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Simvastatin induces apoptosis of nasopharyngeal carcinoma cells through NF-κB signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to investigate the mechanism of simvastatin-induced apoptosis in nasopharyngeal carcinoma (NPC) cells.

MATERIALS AND METHODS: CNE1 and HK1 cell lines were treated with different concentrations of simvastatin for different time course. Subsequently, Cell Counting Kit-8 (CCK-8), colony formation assay, and flow cytometry were conducted to evaluate cell activity, colony formation ability, as well as cell cycle of NPC cells, respectively. The mRNA expressions of p21, Bim, and cyclin D1 were examined by qPCR. Meanwhile, the protein expression levels of apoptosis-related proteins (including caspase-3, Bax, Bcl-2) were detected by Western blot. Caspase-3 activity was determined to estimate cell apoptosis. An NPC xenotransplantation model was constructed to further determine the role of simvastatin *in vivo*. In addition, NF-κB activity was assessed through Luciferase reporter gene assay and Western blot.

RESULTS: Simvastatin treatment lead to significantly reduced viability of NPC cells and the number of cell colonies dose-dependently and time-dependently. Meanwhile, simvastatin treatment caused cell cycle arrest in G0/G1 phase, remarkably downregulated expression of cyclin D1, and upregulated expressions of p21 and Bim. In addition, simvastatin induced apoptosis of NPC cells and enhanced the Luciferase activity of caspase-3. Western blot results indicated that simvastatin promoted the protein level of Bax and caspase-3, whereas suppressed the protein expression of Bcl-2. *In vivo* experiments showed that simvastatin was able to suppress the growth of NPC cells. Further studies demonstrated that simvastatin remarkably attenuated the Luciferase activity of pNF-κB-Luc, thereby specifically inhibiting the NF-κB signaling pathway.

CONCLUSIONS: Simvastatin inhibits proliferation and promotes apoptosis of NPC cells by inhibiting the NF-κB pathway.

Key Words:
Simvastatin, Nasopharyngeal carcinoma (NPC), NF-κB, Apoptosis.

Introduction

Nasopharyngeal carcinoma (NPC) is a tumor originated from the mucous epithelium of the nasopharynx. Due to its insidious growth and early lymph node metastasis, NPC is difficult to be diagnosed at the early stage. Therefore, it poses a serious threat to human life and health[1]. Currently, the postoperative radiotherapy has achieved great improvements. However, the survival rate of NPC patients within 5 years after surgery is only 50%-70%. This is due to the high incidence of recurrence or metastasis or serious complications caused by radiotherapy or chemotherapy drugs. All these outcomes may reduce the clinical efficacy[2,3]. Therefore, searching for drugs with high efficiency, low toxicity, and enhanced sensitivity of radiotherapy and chemotherapy is one of the keys to improve the survival of NPC patients.

Statins are currently the first-line drugs for hyperlipidemia; they possess inhibition effects on the growth of many tumors, including breast cancer, melanoma, colon cancer[4-6], pancreatic cancer, and bladder cancer by inhibiting cell differentiation, proliferation, apoptosis, and

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reducing capillary generation, invasion, and metastasis. As one of the most classic anti-hyperlipidemia drugs, simvastatin exerts good inhibitory effects on 3-hydroxy-3-methylglutaryl coenzyme A reductase. It can effectively reduce LDL cholesterol in patients with cardiovascular diseases[7]. Additionally, Saito et al[8] have reported that simvastatin inhibits human melanoma cell proliferation, blocks cell cycle, and induces cell apoptosis. Ma et al[9] have demonstrated that simvastatin can inhibit the growth of NPC cells. However, the specific mechanism of simvastatin action on NPC cells remains to be fully elucidated.

