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Research Article

Differential proteomic analysis reveals that EGCG inhibits HDGF and activates apoptosis to increase the sensitivity of non-small cells lung cancer to chemotherapy

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Purpose: To search for regulated proteins in response to green tea (–)-epigallocatechin-3-gallate (EGCG) in A549 lung cancer cells.

Experimental design: 2DE and ESI/multistage MS (ESI-MS/MS) were performed to identify modulated proteins in A549 cells treated with EGCG. Cell migration was evaluated by transwell assays. RNA interference was used to silence the hepatoma-derived growth factor (HDGF). Caspase-3, caspase-9, and HDGF were immunodetected by Western blot assays. Flow cytometry was used for detection of mitochondrial membrane potential and apoptosis.

Results: We found that HDGF expression was threefold suppressed by EGCG treatment. Downregulation of HDGF by EGCG was confirmed using anti-HDGF antibodies in three lung cancer cell lines. EGCG treatment and HDGF abrogation by RNA interference resulted in a decreased migration of A549 cells. In addition, EGCG induced a marked synergistic effect with cisplatin in cell death. Consistently, an enhanced cytotoxicity in HDGF-silenced cells was also found. Cell death was associated to increased apoptosis, disruption of the mitochondrial membrane potential, and activation of caspase-3 and caspase-9.

Conclusion and clinical relevance: Our data suggest for the first time that abrogation of HDGF by EGCG enhances cisplatin-induced apoptosis and sensitize A549 cells to chemotherapy. Therefore, we propose that decreasing the HDGF levels by using EGCG may represent a novel strategy in lung cancer therapy.

Keywords:
Apoptosis / EGCG / Lung cancer / HDGF / Migration / Therapy

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Abbreviations: **EGCG**, (–)-epigallocatechin-3-gallate; **ERP29**, endoplasmic reticulum protein 29; **HDGF**, hepatoma-derived growth factor; **MTT**, (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium); **NSCLC**, non-small cells lung cancer; **PI**, propidium iodide; **shRNA**, short hairpin RNA; **SOD1**, superoxide dismutase 1

Clinical Relevance

Green tea polyphenol (–)-epigallocatechin-3-gallate (EGCG) exhibits potential preventive and chemotherapeutic effects against a wide range of human malignancies. In this study, our aim was to search for modulated proteins in response to EGCC in human lung adenocarcinoma A549 cells that may represent novel potential therapeutic targets. Particularly, we found that the expression of the hepatoma-derived growth factor (HDGF) was threefold suppressed by EGCG. Remarkably, both EGCG treatment and abrogation of HDGF by RNA interference enhanced cisplatin-induced apoptosis and sensitize A549 cells to chemotherapy. We propose that decreasing HDGF levels by EGCG may represent a novel strategy in chemoprevention and therapy of lung cancer. To our best knowledge, this is the first report that treatment with EGCG targets HDGF and sensitizes lung cancer cells to cisplatin therapy.

1 Introduction

The natural dietary molecules represent a promising strategy in the search for novel therapeutic agents in cancer. Green tea is the second most commonly consumed beverage in the world. The infusion of leaves from the *Camellia sinensis* plant is rich in polyphenolic flavonoids known as catechins, with the most abundant being (-)-epigallocatechin-3-gallate (EGCG). Although green tea has been consumed for centuries, it has only recently been studied as a health-promoting beverage that may act to prevent several human diseases [1]. A number of epidemiological, preclinical, and in vitro studies have demonstrated that green tea exhibits potential preventive and chemotherapeutic effects against a wide range of human malignancies [2]. These studies indicate that EGCG may alter the hallmarks of cancer by suppressing apoptosis [3], cell proliferation [4], angiogenesis [5], and invasion [6, 7]. All these EGCG-induced cellular effects are mainly due to the modulation of various biological pathways including the growth factor-mediated pathway, the mitogen-activated protein kinase-dependent pathway, and the ubiquitin/proteasome degradation pathway [8]. Therefore, the characterization of biological activities of EGCG could lead to the identification of novel molecular targets that may be important in cancer prevention and treatment.

Lung cancer is the most common cancer in the world; there were an estimated 1.61 million new cases, representing 12.7% of all new cancers in 2008 [9]. Because of its inhibitory effects on oncogenic signaling pathways and tumor development, EGCG has gained increased attention in lung cancer research. In consequence, potential therapeutic applications of EGCG have been recently reported for this neoplasia. EGCG enhances the antiproliferative activity of c-MET and EGFR inhibitors in non-small cells lung cancer (NSCLC) [10]. In addition, EGCG induces the reversion of cisplatin resistance mediated by downregulation of AXK and TYRO 3 receptor tyrosine kinases in chemoresistant lung cancer cells [11]. On the other hand, a schedule-dependent effect of EGCG and paclitaxel on growth inhibition of NCI-H460 lung cancer cells was also reported [12]. In this study, we have performed a proteomic-based approach to identify proteins modulated by EGCG in A549 lung cancer cells. Our results allowed us to identify hepatoma-derived growth factor (HDGF) as a novel target of EGCG. They also revealed that use of EGCG could represent a novel strategy to improve lung cancer therapy.

2 Materials and methods

2.1 Cell cultures and reagents

Human lung carcinoma A549 cells were grown in DMEM supplemented with 10% FBS and penicillin–streptomycin (10 000 unit/mL; Invitrogen) in 5% CO_2 at 37°C. EGCG was purchased from Sigma-Aldrich (E4-143). Doxorubicin (D1515) and cisplatin (cis-diammineplatinum(II) dichloride) (P4394) were purchased from Sigma-Aldrich.

