95 | 49 | 119612724 |
| 10 | Eukaryotic translation initiation factor 3 subunit 2β, 36-kDa [*H. sapiens*] | 36,878 | 5.38 | 138 | 50 | 4503513 |
| 11 | Heterogeneous nuclear ribonucleoprotein A2/B1 isoform A2 [*H. sapiens*] | 36,041 | 8.67 | 74 | 42 | 4504447 |
| 12 | Prohibitin [*H. sapiens*] | 29,843 | 5.57 | 97 | 39 | 4505773 |

Spots that were excised from the gel shown in Fig. 4 were identified by MALDI-TOF MS.

*Immunoblot confirmation of p68 as an EGCG-binding target*

We confirmed the binding of p68 with EGCG in cells exposed to EGCG by immunoblot analysis with anti-p68 antibody. After exposure of AZ521 cells to various concentrations of EGCG for 3 h, proteins binding with PBA from EGCG-treated cells were separated under the same conditions as described above, and then p68 was detected by

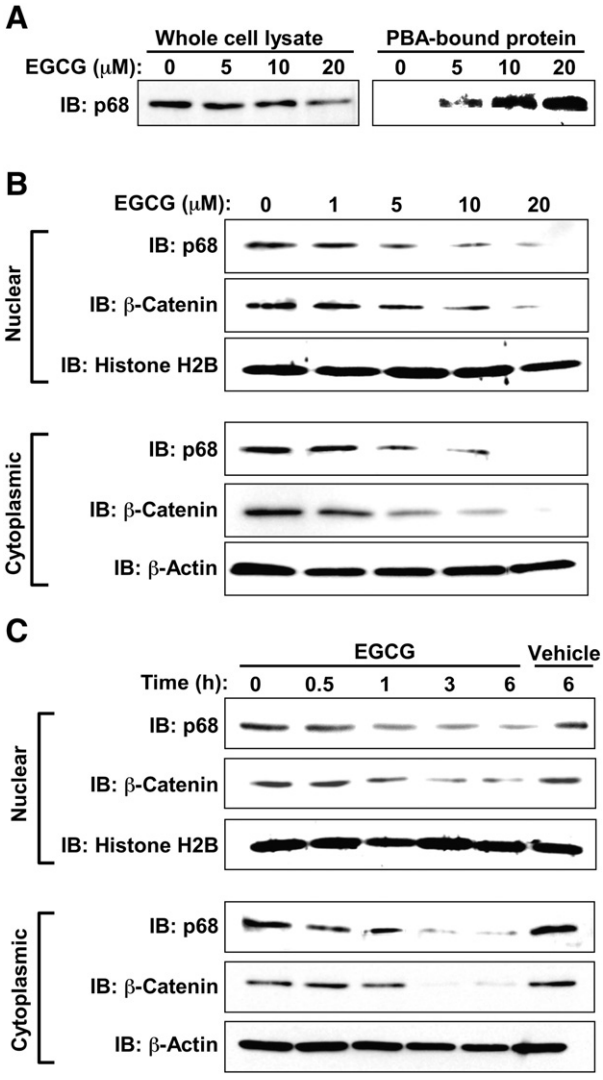


**Fig. 5.** Immunoblot analysis of p68 and β-catenin in EGCG-treated AZ521 cells. (A) Detection of EGCG-modified p68 from EGCG-treated AZ521 cells. AZ521 cells were exposed to the indicated concentrations of EGCG (0–20 μM) for 3 h, and then the EGCG-bound proteins in the cell lysate were separated by the boronate-affinity method. p68 from whole-cell lysates (left) and EGCG-modified p68 in PBA eluates (right) were determined by immunoblotting with anti-p68 antibody as described under Materials and methods. (B) Dose-dependent decreases in p68 and β-catenin by the treatment with EGCG. AZ521 cells were incubated with the indicated concentrations of EGCG (0–20 μM) for 6 h, and then the nuclear and cytoplasmic levels of p68 and β-catenin were determined by immunoblotting. The antibodies against histone H2B and β-actin were used as the loading controls for the nuclear and cytoplasmic proteins, respectively. (C) Time-dependent decreases in p68 and β-catenin by the treatment with EGCG. AZ521 cells were cultured with 5 μM EGCG for 0–6 h, and then the nuclear and cytoplasmic levels of p68 and β-catenin were determined by immunoblotting.