Nuclear factor-κB (NF-κB) is a highly conserved family of important transcription factors. It was first reported by Sen and Baltimore in 1986[10]. Hence, NF-κB was named for its involvement in the regulation of B cell kappa light chain expression. NF-κB forms a homologous or heterodimeric dimer that functions as a transcriptional function. Among them, p65 and p50 are the two most important subunits[10]. It has been confirmed that NF-κB activation is regulated by two pathways, including: the IκB-dependent classical pathway and the non-canonical pathway. In the classical pathway, NF-κB inhibitory protein (inhibitor of NF-κB, IκB) is phosphorylated and ubiquitinated by exogenous stimuli, and dissociated from p50/p65 heterodimer/IκBα complex. After that, it is trans-located into the nucleus through the nuclear membrane and binds to the κB binding site composed of 9-11 nucleotides of the target gene promoter region. Eventually, this may induce the transcription of the target genes[11]. The activation of apoptosis-related pathways during tumor genesis is one of the main factors leading to various malignancies[12,13]. Meanwhile, NF-κB signaling pathway is engaged in the regulation of cell apoptosis[14-17]. In addition, Ouyang et al[18] have demonstrated that NF-κB pathway plays a pivotal part in NPC development and progression. However, whether simvastatin affects the progression of NPC through NF-κB still remains elusive.

Therefore, in this study, we explored the anticancer effect of simvastatin on NPC both *in vitro* and *in vivo*. At the same time, the influence of simvastatin on the phosphorylation of p65 was explored to figure out its relationship with NF-κB signaling pathway. Our findings might help to elucidate the mechanism of simvastatin-induced apoptosis in NPC cells.

Materials and Methods

Cell Culture

NPC cells (CNE1 and HK1) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin in an incubator with 5% CO_2 at 37°C. Simvastatin was obtained from Hangzhou MSD Pharmaceutical Company (Hangzhou, China).

Cell Counting Kit-8 (CCK-8) Assay

After incubation with 5, 10, or 20 μM of simvastatin for 24 hours, cell viability was evaluated according to the instructions of CCK-8 assay (Dojindo Molecular Technologies, Kumamoto, Japan).

Colony Formation Assay

An appropriate number of cells were treated with simvastatin for 24 hours and then maintained in the fresh medium for 10-14 days. Subsequently, cell colonies were fixed with 4% paraformaldehyde, and stained with 1% crystal violet dye. Finally, formed colonies were observed under a microscope, and the number of colonies was counted.

Flow Cytometry

Cells were first trypsinized, centrifuged, and fixed with 75% cold ethanol (2 mL). Subsequently, the cells were added with Propidium Iodide (PI) solution and Rnase A, followed by incubation in the dark for 15 minutes at room temperature. Cell cycle was finally analyzed using a flow cytometer (FACSCalibur; BD Biosciences, Detroit, MI, USA).

Western Blot

Simvastatin-treated CNE1 cells were harvested and lysed with radio-immunoprecipitation assay (RIPA; Beyotime, Shanghai, China). Equal amounts of total protein were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibodies overnight. On

the next day, the membranes were incubated with horse radish peroxidase (HRP)-conjugated secondary antibody. Immuno-reactive bands were finally exposed by enhanced chemiluminescent substrates (ECL) *via* ImageQuant LAS 4000.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from NPC cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using a reverse transcriptase kit. QRT-PCR was performed to determine the expression of target gene. The primer sequences used in this study were as follows: cyclin D1: 5'-GCGAGAACAGAAGTGCG-3'(F), 5'-TGGAGTTGTCGTGTAGATGC-3' (R); P21: 5'-CCATCGGAATATGTACCGACTG-3' (F), 5'-CTCAGCGGTCGTAATCTGTCA-3' (R); Bim: 5'-CATATAACCCCGTCAACGCAG-3' (F), 5'-GCAGCCGCCACAAACATAC-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5'-GCACCGTCAAGGCTGAGAAC-3' (F), 5'-TGGTGAAGACGCCAGTGGA-3' (R).

Caspase-3 Activity Assay

Caspase-3 assay kit was used to evaluate the activity of Caspase-3-like protease in CNE1 cells. The value of optical density (OD) 405 relative to the control was finally calculated.

