Ellagic acid induces cell cycle arrest and apoptosis via the TGF-β1/Smad3 signaling pathway in human colon cancer HCT-116 cells

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Abstract. Colorectal carcinoma (CRC) is a major type of malignancy worldwide. Ellagic acid (EA), a natural phenolic constituent, has been shown to exhibit anticancer effects. In our previous study, it was shown that EA inhibited proliferation of CRC cells. Additionally, microarray analysis revealed 4,738 differentially expressed genes (DEGs) which were associated with multiple cellular events, including cell growth, apoptosis and angiogenesis. However, the associated pathways had not been validated. In the present study, it was shown that EA induced G0/G1 cell cycle arrest in HCT-116 cells, and increased apoptosis. Furthermore, DEGs identified by cDNA microarray analysis were investigated, and showed changes in five genes which were associated with the TGF-β1/Smad3 signaling pathway. TGF-β1 small interfering RNA and SIS3, a Smad3 inhibitor, were used to assess the role of TGF-β1 and Smad3, respectively, and it was shown that they reduced the effects of EA on HCT-116 CRC cells. In addition, the expression patterns of downstream DEGs of the TGF-β1/Smad3 pathway were altered. Thus, this pathway may underlie the molecular mechanism by which EA exhibits its effects in vitro in CRC cells. Accordingly, targeting the TGF-β1/Smad3 pathway with anticancer agents such as EA may be potentially useful to treat CRC.

Introduction

Among the most prevalent tumor types, colorectal carcinoma (CRC) is third most common, and is the second leading cause of cancer-associated deaths worldwide (1). Although the 5-year overall survival rate of patients with CRC is 64%, this rate decreases to ≤10% in patients who have developed metastases, and several patients with CRC will develop local or distant relapses or metastasis (2). It is suggested that only the application of more advanced drugs directed at novel targets may improve the survival rate of patients with CRC (3). However, the pathogenetic mechanisms of CRC are insufficiently understood, hampering drug development. Thus, identifying the molecular mechanism underlying development and/or progression of CRC may facilitate the discovery of potentially novel therapeutic targets.

Previously, it was shown that chemo-prevention is a suitable and effective treatment against cancer (4). Ellagic acid [EA; 2,3,7,8-tetrahydroxy-chromeno (5,4,3-cde) chromene-5,10-dione; International Union of Pure and Applied Chemistry] is a polyphenolic compound abundantly found in woody plants, berries, grapes and nuts (5). EA is considered a potential chemo-preventive agent, and has been shown to inhibit proliferation in a variety of cancer types (5,6). In previous studies, both in vitro and in vivo, EA exhibited notable inhibitory effects against CRC, suggesting its anti-tumor role against CRC (7-9). However, the molecular mechanisms associated with the cellular responses induced by EA, particularly the regulatory mechanisms involved in altered transcription and protein interactions, have not been determined, to the best of our knowledge. Additionally, previous studies have not assessed the involvement of any relevant pathways. Therefore, the aim of the present study was to identify the molecular targets of EA which underlie the effects of EA on HCT-116 CRC cells, and thus provide a theoretical basis for precision therapy in CRC.

In our previous study, using cDNA microarray analysis, it was shown that treatment with EA reduced proliferation of CRC cells. Furthermore, a total of 4,738 genes were shown to be significantly differentially expressed (1.2 fold change) after 72 h of treatment with EA (10). EA was shown to be associated with G0/G1 cell cycle arrest in HCT-116 cells and thereby induced apoptosis. Several genes involved in the TGF-β1 and Smad3 signaling pathways were upregulated by treatment with EA. Additionally, in another of our previous studies, it was shown that EA can regulate breast cancer cell cycle arrest in vitro via
TGF-β/Smad signaling (11), although this has not been shown in CRC yet, to the best of our knowledge. Following on from our previous study, in the present study, it was shown that EA induced cell cycle arrest in HCT-116 CRC cells by enhancing TGF-β1-induced phosphorylation of Smad3, thereby inducing subsequent apoptosis.