2.2 Cell treatments

For lethal doses (LD) determinations, A549 cells (1×10^5) were incubated with increasing concentrations of EGCG (200, 400, 600, and 800 μM) for 24 and 48 h and MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assays were performed as described in Section 2.3. Doxorubicin (1 μM) was used as positive control for cell death. For cell viability assays A549 cells (1×10^5) were treated with EGCG at 174 μM (LD_{25}), 348 μM (LD_{50}), or 522 μM (LD_{75}) together with cisplatin at 32.5 μM (LD_{25}) for 24 and 48 h. As control, cisplatin and EGCG alone were used at 65 μM (LD_{50}) and 348 μM (LD_{50}), respectively.

2.3 MTT assays

MTT reagent was added to A549 cells (1×10^5) and incubated for 3.5 h at 37°C. Dissolution buffer (99% isopropanol, 0.3% HCl, 0.7% NP-40) was added for 15 min. Absorbance was recorded using a spectrophotometer at 570–630 nm and sensitivity to treatments was calculated based on cell

proliferation measurements at 24 and 48 h. Data were analyzed using the BioStat software.

2.4 2DE

Procedures were performed as previously described [13] with minimal changes. Briefly, total proteins were obtained from A549 cell monolayers treated with EGCG (348 μM, LD_{50}) or water vehicle for 48 h using the TNTE buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 5 mM EDTA) in the presence of Complete proteases inhibitor cocktail (Roche). Proteins (300 mg) were mixed with rehydration solution (7 M urea, 2% CHAPS, 40 mM DTT 0.5%, v/v IPG buffer pH 3–10, and 0.002% bromophenol blue), and loaded onto 11 cm ReadyStrip IPG strips with a linear pH 4–7 gradient (Bio-Rad). After IEF using the Protean IEF Cell (Bio-Rad), IPG strips were equilibrated for reduction and alkylation, and proteins were separated through 12% SDS-PAGE. Gels were stained with Sypro-Ruby dye (Invitrogen). Images were captured and analyzed by the PD-Quest software (Bio-Rad) using total spot ratios from biological replicates for protein abundance comparisons. The criterion of a differential expression between conditions was a twofold change ($p < 0.05$) in spot volume between matched sets in triplicate. The spots exhibiting significant changes in abundance were selected for identification by MS.

2.5 ESI/multistage MS (ESI-MS/MS)

Mass spectrometric analysis was performed using a 3200 Q TRAP hybrid tandem mass spectrometer (Applied Biosystems/MDS SCIEX) coupled online to a nanoAcquity ultraperformance LC system (Water Corporations, Milford, MA). Peptides were separated on a BEH, C18 UPLC column (1.7, 75 μm × 100 mm, Waters Corporations) equilibrated with 2% acetonitrile, 0.1% formic acid using a linear gradient of 2–70% acetonitrile, 0.1% formic acid over a 60 min period, at a flow rate of 0.25 μL/min. Spectra were acquired in automated mode using information-dependent acquisition, which involves switching from MS to MS/MS mode on detection of +2 to +4 charged ions. The precursor ions were fragmented by collisionally activated dissociation in the Q2 collision cell. Data interpretation and protein identification were performed with the MS/MS spectra data sets using the Mascot search algorithm. Carbamidomethyl-cysteine and methionine oxidation were used as the fixed and variable modifications, respectively. A protein "hit" was accepted as a valid identification when at least two MS/MS spectrum matched at the 95% level of confidence ($p < 0.05$). Ion score is $-10 \times \log(p)$, where p is the probability that the observed match is a random event. The threshold ion score for the above conditions was 41 for $p < 0.05$.

2.6 Design of short hairpin RNA (shRNA)

To silence the expression of HDGF, two complementary oligonucleotides (21–23 nt) encoding a shRNA targeting the HDGF gene (336–357 coding region) were designed as follows: 5′-GATCCCCGGCAGAAGGAGTACAAATT CAAGAGATTTGTACTCCTTCTGCCGGTTTTTTGGAAA-3′ (sense), and 5′-AGCTTTTCCAAAAAACCGGCAGAAGGA GTACAAATGTGTTGAATTTGTAC TCCTTCTGCCGGG-3′ (antisense). Both oligonucleotides were annealed and cloned into pSilencer 5.1 U6 retro vector (Ambion) resulting in shHDGF vector and nucleotide sequence was confirmed by automatic sequencing.

2.7 Transfection assays

A549 cells (3×10^5) were seeded into a 6-well tissue culture plate and grown to 80% confluence. Then cells were transfected with shHDGF vector (5 μg) or control scramble vector containing a shRNA sequence without target sequences in human genome, using lipofectamine 2000 reagent (Invitrogen) for 5 h. Transfected cells were incubated at 37°C for 24 h and harvested for further assays.

2.8 Western blot assays

Protein extracts (30 μg) were separated by 15% SDS-PAGE and electrotransferred to a nitrocellulose membrane (Bio-Rad). After overnight blocking with 5% nonfat milk, membrane was incubated with primary antibodies raised against caspase-3, caspase-9 (1:1000, Cell Signaling), and HDGF (1:2500, Santa Cruz Biotechnology) overnight at 4°C. Secondary antibodies conjugated to horseradish peroxidase (Sigma) were used at a dilution of 1:2500, and membranes were developed using ECL Western blotting detection system (GE Healthcare, Amersham).

2.9 Cell migration assays

For scratch/wound–healing assays, A549 cells (3.5×10^5) transfected with the shHDGF vector, scramble vector, or treated with EGCG (LD_{50}) were seeded in a 6-well plate. At 24 h, a wound was traced in the cell monolayer. After 24 h, cells were fixed with 4% paraformaldehyde and monolayer restoration was quantified by determination of the number of cells migrating to wound area. For transwell assays, chambers (Corning) with 8 μm pore size polycarbonate membrane were used. A549 cells (1×10^5) treated with EGCG (LD_{50}) or without treatment were transferred to 0.5 mL serum-free medium and placed in the upper chamber, whereas the lower chamber was loaded with 0.8 mL medium containing 10% FBS. After 24 h incubation at 37°C the total number of cells that migrated through the membrane was counted.

