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ORIGINAL PAPER

(−)-Epigallocatechin-3-gallate induces apoptosis and suppresses proliferation by inhibiting the human Indian Hedgehog pathway in human chondrosarcoma cells

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Abstract
Purpose Chondrosarcoma is a soft tissue sarcoma with a poor prognosis that is unresponsive to conventional chemotherapy. The regulatory mechanisms for the rapid proliferation of chondrosarcoma cells and the particular aggressiveness of this sarcoma remain poorly understood. In this study, we investigate the effect of epigallocatechin-3-gallate (EGCG) on growth and apoptosis of chondrosarcoma cells.
Methods The chondrosarcoma cell lines, SW1353 and CRL-7891, were cultured with and without EGCG. The MTT assay was used to test the cytotoxicity of EGCG. Flow cytometry and DAPI staining were used to observe cell apoptosis caused by EGCG. To explore the effect of EGCG on the Indian Hedgehog signaling pathway and apoptosis-related proteins, RT-PCR and Western blotting were used to detect the expression of PTCH and Gli-1 in the Indian Hedgehog signaling pathway. Meanwhile, expression of Bcl-2, Bax, and caspase-3 were also evaluated by Western blot analysis.
Results EGCG effectively inhibited cellular proliferation and induced apoptosis of SW1353 and CRL-7891. EGCG inhibited the human Indian Hedgehog pathway, down-regulated PTCH and Gli-1 levels, and induced apoptosis as confirmed by DAPI staining followed by flow cytometry. Protein expression levels of caspase-3 were unchanged in response to EGCG treatment in chondrosarcoma cells; however, the expression levels of Bcl-2 were significantly decreased and the levels of Bax were significantly increased.
Conclusions Our findings demonstrate that EGCG is effective for growth inhibition of a chondrosarcoma cell lines in vitro, and suggest that EGCG may be a new therapeutic option for patients with chondrosarcoma.

Keywords Chondrosarcoma · EGCG · Indian Hedgehog pathway · Apoptosis

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Introduction

Over the past several decades, long-term survival of malignant primary bone tumors such as osteosarcoma and Ewing's sarcoma has dramatically increased with the advent of systemic chemotherapy (Riedel et al. 2009), whereas chondrosarcoma, the second most frequent malignant primary bone tumor in humans, continues to have a poor prognosis because it is resistant to both ionizing radiation and chemotherapy (Katoh and Katoh 2006). To date, the only curative therapy is surgical resection at an early stage of the tumor, before the tumor has metastasized (Lee et al. 1999; Fiorenza et al. 2002).

Recently, evidence has accumulated that the human Indian Hedgehog (hIHH) pathway may be a promising target for cancer therapy. The hIHH pathway is associated with tumor development and progression (Benoist-Lasselin et al. 2006). The mammalian hedgehog family of genes, the transmembrane protein Patched (PTCH), and the GLI transcription factors are overexpressed in chondrosarcomas, and are hypothesized to contribute to the malignant

potential of this tumor (Ruiz i Altaba et al. 2002). Since the hIHH pathway is over-expressed in the majority of patients with chondrosarcomas, treatment with hIHH pathway inhibitors may represent an appropriate alternate therapy for these patients.

Green tea is one of the most widely consumed beverages in the world (Graham 1992). The beneficial effects of green tea are well-known, including cancer preventive activity and other medical applications (Lambert and Yang 2003; Fujiki 2005). (–)-Epigallocatechin-3-gallate (EGCG), a major polyphenolic component in green tea, has shown numerous health-promoting effects including a decrease in the risk of developing various cancers and cardiovascular diseases. It is also reported that it inhibits multiple components of several downstream signaling pathways (Shimizu and Weinstein 2005; Hou et al. 2004). Islam reported that EGCG inhibited the growth of the chondrosarcoma cells (Islam et al. 2000). However, the antitumor effect of EGCG on chondrosarcoma cells has not been well investigated, which impelled us to investigate whether EGCG could target the hIHH pathway and inhibit cell growth. In this study, we report our findings on the ability of EGCG to inhibit cell growth and the hIHH pathway in SW1353 and CRL-7891 cell lines in vitro.