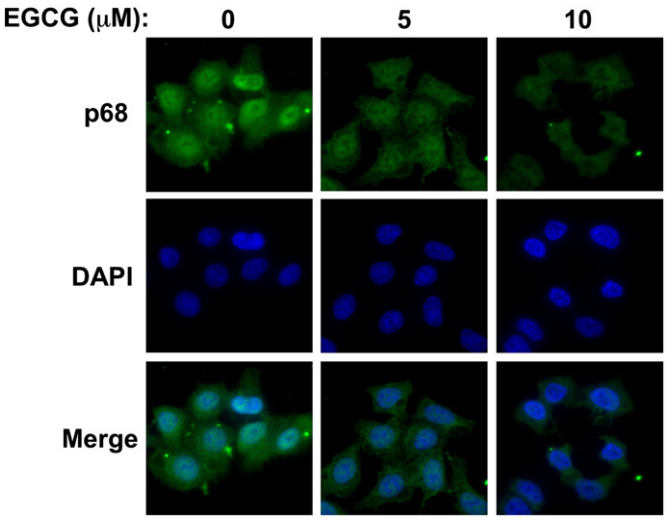


**Fig. 6.** Immunofluorescence microscopic analysis of p68 in EGCG-treated AZ521 cells. AZ521 cells were treated with the indicated concentrations of EGCG (0–10 μM) for 3 h and then were stained with anti-p68 antibody and DAPI as described under Materials and methods.

immunoblotting. As shown in Fig. 5A (right), immunoblot analysis of PBA eluate demonstrated the dose-dependent binding of EGCG to p68 in AZ521 cells. On the other hand, it is noteworthy that total protein levels of p68 in AZ521 cells were significantly decreased after EGCG treatment (Fig. 5A, left). Based on these results, we speculated that the interaction of EGCG with p68 might induce the reduction of p68 levels.

*EGCG-related reduction of p68 and β-catenin levels in cells*

A possible mechanism by which p68 contributes to carcinogenesis is through coactivation of β-catenin-mediated transcription independent of Wnt activation. Recently, it has been shown that tyrosine-phosphorylated p68 binds to β-catenin in the cytoplasm, inhibits its degradation by blocking the binding of glycogen synthase kinase-3β, and then facilitates its nuclear translocation [28,39,40]. Therefore, we evaluated the cytoplasmic and nuclear levels of p68 and β-catenin in AZ521 cells exposed to EGCG by immunoblotting. As shown in Fig. 5B, the exposure to EGCG at concentrations from 1 to 20 μM for 6 h significantly decreased the cytoplasmic and nuclear levels of both p68 and β-catenin in a dose-dependent manner. The cytoplasmic and nuclear levels of both p68 and β-catenin also decreased with the time after treatment with 5 μM EGCG (Fig. 5C). We also examined the intracellular localization of p68 in EGCG-treated AZ521 cells by immunofluorescence microscopy. As shown in Fig. 6, p68 was predominantly localized in the nucleus in intact cells. However, both nuclear and cytoplasmic levels of p68 were significantly decreased in the cells treated with EGCG for 3 h. These data suggest that interaction of EGCG with p68 induces the reduction of p68 levels, resulting in the down-regulation of β-catenin.

*EGCG-induced down-regulation of p68/β-catenin-target gene products and inhibition of cell proliferation*

In the nucleus, the p68/β-catenin complex acts as a cofactor for transcription factors of the T cell factor/lymphoid-enhancing factor (TCF/LEF), modulating the expression of a broad spectrum of target genes [39,40]. Two of the immediate downstream targets of nuclear p68/β-catenin in complex with LEF/TCF are the proto-oncogenes *cyclin D1* and *c-Myc,* which promote cellular proliferation [41]. We examined whether the down-regulation of p68/β-catenin prompted by EGCG affected cyclin D1 and c-Myc expression. After treatment of AZ521 cells with EGCG for 24 h, the expression of cyclin D1 and c-Myc was determined by immunoblotting. As shown in Fig. 7A, the cellular