Luciferase Reporter Gene Assay

CNE1 cells were first seeded into 6-well plates. Luciferase reporter gene or empty vector driven by the NF-κB response element (pNFκB-LUC) was transfected into CNE1 cells together with the internal control plasmid by Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA). Luciferase activity was finally determined in accordance with Luciferase assay kit from Promega Corporation (Promega, Madison, WI, USA).

Allograft Study

Female nude mice were purchased from Shanghai SLAC Experimental Animal Co. (Shanghai, China). Human NPC cells CNE1 (8 x 10^6 cells/site) were subcutaneously injected to the right side of mice. When the tumor became palpable, the mice were randomly divided into two groups. One group received daily subcutaneous injections of simvastatin (20 μg/g body weight/day), while the other group received treatment with vehicle control for two consecutive weeks. All mice were closely observed daily, and tumor size was measured weekly by calipers. This study was approved by the Animal Ethical Committee of Linyi Central Hospital Animal Center.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. Experimental data were expressed as mean ± SD (standard deviation). Differences between two groups were analyzed by using the Student's *t*-test. One-way ANOVA was performed to compare the differences among different groups, followed by post-hoc test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

Simvastatin Inhibits NPC Cell Proliferation and Colony Formation Ability

To explore the influence of simvastatin on NPC cells, CCK-8 assay was conducted to examine the viability of NPC cells treated with different doses of simvastatin. The results showed that cell viability was markedly inhibited with increased concentration of simvastatin and prolonged treatment time (Figure 1A). Subsequently, colony formation assay indicated that the number of colonies formed by CNE1 and HK1 cells remarkably decreased as the concentration of simvastatin increased (Figure 1B). All these results indicated that simvastatin might be capable of suppressing the proliferative ability of NPC cells.

Simvastatin Blocks NPC Cell Cycle and Induces Apoptosis

Further, we investigated whether simvastatin could affect NPC cell cycle. Flow cytometry assay showed that the number of cells in G0/G1 stage was significantly elevated, while that in S stage significantly decreased after 24 hours of treatment with 20 μM simvastatin (Figure 2A). Subsequent qPCR results revealed that simvastatin treatment remarkably downregulated the mRNA level of cyclin D1, while upregulated that of P21 and Bim (Figure 2B). To explore the promoting effect of simvastatin on CNE1 cell apoptosis, caspase-3 activity was detected. The results demonstrated that caspase-3 activity was

