RESEARCH ARTICLE

Ellagic Acid Exerts Anti-proliferation Effects via Modulation of Tgf-B/Smad3 Signaling in MCF-7 Breast Cancer Cells

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Abstract

Ellagic acid has been shown to inhibit tumor cell growth. However, the underlying molecular mechanisms remain elusive. In this study, our aim was to investigate whether ellagic acid inhibits the proliferation of MCF-7 human breast cancer cells via regulation of the TGF-β/Smad3 signaling pathway. MCF-7 breast cancer cells were transfected with pEGFP-C3 or pEGFP-C3/Smad3 plasmids, and treated with ellagic acid alone or in combination with SIS3, a specific inhibitor of Smad3 phosphorylation. Cell proliferation was assessed by MTT assay and the cell cycle was detected by flow cytometry. Moreover, gene expression was detected by RT-PCR, real-time PCR and Western blot analysis. The MTT assay showed that SIS3 attenuated the inhibitory activity of ellagic acid on the proliferation of MCF-7 cells. Flow cytometry revealed that ellagic acid induced G0/G1 cell cycle arrest which was mitigated by SIS3. Moreover, SIS3 reversed the effects of ellagic acid on the expression of downstream targets of the TGF-β/Smad3 pathway. In conclusion, ellagic acid leads to decreased phosphorylation of RB proteins mainly through modulation of the TGF-β/Smad3 pathway, and thereby inhibits the proliferation of MCF-7 breast cancer cells.

Keywords: Smad3 - SIS3 - TGF-β/Smad3 pathway - ellagic acid - chemoprevention - MCF-7breast cancer cells

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Introduction

Ellagic acid is a naturally occurring polyphenolic compound present in pomegranate plants, strawberries, grapes, apples, tendril blueberries, blackberries and walnuts. Ellagic acid exhibits a variety of effects such as anti-bacterial, anti-inflammatory, immune regulatory and anti-tumor effects (Chen et al., 2011). In particular, ellagic acid has an inhibitory effect on many tumors, and is currently one of the most promising natural drugs for cancer therapy. However, the molecular mechanisms by which ellagic acid exhibits anti-tumor effects are not completely understood. Uncontrolled cell proliferation and the resistance to apoptosis are important features of cancer (Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646-74). Thus we speculated that ellagic acid may suppress the proliferation of cancer cells and induce apoptosis in cancer cells. In this study we used MCF-7 breast cancer cells as the experimental model and examined the effects of ellagic acid these cells. Based on our preliminary microarray data that ellagic acid regulated TGF-β signaling pathway, we constructed smad3 overexpression plasmids and explored whether ellagic acid inhibited the proliferation of MCF-7 breast cancer cells via the regulation of the TGF-β/Smad3 pathway, which involves the activation of a variety of downstream target molecules such as MEKK1, TAK1, MAPK, PI3K, Ras, RhoA, PP2A and SMADs (Derynck and Zhang, 2003).

Materials and Methods

Cell culture

Human mammary epithelial tumor MCF-7 cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI1640 medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin at 37°C in 5% CO_2. MCF-7 cells in the logarithmic growth phase were passaged every other day at a ratio of 1:6. Smad3 inhibitor SIS3 (cat# 566405) was purchased from Merck and dissolved in DMSO at a final concentration of 2.5 μM. MCF-7 cells were treated with different concentrations of SIS3.