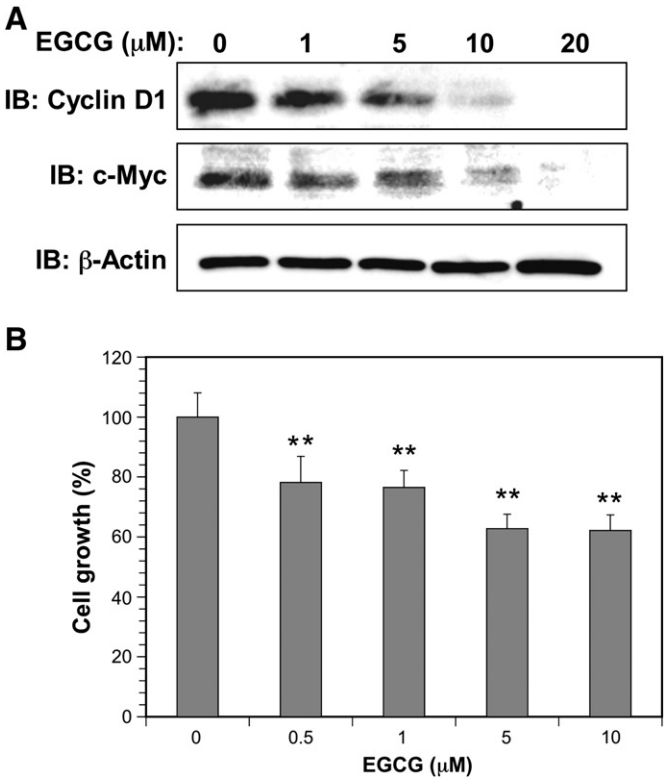


**Fig. 7.** Inhibition of cell proliferation and down-regulation of cyclin D1 and c-Myc by EGCG treatment. (A) Expression levels of cyclin D1 and c-Myc in EGCG-treated cells. AZ521 cells were incubated with the indicated concentrations of EGCG (0–20 μM) for 24 h, and then cyclin D1, c-Myc, and β-actin were determined by immunoblotting. β-Actin was used as a quantitative loading control. (B) Inhibition of cell proliferation by EGCG treatment. AZ521 cells were treated with the indicated concentrations (0–10 μM) of EGCG for 24 h. After the medium was replaced, the cells were further incubated for 48 h, and then cell growth was determined by WST-8 reduction assay as described under Materials and methods. Cell growth is expressed as a percentage of the control value in vehicle-treated cells. Data are the means ± SD of five replicates. The significance of differences was evaluated using Student's *t* test. $^{**}p<0.001$.

levels of both cyclin D1 and c-Myc were down-regulated in AZ521 cells upon EGCG treatment (1–20 μM). We also observed that the treatment of AZ521 cells with EGCG (0.5–10 μM) significantly inhibited cell growth (Fig. 7B). These results suggest that the inhibition of proliferation by EGCG can be attributed to the down-regulation of cyclin D1 and c-Myc.

*EGCG-induced proteasomal degradation of p68*

To determine whether p68 degradation is accelerated by EGCG, we treated AZ521 cells with the translation inhibitor cycloheximide (CHX) and then measured the relative p68 levels in these cells. As shown in Fig. 8A, the level of p68 decreased much faster in cells treated with 100 μg/ml CHX and 10 μM EGCG than in cells treated with CHX only. This result suggests that a posttranslational mechanism contributes to the EGCG-induced p68 decline in AZ521 cells. To investigate the mechanism underlying EGCG-induced p68 degradation, we incubated AZ521 cells with EGCG in the presence or absence of the proteasome inhibitor MG-132. As shown in Fig. 8B, p68 levels were reduced after treatment with EGCG for 6 h, but not in the presence of 20 μM MG-132, accompanied by the marked accumulation of ubiquitinylated proteins. We carried out an immunoprecipitation assay with anti-p68 antibody, followed by sequential immunoblotting with anti-ubiquitin antibody. The assay revealed the accumulation of higher molecular weight, polyubiquitinated p68 in EGCG-exposed AZ521 cells (Fig. 8C). Moreover, cotreatment with EGCG and MG-132 additively promoted the accumulation of