Materials and methods

Reagents and cell culture. EA was purchased from Sigma-Aldrich (Merck KGaA) and diluted to a working concentration in DMSO (≤1%). The solution was sterilized using a 0.22 µm filter and stored at -20°C. HCT-116 cells were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, and were cultured using a 0.22 µm filter and stored at ‑20˚C. HCT‑116 cells were synchronised (10). After a further 6 h incubation in supplemented media, cells were treated EA with 0, 25, 50, 100 or 150 µM. DMSO diluted to <0.1% was used to treat the negative control cells.

Treatment with EA. Cells were cultured in DMEM supplemented with FBS initially. After incubation for 24 h, cells were cultured in DMEM without FBS, as described previously (10). Cells were plated in a T25 flask at a density of 5x10⁵ cells/ml and reached a confluence of 50-60% after incubation for 6 h. As cells had been grown in serum-free media, the cellular growth had been synchronized (10). After a further 6 h incubation in supplemented media, cells were treated with 0, 25, 50, 100 or 150 µM. DMSO diluted to <0.1% was used to treat the negative control cells. After treatment with EA or negative control at 37°C for 24 or 72 h, the cells were harvested, washed in ice-cold PBS and fixed with 70% ethanol at 4°C for at least 12 h. The samples were subsequently adjusted to a density of 1x10⁶ cells/ml, and then stained with 80 mg/ml RNase A and 50 µg/ml PI at a density of 1x10⁶ cells/ml for 30 min, and the stained cells were analyzed using a FACScan cytometer (Becton, Dickinson, Company).

Reverse transcription-quantitative (RT-qPCR). After 24 h of treatment with EA, total RNA was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.) and an RNeasy kit (Qiagen GmbH) according to the manufacturer's protocol. Specifically, for ribosomal RNA, purity and integrity were further evaluated as described previously (10,11).

Total RNA from HCT-116 cells treated with 100 µM EA, an optimal concentration of EA determined in our previous study (10), was used for transcriptomics analysis of the selected target genes using RT-qPCR. RNA (2 µg) was reverse transcribed to cDNA using oligo(dT) primers and SuperScript II reverse transcriptase kit (Thermo Fisher Scientific, Inc.). The thermocycling conditions used were: 30 sec at 95°C; followed by 40 cycles of 5 sec at 95°C and 34 sec at 60°C. A melt curve was plotted between 60-95°C. Primers were purchased from Sangon Biotech Co., Ltd. The sequences of the primers are stated in Table I. qPCR was performed using an ABI Prism 7900HT sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Data were analyzed using the comparative 2-ΔΔCq method (12). The normalization of results was based on β-actin levels respectively (10,11).

Western blotting analysis. To analyze protein expression, total protein was extracted using lysis buffer (Beyotime Institute of Biotechnology) and western blotting was performed. Briefly, 20 µg protein was loaded on a 10-15% SDS gel, resolved using SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% skimmed milk in PBS-Tween for 1 h at room temperature with shaking and subsequently incubated with the primary antibody at 4°C overnight. The antibodies used were: TGF-β1 (cat. no. 3709), Smad3 (cat. no. 9513), p-Smad3 (cat. no. 9520), β-Actin

<table>
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<th>Gene</th>
<th>Primer sequence</th>
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<tr>
<td>β-actin</td>
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<tr>
<td>Forward</td>
<td>CTCACCATGGTAGTAGATATCGGC</td>
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<tr>
<td>Reverse</td>
<td>AGGAATCCCTTCTGACCCATGC</td>
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<tr>
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<tr>
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<td>Reverse</td>
<td>TCGTCCGTTGCAATCCCTC</td>
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Apoptosis analysis. The rate of apoptotic cells was analyzed using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide kit (Becton, Dickinson and Company) according to the manufacturer's protocol. Briefly, upon following treatment with various concentration of EA for 24 h, the cells were digested with 0.25% trypsin and harvested. The samples were washed three times using ice-cold PBS and re-suspended in binding buffer (500 µl) at a density of 1x10⁶ cells/ml, from which 500 µl was transferred to a flow cytometry tube, and 5 µl each Annexin V-FITC (50 µg/ml) and propidium iodide (50 µg/ml) was added. Cells were left to stain in the dark for 30 min at room temperature. Using flow cytometry, the proportion of apoptotic cells per 10,000 cells was detected to calculate the apoptotic rate (11).