2.10 Flow cytometry analysis for apoptosis detection

For apoptosis assays, A549 cells (2.5×10^5) were treated with different EGCG concentrations (LD_{25}, LD_{50}, and LD_{75}). To evaluate the synergism between cisplatin and EGCG or shHDGF on cell death, A549 cells were treated with EGCG (LD_{50}) or shHDGF vector in combination or cisplatin (LD_{25}) for 48 h, and apoptosis assays were performed. Cells treated with cisplatin (LD_{50}) alone were used as positive control. Then, 20 μL annexin-V-Fluos labeling reagent and 20 μL propidium iodide (PI) solution were added to cells treated as described above. Cell pellets were resuspended in 100 μL of labeling solution (annexin-V/PI), and incubated for 10–15 min at room temperature. Annexin V and PI emissions were analyzed using a FACS Calibur flow cytometer (Becton Dickinson). Data from 20 000 cells were acquired in list mode and analysis was performed with the Cell Quest software.

2.11 Flow cytometry analysis for mitochondrial membrane potential detection

A549 cells were treated with cisplatin (LD_{50}), EGCG (LD_{50}), and the combination of cisplatin plus EGCG for 48 h. Alternatively, cells transfected with shHDGF were treated with cisplatin for 48 h. After treatment, the medium was replaced with serum-free medium containing MitoTracker Deep Red FM (100 nM) and cells were incubated for 30 min at 37°C. The fluorescence emissions were analyzed by flow cytometry (excitation wavelength 635 nm, emission filter 661/16 nm) using a FACS Calibur flow cytometer (Becton Dickinson).

2.12 Statistical analysis

Three biological replicas were performed for each point data. The results were presented as mean ± standard deviation (SD). One-way ANOVA followed by Turkey's test were used to compare differences between means. A $p < 0.05$ was considered as statistically significant.

3 Results

3.1 EGCG inhibits viability of A549 lung adenocarcinoma cells

To evaluate the effect of EGCG on viability of A549 lung adenocarcinoma cells, an MTT assay was performed. A549 cells were incubated with increasing concentrations of EGCG (200, 400, 600, and 800 μM) for 24 and 48 h. Results showed that cell viability was significantly decreased by EGCG in a time-dose dependent manner (Fig. 1). Reduction in cell viability was more evident when A549 cells were incubated with EGCG for 48 h. At 200, 400, 600, and 800 μM EGCG, cell viability was reduced by about 25, 68, 80, and 90%, respectively. From these data the LD of EGCG were calculated as LD_{25} (174 μM), LD_{50} (348 μM), and LD_{75} (522 μM), respectively.

3.2 EGCG induces significant changes in protein abundance in A549 cells

To investigate the global changes in protein expression, we performed 2DE analysis of total protein extracts (500 mg) obtained from EGCG (LD_{50}) treated and nontreated A549 cells. Protein extracts were resolved by IEF using immobilized pH 4–7 gradient strips in the first dimension, and 12% SDS-PAGE in the second dimension as described in the Section 2. Two representative gels corresponding to EGCG-treated and control cells are illustrated in Fig. 2A. A total of 23 proteins were detected as differentially expressed between both conditions using the PDQuest software (fold change > 2.0; $p < 0.05$). Proteomic profiles were highly reproducible and changes in spot abundance were consistently observed in three biological replicates (Fig. 2B and D). After spot excision, trypsinization, and ESI-MS/MS analysis seven proteins corresponding to the most abundant spots were identified. An overview of MS/MS peptide sequences (ion scores), Mascot scores, and sequences coverage is shown in Table 1. The modulated proteins identified in EGCG-treated A549 cells corresponded to: (i) endoplasmic reticulum protein 29 (ERP29), and (ii) enoyl CoA hydratase 1 (ECHS1); whereas downregulated proteins were: (iii) HDGF, (iv) ubiquitin carboxyl-terminal esterase L1 (UCHL1); (v) peroxiredoxin 3 (PRDX3), (vi) superoxide dismutase 1 (SOD1), and (vii) cytidine monophosphate kinase 1 (CMPK1).

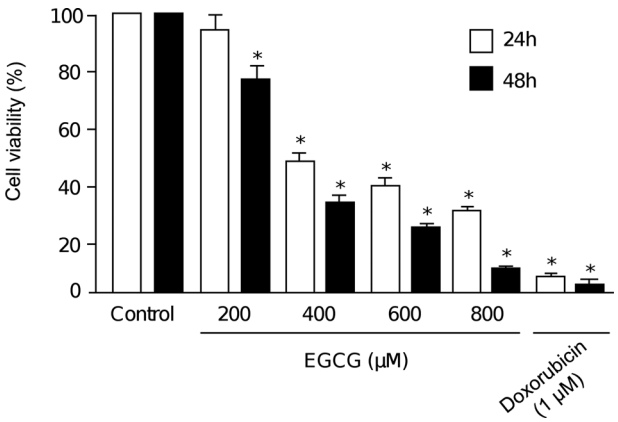

Figure 1. EGCG reduces cell viability of A549 lung cancer cells. Graphic representation of MTT assays for cell viability in response to treatment with 200, 400, 600, and 800 μM EGCG for 24 and 48 h. Doxorubicin (1 μM) was used as positive control of cell death. Control refers to nontreated cells. Bars represent the means of three independent experiments ± SD. *$p < 0.05$ compared to nontreated cells (control).