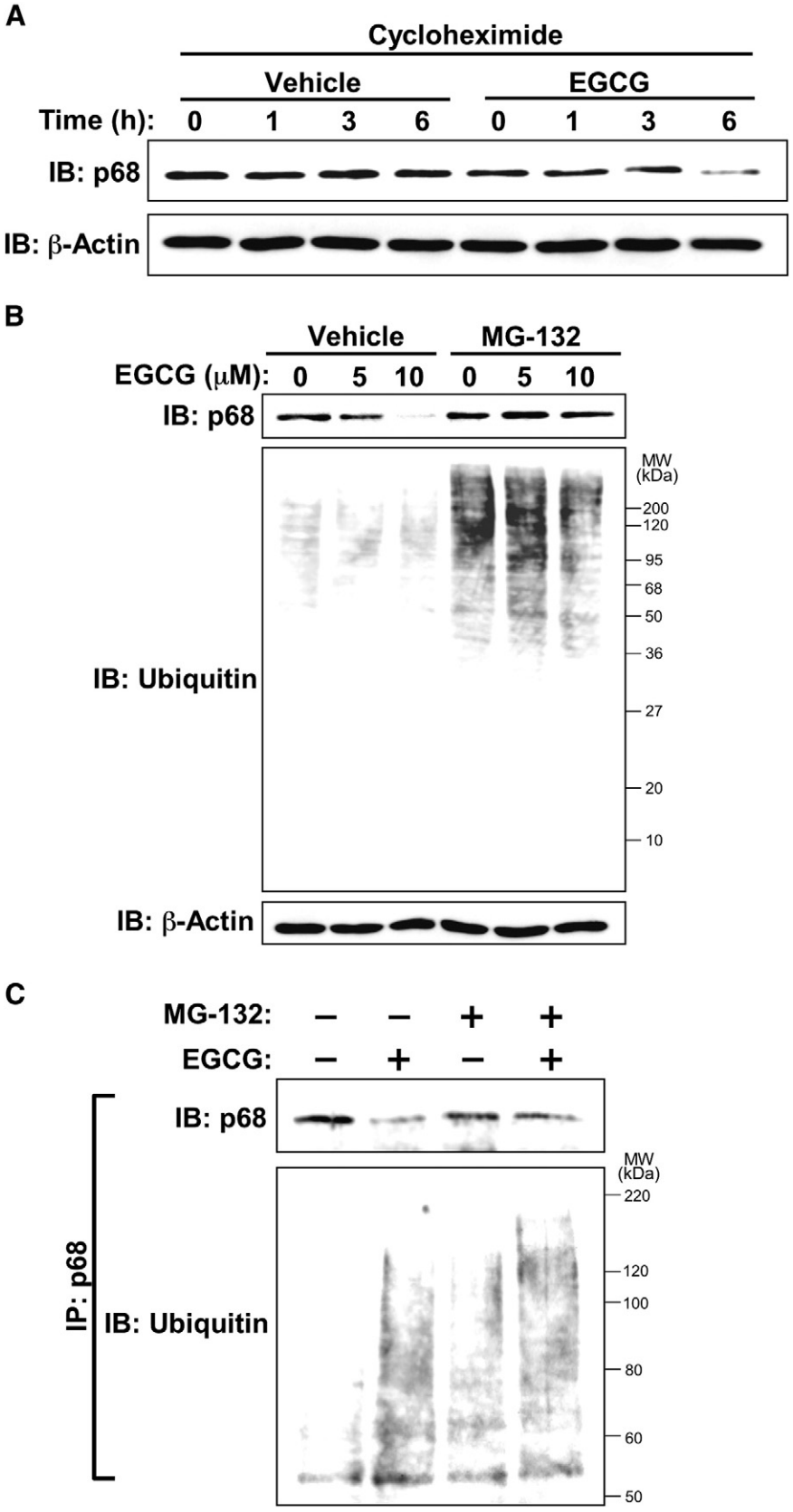


**Fig. 8.** EGCG-induced ubiquitination of p68 in AZ521 cells. (A) Protein levels of p68 in AZ521 cells treated with the translation inhibitor CHX and EGCG. AZ521 cells were incubated with or without 10 μM EGCG in the presence of 100 μg/ml CHX for 0–6 h, and then p68 and β-actin were determined by immunoblotting. β-Actin was used as a quantitative loading control. (B) p68 and ubiquitinylated protein levels in AZ521 cells treated with the proteasome inhibitor MG-132 and EGCG. After treatment with or without 20 μM MG-132 for 2 h, AZ521 cells were incubated with the indicated concentrations of EGCG (0–10 μM) for 6 h. Then, p68, ubiquitinylated proteins, and β-actin were determined by immunoblotting. (C) Ubiquitinylated p68 levels in AZ521 cells treated with the proteasome inhibitor MG-132 and EGCG. After treatment with or without 20 μM MG-132 for 2 h, AZ521 cells were incubated for 6 h in the presence or absence of 10 μM EGCG. Then, the protein was immunoprecipitated from each cell lysate with the anti-p68 antibody. The immunoprecipitates were analyzed by immunoblot using antibodies against ubiquitin and p68.

polyubiquitinated p68 in AZ521 cells. Taken together, these data indicate that EGCG induces ubiquitination of p68 in AZ521 cells and consequently reduces the cellular p68 level by proteasomal degradation.

