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ORIGINAL ARTICLE

Green tea polyphenol EGCG suppresses osteosarcoma cell growth through upregulating miR-1

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Abstract (−)-Epigallocatechin-3-gallate (EGCG), the most abundant and active polyphenol in green tea, has been demonstrated to have anticancer effects in a wide variety of human cancer. MicroRNAs (miRNAs) are a class of short noncoding RNAs and play important role in gene regulation and are critically involved in the pathogenesis and progression of human cancer. This study aims to investigate the effects of EGCG on osteosarcoma (OS) cells and elucidate the underlying mechanism. Cellular function assays revealed that EGCG inhibited cell proliferation, induced cell cycle arrest and promoted apoptosis of OS cells in vitro, and also inhibited the growth of transplanted tumors in vivo. By miRNA microarray and RT-qPCR analysis, miR-1 was found to be significantly upregulated in MG-63 and U-2OS treated by EGCG in dose- and time-dependent manners, and miR-1 downregulation by inhibitor mimics attenuated EGCG-induced inhibition on cell growth of OS cells. We also confirmed that miR-1 was also frequently decreased in clinical OS tumor tissues. Moreover, both EGCG and miR-1 mimic inhibited c-MET expression, and combination treatment with EGCG and c-MET inhibitor (crizotinib) had enhanced inhibitory effects on the growth of MG-63 and U-2OS cells. Taken together, these results suggest that EGCG has an anticancer effect on OS cells, at least partially, through regulating miR-1/c-MET interaction.

Keywords (−)-Epigallocatechin-3-gallate (EGCG) · Osteosarcoma · MicroRNA-1 · c-MET · Crizotinib

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Introduction

OS is the most common primary malignant bone tumor with a higher incidence in children and adolescents. Over the past decades, therapeutic efficacy for OS patients has significantly improved due to the introduction of combinatorial chemotherapy [1]; however, many OS patients respond poorly to chemotherapy, and a significant proportion of patients who initially respond well to chemotherapy develop chemoresistance, and thus the long-term survival rate for OS patients remains poor [2]. Recently, green tea polyphenols such as (−)-epigallocatechin-3-gallate (EGCG) have been demonstrated to have anticancer effects in many kinds of human cancers [3, 4]. More importantly, this plant-derived polyphenol is pharmacologically safe [5], and increasing evidences have demonstrated that combined administration of EGCG and other interfering approaches result in improved therapeutic efficacy [6, 7]. However, the effects of EGCG on OS cells and the underlying mechanisms are still unclear.

MicroRNAs (miRNAs) are a class of short noncoding RNAs, which is an effective mechanism to negatively regulate gene expression by directly binding to the 3′ untranslated regions (3′UTR) of target messenger RNAs and inducing their degradation or transcriptional repression [8]. Increasing evidence has revealed that miRNAs are extensively involved in biological processes such as differentiation and development, as well as in human diseases including cancers. In the initiation and progression of a certain cancer, the whole miRNA

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profile is changing with some miRNAs downregulated (usually taken as tumor suppressor miRNAs) and some other miRNAs upregulated (usually taken as oncogenic miRNAs). Thus, the modulation of these cancer-related miRNAs may provide therapeutic benefits [9]. Since many reports have demonstrated miRNAs as molecular targets of tea polyphenols underlying their biological effects [10–12], we then asked whether EGCG exerts its anticancer effects on OS cells through modulation of specific miRNAs.

In this study, we firstly validated the anticancer effects of EGCG on OS cells. Then, using miRNA microarray analysis, we identified that miR-1 was significantly upregulated and closely involved in EGCG-inhibited cell growth of OS cells, at least partially, through targeting c-MET. Moreover, combination treatment with EGCG and c-MET inhibitor (crizotinib) had enhanced inhibitory effects on the growth of OS cells and also provided a promising approach of combination with EGCG and other interfering methods, such as crizotinib for patients with OS.

Material and methods

Cell culture and treatment

Human OS cell lines MG-63 and U-2OS were purchased from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) (Biochrom, Berlin, Germany) at 37 °C with 5 % CO_2. (−)-Epigallocatechin-3-gallate (EGCG) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) as a stock solution of 50 g/L and further diluted in culture medium to appropriate concentration when used. Crizotinib (PF-02341066) was purchased from Selleck Chemicals (Houston, TX, USA).

Patients and tissue samples

Forty-one OS samples and 12 normal bone tissue samples were collected from Xiangya Hospital from March 2013 to December 2014, and the information of these samples is found in Table S1. Use of these samples for all experiments was approved by the Ethics Committee of The 2nd Xiangya Hospital of Central South University.

Cell proliferation, cell cycle, and apoptosis assay

Cell proliferation was evaluated by MTT assay after 24, 48, and 72 h of seeding. The optical density at 570 nm of each well was measured with an enzyme-linked immunosorbent assay (ELISA) reader (ELX-800 type, BioTek Winooski, VT, USA). For cell cycle analysis, cells were digested with trypsin (Auragene Bioscience Corporation, Changsha, China) and collected after treatment for 48 h, and washed with phosphate-buffered saline (PBS) twice. The cells were resuspended in PBS and then fixed in 70 % ethanol at 4 °C for 18 h. The cells were washed with PBS and resuspended in staining solution (50 μg/mL of PI, 1 mg/mL of RNase A, 0.1 % Triton X-100 in PBS). The stained cells (1×10^5) were then analyzed with a flow cytometer (Beckman Coulter, Brea, CA, USA). For cell apoptosis assay, cells were stained with annexin-V-FITC and propidium iodide (PI) labeling solution using annexin-V Apoptosis Detection kit FITC (eBioscience, Affymetrix, San Diego, CA, USA) according to the suggested procedure.