Materials and methods

Cell and reagents

Two chondrosarcoma cell lines was used. SW1353 and CRL-7891 were obtained from American Type Cell Collection (Manassas, VA, USA). The SW1353 cells were cultured in L-15 medium containing 10% fetal bovine serum and 1% antibiotics–antimycotics (Invitrogen), and incubated in a non-CO_2 humidified atmosphere at 37°C. The CRL-7891 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, and incubated in a 5% CO_2 humidified atmosphere at 37°C. Anti-PTCH, anti-Gli-1, anti-BAX, anti-BCL-2, anti-caspase-3, and anti-actin antibodies were purchased from Santa Cruz Biotechnology. EGCG (more than 98% purity) was purchased from Sigma. Chemicals were dissolved in dimethyl sulfoxide (DMSO) and kept at −20°C until just before use.

Cytotoxicity test

The cytotoxicity of EGCG was investigated using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay. The cells were suspended in 0.2 ml of medium at a concentration of 1×10^6 ml^{-1} in 96-well plates. After 24 h incubation, 0.2 ml of medium containing different concentrations of EGCG was added to the cells, followed by another 24 h incubation. To evaluate the cell viability, 20 μl MTT solution (5 mg/ml in medium) was added to the cultured cells, which were further incubated for 4 h at 37°C. After removing the remaining medium, 150 μl DMSO was added to each well to solubilize the precipitate. The resulting color intensity, which is proportional to the number of viable cells, was measured at 490 nm using a microplate reader (Versa Max, Molecular Devices Co., CA, USA).

Apoptosis assay

Cells with morphological changes indicative of cell death by apoptosis were identified and quantified by fluorescence microscopy after staining with 4,6-diamidino-2-phenylindole (DAPI). Fluorescence was visualized using a Nikon Eclipse TE300 inverted fluorescence microscope (Nikon Corp., Tokyo, Japan). Apoptotic nuclei were identified by condensed chromatin as well as nuclear fragmentation.

Apoptosis analysis by flow cytometer

Apoptotic cells were measured with annexin V/FITC kit (BD Biosciences, USA) according to the manufacturer's instructions and analyzed by flow cytometry after compound treatment. Briefly, 1×10^6 cells were collected, washed three times with 4°C pre-cooled PBS, then the cells were resuspended in 300 μl of binding buffer. Five microliters of annexin V-FITC solution and PI (1 μg/ml) were added to these cells at 37°C for 15 min in the dark. Then, 200 μl binding buffer was added again. Using flow cytometry (FACS) to detect apoptosis through channels two and three, 10,000 cells were evaluated.

RNA extraction and RT-PCR analysis

SW1353 and CRL-7891 cells were cultured in medium containing different concentrations of EGCG (0, 1, 2, or 4 μM). After 24 h, total RNA was isolated from cells using TRIZOL reagent (Invitrogen, US) according to the manufacturer's instructions. The cDNA was amplified from 1 μg of total RNA using SuperScript one-step RT-PCR with the platinum *Taq* system (Invitrogen). The primers used for amplification of PTCH, Gli-1, and GAPDH transcripts were as follows: PTCH forward, 5′-CCC AAG CAA ATG TAC GAG CAC-3′; PTCH reverse, 5′-TGC GAC ACT CTG ATG AAC CAC-3′; Gli-1 forward, 5′-GAA CCC TTG GAA GGT GAT ATG TC-3′; and Gli-1 reverse, 5′-GGC AGT CAG TTT CAT ACA CAG AT-3′; GAPDH forward, 5′-GAA GGT CGG AGT CAA CGG ATT T-3′; and GAPDH reverse 5′-ATG GGT GGA ATC ATA TTG GAA C-3′. The reactions were performed in a 96-well

optical plate (Applied Biosystems) at 94°C for 2 min, followed by 38 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 40 s.

Western blotting analysis

The procedure for Western blotting analysis is briefly described below. After treating with different concentrations of EGCG (0, 1, 2, or 4 μM) for 24 h, Chondrosarcoma cells were washed twice with ice-cold PBS, collected in RIPA lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 5 mM EDTA, and 1 mM Na_3VO_4, pH 7.5) supplemented with a protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO), and incubated on ice for 30 min. Afterward, the cell lysate was centrifuged at 12,000 rpm for 15 min, and the supernatant was recovered. The protein concentration was determined with BCA Protein Assay Reagents (Pierce, Rockford, IL). Equal amounts of total protein were subjected to SDS-PAGE, transferred to PVDF membranes (BioRad, Richmond, CA), then probed with the appropriate antibodies.