## Discussion

EGCG, the primary active ingredient in green tea, has been shown to modulate multiple signal transduction pathways in a fashion that controls the unwanted proliferation of cancer cell lines and animal models of cancer, thereby imparting strong cancer chemopreventive as well as therapeutic effects [1–4]. Recently, protein interactions with EGCG have been proposed to be related to the anti-cancer effects. The intermediate filament protein vimentin has been identified as a target of EGCG, and the binding of EGCG to phosphorylation sites of vimentin has been proposed to inhibit a cascade of vimentin-mediated cell proliferation [13]. Tachibana et al. reported that the inhibitory effect of EGCG on tumor cell proliferation is exerted by its binding to 67LR, which is expressed on a variety of tumor cells, and it activates multiple interrelated pathways of apoptosis and cell-cycle arrest [11,12]. More recently, Shim et al. reported that EGCG suppresses the growth of lung cancer cells by inhibition of Ras downstream signaling through its binding with the Ras–GAP-binding region and the glycine-rich domain of G3BP1 [17]. Therefore, protein interaction with EGCG seems to be a critical step for EGCG to act on various key proteins involved in cellular proliferation and apoptosis. Nevertheless, the molecular targets of EGCG and the precise mechanism by which the binding of EGCG to the targets induces the anti-cancer effects remains to be fully clarified. Therefore, the discovery of the EGCG-binding proteins is an important step toward understanding the molecular mechanism of the anti-cancer effects of EGCG. Understanding the mechanism will contribute to the development of more specific preventive strategies against cancer.

Recently, we demonstrated that EGCG quinone formed via autoxidation undergoes electrophilic addition to protein thiol groups, yielding EGCG–protein adducts [19]. In general, polyphenols are easily oxidized to the corresponding quinones with dioxygen ($O_2$) and a transition metal ion, concomitant with the evolution of reactive oxygen species such as $O_2^-$ and hydrogen peroxide ($H_2O_2$) via autoxidation [22,42]. The generated $O_2^-$ also can participate in the autoxidation of polyphenols. EGCG has been reported to autoxidize in neutral and alkaline buffers or human or mouse plasma [20,22,43]. Moreover, several studies have shown that oxidation of catechol-type polyphenols and dopamine leads to the formation of quinones, which rapidly react with thiol groups in glutathione and protein cysteine residues to form *S*-cysteinyl adducts in mouse and cellular systems [44–49]. Several lines of evidence indicate that electrophiles act as second messengers, representing an integral part of the cellular signal transduction network [50–52]. The downstream effect of electrophiles is the oxidation of redox-sensitive proteins through the direct modification of the thiol group of reactive cysteines. In this study, we investigated whether the covalent binding of green tea catechins with protein nucleophiles can occur in cultured cells and then block or activate signal transduction pathways. After treatment of AZ521 cells with EGCG, EGCG-bound proteins were determined by SDS–PAGE/electroblotting followed by redox-cycle staining. Thus, we demonstrated the direct binding of EGCG with cellular proteins in EGCG-treated AZ521 cells (Fig. 3). This is the first report to determine the EGCG-modified proteins from EGCG-treated cells. We also observed the binding of EGCG to cellular proteins by redox-cycle staining when several other cell lines were treated with EGCG (data not shown). The EGCG–protein binding was sufficiently stable to exist under the reducing and denaturing conditions of SDS–PAGE. This suggests that a covalent interaction mediates EGCG binding to these cellular proteins.

In this study, we developed a new strategy for the biochemical and mechanistic understanding of the action of EGCG. Using the affinity pull-down method, 2D gel electrophoresis, and MALDI-TOF MS analysis, we identified EGCG-modified proteins from EGCG-treated AZ521 cells. Previously, we demonstrated the availability of the boronate-affinity method for the isolation of EGCG-binding protein using EGCG-modified GAPDH by SDS–PAGE, redox-cycle staining, and MALDI-TOF MS analysis [19]. The boronate-affinity method was also valuable for the isolation of EGCG-bound proteins from EGCG-treated cells (Fig. 4A) and enabled us to determine EGCG-target proteins using a proteomic strategy. Thus, MALDI-TOF MS analysis provided identification of 12 EGCG-binding targets from 2D gel spots (Fig. 4B and Table 1). EGCG-binding targets were two key enzymes involved in ATP production (pyruvate kinase and ATP synthase), three 70-kDa heat shock proteins (HSP70 and GRP78), one translation initiation factor, two major cytoskeleton proteins (vimentin and α-actin), one nucleic acid-binding protein (heterogeneous nuclear ribonucleoprotein), one potential tumor suppressor protein (prohibitin) [53], and one Ras signaling molecule (G3BP). In addition, we also identified p68 as a novel and important molecular target of EGCG. Of note, EGCG has been previously shown to bind to vimentin, GRP78, and G3BP1 [13,16,17], all of which were also identified in this analysis. All of these proteins are important for the suppression of cell proliferation by EGCG in cancer cell lines.