miRNA microarray assay

MG-63 and U-2OS cells treated with EGCG at a concentration of 0.08 g/L for 48 h and corresponding nontreated cells were subjected to miRNA microarray analysis in KangChen Bio-tech Company (Shanghai, China). Briefly, miRNAs were isolated and labeled using the miRCURY™ Hy3™/Hy5™ Power labeling kit and hybridized on the miRCURY™ LNA Array, which contains 3100 capture probes, covering all annotated microRNAs in miRBase 18.0. The slides were washed and scanned by the Agilent Scanner G2505C; then, the scanned images were then imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. After normalization, significant differentially expressed miRNAs were identified through Volcano Plot filtering.

RT-qPCR

Total RNAs were prepared using TRIzol reagent (Invitrogen, USA) and reverse-transcribed to cDNA using the Prime Script Reagent Kit (Promega, Madison, WI, USA). The relative expression levels of c-MET mRNA were detected by SYBR green qPCR assay (Bio-Rad, USA) and normalized to β-actin. The specific primers are as follows: c-MET-F: 5′-AGGTTGTGGTTTCTCGATCAG-3′, c-MET-R: 5′-CAGTGATAACCAGTGTGTAGCC-3′; β-actin-F: 5′-AGGGGCCGGACTCGTCATACT-3′, β-actin-R: 5′-GGCGGCACCACCATGTACCCT-3′. For miRNA real-time quantitative polymerase chain reaction (RT-qPCR), the specific primer sets for miR-1 (HmiRQP4647) and U6 RNA (HmiRQP9001), and the PCR mix were purchased from GeneCopoeia, and the relative expression levels of miR-1 were normalized to U6 and calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot

Total cellular extracts were prepared with 200 μl of lysis buffer, and approximately 50 μg of total protein was subjected to western blot analysis using a standard procedure, as described

previously [13]. β-Actin was used for normalization. Antibodies against c-MET and β-actin were obtained from Abzoom (Abzoom, USA).

miRNA in situ hybridization

The expression level and subcellular localization of miR-1 in 41 OS tumor tissues and 12 normal bone tissues were detected by in situ hybridization. Briefly, the tissue slides were hybridized with 300 nM of 5′-digoxigenin (DIG) LNA-modified-miR-1 (Exiqon, Copenhagen, Denmark) using IsHyb in situ Hybridization kit (Biochain, Eureka Drive, Newark, CA, USA), according to the manufacturer's instructions. The expression signal of miR-1 was scanned, and the mean density was calculated by Image Pro-Plus 6.0 software.

Animal treatments

Nude mice of 20–25 g in weight were purchased from Shanghai Laboratory Animal Center (SLAC, Shanghai, China) and