Cell cycle analysis. HCT-116 cells (5x10⁵) were seeded in T25 culture flasks and grown for 6 h to a confluence of 50-60%. Cells were starved in serum-free medium for 24 h to achieve synchronization. Cells were subsequently grown for a further 6 h in supplemented media for 6 h, and treated with EA as described above. After treatment for 24 h at 37°C, floating and adherent cells were collected, washed with ice-cold PBS and fixed with 70% ethanol for at least 12 h at 4°C. The cells were then treated with 80 mg/ml RNase A and 50 µg/ml PI at a density of 1x10⁶ cells/ml for 30 min, and the stained cells were analyzed using a FACScan cytometer (Becton, Dickinson, Company).

Western blotting analysis. To analyze protein expression, total protein was extracted using lysis buffer (Beyotime Institute of Biotechnology) and western blotting was performed. Briefly, 20 µg protein was loaded on a 10-15% SDS gel, resolved using SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% skimmed milk in PBS-Tween for 1 h at room temperature with shaking and subsequently incubated with the primary antibody at 4°C overnight. The antibodies used were: TGF-β1 (cat. no. 3709), Smad3 (cat. no. 9513), p-Smad3 (cat. no. 9520), β-Actin
(cat. no. 4967) and p15 (cat. no. 4138) were obtained from Cell Signaling Technology, Inc. All antibodies were used at a 1:1,000 dilution. Signals were developed using an Enhanced Chemiluminescence Plus Detection kit (Pierce; Thermo Fisher Scientific, Inc.). Signals were visualized using a digital camera and densitometry analysis was performed using ImageJ version 1.41q (National Institutes of Health). Protein expression was normalized to \( \beta \)-actin levels.

Small interfering (si)RNA transfection and specific inhibitors. si-TGF-\( \beta \)1 (5'-CAC UGC AAG UGG ACA UCA ATT-3' and 5'-UUG AUG UCC ACU UGC AGU GTT-3') and a negative control scrambled siRNA (5'-GCC TAA CTG TGT CAG AAG GAA-3') were purchased from Shanghai GenePharma, Co., Ltd. and 20 nM transfected into HCT-116 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). CRC cells were pretreated with 10 ng/ml TGF-\( \beta \)1 siRNAs for 16 h, prior to treatment with EA (100 \( \mu \)M) for 24 h. To inhibit Smad3, cells were pretreated with 3 \( \mu \)mol/l of the specific inhibitor SIS3 for 6 h, prior to a 24 h EA treatment.

**Statistical analysis.** Data are presented as the mean ± the standard error of the mean. Data were analyzed using GraphPad Prism version 5.0 (GraphPad Software, Inc.). The statistical differences between >2 groups were determined using one-way ANOVA followed by a Tukey's post-hoc test or a two-way ANOVA followed by a Bonferroni post-hoc test. Statistical differences between 2 groups were determined using a Student's t-test. \( P < 0.05 \) was considered to indicate a statistically significant difference.

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Figure 1. Effect of treatment with various concentrations of EA on cell cycle distribution and apoptosis. (A) HCT-116 cells were treated with increasing concentrations of EA for 24 h before assessment using flow cytometry. The distribution of cells in the different phases of the cell cycle are presented. Experiments were repeated independently three times. *\( P < 0.05 \) vs. control. (B) HCT-116 cells were treated with various EA concentrations for 24 h. The proportion of apoptotic cells was detected using Annexin V-FITC and propidium iodide staining. ***\( P < 0.001 \) vs. control. EA, ellagic acid; FITC, fluorescein isothiocyanate.
Results

**EA reduces cell proliferation and results in G0/G1 cell cycle arrest.** The effect of EA treatment on the cell cycle was investigated using flow cytometry to determine whether the EA-related decrease in the quantity of cells was the result of cell cycle arrest. Treatment with 100 and 150 µM EA resulted in a significant increase in the proportion of G0/G1 cells, and a significant decrease in the proportion of cells in the S and G2/M phase (Fig. 1A). These results suggest that EA treatment was associated with G0/G1 arrest in HCT-116 cells.

