Casticin inhibits the activity of transcription factor Sp1 and the methylation of *RECK* in MGC803 gastric cancer cells

FAN YANG[1,2], KEFEI HE[1], LI HUANG[3], LINGYAN ZHANG[4], AIXUE LIU[5] and JIREN ZHANG[1]

[1]Department of Oncology, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong 510280; [2]Department of Basic Medicine, Xiangnan University, Chenzhou, Hunan 423000; [3]Department of Oncology, The First Affiliated Hospital of Gannan Medical University, Ganzhou, Jiangxi 341000; [4]Medical Department of Chongqing Bishan People's Hospital, Chongqing 402760; [5]Department of Oncology, The Second People's Hospital of Shenzhen, Shenzhen, Guangdong 518000, P.R. China

Received July 17, 2015; Accepted September 27, 2016

DOI: 10.3892/etm.2016.4003

Abstract. The present study investigated the effect of casticin on reversion-inducing-cysteine-rich protein with kazal motifs (*RECK*) gene expression and intracellular methylation levels in MGC803 gastric cancer cells. Cells were treated with 1, 10 and 30 μmol/l casticin. Western blotting and reverse transcription-quantitative polymerase chain reaction assays were performed to determine the protein expression and mRNA levels of RECK and DNA methyltransferase 1 (DNMT1), respectively. High-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry was used to detect *RECK* methylation. In addition, MGC803 cell proliferation was measured by an MTT assay and the DNA-binding activity of transcription factor Sp1 was determined using an enzyme-linked immunosorbent assay. The results demonstrated that treatment with 1, 10 and 30 μmol/l casticin significantly increased RECK protein expression and mRNA levels. In addition, casticin (30 μmol/l) decreased *RECK* promoter methylation levels by 31%, global DNA methylation levels by 39% and nuclear methylation activity by 71.6%. Furthermore, casticin downregulated the mRNA levels and protein expression of DNMT1. The MTT assay demonstrated that MGC803 cell proliferation was inhibited by casticin treatment and DNA binding assays indicated that casticin reduced the DNA-binding activity of Sp1. The present study therefore indicated that casticin inhibits the proliferation of gastric cancer MGC803 cells by upregulating *RECK* gene expression and reducing intracellular methylation levels.

Correspondence to: Professor Jiren Zhang, Department of Oncology, Zhujiang Hospital, Southern Medical University, 253 Industrial Road, Guangzhou, Guangdong 510280, P.R. China
E-mail: zhangjiren2015@163.com

Key words: gastric cancer, methylation, casticin, reversion-inducing-cysteine-rich protein with kazal motifs, transcription factor Sp1

Introduction

Gastric cancer is the most common tumor of the human digestive system and has relatively high morbidity and mortality rates compared with other types of digestive system cancer (1). Patients with gastric cancer usually exhibit ambiguous symptoms, high degrees of malignancy and early distal metastasis (2). Similar to other malignant tumors, gastric cancer develops by a complex process involving multiple steps and factors, including genetic and phenotypic changes in cells (3).

Epigenetic changes, such as DNA methylation, have attracted increasing attention in the study of tumor occurrence (4). Under normal conditions, DNA methylation is a basic physiological process in biological organisms, which transforms cytosine into 5'-methylcytosine using DNA methyltransferases (DNMTs). Therefore, DNA methylation serves an important role in maintaining chromatin structures, DNA conformation, DNA stability and DNA-protein interactions (5). However, abnormal methylation of DNA in certain pathological conditions may deactivate tumor suppressor genes and activate oncogenes (6,7). The deactivation of tumor suppressor genes, including *RECK* and Ras association domain family 1 isoform A (*RASSF1A*), is closely associated with the occurrence of gastric cancer (8-10). The *RECK* gene, first identified by Takahashi *et al* (11) is located on chromosome 9 and encodes a membrane-anchoring protein that inhibits matrix metalloproteinases (MMPs). MMPs can degrade basal lamina and extracellular matrix, facilitating the metastasis of tumor cells to connective tissues and the vascular wall (3).