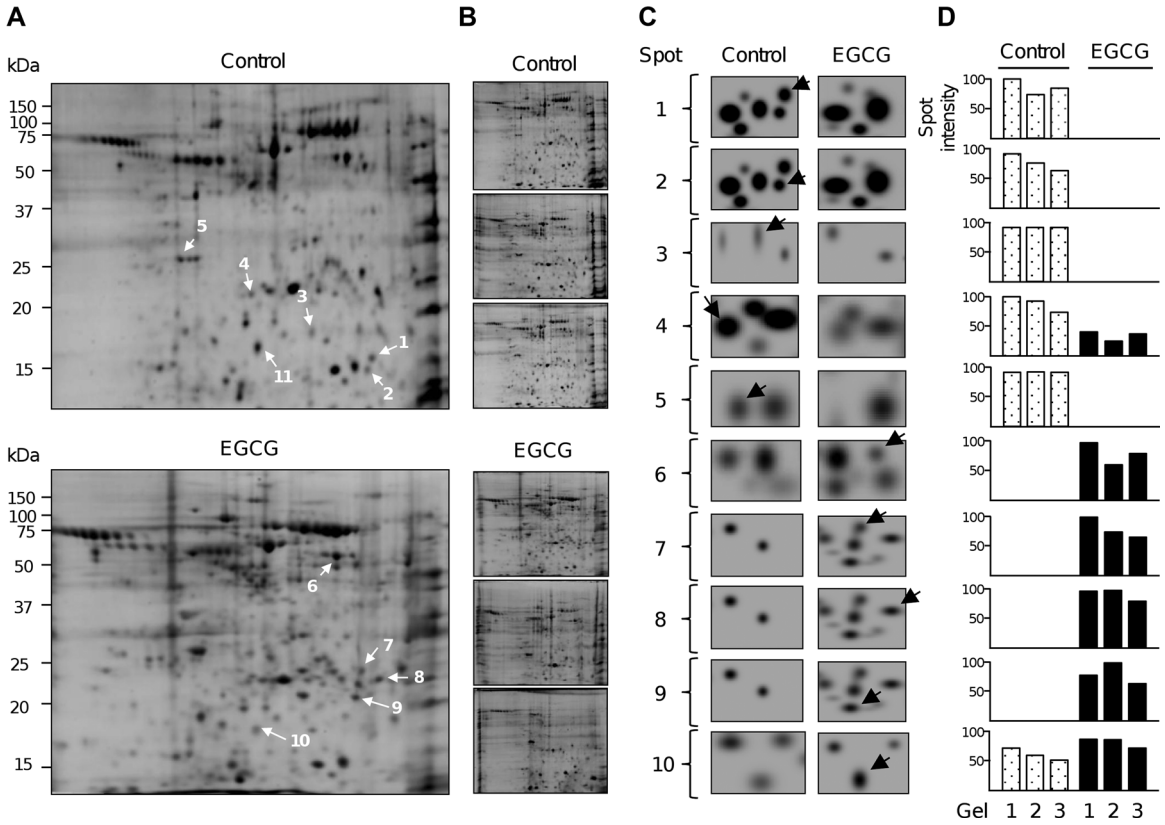

Figure 2. Proteomic profiles of A549 cells treated with EGCG. (A) Proteomic profiles of A549 cells nontreated and treated with EGCG (LD_{50}) for 48 h. (B) Images from biological triplicated 2DE gels. (C) Ten representative spots showing differential expression between A540 cells treated with EGCG and nontreated control. Arrows indicate the selected proteins for identification by ESI-MS/MS. (D) Densitometric analysis of spots abundance observed in (C). The spots quantification was performed using the PDQuest program (BioRad) and data from three independent gels.

3.3 EGCG inhibits HDGF in various lung cancer cell lines

As HDGF have been considered as promising therapeutic target in lung cancer, we decided to further analyze the biological relevance of its inhibition by EGCG. We first validated the EGCG-mediated inhibition of HDGF expression using anti-HDGF antibodies in two additional cancer cell lines. Western blot analysis of protein lysates obtained from H209, H187, and A549 lung cancer cells treated with EGCG (LD_{50}) for 48 h indicated that HDGF expression was reduced by about 70, 81, and 88%, respectively, in comparison with nontreated cells (Fig. 3A and B). β-actin used as control did not show significant changes in expression.

3.4 EGCG treatment and HDGF abrogation inhibit cell migration

Our results described above indicated that EGCG inhibits HDGF in A549 cells. To investigate the biological relevance of HDGF suppression, we generated HDGF-silenced A549 cells and evaluated the effects on cell migration by scratch/wound-healing and transwell assays. Western blot assays showed that both shHDGF and EGCG (LD_{50}) treatments diminished the HDGF levels (Fig. 3C and D). Then, monolayers of A549 cells were treated with EGCG, grated and the wounded areas were analyzed after 24 h. Results showed that the restoration of monolayer was significantly delayed (37.5%, $p < 0.05$) in EGCG-treated cells in comparison with control (Fig. 3E and F). Congruently, transwell chambers assays showed that the number of migratory cells was reduced (42.1%, $p < 0.05$) in EGCG-treated A549 cells confirming that EGCG has negative effects in cell migration (Fig. 3G). Then, we sought to determine if targeted HDGF abrogation may result in the inhibition of migration of A549 cells. We proceeded to inhibit the expression of HDGF using a specific sequence coding for a short-hairpin RNA (shHDGF), which was cloned in pSilencer vector as described in Section 2. The construct was transfected into A549 cells and HDGF expression was analyzed at 48 h after transfection. Western blot assays showed that shHDGF downregulated the expression of HDGF by about 51.6% ($p < 0.05$) similar to the levels found for EGCG treatment alone (Fig. 3C and D). No significant changes were

Table 1. Regulated proteins in EGCG-treated A549 lung cancer cells identified by ESI/multi-stage MS