**EA induces apoptosis in HCT-116 cells.** Flow cytometry was used to assess the effects of EA treatment on HCT-116 with regards to apoptosis. The results showed that treatment with 100 and 150 µM EA for 24 h increased apoptosis in a dose-dependent manner.

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Table II. Relative expression changes in the expression of five genes associated with the TGF-β1/Smad3 signaling pathway in HCT-116 cells treated with 100 µM EA for 24 or 72 h, determined using Affymetrix microarray analysis (72 h) and RT-qPCR (24 h).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change, Microarray</th>
<th>P-value, Microarray</th>
<th>Fold change, RT-qPCR</th>
<th>P-value, RT-qPCR</th>
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<tr>
<td>TGFβ1</td>
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<td>0.003244&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>E2F5</td>
<td>-1.626492</td>
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<td>-1.85462</td>
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<tr>
<td>CDKN2B/p15</td>
<td>1.740624</td>
<td>0.003070&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.10236</td>
<td>0.012624&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>P<0.05, <sup>b</sup>P<0.01. RT-qPCR, reverse transcription-quantitative PCR; CDKN2B, cyclin-dependent kinase inhibitor 2B.

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Figure 2. Changes in the expression of genes and proteins associated with TGF-β1/Smad signaling pathways in HCT-116 cells treated with EA and with TGF-β1 expression knocked down. (A) Relative mRNA expression levels of TGF-β1, Smad3 and p15 in HCT-116 cells treated with DMSO+scrambleRNA, EA+scrambleRNA, EA+TGF-β1 siRNA, or DMSO+TGF-β1 siRNA for 24 h. **P<0.01, ***P<0.001 vs. DMSO+scramble; ###P<0.001 vs. EA+scrambleRNA (B) Western blotting showed that TGF-β1, Smad3, and p15 expression patterns were altered in HCT-116 cells treated with DMSO+scrambleRNA, EA+scrambleRNA, EA+TGF-β1 siRNA, or DMSO+TGF-β1 siRNA for 24 h. **P<0.01, vs. ***P<0.001 vs. DMSO+scramble; ##P<0.01, ### P<0.001 vs. EA+scrambleRNA, EA, ellagic acid; si, small interfering; p-, phospho; t-, total.
These results showed that EA was associated with the activation of apoptosis in HCT-116 cells, and this effect was positively associated with concentration of EA (Fig. 1B).

**TGF-β1/Smad signaling is involved in the inhibitory effects of EA.** Based on the further analysis of the microarray data, the TGF-β1/Smad3 pathway was considered to be associated with the induction of G0/G1 cell cycle arrest as well as reduction of HCT-116 cell proliferation.

Subsequently, the expression levels of five differentially expressed genes (DEGs) (TGF-β1, Smad3, E2F4, E2F5 and p15), which were enriched in the TGF-β1/Smad signaling pathway, were assessed using RT-qPCR. These findings were consistent with those of the microarray analysis, indicating the 100% accuracy of the array data (Table II).

**TGF-β1 is a key factor involved in EA-mediated regulation of cell cycle arrest and apoptosis.** si-TGF-β1 were transfected into HCT-116 cells, to determine the role of TGF-β1 in the EA-mediated effects on cell behavior. The results showed that, si-TGF-β1 effectively reduced TGF-β1 mRNA and protein expression levels. Additionally, si-TGF-β1 reduced the expression of Smad3 and P15 (Fig. 2). si-TGF-β1 abrogated the effects of EA on cell cycle arrest and apoptosis (Fig. 3). Thus, it was demonstrated that TGF-β1 was a crucial factor involved in the EA-mediated effects on HCT-116 cells.
Smad3 phosphorylation is involved in EA-mediated regulation of cell cycle arrest and apoptosis. The specific inhibitor SIS3 was used to treat CRC cells, which had been pretreated with EA (100 µM) for 6 h. After 24 h of treatment, SIS3 effectively reduced the mRNA and protein expression levels of P15 and the levels of phospho-Smad3 (Fig. 4). In addition, the regulatory function of EA on the cell cycle and apoptosis were reduced (Fig. 5). Therefore, Smad3 may be crucial in the EA-mediated effects on HCT-116 cells.