Plasmid construction and transfection

pEGFP-C3 plasmid was provided by the experimental center of the Fourth Affiliated Hospital of Harbin Medical University. pEGFP-C3/Smad3 recombinant plasmid was constructed and sent for sequencing. pEGFP-C3 and pEGFP-C3/Smad3 plasmids were purified by using a

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Table 1. The Effect of Ellagic Acid and SIS3 on the Proliferation of MCF-7 Cells (survival rate%)

SIS3 EA	-	+	-	+	-	+
Concentration (ug/ml)	10		20		30	
MCF-7	83.47±2.68	87.76±2.34	40.31±2.64	64.21±1.89**	16.13±2.17	46.78±2.34**
pEGFP-C3/Smad3	76.09±2.57	83.21±2.55	28.20±2.49*	56.62±2.47***	10.75±2.16*	38.54±2.65***
pEGFP-C3	84.57±2.54	89.88±1.97	41.23±2.88	63.78±2.02**	17.63±1.98	47.02±2.03**

*P<0.05; **P<0.05

Table 2. Effects of ellagic acid and SIS3 on cell cycle progression of MCF-7 cells

SIS3	-	+	-	+	-	+
Cells	MCF-7		pEGFP-C3/Smad3		pEGFP-C3	
G0/G1	62.47±3.87**	54.51±2.47	69.04±2.87***	57.28±2.13	63.36±3.65**	54.90±2.45
S	18.22±3.21	23.02±3.12	14.29±3.41*	22.77±1.96	17.76±4.01*	23.45±2.23
G2/M	19.31±2.89	22.47±2.56	16.67±2.38*	19.95±2.43	18.88±2.34	21.65±3.02

*P<0.05; **P<0.05

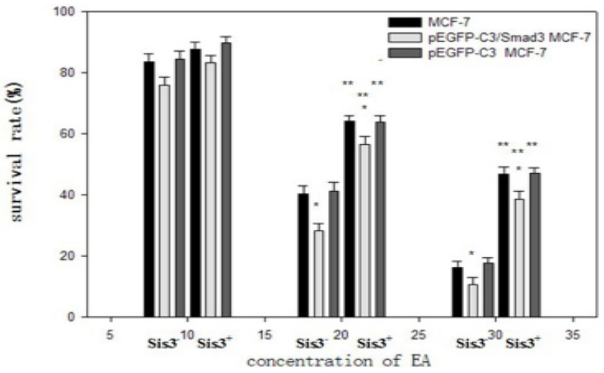

Figure 1. The Survival Rate of Mcf-7 Cells in Different Groups

UNIQ-10 spin column DNA gel extraction kit (Sangon, Shanghai, China) and transfected into MCF-7 cells in the logarithmic phase by using Lipo2000 (Invitrogen) following the manufacturer's protocol.

MTT assay

MCF-7 cells were seeded into 96-well culture plates at a density of 5,000 cells/well and cultured in a humidified chamber at 37˚C overnight. The cells were then treated with SIS3 and viable cells were evaluated with the CCK-8 Assay (Dojindo, Japan) according to the manufacturer's instructions. CCK-8 solution was added to the cells in the 96-well plates, and the cells plates were incubated at 37°C for 4 hours. The optical density of each well was then read at 450 nm using a microplate reader.

Flow cytometry

MCF-7 cells were prepared for flow cytometry to assess the relative distribution of cells in different phases of the cell cycle. Cells were transfected with plasmid and/or treated with SIS3, and then the cells were collected by centrifugation, washed in PBS and fixed overnight at 4°C in 70% ethanol. After being washed twice with PBS, DNA was stained with propidium iodide (50 ug/ml) in the presence of 1 mg/ml RNase A for 30 minutes. Analysis was performed using a BD FACSCanto flow cytometer.

Real-time PCR

Total RNA was isolated from MCF-7 cells using Trizol (Invitrogen). The mRNA expression was quantified by quantitative real-time RT-PCR (qPCR) on an I-Cycler (Bio-Rad, München, Germany) with I-Cycler software (Bio-Rad). Primers were as follows: smad3: ATGGCCGGTTGCAGGTGTC; GGTTCATCTGGTGG TCACTGGTTTC. p21Cip1: TTAGCAGCGGAACAAG GAGT; AGAAACGGGAACCAGGACA. RB1: AAAGG ACCGAGAAGGACCAACT; CAGACAGAAGGCGTT CACAAAGT. GAPDH: GGTGAAGGTCGGAGTCAA CGG; CCTGGAAGATGGTGATGGGATT. PCR amplification parameters were 57°C (melting temperature, Tm) for SMAD3, 52°C (Tm) for P21, and 55°C (Tm) for RB. Each sample was normalized to GAPDH. Statistical analyses were performed by comparing Ct values.