Protein name	Molecular weight/p*I*	Gene symbol	Access number	Mascot score	Number of peptides	Sequence coverage (%)	Peptide sequence MS/MS (ion scores)	Function
Overexpressed proteins in A549 cells exposed to 348 μM EGCG for 48h								
Endoplasmic reticulum protein 29	28 975/6.77	ERP29	NP_006808	144	45	17	GALPLDTVTFYK (76) LNMELSEK (17) ILDQGEDFPASEMTR (93) SLNILTAFQK (52)	Processing and transport of proteins in the early secretory pathway. Induces cell-growth arrest and metastasis in cancer
ECHS1, short Chain, 1, mitochondrial	31 351/8.34	ECHS1	CAA66808	228	106	36	ASGANFEYIIAEK (87) NNTVGLIQLNRPK (57) IFEEDPAVGAIVLTGGDK (92) AFAAGADIK (38) AQFAQPEILIGTIPGAGGTQR (50) SLAMEMVLTGDR (67) LFYSTFATDDR (60) EGMTAFVEK (49)	Involved in fatty acid beta-oxidation pathway
Downregulated proteins in A549 cells exposed to 348 μM EGCG for 48h								
Hepatoma-derived growth factor	26 903/4.65	HDGF	BAG37895	96	72	30	IDEMPEAAVK (56) YQVFFFGTHETAFLGPK (22) LVIDEPAK1 (49) EAATLEVERPLPMEVEK (74) NSTLSEPGSGR (20) EDAEAPGIR (18)	Role in cellular proliferation, migration and differentiation
Ubiquitn carboxy-terminal esterase L1	24 808/5.33	UCHL1	NP_004172	105	94	42	MQLKPMEINPEMLNK (33) LGVAGQWR (38) QIEELKGQEVSPK (45) VYFMK (27) LGFEDGSVLK (59) QFLSETEK (59) NEAIQAAHDAVAQEGQCR (45) MPFPVNHGASSEDTLLK (51)	Ubiquitination, proliferation and cell differentiation. Involved in oncogenic transformation and tumour invasion
PRDX3	27 675/7.67	PRDX3	NP_006784	57	30	11	GLFIIDPNGVIK HLSVNDLPVGR SVEETLR	Cellular proliferation, differentiation, and antioxidant functions
SOD1	15 747/5.70	SOD1	AAB05662	115	26	16	GDGPVQGIINFEQK (67) HVGDLGNVTADK92 (94)	Binds copper and zinc ions destroying free superoxide radicals
UMP-CMP kinase 1	25 838/8.14	CMPK1	NP_057392	76	36	15	YGYTHLSAGELLR (15) FLIDGFPR (54) SVDEVFDEVVQIFDK (62)	Catalyzes the transfer of a phosphate group from ATP to CMP, UMP or dCMP, to form diphosphate nucleotide

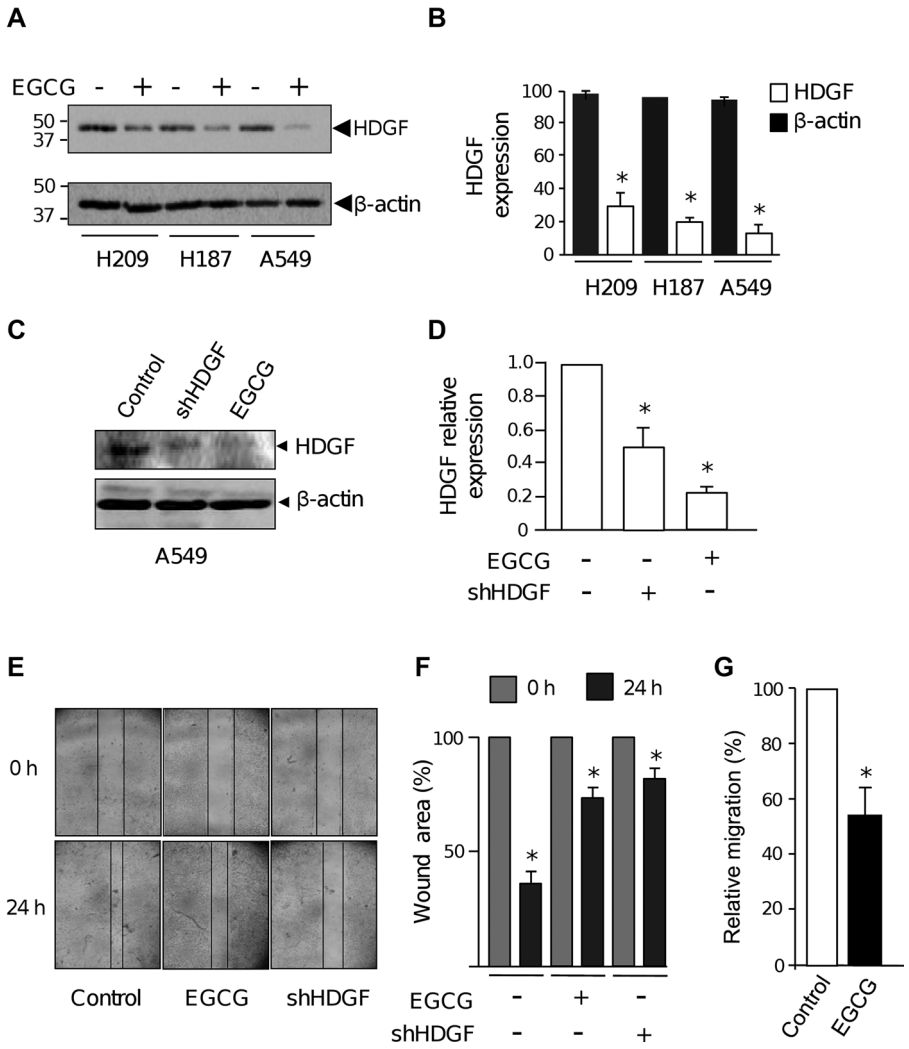

Figure 3. EGCG treatment and HDGF silencing suppress cell migration. (A) Immunodetection of HDGF and β-actin proteins by Western blot in H209, H187, and A549 lung cancer cell lines exposed to EGCG (LD_{50}). (B) Graphic representation corresponding to the densitometric quantification of immunodetected bands in (A). Pixels corresponding to β-actin expression were taken as 100%. $*p < 0.05$ compared to β-actin control. (C) Western blot assay for HDGF and β-actin expression in A549 cells exposed to EGCG (LD_{50}), treated with shHDGF or with transfectant agent as control. (D) Graphic representation corresponding to the densitometric quantification of immunodetected bands in (C). $*p < 0.05$ compared to nontreated control. (E) Scratch/wound-healing assays for A549 cells treated with EGCG (LD_{50}) or shHDGF for 24 h. (F) Graphical representation of wound-healing assay indicating the percentage of migrating A549 cells (closure of wound area) in (E). $*p < 0.05$ compared to control (0 h). (G) Graphical representation of transwell cell migration assays using A549 cells treated with EGCG (LD_{50}) and without treatment. $*p < 0.05$ compared to nontreated controls. In all experiments bars represent the mean of three independent experiments ± SD.