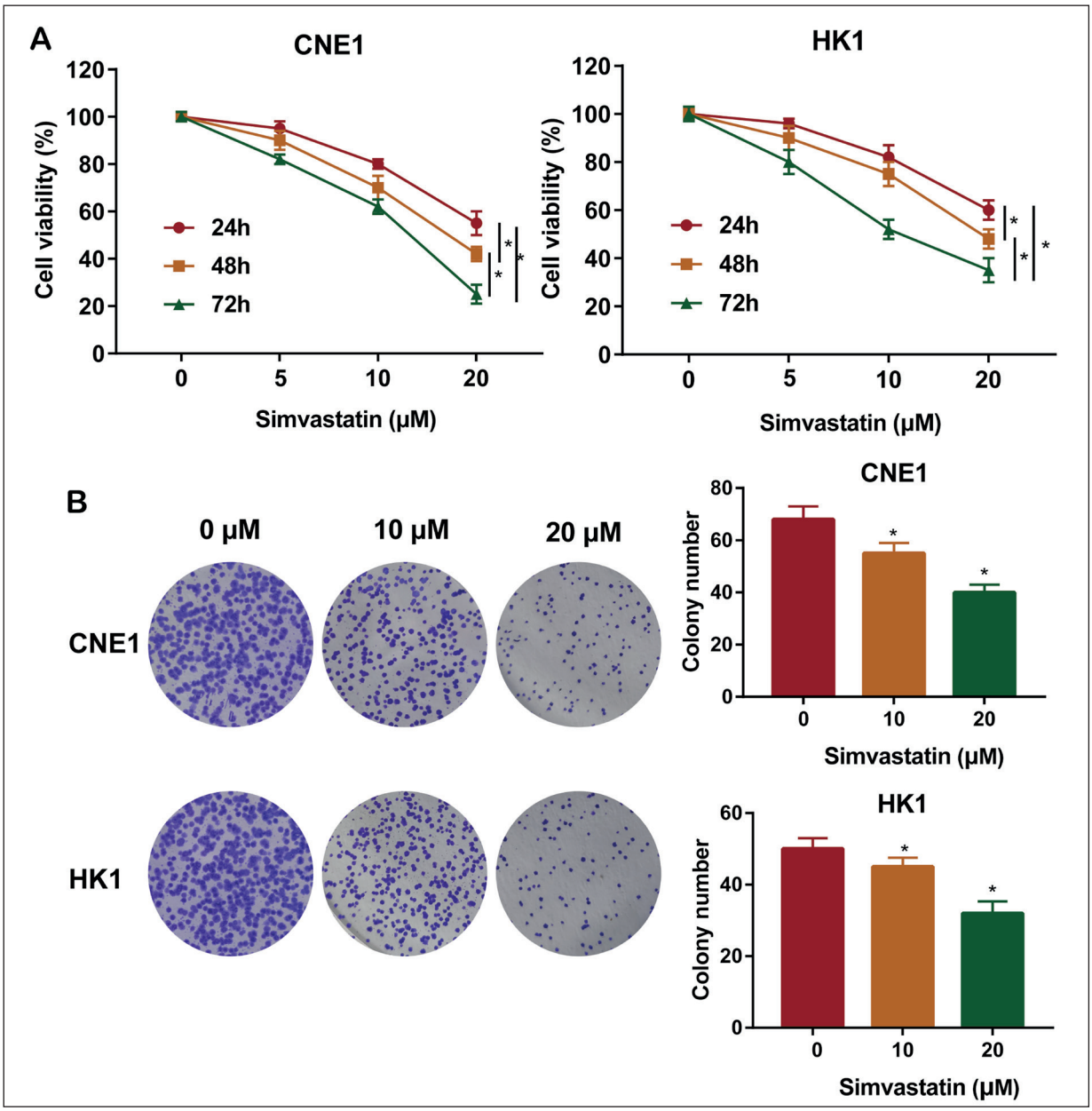

Figure 1. Simvastatin inhibits NPC cell proliferation and colony formation ability. **A,** CNE1 and HK1 cells were treated with different concentrations of simvastatin (5 μM, 10 μM, 20 μM) for 24, 48, and 72 hours, and cell viability was measured by CCK-8 assay. Cell viability decreased in a time-dependent and dose-dependent manner. B, After treatment of CNE1 and HK1 cells with 10 μM and 20 μM simvastatin for 24 hours, colony formation assay showed that simvastatin reduced the number of colonies of CNE1 and HK1 cells in a dose-dependent manner (magnification: 40×).

remarkably enhanced in the simvastatin-treated group compared with the normal control group (Figure 2C). In addition, Western blot displayed that the protein levels of Bax and caspase-3 was markedly upregulated, while Bcl-2 was significantly downregulated (Figure 2D). The above results demonstrated that simvastatin could inhibit cell cycle and induce cell apoptosis in NPC.

Simvastatin Inhibits the Growth of NPC Cells In Vivo

To explore the anti-tumor activity of simvastatin *in vivo*, we established a xenograft model. *In vivo* experiments found that simvastatin remarkably suppressed tumor growth on day 2. Meanwhile, the simvastatin-treated group exhibited significantly smaller tumor volume than the

control group (Figure 3A). Further examination demonstrated that simvastatin-treatment reduced tumor weight than that of the control group (Figure 3B). These results suggested that simvastatin inhibited the tumor growth of mice model of NPC xenografts.

