Brief Report

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Simvastatin downregulated C35 expression and inhibited the proliferation of colon cancer cells Lovo and HT29 *in vitro*

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Summary **The aim of this study was to investigate the antitumor effect of simvastatin in human colon cancer and the possible underlying mechanism. We found that simvastatin dose-dependently inhibited the proliferation of human colon cancer cells Lovo and HT29 using a MTT assay. Real-time PCR and Western blotting assays showed that simvastatin significantly suppressed C35 expression at both mRNA and protein levels. Since C35 is known to have a significant oncogenic role in cancer development *via* promoting cell proliferation and migration, results obtained in the current study imply that downregulation of C35 expression might be involved in the antitumor effect of simvastatin on colon cancer.**

Keywords: Colon cancer, C35, simvastatin, mevalonate, HMG-CoA

1. Introduction

Colon cancer is one of the leading cancer deaths worldwide (*1*). Chemotherapy drugs including cytotoxic agents such as irinotecan and capecitabine and targeted drugs such as cetuximab and panitumumab are usually recommended for advanced colon cancer, however, most of the patients die from metastatic diseases eventually (*1*). Clarification of mechanisms underlying the initiation and progression of colon cancer and thus development of novel targeted drugs may lead to better treatment of this disease.

Statins such as hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors, are commonly used to treat hyperlipidemia and show benefits of reducing cardiovascular events (*2*). Studies in recent years showed that this type of drug also has anti-tumor effects through inhibiting cell proliferation, inducing cell differentiation, or soliciting cell apoptosis (*3,4*). In colon cancer, it was reported that simvastatin induced apoptosis in human colon cancer cells and in tumor xenografts, and attenuated colitis-associated colon cancer in mice (*5*). Simvastatin could synergize with irinotecan in suppressing growth of colon cancer cells as well (*6*). In a colorectal cancer epidemiology survey, it was found that long-term treatment with statins reduced the prevalence of colorectal cancer by 47% (*7*). These studies reveal the usefulness of simvastatin against colon cancer. However, the antitumor mechanisms of this drug have not yet been fully elucidated.

C35 (also termed C17orf37) is a novel tumor biomarker abundantly expressed in breast cancer (*8*). Our previous studies demonstrated that C35 was also overexpressed in colorectal cancer and correlated with tumor serosal invasion, lymph node metastasis, and an advanced Dukes stage (*9*), implying C35 might serve as a potential biomarker for colorectal cancer. The conserved canonical immunoreceptor tyrosine-based activation (ITAM) motif located in its C-terminal end and the last four amino acids CVIL of C35 were proved to have an important role in cancer progression and metastasis (*10,11*), suggesting C35 functions as an oncogene and thus can serve as a potential therapeutic drug target. It is worth noting that the maturation of C35 involves polyisoprene groups such as farnesyl and geranylgeranyl which are dependently generated on

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HMG-CoA (*12*). We speculate that simvastatin may suppress the maturation and expression of C35 and thus inhibit the proliferation of colon cancers. This hypothesis was explored in the present study.

2. Materials and Methods

2.1. *Agents*

Simvastatin was obtained from Lukang Pharmaceutical Co., Jining, Shandong, China.

2.2. *Cell lines and cell culture*

Human colon cancer cell lines Lovo and HT-29 were obtained from the cell bank of Chinese Academy of Sciences. Cells were maintained in Dulbecco Modified Eagle Medium (DMEM, Hyclone, Thermo scientific, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Evergreen Biotechnology, Hangzhou, Zhejiang, China) at 37°C in a humid atmosphere (5% CO_2-95% air).

2.3. *Cell proliferation assay*

Cells (5×10^3 per well) seeded in 96-well plates were exposed to simvastatin for a specified time. Then the medium was removed and the wells were washed with PBS. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by adding 20 μL of MTT (5 mg/mL, Sigma, USA) for 4 h. Light absorbance of the solution was measured at 570 nm on a microplate reader (Perkin-Elmer, USA).