It has been demonstrated that the *RECK* gene is expressed in the majority of normal tissues and cell lines, but is either not expressed or expressed at very low levels in tissues or cell lines from tumors including gastric cancer (12). In tissues from pancreatic, colon and breast cancer, levels of *RECK* gene expression are lower than those in adjacent healthy tissue (13). In addition, patients with increased *RECK* gene expression exhibit lower tumor invasion and higher survival rates than those with lower *RECK* gene expression (14). In tumor tissue with low *RECK* gene expression, the promoter region usually exhibits abnormal methylation (15,16). It is widely accepted that the existence of oncogenes is necessary for the occurrence

of cancer, however the deactivation of tumor-suppressor genes may be more common and important than the activation of oncogenes (17). Therefore, the *RECK* gene may suppress the expression of multiple MMPs at post-transcriptional levels and hence inhibit tumor invasion and metastasis.

Casticin is a type of polymethoxylated flavone containing C-3, C-6, C-7 and C-4' methoxy and C-3' and C-5 hydroxy substituents. It has been identified that the C-3 and C-4' methoxy and C-3' and C-5 hydroxy functional groups provide the strong anti-proliferative activity of this flavonoid (18). Casticin exhibits weaker cytotoxicity but greater selectivity than taxol, (the IC_{50} of taxol for tumor cells is at the nmol/l level, while the IC_{50} of casticin is at the μmol/l level) (19). Although casticin has little or no effect on the apoptosis of normal cells and tissues, it is reported to have an inhibitory effect on the proliferation of malignant tumors (20-23). However, it is currently unclear whether casticin affects methylation of the *RECK* gene in gastric cancer cells. The current study aimed to investigate the effect of casticin on the methylation and expression of the *RECK* gene in MGC803 gastric cancer cells.

Materials and methods

Cells and cell culture. Human MGC803 gastric cancer cells (American Type Culture Collection, Manassas, VA, USA) were cultured using 6-well plates (10^4 per well) in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; both Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 μg/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) at 37˚C in 5% CO_2 for 24 h. For stimulation experiments, MGC803 cells were seeded in serum-free medium in 6-well plates ($1x10^5$/well) and cultured overnight. Cells were stimulated with 1, 10 and 30 μmol/l casticin (Sigma-Aldrich; Merck Millipore) for indicative time intervals. Equal volumes of DMSO (Sigma-Aldrich; Merck Millipore) were used as the controls.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Following treatment, total RNA was extracted from the cells and purified using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. RNA was reversely transcribed into cDNA by a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). A total of 2 μg RNA was subject to RT-qPCR using SuperReal PreMix kit (Tiangen Biotech, Co., Ltd., Beijing, China). The primers used were as follows: *RECK*, forward, 5'-ATCATTCCCGTCGATCACTATC-3' and reverse, 5'-ATATGTCCAGAGCAAGTGCAAG-3'; DNA methyltransferase 1 (*DNMT1*) forward, 5'-AACCTTCACCTAGCCCCAG-3' and reverse, 5'-CTCATCCGATTTGGCTCTTCA-3'; β-actin forward, 5'-CATCCTGCGTCTGGACCTGG-3' and reverse, 5'-TAATGTCACGCACGATTTCC-3'. PCR amplification was performed on an ABI Prism® 7700 (Applied Biosystems; Thermo Fisher Scientific, Inc.) as follows: 5 min at 95˚C; 40 cycles of 30 sec at 95˚C, 30 sec at 60˚C and 1 min at 72˚C. The $2^{-\Delta\Delta Ct}$ method (24) was used to calculate the relative levels of target mRNAs and β-actin was used as a reference gene.

Western blotting. Following treatment, cells were centrifuged at 1,000 x *g* and 4˚C (Centrifuge 5418R; Eppendorf, Hamburg, Germany) for 5 min and supernatants were subsequently discarded. Following two washes with ice-cold phosphate-buffered saline, cells were re-suspended using 200 μl cell lysis buffer (P0013; Beyotime Institute of Biotechnology, Shanghai, China) for lysis on ice for 40 min. Following centrifugation at 1,000 x *g* and 4˚C for 15 min, supernatants were collected. Protein samples were mixed with 2X sodium dodecyl sulfate (SDS) loading buffer and boiled for 5 min to induce denaturation. A total of 80 μg protein was resolved using 12% SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes (Sigma-Aldrich; Merck Millipore) under 30 V at 4˚C. Following blocking at 4˚C and washing with Tris-buffered saline with Tween-20 (TBST) for 30 min, the membranes were incubated with polyclonal rabbit anti-human RECK (1:400; sc-28918) or DNMT1 antibodies (1:400; sc-20701) or rabbit anti-β-actin antibodies (1:1,000; sc-7210; all Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4˚C for 8 h. After washing with TBST for 30 min, the membrane was incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin G antibody (1:5,000; sc-2054; Santa Cruz Biotechnology, Inc.) for 4 h at room temperature. After washing with TBST for 30 min, bands were visualized by enhanced chemiluminescence (sc-2048; Santa Cruz Biotechnology, Inc.). The level of target protein expression in each sample was determined by normalizing protein band intensity to β-actin band intensity using ImageJ analysis software (version 1.46; National Institutes of Health, Bethesda, MD, USA). Tests were performed in triplicate.