Discussion

CRC is a serious malignant disease, ranking fifth and second among the most common causes of cancer-associated death in China (13) and western countries (14), respectively. Although effective therapeutic strategies have been developed over the previous decades, the 5-year overall survival of patients with CRC has remained unsatisfactory, owing to limitations of currently available prognostic factors (such as, vascular and neural invasion, a low lymphocyte-to-monocyte ratio and tumor stage III/IV) (3). Chemo-prevention is an effective method for the inhibition of cancer cell growth. However, our understanding regarding CRC pathogenesis remains limited. Thus, it is crucial to further determine the molecular mechanisms underlying CRC, with the aim of facilitating the discovery of novel therapeutic targets.

EA may be a potential chemo-preventive agent, which has been shown to inhibit proliferation in various types of cancer (15). Based on in vitro and in vivo experiments, EA was shown to significantly delay progression of CRC, suggesting that it may serve an anti-tumor role in CRC (8,16,17). However, the relevant molecular pathways underlying the cellular response to EA, especially those associated with transcriptional regulation and protein production, have not been determined. Therefore, it is clinically significant to determine the molecular mechanisms and targets of EA for the inhibition of growth in HCT-116 CRC cells. Although several studies have hypothesized the involvement of various pathways in this process, few of these have investigated these pathways further (7,8,16).

Microarray profiling was used to investigate the molecular mechanisms underlying EA-induced inhibition for HCT-116 CRC cell proliferation. In our previous study, it was shown that EA reduced cancer cell proliferation. Furthermore, the cDNA microarray analysis showed that, after 72-h EA treatment, a total of 4,738 genes exhibited a >1.2-fold change in their expression levels (10). Based on the present study, EA was shown to arrest the HCT-116 cell cycle in the G0/G1 phase, which resulted in apoptosis. Furthermore, among the DEGs identified in the cDNA microarray analysis, the changes in TGF-β1, Smad3, E2F4, E2F5 and p15, involved in the TGF-β1/Smad3 pathway, were verified using RT-qPCR. Treatment with si-TGF-β1 and a Smad3 inhibitor were used to assess the function of TGF-β1 and Smad3, respectively, the corresponding regulatory functions of EA were abrogated in HCT-116 cells, and the expression patterns of downstream DEGs in TGF-β1/Smad3 pathway, including the cyclin-dependent kinase inhibitor 2B (CDKN2B) (also known as p15), were altered.

TGF-β is a family of multifunctional polypeptides that promotes differentiation and inhibits growth and proliferation in most epithelial cell types in vitro as well as in vivo (18).
TGF-β1 is one of the most predominant cytokines which participates in various biological pathways in critical physiological functions (19). Following hetero-oligomerization of the type I and type II transmembrane TGF-β receptors, signals are transmitted by TGF-β from the cellular membrane to the nuclear targets via a signaling cascade involving Smad proteins (20). Smad3, one mediator involved in the signaling cascade, is critically responsible for the transduction of TGF-β signals to its nuclear targets, thereby inhibiting cellular growth. Once a TGF-β signal is activated, the carboxyl-terminal serine amino acids of two important downstream targets, Smad2 and Smad3, are phosphorylated allowing them to bind to Smad4 to form heteromeric complexes. Smad2/3/4 complexes are translocated to the nucleus to regulate transcription of target genes, such as CDKN2B (21).

TGF-β induces G1 cell cycle arrest via its regulatory function on CDKN2B (21). TGF-β also induces apoptosis in several types of cancer cells through multiple mechanisms (21,22). Based on in vitro experiments using breast cancer cells, our previous study showed that EA induced cell cycle