Statistical analysis

Data are shown as mean ± SD. Student's test or one-way analysis of variance (ANOVA) was used for statistical analyses. A value of $P < 0.05$ was considered statistically significant.

Results

Affects of EGCG on SW1353 and CRL-7891 cell growth and proliferation

The growth suppressive effect of EGCG on the human chondrosarcoma cell lines, SW1353 and CRL-7891 were evaluated using the MTT assay. After 24 h exposure to different concentrations of EGCG, the proliferation of SW1353 and CRL-7891 was dramatically decreased in a dose-dependent manner, as shown in Fig. 1. The concentration required to inhibit growth of SW1353 and cells by 50% (IC50) was 1.8 and 2.4 μM after 24 h of incubation.

Induction of apoptosis by EGCG in SW1353 and CRL-7891

To determine whether EGCG can induce apoptosis of human chondrosarcoma cells, we examined EGCG-induced nuclear fragmentation. Cells were treated with 0, 1, 2, or 4 μM EGCG for 24 h then nuclei of the cells were stained with DAPI. Control cells exhibited intact nuclei, but EGCG-treated cells showed significant nuclear

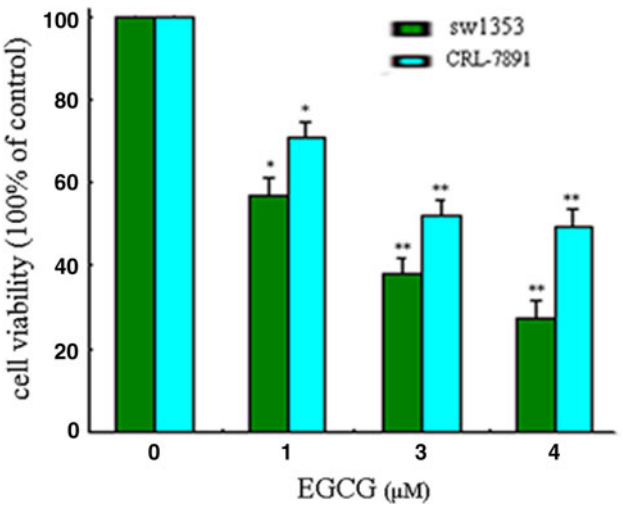

Fig. 1 Growth suppression effect of EGCG in chondrosarcoma cell lines. Cells were incubated with 0, 1, 2 and 4 μM of EGCG for 24 h. The experiments were performed at least three times and data are presented as mean ± SD (*$P < 0.05$ and **$P < 0.01$)

fragmentation (Fig. 2), which is a typical marker of apoptosis. The induction of apoptotic cell death was accompanied by characteristic morphological and structural changes, including a condensed and fragmented nuclear structure and decreased cell size.

To further confirm whether EGCG could induce apoptosis of chondrosarcoma cells, apoptotic death assays were done by employing annexin V/PI staining followed by FACS analysis. As shown in Fig. 3, EGCG can induce apoptosis of human chondrosarcomas in a dose-dependent manner. It is important to mention here that in this assay, double-negative (unstained) cells show uniform viability (marked as LL in Fig. 3), annexin V-positive and PI-negative stained cells show early apoptosis (marked as LR in Fig. 3), annexin V/PI double-stained cells show late apoptosis (marked as UR in Fig. 3), and finally, PI-positive and annexin V-negative stained cells account for apoptosis (marked as UL in Fig. 3). As evidenced in representative FACS analysis scatter-grams, annexin V/PI staining of control cells showed a large viable cell population with some staining for early apoptotic, late apoptotic, and dead cells (Fig. 3). However, treatment of cells with EGCG at 4 μM doses for 24 h resulted in a strong shift from live cells to a selectively early and late apoptotic cell population, without a considerable change in the dead cell population.