Methylation activity determination. Following treatment with casticin, MGC803 cells ($1x10^7$) underwent genomic DNA extraction and purification using the DNeasy Blood & Tissue kit (Qiagen GmbH, Hilden, Germany). A total of 200 ng genomic DNA was denatured at 100˚C for 3 min and placed on ice for cooling. Afterwards, 1/10 (volume ratio) ammonium acetate (0.1 mol/l, pH 5.3) and 2 units nuclease P1 were added. After 2 h incubation at 45˚C, 1/10 (volume ratio) NH_4HCO_3 (1 mol/l) and 0.002 units venom phosphodiesterase I were added prior to incubation at 37˚C for 1 h. Subsequently, 0.5 units alkaline phosphatase were added, followed by further incubation at 37˚C for 1 h. An EpiQuik DNMT Activity/Inhibition Assay Ultra Kit (EpiGentek, Farmingdale, NY, USA) was used to determine the methylation activity of nucleoprotein in MGC803 cells, following the manufacturer's protocol. For high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) analysis of a 10-μl sample, an Agilent 1100 series HPLC system was used (Agilent Technologies, Inc., Santa Clara, CA, USA). The Atlantis™ dC_{18} column (inner diameter, 2.1 mm; length, 150 mm; particle size, 3 μm) was purchased from Waters Corporation (Milford, MA, USA) and the mobile phase was 0.1% formic acid-methanol with a flow rate of 0.2 ml/min. The mode of electrospray ionization was positive ions, with the following conditions: Scanning range, 100-2,000 m/z; ion source temperature, 450˚C; electrospray voltage, 415 kV; cluster breaking voltage, 55 V; entrance voltage, 6 V; collision energy, 13 V; air curtain pressure, 138 kPa; gas 1 pressure, 221 kPa; gas 2 pressure, 379 kPa; collision gas pressure, 41 kPa (25). Analyst Software version 1.3.1 (AB SCIEX, Framingham, MA, USA) was used for data analysis.

Table I. Effects of casticin on promoter methylation level, global DNA methylation level and methylation activity in nuclear extracts.

Group	Promoter methylation (% of control)	Global DNA methylation (% of control)	Methylation activity in nuclear extracts (activity unit)
Control	100	100	8.46±1.08
CAS (1 μmol/l)	87.14±6.31[a]	86.81±10.41[a]	6.06±1.36[a]
CAS (10 μmol/l)	77.56±5.47[a]	71.38±4.22[a]	3.2±1.24[b]
CAS (30 μmol/l)	69.04±10.62[b]	61.13±7.08[b]	2.8±1.32[b]

[a]P<0.05; [b]P<0.01 vs. control. CAS, casticin.

MTT assay. MGC803 cells (100 μl) were seeded onto 96-well plates (~3x10^3 cells per well) containing 100 μl RPMI-1640 medium supplemented with 5% fetal bovine serum (both Thermo Fisher Scientific, Inc.). On the second day, the medium was changed to RPMI-1640 supplemented with 5% FBS, 1% penicillin/streptomycin and 1, 10 or 30 μmol/l casticin (98% purity; Sigma-Aldrich; Merck Millipore) prior to incubation for 72 h. Untreated cells served as the control. A total of 4 h before the end of incubation, 20 μl MTT (5 mg/ml; Sigma-Aldrich; Merck Millipore) was added. Supernatants were subsequently discarded and 200 μl dimethylsulfoxide was added to each well before shaking gently for 10 min to dissolve crystals. The absorbance of each well was measured at 570 nm using a Synergy HT microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The following formulae were used to calculate the survival and inhibitory rates of the tumor cells. Survival rate of tumor cells (%) = (absorbance of treatment wells / absorbance of control well) x 100. Inhibitory rate of tumor cells (%) = (absorbance of control well - absorbance of treatment wells) / absorbance of control well x 100. Tests were performed in triplicate.