2.4. *Real-time PCR*

Total RNA was extracted with Trizol reagent (Invitrogen, USA), and reverse-transcribed with oligo-dT using a reverse transcription kit (TaKaRa, Dalian, Liaoning, China) according to the manufacturer's protocol. Quantitative real time PCR was carried out by monitoring the increase in fluorescence of SYBR green (SYBR Green Kit, Genstar, Beijing, China). The primer sets were synthesized by Sangon Biotech (Shanghai, China). C35: upstream primer 5'-GCCATCCGAAGAGCCAGTA-3'; downstream primer 5'-ATTACCGAGGCGAAGAGTGG-3'. GAPDH: upstream primer 5'-GACTCACCCTGCCCTCAATA-3'; downstream primer 5'-CCCTGTAGCCTGGACCTGAT-3'. PCR reaction conditions: denaturation at 95°C for 10 min, denaturation at 95°C for 15 sec, annealing at 60°C for 15 sec, extension at 72°C for 35 sec, for a total of 40 cycles. Each sample was amplified in triplicate for quantification. Data were analyzed by relative quantitation using the $\Delta\Delta C_t$ method and normalized to GAPDH.

2.5. *Western blotting*

Cells seeded in 6-well plates were exposed to simvastatin for a specified time period. Cells were harvested and cell lysates (30 μg of protein per lane) were fractionated by 10% SDS-PAGE. The proteins were electro-transferred onto nitrocellulose membrane and the protein levels were detected using the primary antibodies against XTP4 and GAPDH (Abcam, Cambridge, England) with appropriate dilution. The bound antibodies were visualized using an enhanced chemiluminescence reagent and quantified by densitometry using ChemiDoc XRS+ image analyzer (Bio-Rad, Hercules, CA, USA). Densitometric analyses of bands were adjusted with GAPDH as loading control. The percentages of increase or decrease of protein were estimated by comparison to the vehicle control (100%).

2.6. *Statistical analyses*

Data was expressed as mean ± S.D. for three different determinations. Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple range tests for the MTT assay and by Mann-Whitney *U* test for real-time PCR and Western blotting assays. $p < 0.05$ was considered as statistically significant. Statistical analysis was performed using the SPSS/Win 17.0 software (SPSS, Inc, Chicago, IL, USA).

3. Results and Discussion

We first examined the effect of simvastatin on cell proliferation of Lovo and HT29 cells. Cells were incubated with increasing concentrations of simvastatin (10, 20, 40, 60, and 80 μM) and subjected to MTT assay at 24, 48, 72, and 96 h, respectively. Results showed that simvastatin inhibited proliferation of Lovo and HT29 cells in a both time- and dose-dependent manner (Figure 1). IC_{50} values of simvastatin determined at 96 h were 45.6 and 33.7 μM, respectively, for Lovo and HT29 cells. Maximum inhibition rates on Lovo and HT29 cells were determined to be 69.8 and 89.7%, respectively, at 96 h by 80 μM simvastatin. These results suggested that simvastatin has certain antitumor effects on colon cancer.

We next determined the effect of simvastatin on C35 expression at both mRNA and protein levels. Lovo and HT29 cells were exposed to 80 μM simvastatin for 48 h. C35 mRNA and protein expression were examined by real-time PCR and Western blotting assays, respectively. Results demonstrated that the mRNA level of C35 was significantly lower in Lovo ($2^{-\Delta\Delta ct} = 0.08 \pm 0.0022$) and HT29 ($2^{-\Delta\Delta ct} = 0.05 \pm 0.0053$) cells exposed to simvastatin than that in cells treated with vehicle (Figure 2A). We found that C35 protein expression was also obviously downregulated in Lovo and HT29 cells