Effect of EGCG on the levels of the pro-apoptotic protein Bax, anti-apoptotic protein Bcl-2, and apoptosis-related protein Caspase-3

The anti-apoptotic proteins are associated with the inhibition of apoptosis and cell survival mechanisms. Bax is a

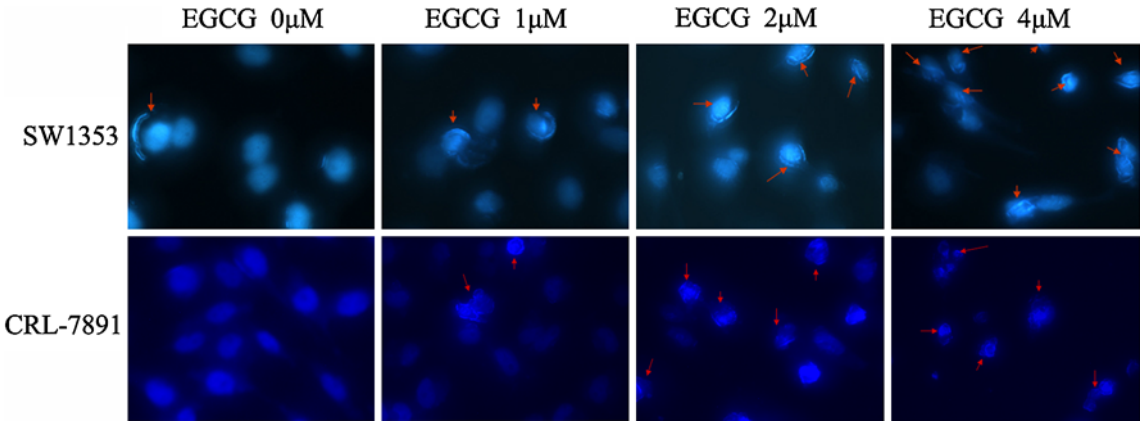

Fig. 2 Nuclear fragmentation was induced by EGCG treatment in SW1353 and CRL-7891 cell lines. Cells were treated with 0, 1, 2 and 4 μM of EGCG for 24 h and stained with 4,6-diamidino-2-phenylindole (DAPI). Cellular morphological changes were observed using a fluorescence microscope at the magnitude of ×600 (*arrows* indicate apoptotic cells)

pro-apoptotic protein, and the increased expression of this protein is often associated with increased apoptosis in target cells (Yip and Reed 2008). Because cell death decisions are tightly regulated by the balance between proapoptotic (e.g., Bax) and antiapoptotic (e.g., Bcl-2) proteins, attempts were made to determine what effects exposure to EGCG might have on such proteins. As shown in Fig. 4, EGCG treatment of chondrosarcoma cells for 24 h had little or no effect on the expression of Caspase-3. However, treatment of SW1353 cells with EGCG resulted in a significant down-regulation of Bcl-2 and a significant up-regulation of Bax.

EGCG inhibits the hIHH pathway in SW1353 and CRL-7891 cells

The hIHH pathway has an important role in cancer cell proliferation. Therefore, we evaluated the effect of EGCG on proteins in the hIHH pathway by RT-PCR and Western blotting (Fig. 5). We compared these genes and proteins in cells treated with various concentrations of EGCG for 24 h. The results of RT-PCR showed that EGCG treatment inhibited Ptch and Gli-1 mRNA expression in chondrosarcoma cells in a dose-dependent manner. The pattern of protein expression of PTCH and Gli-1 correlated well with their respective mRNA levels. These results were consistent with growth suppression caused by EGCG-treatment.

Discussion

Chondrosarcomas represent a heterogeneous group of neoplasms ranging from indolent, low-grade tumors to aggressive, high-grade forms. Although surgery and radiation therapy have achieved excellent local control, the treatment of advanced, metastatic disease is still ineffective. These tumors are largely unaffected by systemic chemotherapy. Thus, there is universal agreement that novel approaches are desperately needed (Damron et al. 2007).