Enzyme-linked immunosorbent assay (ELISA). MGC803 cells were left untreated or treated with 1-30 μmol/l casticin and nuclear protein was extracted using a NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce; Thermo Fisher Scientific, Inc.). Nuclear protein concentrations were determined with the Bradford assay using the Coomassie Plus reagent (Thermo Fisher Scientific, Inc.). A total of 15 μg nuclear protein from each treatment group was analyzed for Sp1 activity using the Transcription Factor ELISA kit (Affymetrix, Inc., Santa Clara, CA, USA). Sp1 antibody was used as the primary antibody and anti-rabbit IgG HRP was used as secondary antibody, which were provided in the kit. The absorbance was measured at a wavelength of 450 nm on a spectrophotometer (BioTek Instruments, Inc.).

Statistical analysis. The results were analyzed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation. Two groups of mean values were compared using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Casticin increases the mRNA levels and protein expression of RECK in MGC803 cells. To determine the effect of casticin on the mRNA levels and protein expression of RECK, RT-qPCR and western blotting were performed, respectively. Western blot analysis indicated that the RECK protein band in the control group was relatively thin, whereas treatment with 1, 10 or 30 μmol/l casticin significantly increased band thickness (P<0.05; Fig. 1A). Results from RT-qPCR showed that treatment with 1, 10 or 30 μmol/l casticin also significantly increased *RECK* mRNA levels in a dose-dependent manner (P<0.05; Fig. 1B). These results suggest that casticin increases RECK mRNA levels and protein expression in MGC803 cells.

Casticin reduces RECK promoter methylation level, global DNA methylation level and nuclear methylation activity. To detect the effect of casticin on methylation, the EpiQuik DNMT kit was used. The results demonstrated that casticin decreased *RECK* promoter methylation level, global DNA methylation level and methylation activity in nuclear extracts in a dose-dependent manner. Treatment with 30 μmol/l casticin reduced *RECK* promoter methylation level by 31%, global DNA methylation level by 39% and nuclear methylation activity by 71.6% (Table I). These results indicate that casticin reduces the *RECK* promoter methylation level, global DNA methylation level and nuclear methylation activity.

Casticin downregulates the mRNA levels and protein expression of DNMT1 in MGC803 cells. RT-qPCR and western blotting were performed to measure the DNMT1 mRNA and protein expression levels, respectively. Western blot analysis demonstrated that the DNMT1 protein band in the control was relatively thick, whereas treatment with 1, 10 or 30 μmol/l casticin significantly decreased band thickness (P<0.05; Fig. 2A). Quantification of mRNA levels and protein expression indicated that treatment with 1, 10 or 30 μmol/l casticin significantly reduced levels of DNMT1 mRNA and protein in a dose-dependent manner (P<0.05; Fig. 2B). These results suggest that casticin downregulates the levels of DNMT1 mRNA and protein in MGC803 cells.

Casticin inhibits the proliferation and DNA-binding activity of transcription factor Sp1 in MGC803 cells. To investigate the effect of casticin on MGC803 cell proliferation and the DNA-binding activity of Sp1 transcription factor, MTT assay and ELISA were performed, respectively. The MTT assay demonstrated that the proliferation rates of MGC803 cells following treatment with 10 and 30 μmol/l casticin were 44