observed in the expression of β-actin protein used as control. Results of scratch/wound healing assays showed that HDGF silencing significantly inhibited the migration of A549 cells (24.9%, $p < 0.05$) in comparison with control (Fig. 3E and F). These data indicate that both EGCG treatment and HDGF abrogation inhibit cell migration of A549 lung cancer cells in vitro.

3.5 EGCG treatment and HDGF silencing activates apoptosis and sensitizes A549 cells to cisplatin therapy

Standard treatment options for patients with advanced NSCLC include the use of adjuvant cisplatin-based therapy. However, the effectiveness of treatment is low and novel therapy strategies are needed to overcome resistance and improve outcome. To investigate if EGCG treatment and HDGF silencing may sensitize lung cancer cells to chemotherapy, we performed MTT assays. First, we evaluated the effect of different EGCG concentrations (LD_{25}, LD_{50}, and LD_{75}) in combination with cisplatin (LD_{25}) on cell viability (Fig. 4A). As expected, cisplatin (LD_{50}) monotherapy, used as control of cell death, resulted in a marked inhibition of cell viability (50.5%) in comparison to A549 cells without treatment. Importantly, a higher inhibition of cell viability was observed when a lower concentration of cisplatin (LD_{25}) was used in combination with EGCG (Fig. 4A). Then, we compared the effect on cell viability of EGCG plus cisplatin combined therapy in HDGF-expressing cells versus cisplatin treatment in HDGF-silenced cells. Results showed that cisplatin (LD_{50}), EGCG (LD_{50}), and shHDGF alone reduced cell viability by about 57, 69, and 65%, respectively, in comparison to control (Fig. 4B). In addition, HDGF inhibition resulted in a significant reduction of cell viability (64%). Notably, a significant increase in cell death (83.8%) was observed in HDGF-deficient cells treated with cisplatin in comparison to controls, as a result of a marked synergistic effect (Fig. 4B). Taken altogether, these data indicate that both EGCG intervention