The hIHH pathway is overexpressed in chondrosarcomas. During bone development, the IHH pathway plays an important role in chondrocyte proliferation, differentiation, and normal longitudinal bone development (Machold et al. 2003; Ingham and McMahon 2001). Hedgehog (HH) activates GLI-mediated transcription through the transmembrane proteins patched (PTCH) and smoothened (SMO). Activation of GLI transcription factors results in transcriptional repression. GLI-mediated transcriptional activation results in the up-regulation of target genes including the transmembrane protein PTCH-1 and the transcription factor GLI-1 (Lum and Beachy 2004). It has been reported that over-expression of this pathway is highly associated with tumor development and progression in several types of human cancers including lung (Ruiz i Altaba et al. 2002), human glioma (Kinzler et al. 1981), biliary (Lauth and Toftgard 2007), and breast cancers (Zhao et al. 2010). In addition, high expression of the Hedgehog target genes PTCH-1 and GLI-1 has been reported in chondrosarcoma cells (Tiet et al. 2006). Treatment of chondrosarcoma organ cultures with Hedgehog protein increased the cell proliferation rate, whereas the addition of chemical inhibitors of Hedgehog signaling decreased the proliferation rate (Kronenberg et al. 1997; Tiet et al. 2006). Therefore, targeting the hIHH pathway is a promising strategy for the treatment of chondrosarcomas.

EGCG, a major polyphenolic component in green tea, has shown numerous health-promoting effects including a reduction in the risk of developing various cancers, and inhibits several downstream signaling molecules in multiple signaling pathways. However, the effects of EGCG on chondrosarcoma have not been clearly elucidated.