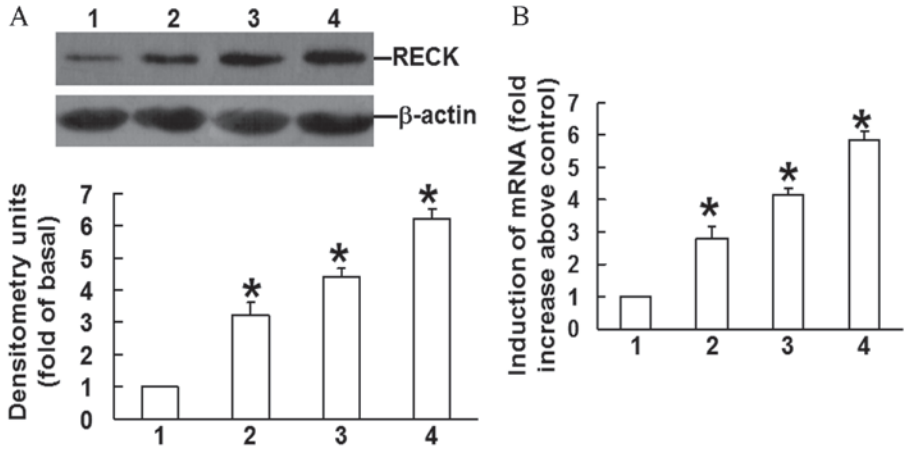

Figure 1. Effect of casticin on RECK mRNA and protein expression in MGC803 cells. (A) RECK protein expression detected by western blotting and quantification of protein expression. (B) Quantification of *RECK* mRNA expression by reverse transcription-quantitative polymerase chain reaction. Data are expressed as mean ± standard deviation. *P<0.05 vs. control. 1, control; 2, treatment with 1 μmol/l casticin; 3, treatment with 10 μmol/l casticin; 4, treatment with 30 μmol/l casticin. RECK, reversion-inducing-cysteine-rich protein with kazal motifs.

and 23% of that in the control, respectively (P<0.05; Fig. 3A). Furthermore, the results from ELISA indicated that treatment with casticin (1, 10 and 30 μmol/l) reduced the DNA-binding activity of Sp1 in MGC803 cells (P<0.05; Fig. 3B). These results suggest that casticin inhibits the proliferation and DNA-binding activity of transcription factor Sp1 in MGC803 cells.

Discussion

RECK is a tumor suppressor gene that exists in multiple tissues and cells and regulates cell proliferation. In gastric, breast, esophageal and pancreatic cancer cells, *RECK* gene expression is low and usually accompanied by abnormal methylation (26,27). The present study demonstrated that casticin upregulated RECK mRNA and protein expression in MGC803 gastric cancer cells. The activation of RECK was found to be associated with reduced methylation of its promoter region. In addition, the present study demonstrated that casticin inhibits DNMT1 expression in MGC803 cells and affects the DNA-binding activity of Sp1. This may subsequently inhibit the methylation of *RECK*, thus inhibiting the proliferation of gastric cancer cells. Similarly, it has been determined in previous studies that some tumor suppressor genes silenced by supermethylation, including *GSTP1* and *MGMT*, are re-activated by certain exogenous drugs (28,29).

Epigenetic chemotherapy is a type of novel therapeutic strategy for cancer that has been developed in recent years (30). In eukaryotic transcriptional regulation, DNA methylation is an important epigenetic mechanism and changes in methylation patterns may promote the occurrence of tumors. It has been over 40 years since abnormal DNA methylation was first identified in tumor cells (31). Hypomethylation and gene-specific hypermethylation have since been observed in all detected tumor types (32). Currently, DNA demethylation drugs including decitabine and azacytidine are clinically used to treat myelodysplastic syndrome (33). However, the clinical application of epigenetic chemotherapy in solid tumors such as gastric tumors requires further investigation. Although the toxicity of taxol in tumor cells is higher than that of casticin, it is less selective than casticin (34). Casticin causes no toxicity in healthy cells and tissues (23); however it significantly inhibits the proliferative activity of numerous malignant tumor cells (20). As a type of demethylation drug, casticin may become a novel preventive means for gastric cancer, due to its few adverse reactions.

In the current study, the activity of transcriptional factor Sp1 was analyzed, in order to further investigate the possible mechanism of action of casticin. Sp1 is the most important transcriptional factor in the Sp family, because it is the most relevant to methylation among all Sp members. Notably, Sp1 specifically binds to the GC box (GGGGCGGGG) or GT box (GGTGTGGGG) in target genes, resulting in the activation of transcription (35). Sp1 promotes the expression of a number of molecules that positively regulate cell cycles and affects the methylation of the DNA CpG island (36). In numerous tumor tissues, Sp1 expression is high and its activity is positively correlated with the degree of tumor invasion, but negatively correlated with patient prognosis (37,38).

The present study demonstrated that Sp1 activity in MGC803 cells in the control group was high, but significantly decreased following treatment with different concentrations of casticin. There are Sp1 binding sites on DNMT1 (39) and Yu *et al* (40) observed that curcumin inhibits DNMT1 expression, possibly by inhibiting Sp1 activity. Similar results were observed by Du *et al* (41). Yie *et al* (42) also demonstrated that overexpression of Sp1 in live cancer cells promotes the expression of DNMT1. Therefore, the inhibition of Sp1 by casticin may lead to the reduced activity of DNMT1 and an inhibitory effect on methylation. However, further studies are necessary to elucidate whether casticin exerts its inhibitory effect on DNMT1 activity via Sp1.