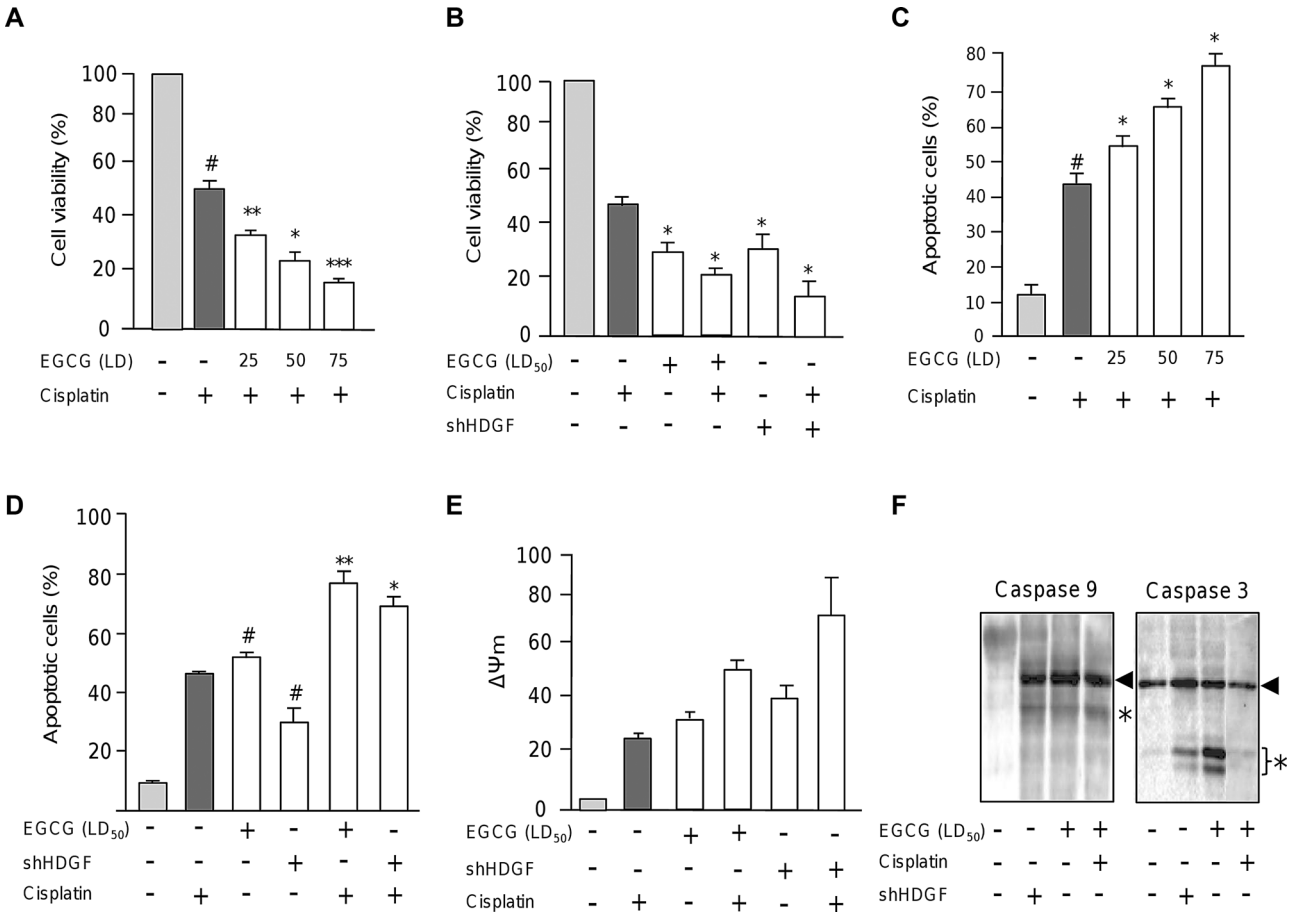

Figure 4. EGCG treatment and HDGF silencing induce apoptosis and sensitize A549 cells to cisplatin. (A) MTT assays for cell viability of A549 cells treated with EGCG in combination with cisplatin. $^{\#}$ $p < 0.005$ compared to nontreated cells. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.005$ indicate significant changes compared with cisplatin alone. (B) MTT assays for cell viability of cells treated with EGCG and shHDGF in combination with cisplatin for 48 h. $^{*}p < 0.05$ compared to cisplatin monotherapy. (C) Induction of apoptosis in A549 cells treated with EGCG alone or in combination with cisplatin. $^{\#}p < 0.01$ compared to nontreated cells. $^{*}p < 0.05$ compared to cisplatin alone. (D) Induction of apoptosis in A549 cells treated with EGCG and shHDGF alone or in combination with cisplatin. $^{\#}p < 0.01$ compared to nontreated cells. $^{*}p < 0.05$ compared to cisplatin alone. (E) Graphical representation for mitochondrial depolarization assays in A549 cells after treatments as described in (D). (F) Immunodetection of caspase-9 and caspase-3 by Western blot using protein extracts from A549 cells treated with EGCG and shHDGF alone or in combination with cisplatin. Results shown are the mean of three independent experiments ± SD.

and targeted HDGF inhibition may sensitize A549 cells to cisplatin therapy.

To evaluate if the reduction of cell viability induced by cisplatin and EGCG treatments, as well as HDGF inhibition was due to apoptosis, an annexin assay was performed. First, A549 cells were treated with EGCG (LD_{25}, LD_{50}, and LD_{75}) in combination with cisplatin (LD_{25}) and apoptosis was determined after 48 h. Results showed that the percentage of apoptotic cells was significantly increased when cisplatin was used in combination with EGCG in a dose-dependent manner (Fig. 4C). Then, we compared the effect on apoptosis of combined cisplatin plus EGCG therapy in HDGF-expressing cells versus cisplatin monotherapy in HDGF-silenced cells. Results showed that cisplatin (LD_{50}), EGCG (LD_{50}), and shHDGF alone induced apoptosis up to 50, 55, and 28%, respectively, in comparison to nontreated cells. Remarkably, the combination of EGCG or shHDGF with cisplatin significantly increased apoptosis up to 78 and 70%, respectively, demonstrating a synergistic effect between EGCG or HDGF deficiency with cisplatin (Fig. 4D). These data indicate that both EGCG treatment and HDGF silencing may induce apoptosis and sensitize A549 cells to cisplatin therapy.

3.6 EGCG treatment and HDGF inhibition result in disruption of the mitochondrial membrane potential and caspase-9 and caspase-3 activation

Apoptosis is associated to membrane depolarization represented by a decrease in membrane potential ($\Delta\Psi m$) followed by an increase in permeability resulting in cytochrome c release, apoptosome formation, and caspases activation. To

further investigate the mechanism behind the apoptosis induced by EGCG and HDGF silencing, we evaluated the involvement of mitochondrial membrane alterations in A549 lung cancer cells after 48 h of treatments. The mitochondrial depolarization was analyzed by flow cytometry in A549 cells labeled with tetramethyl rhodamine ethyl ester. Our data showed that the mitochondrial membrane potential was significantly decreased by 23.6 and 20.1% in cells treated with EGCG and cisplatin alone, respectively, in comparison to nontreated cells (Fig. 4E). The combination of EGCG plus cisplatin potentiated the effect of the drug resulting in an increased loss of membrane potential (45.3%) in comparison to cisplatin monotherapy. Similarly, the inhibition of HDGF induced loss of mitochondrial membrane potential (40.4%) in comparison to nontreated cells. Importantly, HDGF abrogation potentiates the cytotoxic effects of cisplatin therapy since loss of membrane potential increased up to 49% in comparison to cisplatin alone (Fig. 4E). In order to evaluate if apoptosis was related to caspase-9 and caspase-3 activation, we performed Western blot assays. Data indicated that both caspase-9 and caspase-3 were activated after EGCG and shHDGF treatments. In addition, combination of EGCG plus cisplatin also resulted in activation of caspase-3 and caspase-9 (Fig. 4F). Taken altogether, these data suggest that EGCG may induce apoptosis in A549 cells through the inhibition of HDGF resulting in dissipation of mitochondrial permeability and activation of caspase-3 and caspase-9.