Western blot analysis

MCF-7 cells were collected and total protein was isolated from the cells and quantitated using the BSA method. 50ug of protein was loaded onto a 10% SDS–PAGE and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). Next, the membrane was incubated with specific primary antibodies for P-SMAD3, SMAD3, P21 and RB at 4ºC over night. The membrane was washed with TBST for 5 minutes 3 times, then incubated with secondary antibody for 1 hour at room temperature. The membrane was developed using an ECL kit (Pierce, Rockford, IL, USA) and exposed to X-ray film. Bands on X-ray films were quantified with Image. plus5.1 software. β-actin was used as the loading control.

Results

Ellagic acid inhibits the proliferation of MCF-7 breast cancer cells

MCF-7 breast cancer cells were transfected with pEGFP-C3 or pEGFP-C3/Smad3 plasmid and treated with ellagic acid alone or in combination with SIS3, an inhibitor of Smad3 phosphorylation. MTT assay showed that ellagic acid could inhibit the proliferation of MCF-7 breast cancer cells in a concentration-dependent manner within a 48 hour timeframe. However, concurrent treatment with SIS3 reduced the inhibitory effect of ellagic acid on the proliferation of MCF-7 breast cancer cells in all groups (Table 1 and Figure 1).

Table 3. Real-time PCR Analysis of mRNA Expression of Smad3, P21 and RB in MCF-7 Cells

	MCF-7				pEGFP-C3/Smad3			
SMAD3	1.02±0.13	4.19±0.36*	0.93±0.26	0.93±0.17	1.12±0.22	5.23±0.28*#	1.07±0.13	1.09±0.17
P21	1.04±0.27	3.22±0.39*	0.92±0.18	0.97±0.22	1.07±0.26	3.56±0.26*	1.11±0.19	1.17±0.26
RB	1.05±0.19	0.51±0.12*	1.03±0.28	0.77±0.24*	1.02±0.15	0.42±0.11*#	1.04±0.24	0.63±0.14*#
Ellagic acid	-	+	-	+	-	+	-	+
SIS3	-	-	+	+	-	-	+	+

	pEGFP-C3			
SMAD3	1.03±0.15	3.98±0.27*	0.93±0.15	0.94±0.23
P21	1.06±0.20	3.19±0.29*	0.94±0.23	0.92±0.31
RB	1.02±0.16	0.49±0.21*	1.07±0.26	0.72±0.15*
Ellagic acid	-	+	-	+
SIS3	-	-	+	+

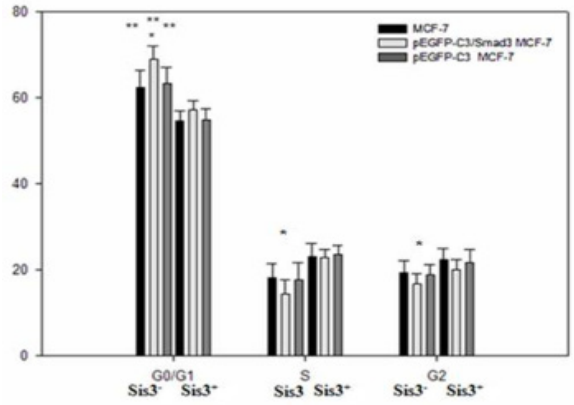

Figure 2. The Distribution of Cell Cycle Phases in Mcf-7 Cells in Different Groups