Simvastatin Affects NF-κB Signaling Pathway

The influence of simvastatin on NF-κB pathway was further explored. Luciferase reporter gene assay revealed that NF-κB activity was remarkably reduced by simvastatin treatment in

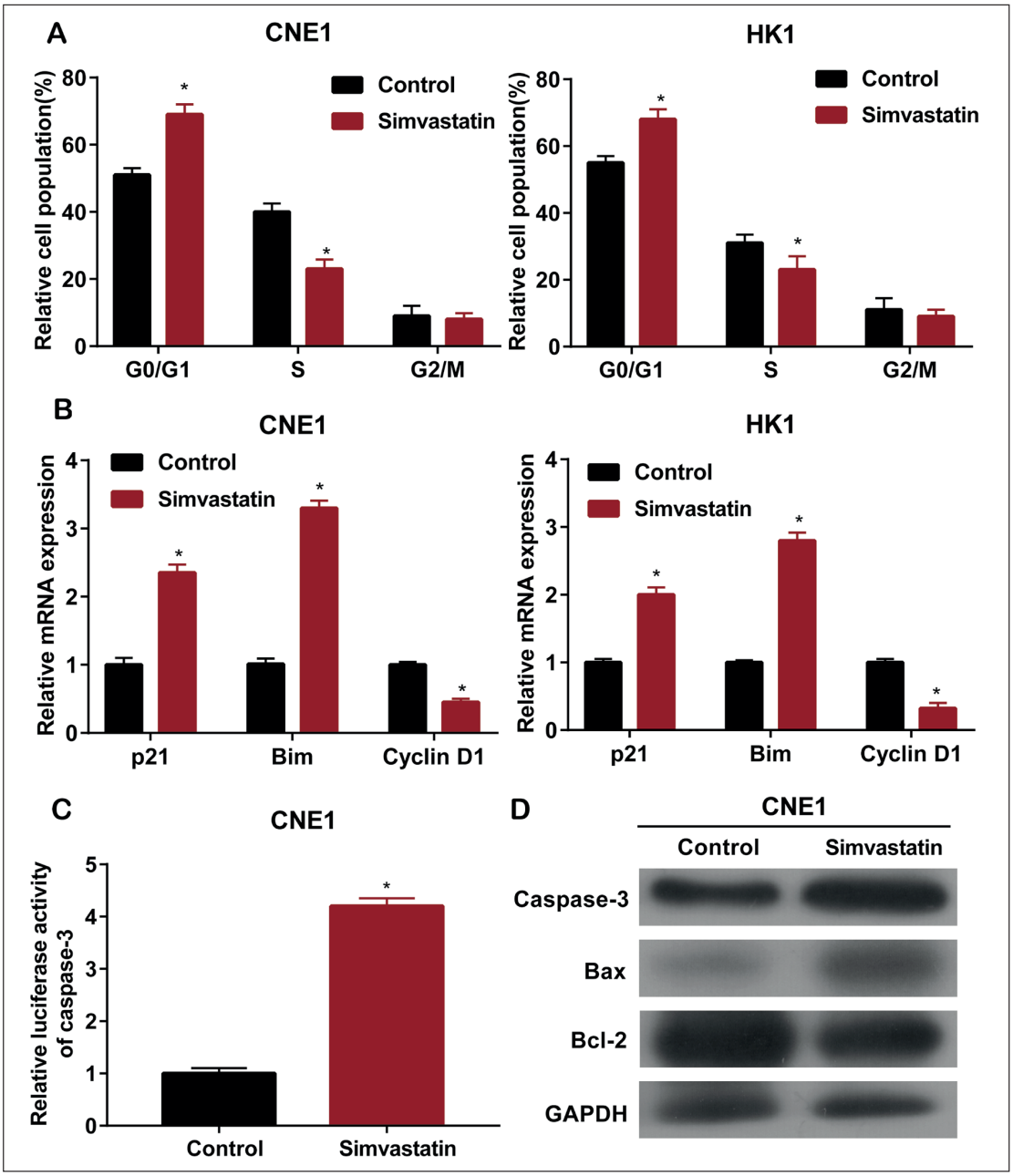

Figure 2. Simvastatin blocks NPC cell cycle and induces apoptosis. A, Flow cytometry revealed that simvastatin significantly induced cell cycle arrest in G0/G1 phase of CNE1 and HK1 cells. B, The expression of cell cycle-related genes was detected by qRT-PCR. After simvastatin treatment, the mRNA expression levels of cyclin D1 in CNE1 and HK1 cells were significantly downregulated, while P21 and Bim were significantly upregulated. C, Caspase-3 activity in CNE1 cells was determined by Luciferase reporter gene assay. D, The results of Western blot analysis of apoptosis-related proteins in CNE1 cells showed that the expression of Bax and caspase-3 increased, while the expression of Bcl-2 decreased after simvastatin treatment.