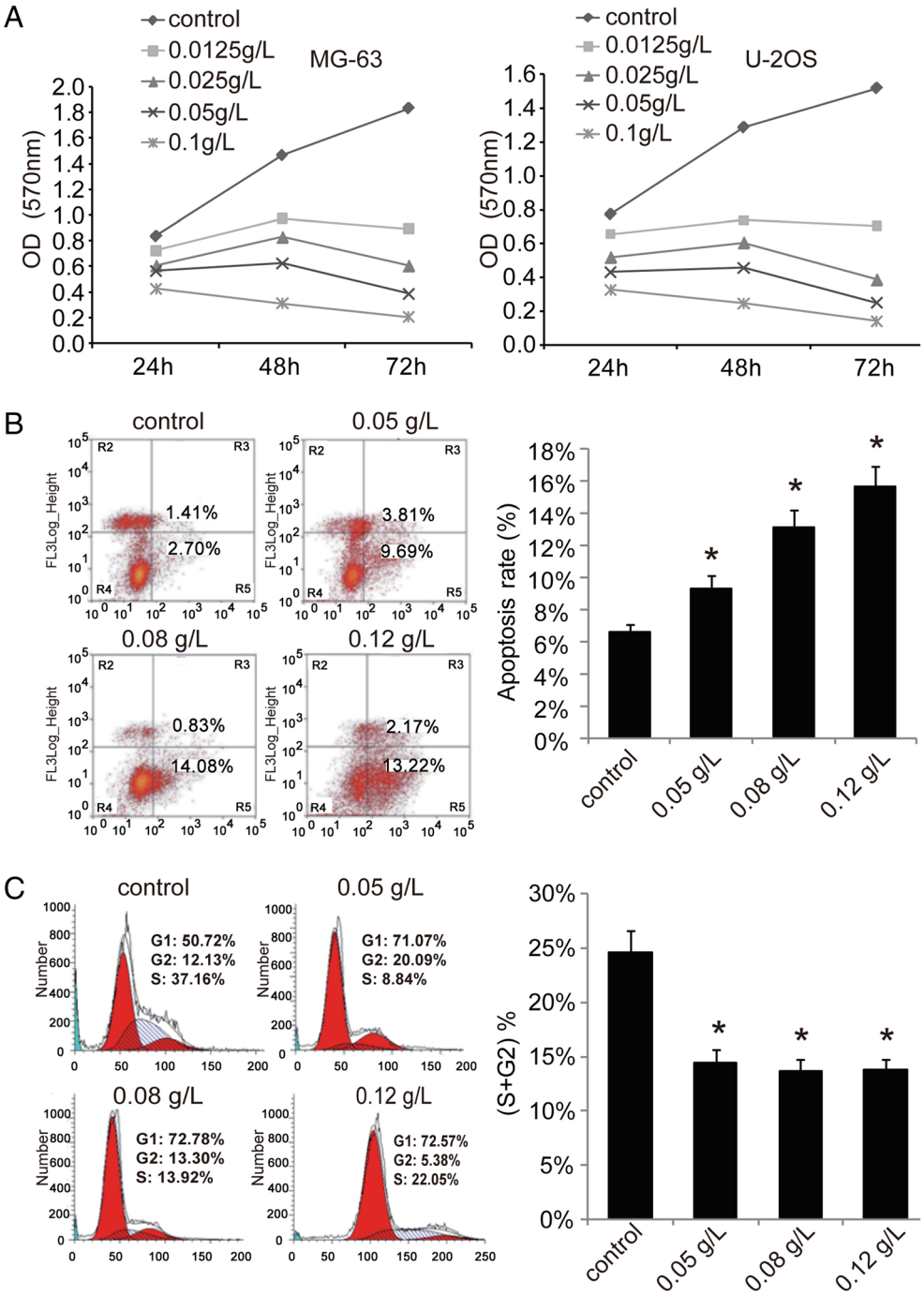


Fig. 1 EGCG inhibits the growth of OS cells in vitro. **a** MG-63 and U-2OS cells were treated with different concentrations of EGCG (from 0.0125 to 0.1 g/L) for 24, 48, and 72 h, respectively; then, the effects of EGCG on cell proliferation were determined using MTT assay. **b**, **c** MG-63 cells were treated with increasing concentrations of EGCG for 48 h, and the effects of EGCG on cell cycle and apoptosis were determined using flow cytometry. **P*<0.05 versus the control; data shown are means±S.D.

maintained under specific pathogen-free conditions. The MG-63 cells were suspended in serum-free medium, and 1000,000 cells in 200 μl were injected into the proximal tibia of each anesthetized nude mice. After 7 days postinoculation, the mice that developed palpable tumors were intraperitoneally injected EGCG (30 mg kg^{-1}) or physiological saline as control (n=3 animals per group) once every 3 days for three times. After EGCG treatment, all of the mice were euthanized, and the tumors were excised and imaged.

Statistical analysis

The data are shown as the mean±SD. Student's t test was used to analyze the differences between experimental and control groups. Statistical analyses were performed using the SPSS11.0 software (SPSS Inc., Chicago, IL, USA), and P<0.05 was considered to indicate a statistically significant difference.

Results

EGCG inhibits the growth of OS cells both in vitro and in vivo

MG-63 and U-2OS cells were treated with different concentrations of EGCG (from 0.0125 to 0.1 g/L) for 24, 48, and 72 h, respectively. MTT assays revealed that EGCG reduced the cell viability in a dose-dependent manner, as well as in a time-dependent manner (Fig. 1a). To investigate the mechanisms of EGCG inhibition on the cell proliferation of MG-63 and U-2OS cells, we analyzed the effects of EGCG on cell apoptosis and cell cycle. MG-63 and U-2OS cells treated with increasing dose of EGCG for 48 h were used for analysis. As shown in Fig. 1b, EGCG promoted apoptosis of MG-63 cells in a significant dose-dependent manner. Cell cycle analysis also indicated that EGCG significantly resulted in cell cycle arrest by increasing the proportion of S and G2 phases in MG-63 cells, but without in a significant dose-dependent manner (Fig. 1c). Similar results were also found in U-2OS cells. To further validate the anticancer role of EGCG on OS cells in vivo. As shown in Fig. 2, the growth of transplanted tumors was significantly inhibited in EGCG (30 mg kg^{-1})-treated group than the control groups. These data suggest that green tea polyphenol EGCG suppresses OS cell growth both in vitro and in vivo.

MiR-1 is induced by EGCG in OS cells and downregulated in OS patients

To determine the involvement of miRNAs in response to EGCG treatment, MG-63 and U-2OS cells treated with EGCG at a concentration of 0.08 g/L for 48 h and corresponding nontreated cells were subjected to miRNA microarray analysis using miRCURY™ LNA Array Chip (version 18.0). After normalizing the expression data with bioinformatical methods, 24 and 38 miRNAs with significant changes (upregulated or downregulated by at least twofold, P<0.05) were found in MG-63 and U-2OS cells, respectively (Fig. 3a) (Tables S2–S5). To confirm the microarray findings, the top five differentially expressed miRNAs from two cell lines with lowest P values were validated by RT-qPCR. The majority (9/10) of miRNAs tested was consistent with the microarray results (data not shown). Of note, only hsa-miR-1-3p (miR-1) was found to be significantly changed in both MG-63 and U-2OS cells, and upregulated by EGCG treatment and validated by RT-qPCR (Fig. 3a). Further study revealed that miR-1 was induced by EGCG in both dose- and time-dependent manners (Fig. 3b, c). Thus, our data demonstrated that miR-1 was the major miRNA that was elevated in response to EGCG treatment in OS cells. Moreover, we