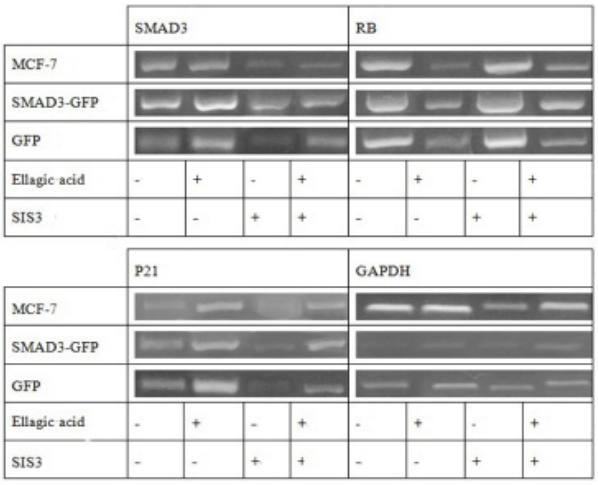

Figure 3. Expression of Genes Involved in the TGF-B/Smad3 Pathway in Mcf-7 Cells after Treatment with Ellagic Acid or Sis3, Either Alone or in Combination. The expression of Smad3, RB and p21 was detected by RT-PCR analysis. GAPDH was the internal control

Ellagic acid regulates cell cycle progression of MCF-7 breast cancer cells

MCF-7 breast cancer cells were transfected with pEGFP-C3 or pEGFP-C3/Smad3 plasmids and treated with ellagic acid alone or in combination with SIS3. Flow cytometry showed that ellagic acid could induce G0/G1 phase arrest of MCF-7 breast cancer cells. However, concurrent treatment with SIS3 relieved G0/G1 phase arrest of the MCF-7 breast cancer cells (Table 2, Figure 2).

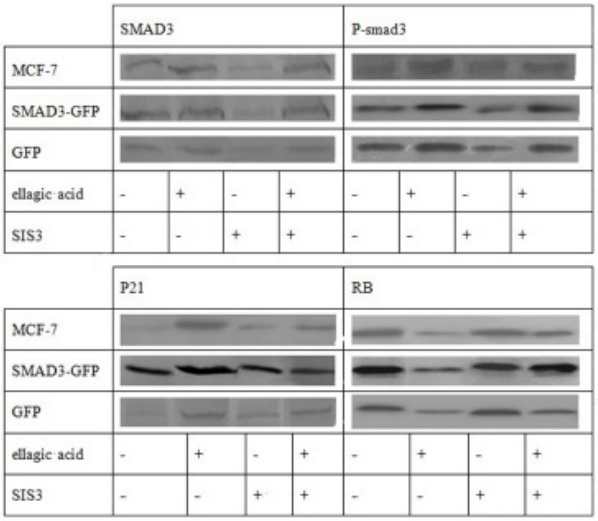

Figure 4. Western Blot Analysis of smad3, p-smad3, p21 and RB Levels in MCF-7 Cells after Treatment with Ellagic acid or SIS3, either Alone or in Combination. β-actin was the loading control

Ellagic acid regulates TGF-β/Smad3 signaling in MCF-7 breast cancer cells

To investigate whether the effects of ellagic acid on the proliferation of MCF-7 cells are mediated by TGF-β/Smad3 signaling, MCF-7 breast cancer cells were transfected with pEGFP-C3 or pEGFP-C3/Smad3 plasmids and treated with ellagic acid alone or in combination with SIS3, and the expression of Smad3, P21 and RB was detected by RT-PCR and real-time PCR analysis. We found that treatment with ellagic acid alone resulted in higher Smad3 and P21 expression and lower RB expression compared to control cells. Treatment with SIS3 alone did not lead to significant changes in the expression of Smad3, P21 or RB in each group. However, combination treatment with ellagic acid and SIS3 resulted in attenuated Smad3 and P21 expression and enhanced RB expression (Figure 3).

In addition, we performed real-time PCR analysis. As shown in Table 3, treatment with ellagic acid alone resulted in higher expression of Smad3 and P21 and lower RB expression. Treatment with SIS3 alone did not lead to significant changes in the expression of Smad3, P21 or RB in each group. Concurrent treatment with ellagic acid and SIS3 led to diminished Smad3 and P21 expression and enhanced RB expression.