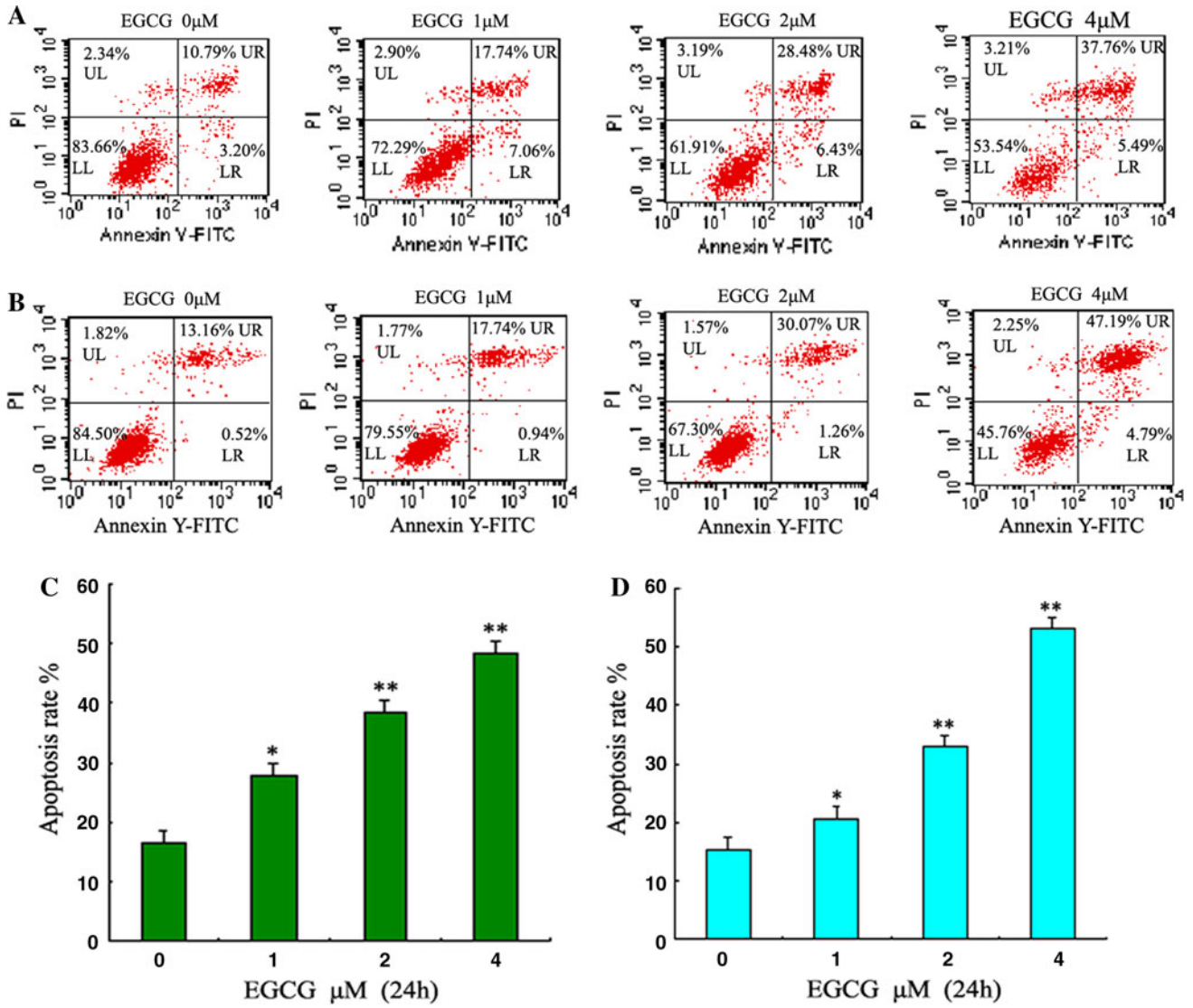

Fig. 3 EGCG caused apoptotic death in SW1353 (**a**, **c**) and CRL-7891 (**b**, **d**) cells. Following 24 h of cell treatments with EGCG detailed in "Materials and methods". In brief, cells were collected and stained with Annexin V/PI followed by FACS analysis. Representative FACS analysis scatter-grams of Annexin V/PI stained 0, 1, 2 and 4 μM EGCG treatment showed four different cell populations marked as: double-negative (unstained) cells showing live cell population (*LL*, *lower left*), Annexin V-positive and PI-negative stained cells showing early apoptosis (*LR*, *lower right*), Annexin V/PI double-stained cells showing late apoptosis (*UR*, *upper right*), and finally PI positive and Annexin V-negative stained cells showing dead cells (*UL*, *upper left*). (*$P < 0.05$ and **$P < 0.01$)

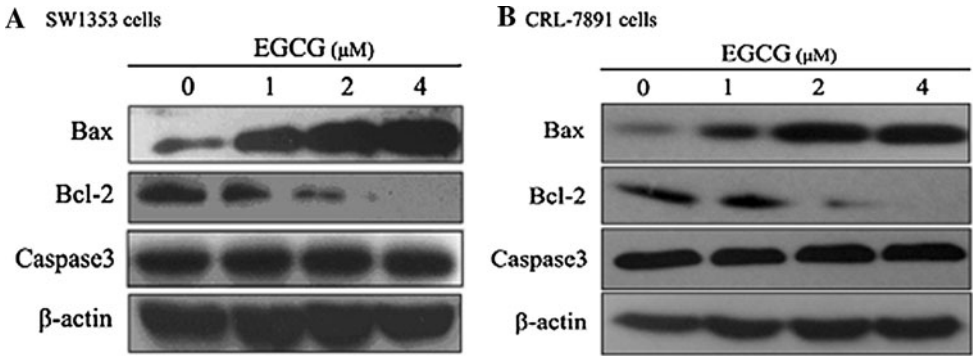

Fig. 4 Changes in the expression of apoptosis-related proteins in response to treatment with EGCG. SW1353 and CRL-7891 cells were treated with 0, 1, 2 and 4 μM of EGCG for 24 h. Cell extracts were subjected to Western blotting to determine levels of apoptosis-related proteins as described in "Materials and methods"