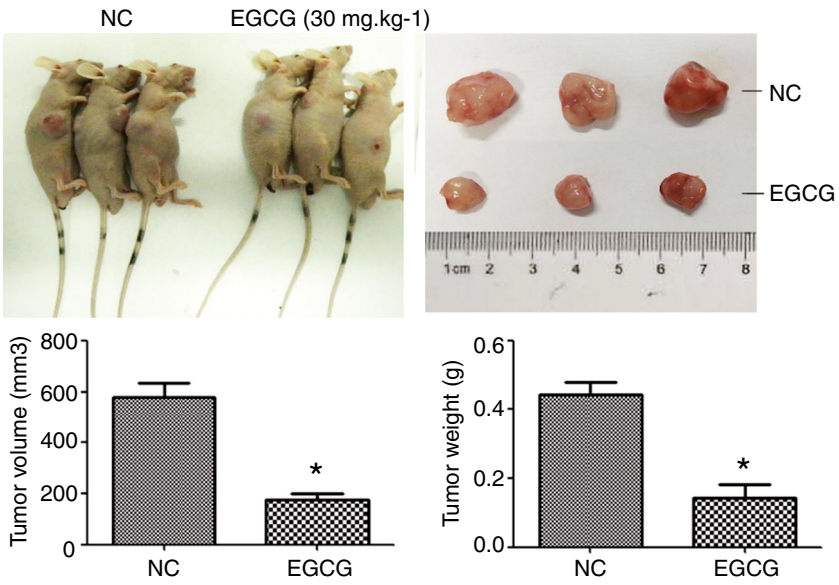


Fig. 2 EGCG inhibits the growth of OS cells in vivo. The MG-63 cells were suspended in serum-free medium, and 1000,000 cells in 200 μl were injected into the proximal tibia of each anesthetized nude mice. After 7 days postinoculation, the mice that developed palpable tumors were intraperitoneally injected EGCG (30 mg kg^{-1}) or physiological saline as control (n=3 animals per group) once every 3 days for three times. After EGCG treatment, all of the mice were euthanized, and the tumors were excised and imaged under a light microscope

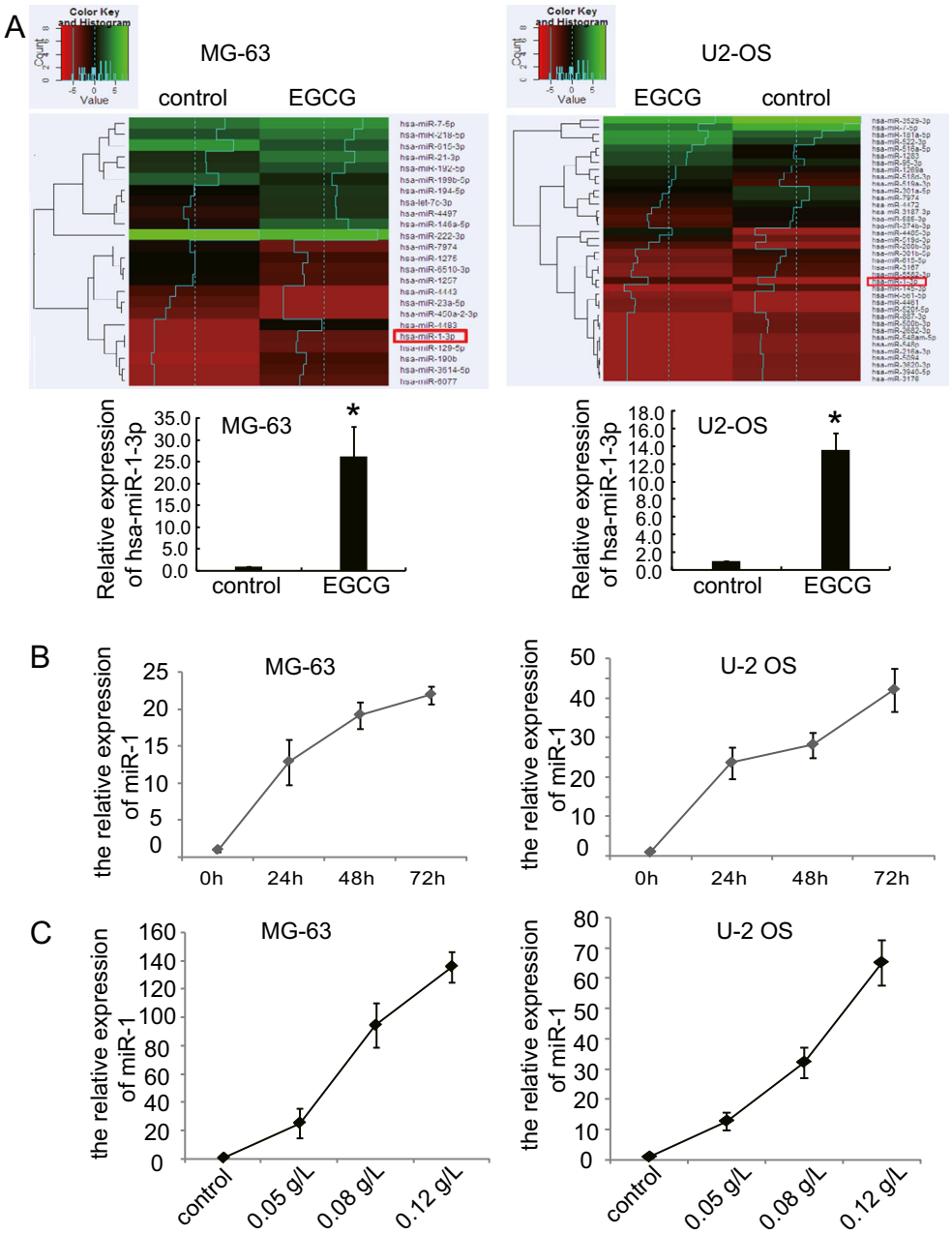


Fig. 3 MiR-1 is induced by EGCG in OS cells. **a** Hierarchical clustering analysis of differentially expressed miRNAs detected by the miRNA microarray analysis in MG-63 and U-2OS cells treated with EGCG at a concentration of 0.08 g/L for 48 h. MiR-1 (hsa-miR-1-3p) marked by *red box* was found to be significantly upregulated in both MG-63 and U-2OS cells by microarray analysis and validated by RT-qPCR. **b** RT-qPCR revealed that miR-1 was significantly induced in MG-63 and U-2OS cells treated with 0.08 g/L EGCG in a time-dependent manner. **c** RT-qPCR revealed that miR-1 was significantly induced in MG-63 and U-2OS cells treated with EGCG for 48 h in a dose-dependent manner. *$P<0.05$ versus the control; data shown are means±S.D.