4 Discussion

Patients with advanced NSCLC are not curable by standard treatments, mainly due to resistance to chemotherapy. Thus, novel therapeutic strategies to overcome this major obstacle are needed. It has been reported that 5–10% of cancers are due to genetic factors, suggesting that environmental factors including nutrition may influences the development of disease. The use of natural dietary or pharmaceutical agents to hinder the development of human malignancies represents a relevant chemopreventive approach to decrease the incidence of cancer. Chemoprevention based on the regular consumption of fruits and vegetables would help to provide protection against cancer. Several epidemiological and animal studies suggest that diverse components of green tea, particularly the polyphenol EGCG, have a chemoprotective effect against a variety of malignancies including breast, prostate, and lung cancers. Indeed, recent studies suggest that chemoprevention could delays the development of lung cancer in current and former smokers [14]. Therefore, tea consumption and EGCG-based treatments could be beneficial for smokers that are at high risk for lung cancer, before the disease develops. Phases I and II clinical trials have been conducted to explore the anticancer effects of diverse chemopreventive dietary agents including green tea in humans [15]. Several clinical trials involving green tea and EGCG intervention in lung, breast, and prostate cancer are undergoing (ClinicalTrials.gov). However, it is important to note that some epidemiological studies have not yielded conclusive results on the cancer-preventive effect of tea consumption in human. Relevant cytotoxic levels of EGCG in plasma may be not reached in vivo through ingestion of green tea, or EGCG. In addition, some discrepancies about the relevant EGCG concentrations to elicit antitumor responses have been reported. For instance, EGCG at 0.1–1 μM concentration is able to inhibit matrix metalloproteinases and angiogenesis. Notably, these EGCG levels are similar to those found in humans after drinking tea [16]. In contrast, the concentrations of EGCG needed to inhibit cancer cell proliferation are 20–100 μM, which are much higher than those observed in blood or tissues of tea consumers. Thus, it is difficult to extrapolate the EGCG concentrations needed to elicit antitumor activities in cell lines studies to human, as they greatly depends on the specific cancer related cellular process to be targeted, and in the variable degree of responsiveness to EGCG between individuals. This may explain, at least in part, the discrepancies in response to EGCG treatments observed in clinical trials. These observations should be examined when trying to extrapolate results from cell lines (this study) to clinical information in human cancer prevention. From our data, is tentative to propose that EGCG at high concentration (348 μM) may be useful to inhibit some hallmarks of cancer in vitro. However, dosage adequation should be defined for studies in in vivo models.

Mechanistic studies showed that EGCG may hamper lung cancer related processes including the anchorage-independent growth and cell cycle by directly targeting EGFR signaling pathway [17]. Therefore, the identification of novel molecular targets for tea polyphenols is a major goal to cancer prevention, and will help us to a better understanding of EGCG anticancer mechanisms. Here, we have performed a proteomic approach in A549 lung cancer cells in order to identify novel targets of EGCG with potential therapeutic applications. Our proteomic analysis evidenced that this flavonoid directly or indirectly regulates the expression of diverse proteins with essential roles in cancer (Table 1). ERP29 protein facilitates transport of proteins in the secretory pathway, and it has been correlated with tumor progression, cell growth arrest, metastasis, and prognosis in breast cancer [18]. ERP29 represents a biomarker for predicting nasopharyngeal carcinoma response to radiotherapy [19], and its overexpression attenuates doxorubicin-induced apoptosis in breast cancer [20]. ECHS1 protein functions in the mitochondrial fatty acid beta-oxidation pathway. Attenuation of EHCS1 inhibits cell proliferation and migration of gastric cancer and hepatocellular carcinoma cells [21]. HDGF is a secreted heparin-binding growth factor that regulates migration and invasion in breast cancer cells, and contributes to carcinogenesis in oral cancer [22]. HDGF is also a novel angiogenic secreted factor in human glioblastoma [23], and a mitogenic growth protein in gliomas [24]. UCHL1 is an ubiquitin-protein hydrolase involved in the processing of ubiquitinated proteins. It is a tumor suppressor frequently silenced by hypermethylation in melanoma, and hepatocellular carcinoma [25, 26].

PRDX3 protein is involved in the redox regulation of the cell. It is overexpressed and affects proliferation of prostate cancer cells; it represents a prognosis biomarker in hepatocellular carcinoma [27]. SOD1 protein is frequently overexpressed in cancer and its activity is essential to maintain cellular reactive oxygen species under critical threshold [28]. Inhibition of SOD1 by the small molecule ATN-224 induces cell death in various NSCLC cells, highlighting the intervention of SOD1 as a novel clinical target [29].

From these EGCG-regulated proteins, we selected HDGF for further analysis due to its key role in cancer. HDGF is frequently overexpressed in several types of cancer, where it is associated to increased migration, apoptosis inhibition, and resistance of cancer cells to therapy. Remarkably, HDGF upregulation has been recognized as an important prognostic marker and correlates with advanced stages and poor survival outcome in liver cancer [30], and esophageal carcinoma [31]. Furthermore, HDGF promotes the invasion and metastasis by inducing epithelial-to-mesenchymal transition of breast cancer cells [32]. As an important factor in cancer development and progression, HDGF have been considered as promising therapeutic target in lung cancer. Remarkably, it was reported that cisplatin treatment in combination with specific antibodies against HDGF, delayed the time relapse of Nu/Nu mice with heterotransplants [33]. However, the upstream factors and signaling mechanisms that modulate HDGF expression affecting tumor progression remain largely unknown. In this study, we demonstrated for the first time as far we known that HDGF is targeted by EGCG. In addition, EGCG treatment and HDGF silencing were able to inhibit cell migration and invasion, in agreement with two previous reports in breast cancer cells [32, 34]. Of therapeutic interest, our data indicate that both EGCG intervention and HDGF abrogation also induced a marked synergistic effect with cisplatin in cell death. Mechanistic studies indicate that cell death was associated to increased apoptosis, and activation of caspase-3 and caspase-9 indicative of intrinsic pathway activation. These data suggested that EGCG mediates, at least in part, HDGF downstream effects in cell migration, apoptosis, and sensitization to therapy. In summary, this study provides evidence supporting the potential use of EGCG for sensitization of lung cancer cells. We propose that the use of pure EGCG in combination with cisplatin, after dosage definition for in vivo studies, may represent an attractive therapeutic combination particularly on earliest stages of disease. In addition, the consumption of green tea as part of regular diet may have important chemopreventive effects in heavy smokers, which are at high risk for lung cancer.

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