In recent years, demethylation treatment has become an important method of tumor therapy. For example, 5-Aza-2'-deoxycytidine has been applied clinically in the treatment of myelodysplastic syndrome (43). In the present study, we demonstrates that casticin inhibits the proliferation of gastric cancer MGC803 cells by upregulating *RECK*

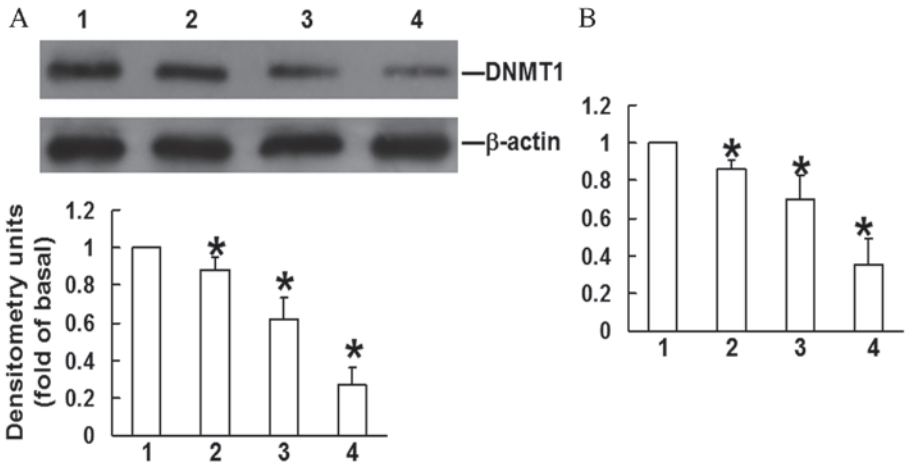

Figure 2. Effect of casticin on DNMT1 mRNA and protein expression in MGC803 cells. (A) DNMT1 protein expression detected by western blotting and quantification of protein expression. (B) DNMT1 mRNA levels were determined by reverse transcription-quantitative polymerase chain reaction and quantified. Data are expressed as mean ± standard deviation. *P<0.05 vs. control. 1, control; 2, treatment with 1 μmol/l casticin; 3, treatment with 10 μmol/l casticin; 4, treatment with 30 μmol/l casticin. DNMT1, DNA methyltransferase 1.

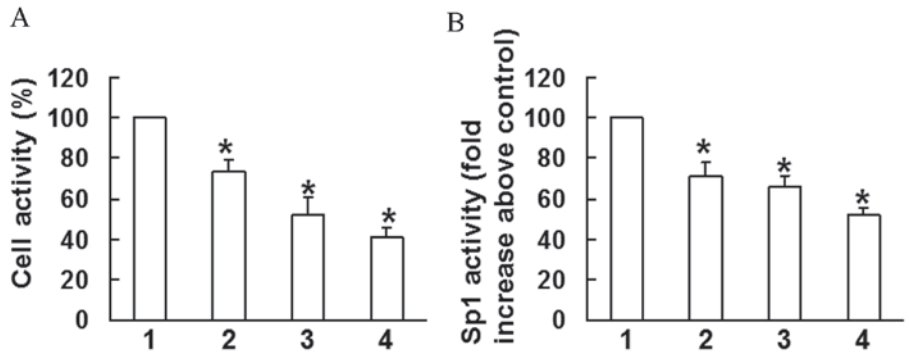

Figure 3. Anti-proliferative activity and Sp1 DNA-binding activity of casticin in MGC803 cells. (A) Cell metabolic activity determined by MTT assay. (B) DNA-binding activity of Sp1 determined by ELISA. *P<0.05 vs. control. 1, control; 2, treatment with 1 μmol/l casticin; 3, treatment with 10 μmol/l casticin; 4, treatment with 30 μmol/l casticin.

gene expression and reducing intracellular methylation levels. Casticin is originated from natural plant, and its toxicity is relatively low. Therefore, casticin may have more extensive application where 5-aza-2'-deoxycytidine has little effect. Further research is required to investigate the effect of casticin on healthy cells and homeostasis, and the toxicity of casticin, in order to determine whether casticin may be developed as a novel methylation inhibitor.

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