evaluated the expression levels of miR-1 in OS tumor tissues ($n=41$) and normal bone tissues ($n=12$). As shown in Fig. 4, the expression levels of miR-1 were significantly decreased in OS tumor tissues compared with normal bone tissues.

MiR-1 is involved in EGCG-inhibited cell growth of OS cells

To further determine whether EGCG exerts its inhibitory effect on the growth of OS cells through upregulation of miR-1, we inhibited that miR-1 expression by miR-1 inhibitor mimics in MG-63 and U-2OS cells treated with EGCG. As expected, miR-1 inhibitor significantly attenuated EGCG-induced inhibition on cell growth of OS cells by regulating cell cycle (Fig. 5a) and cell apoptosis (Fig. 5b). Taken together, these results suggest that miR-1 is a functional target of EGCG, involved in EGCG-inhibited cell growth of OS cells.

EGCG and miR-1 inhibit c-MET expression in OS cells

We then examined whether the c-MET expression was changed in response to EGCG treatment in OS cells. RT-qPCR revealed that c-MET was significantly inhibited by EGCG treatment for 48 h in a dose-dependent manner,

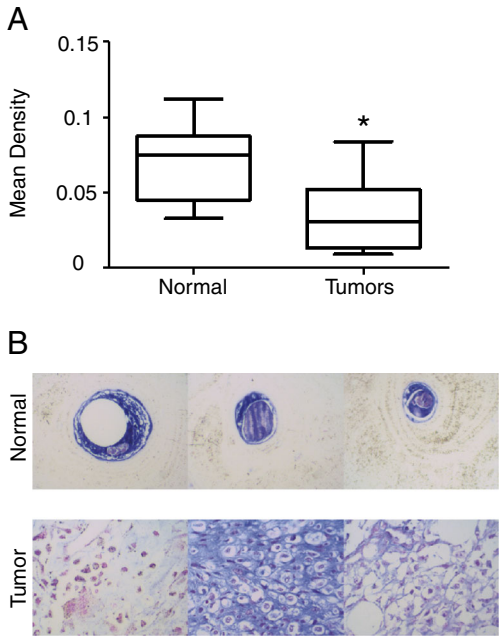


Fig. 4 MiR-1 is frequently downregulated in OS patients. **a** The relative expression levels of miR-1 were evaluated by in situ hybridization (ISH) in 41 tumor tissues and 12 normal tissues. **b** Representative micrographs of miR-1 expression in three tumor tissues and three normal bone tissues with blue violet staining as positive miR-1 probe under. *$P<0.05$ versus the normal; data shown are means±S.D.

especially in U-2OS cells (Fig. 6a). Based on the observation of inverse alteration of miR-1 and c-MET expression in response to EGCG treatment, we speculated that EGCG might inhibit c-MET expression by inducing miR-1. This was partially supported by the observation that miR-1 mimics inhibited c-MET expression in MG-63 and U-2OS cells, while miR-1 inhibitor mimics treatment resulted in increased c-MET expression compared to the controls (Fig. 6b, c). Together with the above findings, our study revealed an important miR-1/c-MET interaction involved in the anticancer activity of EGCG on OS cells.

Combination of EGCG with c-MET inhibitor crizotinib has enhanced inhibitory effects on the growth of OS cells

We then asked whether the combination of EGCG and crizotinib which is widely used as a c-MET/ALK inhibitor would have a better antitumor effect on OS cells. MG-63 and U-2OS cells were treated with crizotinib (0.05 mM) and/or EGCG (0.08 g/L) for 48 h. As expected, the combinational treatment of EGCG and cetuximab had more enhanced inhibitory effects on the growth of MG-63 and U-2OS cells than either EGCG or crizotinib used alone, by inducing cell cycle arrest (Fig. 7a) and promoting apoptosis (Fig. 7b). These data suggest a promising choice of a combinational therapy for patients with OS.