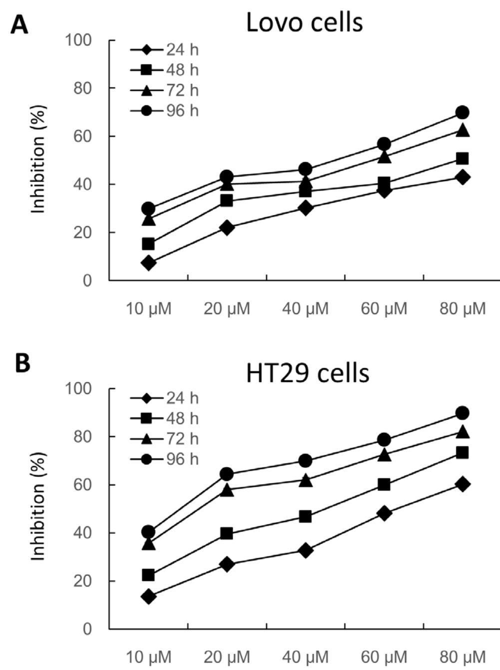

Figure 1. Simvastatin dose- and time-dependent inhibition of proliferation of Lovo (A) and HT29 cells (B). Cells were incubated with increasing concentrations of simvastatin for 96 h and subjected to MTT assay at an interval of 24 h.

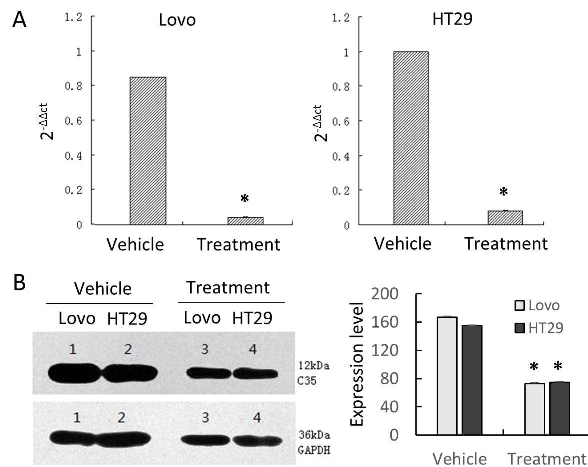

Figure 2. Simvastatin inhibited C35 expression at both mRNA (A) and protein (B) levels in Lovo and HT29 cells. Cells were incubated with 80 μM simvastatin for 48 h and then subjected to real-time PCR and Western blotting assays, respectively. $p < 0.05$ *vs*. vehicle control.

exposed to simvastatin compared to control cells (Figure 2B). These results suggested that simvastatin is capable of inhibiting the expression of C35 at both mRNA and protein levels in Lovo and HT29 cells.

The present study provides a possible mechanism underlying the antitumor effect of simvastatin. Previous studies showed that C35 has a significant oncogenic role in cancer, promoting cell growth, cell migration, and transformation, and that knockdown of C35 inhibited cell motility and cell growth (*13*). Therefore, it is reasonable that downregulation of C35 might be involved in the effect of simvastatin in suppressing the proliferation of Lovo and HT29 cells. Sequencing of C35 revealed a 'CaaX' prenylation motif consisting of the last four amino acids, 'CVIL,' at the C-terminal end (*10*). The 'CaaX' group of proteins are known to be farnesylated by the enzyme farnesyltransferase or geranylgeranylated by the enzyme geranylgeranyl transferase type I (GGTase-I) (*14*). This post-translational modification of the C-terminal prenylation domain of C35 is essential for its membrane association, which facilitates the induction of filopodia formation (*15*). Because farnesyl and geranylgeranyl are generated from mevalonate (MVA) which is synthesized by HMG-CoA (*16*), inhibition of HMG-CoA will reduce the production of MVA and in turn decrease the generation of farnesyl and geranylgeranyl, which finally influences the maturation and expression of C35. These studies provide an explanation of the effect of simvastatin in reducing the expression of C35 in Lovo and HT29 cells.

In conclusion, the present study found that HMG-CoA inhibitor simvastatin inhibited the proliferation of colon cancer cells Lovo and HT29, which might be associated with the activity of simvastatin in suppressing C35 expression. Further detailed mechanisms underlying the anticancer effect of simvastatin is currently undergoing investigation.

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