The p68 DEAD-box RNA helicase is involved in pre-mRNA, rRNA, and microRNA processing, but also functions in the regulation of gene transcription [54]. p68 has been shown to be overexpressed in some cancer cells and promote the nuclear translocation and the ability of β-catenin to activate TCF/LEF transcription of β-catenin-regulated genes, *c-Myc, cyclin D1, c-Jun,* and *Fra-1,* all of which are proto-oncogenes [28,39,40]. Furthermore, knockdown of p68 in colon cancers leads to reduced expression of these proto-oncogenes and inhibits their proliferation [28]. Hence, p68 may contribute to cancer formation and progression by directly up-regulating proto-oncogenes. In fact, p68 is considered a potential marker of cancer and a significant molecular target for cancer-preventive agents [55]. In this study, the exposure of AZ521 cells to EGCG caused a marked decrease in cellular levels of p68, accompanied by a significant decrease in β-catenin levels and a significant inhibition of cell growth (Figs. 5–7B). In addition, the concurrent reduction of p68 and β-catenin levels in EGCG-treated AZ521 cells resulted in the down-regulation of c-Myc and cyclin D1 expression that enhances cell cycle progression (Fig. 7A). Many researchers have reported that EGCG administration decreases nucleus β-catenin level and inhibits the activity of β-catenin/TCF signaling in vitro and in vivo [56–58]. Our findings imply that interaction of EGCG with p68 can induce the degradation of p68 levels and prevent translocation of β-catenin to the nucleus, leading to the inhibition of β-catenin/TCF signaling.

The regulated degradation of cellular proteins by the ubiquitin–proteasome system influences a range of vital cellular processes in both normal and cancer cells [59]. Ubiquitination is an ATP-dependent posttranslational modification that requires E1, E2, and E3 ligases. E3 ligases confer specificity to ubiquitination by recognizing target substrates and mediating transfer of ubiquitin from an E2 ligase by posttranslational modification, including phosphorylation, glycosylation, and proline hydroxylation [59]. Furthermore, recent studies suggest that specific covalent modifications of cysteine residues by some thiol-reactive electrophiles can trigger polyubiquitination of Keap1, leading to the degradation of Keap1 and the stabilization and activation of Nrf2 [60,61]. Exposure of AZ521 cells to EGCG promoted ubiquitination of p68, resulting in a proteasome-dependent degradation of p68 (Fig. 8). Several studies suggest that the p68 level may be regulated by proteasomal degradation [54,62]. Although the exact mechanism of EGCG-triggered ubiquitination of p68 remains unknown, the present results strongly suggest that EGCG can covalently bind to the cysteinyl thiol residue of p68. We could not detect EGCG-bound protein in the nucleus fraction from EGCG-treated AZ521 cells by redox-cycle staining (Fig. 3D). As shown in Fig. 6, p68 was

predominately localized in the cell nucleus and partially in the cytoplasm. However, p68 has been recently shown to shuttle between the nucleus and the cytoplasm [63]. Therefore, we speculate that the covalent modification of p68 by EGCG in the cytoplasm can trigger its ubiquitination and then cause proteasomal degradation of p68. These events can consequently lead to the down-regulation of nuclear p68 and inhibition of tumor cell growth. Future studies need to elucidate the molecular mechanism of EGCG-triggered ubiquitination of p68.

In conclusion, we identified EGCG-binding proteins by the application of proteomics techniques combined with the boronate-affinity method. We demonstrated for the first time that p68 is one of the major targets of cellular protein modification by EGCG, providing a new perspective on the molecular mechanism underlying the action of EGCG. Indeed, our results showed that EGCG inhibits cancer cell proliferation by preventing β-catenin oncogenic signaling through proteasomal degradation of p68. In future clinical applications, the identification of p68 as the target for EGCG action would aid in the design of prospective studies for cancer chemoprevention and therapy.

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