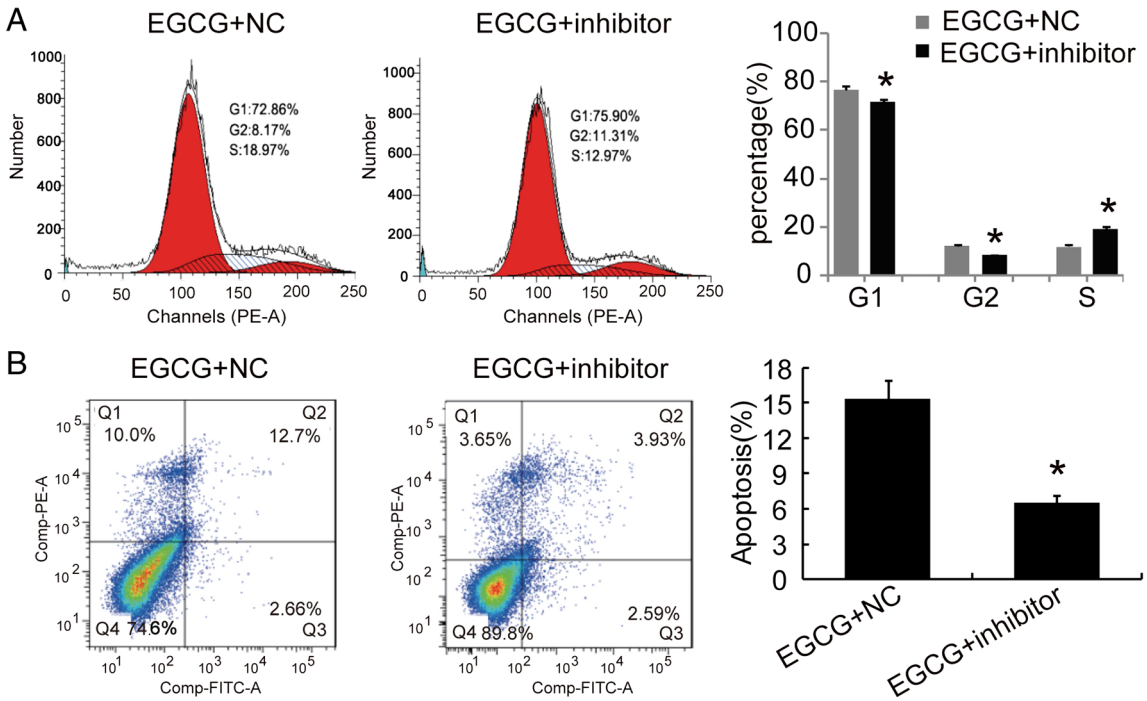


Fig. 5 MiR-1 inhibition attenuates EGCG-induced inhibition on cell growth of OS cells. MG-63 cells were treated with EGCG and miR-1 inhibitor mimics (inhibitor) or negative control (NC) mimics. Then, the cell cycle analysis by flow cytometer (**a**) and apoptosis analysis by flow cytometry analysis using Annexin V and PI staining (**b**) revealed that miR-1 inhibition attenuated EGCG-induced cell cycle arrest and cell apoptosis promotion. The similar result was also observed in U-2OS cells. *$P<0.05$ versus the normal; data shown are means±S.D.