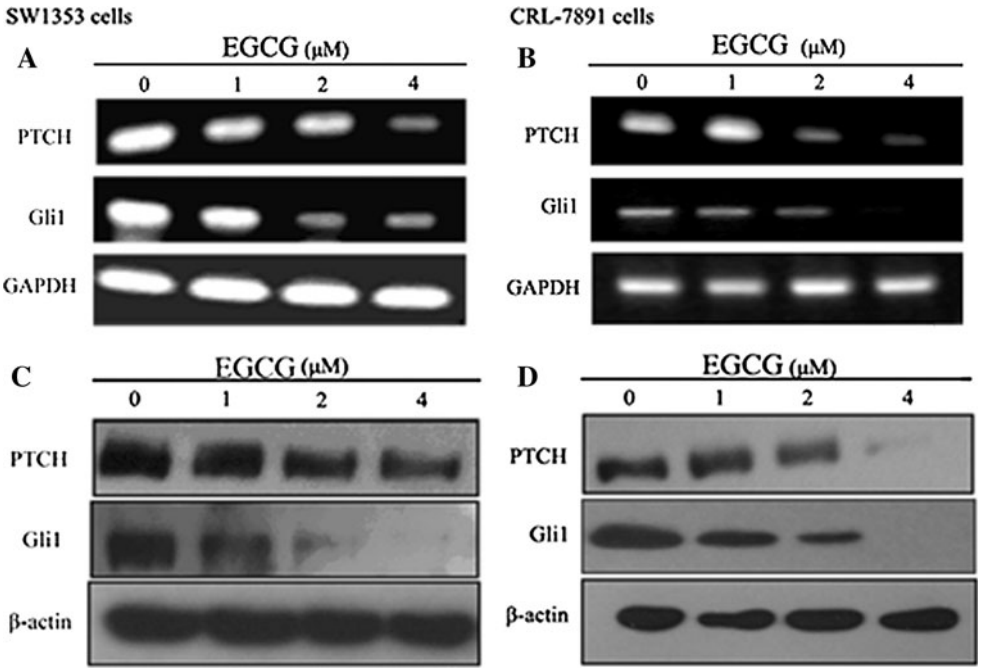

Fig. 5 Changes in the expression of PTCH and Gli1 genes expression and proteins levels in response to treatment with EGCG. **a** and **b** Expressions of PTCH and Gli1 genes were examined by RT-PCR. PTCH and Gli1 genes were decreased in a dose-dependent manner. **c** and **d** Decrease of PTCH and Gli1 protein levels were confirmed by Western blot. The pattern of protein expression for PTCH and Gli1 was correlated well with their respective mRNA

In the present study, we first examined the hIHH pathway in SW1353 cells after EGCG treatment. Interestingly, EGCG inhibited the hIHH pathway in a concentration-dependent fashion in SW1353. The IHH pathway has been implicated in a wide variety of processes in cancer cells, including the regulation of cell proliferation and survival. Therefore, we hypothesized that the inhibition of activation of the hIHH pathway after exposure to EGCG might lead to the suppression of tumor cell growth and induce apoptosis. To test this hypothesis, the effect of EGCG on cell proliferation was measured by MTT (24 h) assay. The result showed that EGCG inhibited SW1353 cell growth in a dose-dependent manner. To observe the possible effect of EGCG on cell apoptosis, we performed experiments to test for apoptosis using flow cytometry and DAPI staining. Significant induction of apoptosis was found in SW1353 cell lines.

The Bcl-2 protein family plays a central role in the control of apoptosis (Merry and Korsmeyer 1997; Adams and Cory 1998). Bcl-2, a 52 kDa protein, is the prototype of this family and inhibits the induction of apoptosis, whereas Bax is pro-apoptotic. High concentrations of Bcl-2 affect the susceptibility of a cell to the induction of apoptosis by altering the ratio of death promoters to suppressors, providing tumor cells with a survival advantage, and permitting the expansion of transformed cells harboring mutations within their genome (Peng et al. 2009). In our study, we detected the expression of caspase-3, Bax, and Bcl-2. Islam reported that EGCG could affect caspase-3 in the chondrosarcoma cell line, HTB-94. In our study, there was no obvious change in caspase-3. We believe the reason was that the concentrations of EGCG we used were much lower than theirs. However, treatment of chondrosarcoma cells with EGCG resulted in a significant down-regulation of Bcl-2 and a significant up-regulation of Bax. A higher Bax/Bcl-2 ratio was observed following EGCG treatment. These results suggest that Bcl-2 and Bax are crucial proteins in EGCG-induced apoptosis.

In most advanced malignancies, cancer cells become resistant to apoptosis and do not respond to the cytotoxic of most chemotherapeutic agents currently available (Molinari 2000). Thus, the development of compounds such as EGCG that could induce apoptosis in chondrosarcoma cells could be worthwhile as potential chemotherapeutic agents. In this study, we demonstrated that EGCG could inhibit the hIHH pathway, induce apoptosis, and suppress proliferation of a human chondrosarcoma cell line. In summary, we demonstrate that EGCG has strong antitumor activity against SW1353 cells in vitro. Our results indicate that EGCG may be a promising therapeutic intervention in the treatment of chondrosarcoma.

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Conflict of interest statement We declare that we have no conflict of interest.

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