Figure 5. SIS3 treatment reverses EA-induced changes in cell cycle distribution and apoptosis in HCT-116 cells. (A) HCT-116 cells were treated with DMSO+scrambleRNA, EA+scrambleRNA, EA+SIS3 or DMSO+SIS3 for 24 h prior to flow cytometry analysis. *P<0.05 vs. DMSO+scramble; †P<0.05 vs. EA+scrambleRNA. (B) HCT-116 cells were treated with DMSO+scrambleRNA, EA+scrambleRNA, EA+SIS3 or DMSO+SIS3 for 24 h. The apoptotic rates were determined using Annexin V-fluorescein isothiocyanate and propidium iodide staining. **P<0.001 vs. DMSO+scramble; †P<0.05 vs. EA+scrambleRNA.
arrest predominantly via modulation of the TGF-β/Smad signaling pathway (11). Although the role of EA on regulating TGF-β/Smad3 pathway has been demonstrated in several types of tumor (9,11,15,23), to the best of our knowledge, the present study is the first to report the anti-tumor role of EA by regulating TGF-β1/Smad3 signaling pathway in CRC.

CDKN2B regulates cell growth control during the G1 phase (24). The cyclin-dependent kinase inhibitor encoded by CDKN2B targets CDK4 or CDK6, binding with them, to prevent CDK activation. Therefore, the protein product of CDKN2B is a regulator of cell growth, specifically controlling cell cycle progression to the G1 phase (25). CDKN2B expression was detected in multiple breast cancer cell lines and epithelial cells of normal breast tissue (26). Furthermore, it was shown to be associated with cell aging and is hypothesized to be a tumor suppressor gene (27). In an in vivo melanoma model, loss of p15 promoted the transition from benign nevus to melanoma, demonstrating its importance in this process (28).

Binding of CDK inhibitors (p15, p21 and p27) to the corresponding cyclin/CDK complexes results in the inactivation of cyclin/CDK complexes, and thus the subsequent restriction of cell growth (29). In HeLa cells, the growth-inhibitory effect of 1-(2-hydroxy-5-methylphenyl)-3-phenyl-1,3-propanedione may be induced by blocking the G1/S transition, through upregulation of these CDK inhibitors (30). In our previous study, EA was shown to upregulate TGF-β1 and Smad3 expression levels and promote the phosphorylation of Smad3 (23). phospho-Smad3 bound to Smad4 in the nucleus, thereby regulating the expression of the p15 target gene (31,32). In the present study, EA inhibited HCT-116 cell proliferation, to a certain extent, via the TGF-β1/Smad3 pathway.

Based on the results of the present study, EA caused G0/G1 cell cycle arrest in HCT-116 cells, thereby inducing apoptosis in vitro. EA was predicted to regulate TGF-β1/Smad3 signaling based on microarray profiling analysis. Furthermore, it was shown that EA regulated TGF-β1/Smad3/CDKN2B signaling via phosphorylation of Smad3, resulting in increased transcriptional activity of CDKN2B. These observations show the relationship between TGF-β1/Smad3 signaling and EA treatment which resulted in reduced cancer cell growth. In the microarray analysis the protein markers of the cell cycle, including Cyclin E, D and B, and apoptosis markers, including caspase-3, were not differentially expressed genes in our previous study (10). Collectively, the present data demonstrated that EA inhibited CRC cell cycle progression via upregulation of CDKN2B, a cell cycle inhibitor.

There are some limitations in the present study. First, only a single cell line was used for in vitro experiments, and in vivo experiments were not performed. Thus, additional studies using different CRC cell lines and in vivo models of CRC are required to verify the role and mechanism of EA further. Secondly, in-depth mechanisms underlying the properties of EA were not explored. Thirdly, the results of present study have not been verified in clinical specimen. Finally, TGF-β1/Smad3 signaling is known to be associated with epithelial to mesenchymal transition. Whether EA affects epithelial to mesenchymal transition in colon cancer cells will be explored in future studies.

In conclusion, the present study provides preliminary evidence showing the anti-growth function of EA in CRC cells. The results suggested that EA treatment may alter TGF-β1/Smad3 signaling pathway and thus upregulate the cell cycle inhibitor, CDKN2B. Therefore, the present study highlights the therapeutic potential of EA for treatment of CRC. However, further research is required to develop a suitable clinical approach for use of EA in the treatment of cancer.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ML, GL and JZ conceived and designed the study. JW, SD and XY analyzed the microarray data. JZ, LD, ZZ, HZ and CS performed the experiments. JZ, GL and HC wrote the article. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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