Finally, we performed Western blot analysis to detect the protein levels of components of the TGF-β/Smad3 pathway in MCF-7 cells treated with ellagic acid and SIS3 alone or in combination. The results showed that treatment with ellagic acid alone resulted in higher levels of Smad3 and P21 and lower RB protein levels compared to the control cells. Treatment with SIS3 alone did not lead to significant changes in the protein levels of Smad3, P21 or RB in each group. Concurrent treatment with ellagic acid and SIS3 led to lower Smad3 and P21 levels and elevated RB level (Figure 4).

Discussion

In this study we showed that ellagic acid could inhibit the proliferation of MCF-7 breast cancer cells in a dose- and time-dependent manner. Moreover, the inhibitory effect of ellagic acid on the proliferation of MCF-7 cells was attributed to the induction of cell cycle arrest. These findings are consistent with earlier studies. Adams et al. found that blueberry extracts, which were rich in ellagic acid, could modulate the PI3K/AKT/NF kappa B pathway and inhibit the growth and metastasis of MDA-MB-231 breast cancer cells (Adams et al., 2010). Li et al. reported that ellagic acid induced G0/G1 cell cycle arrest and apoptosis in bladder cancer T24 cells (Li et al., 2005). Furthermore, Narayanan et al. (2001) found that ellagic acid induced apoptosis and G1/S cell cycle arrest in SW480 colon cancer cells at a concentration of 5-10 mol/L.

Our preliminary data suggested that ellagic acid could activate the TGF-β/Smad signaling pathway. TGF-β mediated signal transduction involves the activation of a variety of downstream targets, including MEKK1, TAK1, MAPK, PI3K, Ras, RhoA, PP2A and SMADs. By using the smad3 overexpression plasmid, we found that ellagic acid inhibited the proliferation of MCF-7 cells at least partly by activating the TGF-β/Smad signaling pathway, and established that Smad3 functioned as a critical mediator of this pathway. Our study found that Smad3 overexpression induced p21 gene expression. Previous studies have shown that increased expression of p21 could suppress the formation of cyclinD/Cdk4/6 complexes and prevent RB protein phosphorylation. Phosphorylation of RB plays a key role in stabilizing E2F1 and prevents the transition from G1 to S phase (Müller et al., 1997; Yu et al., 2002; Maiti et al., 2005).

In addition, we observed that the Smad3 protein itself had no enzymatic activity, and overexpression of Smad3 alone would not lead to significant inhibition of MCF-7 cell proliferation. Moreover, MCF-7 cells overexpressing Smad3 were more sensitive to ellagic acid. Previous studies have suggested that SMAD mutation and dysfunction plays an important role in the development of tumors (Tian et al., 2003; Leaslc and Abraham, 2004; Xu and Pasche, 2007; Su et al., 2010). Some downstream targets of the TGF-β signaling pathway are key regulators of cell cycle progression, including p21, p27 and p15. The activation of these genes will suppress tumor cell growth (Xu and Pasche, 2007). SIS3 is a specific inhibitor of Smad3 phosphorylation without affecting Smad2 and Smad4 (Jinnin et al., 2006). Our results showed that the expression of the downstream targets of the TGF-β/Smad signaling pathway was suppressed, and the inhibitory effect of ellagic acid on the proliferation of tumor cells was mitigated in SIS3-treated cells. These results suggest that ellagic acid modulates the proliferation of MCF-7 cells via the TGF-β/Smad signaling pathway, and this effect could be attenuated by SIS3.

However, other studies have shown that TGF-β may promote tumor development in later stages. TGF-β up-regulation is associated with angiogenesis, tumor metastasis and poor prognosis of various cancers. Further studies are needed to investigate the consequences of TGF-β/Smad activation induced by ellagic acid in cancer development. These will provide important information on the potential application of ellagic acid in cancer therapy.

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