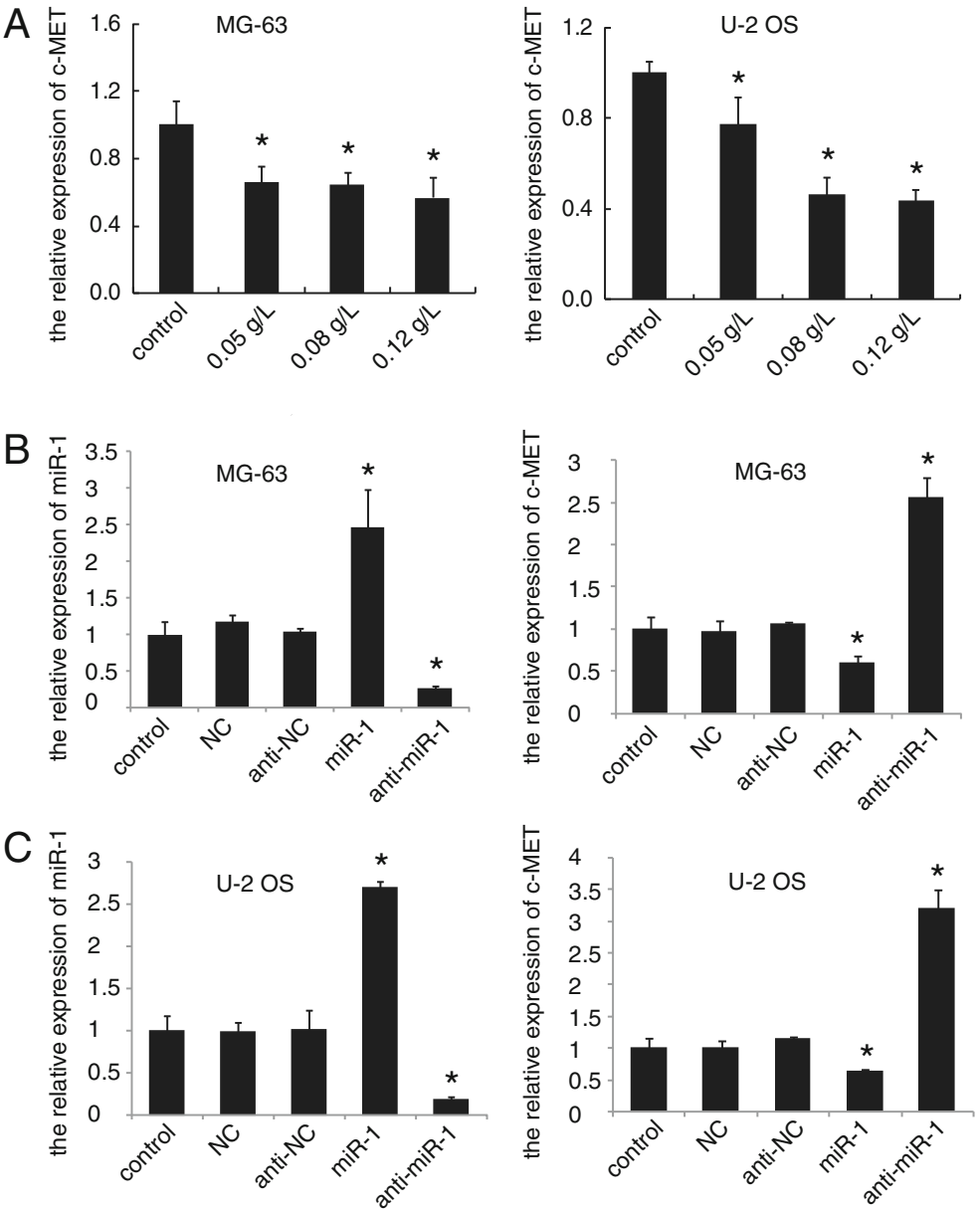
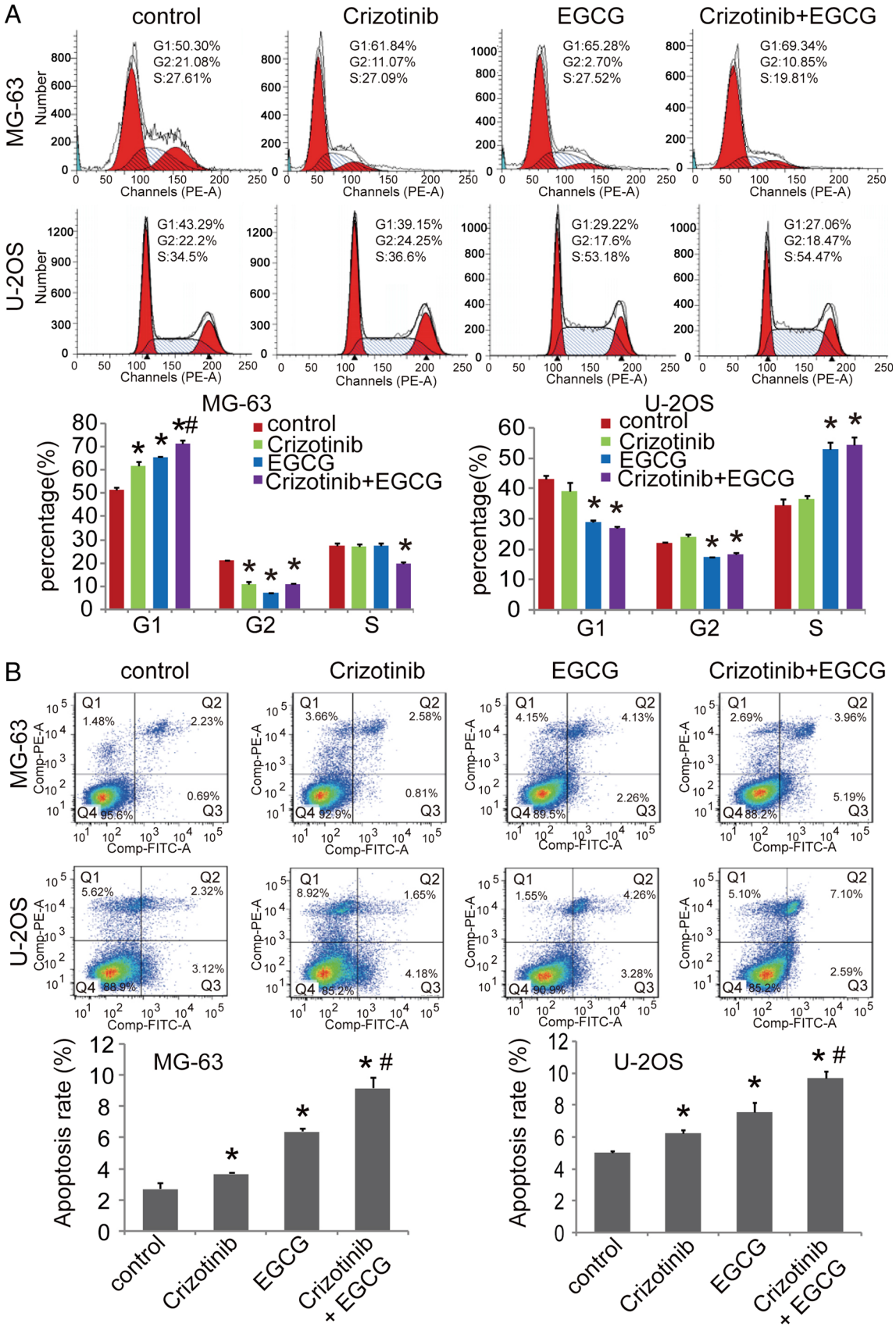


Fig. 6 MiR-1 inhibits c-MET expression in OS cells. **a** MG-63 and U-2OS cells were treated by increasing concentrations of EGCG for 48 h, and the expression levels of c-MET were determined by RT-qPCR. **b**, **c** MG-63 and U-2OS cells were transfected with miR-1 mimics (pre-miR-1), scrambled mimics as negative control (pre-NC), miR-1 inhibitor (anti-miR-1), or negative control (anti-NC), and the expression levels of miR-1, as well as c-MET, were determined by RT-qPCR

Discussion

In this study, we demonstrated, for the first time, that the anticancer activity of EGCG on OS cells is associated with alteration of many miRNAs, of which miR-1 is a key one to be stably upregulated, and substantially involved in EGCG-inhibited cell growth of OS cells. By evaluating the expression levels of miR-1 in clinical OS tumors, we confirmed that miR-1 was frequently decreased in OS tumor tissues compared with those in adjacent normal tissues. We also revealed that both EGCG and miR-1 resulted in a significant reduction of c-MET expression. The result revealed a new mechanism by which EGCG exerts its anticancer activity by regulating miR-1/c-MET interaction in OS cells. Moreover, combinational treatment with EGCG and c-MET inhibitor (crizotinib) has enhanced inhibitory effects on the growth of OS cells.

EGCG, a major green tea polyphenol, has been reported to have a broad anticancer activity in human cancer cells and is pharmacologically safe for patients [5, 14]. In this study, we found that EGCG reduced the cell growth in dose- and time-dependent manners, mechanistically by promoting cell apoptosis and increasing the proportion of S and G2 phases of cell cycle. Our result is consistent with the anticancer activity of EGCG found in other human cancers, such as gastric [15], colorectal [16], lung [17], breast [18], ovarian [19], and prostate cancer [20]. Of note, in most of these cancers, EGCG exhibits inhibitory effects on the growth of cancer cells by regulating cell apoptosis and associated genes, for example, in colon cancer cells, EGCG promoted apoptosis via AMPK


A
control
Crizotinib
EGCG
Crizotinib+EGCG
MG-63
Number
G1:50.30%
G2:21.08%
S:27.61%
G1:61.84%
G2:11.07%
S:27.09%
G1:65.28%
G2:2.70%
S:27.52%
G1:69.34%
G2:10.85%
S:19.81%
Channels (PE-A)
U-2OS
G1:43.29%
G2:22.2%
S:34.5%
G1:39.15%
G2:24.25%
S:36.6%
G1:29.22%
G2:17.6%
S:53.18%
G1:27.06%
G2:18.47%
S:54.47%
MG-63
U-2OS
percentage(%)
control
Crizotinib
EGCG
Crizotinib+EGCG
G1
G2
S
B
Q1
Q2
Q3
Q4
1.48%
2.23%
0.69%
95.6%
3.66%
2.58%
0.81%
92.9%
4.15%
4.13%
2.26%
89.5%
2.69%
3.96%
5.19%
88.2%
5.62%
2.32%
3.12%
88.9%
8.92%
1.65%
4.18%
85.2%
1.55%
4.26%
3.28%
90.9%
5.10%
7.10%
2.59%
85.2%
Comp-PE-A
Comp-FITC-A
Apoptosis rate (%)
control
Crizotinib
EGCG
Crizotinib + EGCG

◀ **Fig. 7** Combination of EGCG with c-MET inhibitor has enhanced inhibitory effects on the growth of OS cells. MG-63 and U-2OS cells were treated with crizotinib (0.05 mM) and/or EGCG (0.08 g/L) for 48 h, and the effects on cell cycle (**a**) and cell apoptosis (**b**) were determined using flow cytometry. *$P<0.05$ versus the control; #$P<0.05$ versus crizotinib-treated groups or EGCG-treated groups

signal pathway [21, 22]. This is consistent with our observation that EGCG promoted cell apoptosis in a significant dose-dependent manner in OS cells, suggesting a common mechanism of EGCG anticancer activity on cancer cell growth.

Concerning many reports that have identified miRNAs as molecular targets of EGCG underlying its biological effects [10, 11], we then used miRNA microarray chip to screen differentially expressed miRNAs upon EGCG treatment in two OS cell lines (MG-63 and U-2OS). Dozens of differentially expressed miRNAs were found in MG-63 or U-2OS cells upon 0.08 g/L EGCG treatment for 24 h, some of which were validated by RT-qPCR, suggesting a reliable result from miRNA microarray analysis. However, only miR-1 was found to be significantly regulated by EGCG in both MG-63 and U-2OS cells. These data demonstrated cell-specific regulation by EGCG on miRNAs, and miR-1 might be a key target of EGCG underlying its biological effect in OS cells. This was further supported by the fact that miR-1 was induced by EGCG in both dose- and time-dependent manners in both MG-63 and U-2OS cells.

MiR-1 has been reported to be downregulated and act as a tumor suppressor in many types of tumors, such as colorectal [23], renal [24], hepatocellular [25], bladder [26], and prostate [27]. In this study, we observed a frequently downregulated miR-1 expression in clinical OS tumors, which is consistent with previous report by Novello et al. [28], suggesting that aberrant decreased miR-1 expression is closely involved in OS development and progression. c-MET is tyrosine kinase growth factor receptor and drives the malignant progression of several tumor types, including OS [29]. Recently, several studies have showed that c-MET is a functional target of miR-1 in several tumor types [30–32], including OS [28], suggesting an important mechanism of miR-1/c-MET interaction during tumor development and progression.

In this study, we observed that miR-1 was upregulated, while c-MET was downregulated, in MG-63 and U-2OS treated by EGCG in dose- and/or time-dependent manner. Thus, we speculated that EGCG might suppress OS cell growth through regulating miR-1/c-MET interaction. This was supported by the observation that miR-1 inhibition significantly attenuated EGCG-induced inhibition on cell growth of OS cells and miR-1 negatively regulated c-MET expression in OS cells. In addition, the combinational treatment of EGCG and c-MET inhibitor (crizotinib) had more enhanced inhibitory effects on the growth of OS cells than either EGCG or crizotinib used alone, by inducing cell cycle arrest and promoting apoptosis.

In summary, we demonstrated that upregulation of miR-1 induced c-MET inhibition is a key anticancer mechanism of EGCG in OS cells. We have further provided the evidence that the combinational treatment with EGCG and c-MET inhibitor has enhanced inhibitory effects on the growth of OS cells in vitro, a promising new therapy for patients with OS, which remains to be investigated in animal models.

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