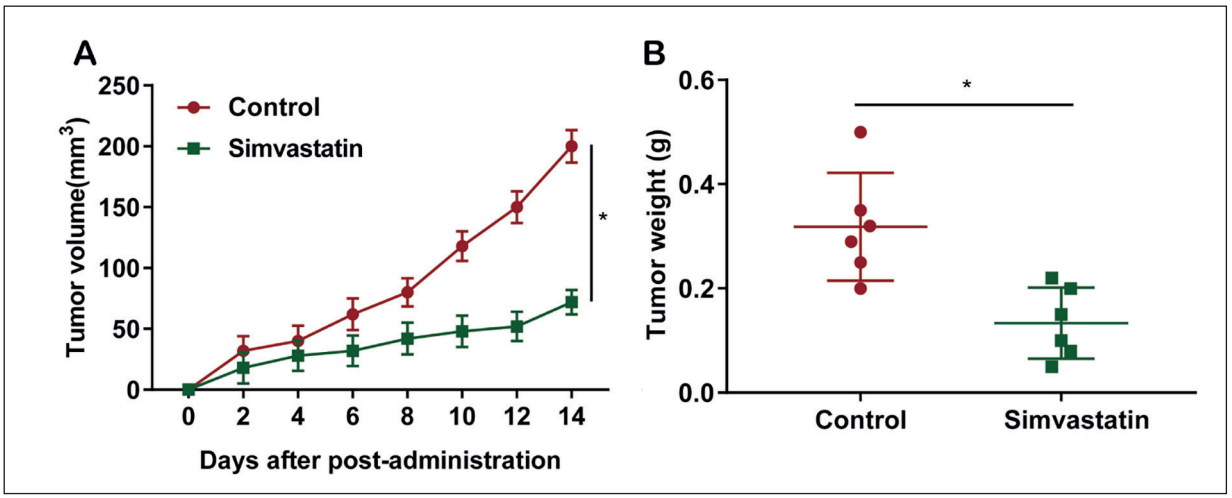

Figure 3. Simvastatin inhibits NPC cell growth in vivo. A, NPC cells CNE1 (8 x 106) were subcutaneously inoculated into nude mice. When the tumor was accessible, the mice were randomized into two groups (n = 6 / group). One group was given a daily subcutaneous injection of simvastatin (20 μg/g body weight/day) for 2 weeks, while the other group was given a vehicle as a control group. Tumor volume was monitored every other day. Simvastatin significantly inhibited tumor growth. B, Tumor body weight was measured, and the weight of the simvastatin-treated group was remarkably lighter than the control group.

a dose-dependent manner. This suggested that simvastatin might be able to inhibit NF-κB signaling (Figure 4A). Subsequently, CNE1 cells were treated with increasing doses of simvastatin for 24 hours. Western blot results suggested that simvastatin remarkably inhibited p65 phosphorylation dose-dependently (Figure 4B). Besdes, qPCR results indicated that the expressions of cyclin D1 and Bcl-2 in the NF-κB signaling pathway remarkably decreased with increased concentration of simvastatin (Figure 4C-4D). All these findings suggested that simvastatin could suppress NF-κB pathway in NPC cells.

Discussion

NPC is a common malignant tumor of the neck in central and southern China. Radiotherapy combined with chemotherapy is the main treatment method for NPC. However, the treatment exhibits large side effects[19]. Therefore, searching for anti-tumor drugs with good efficacy and small side effects is an urgent problem to be solved in clinical practice.

Simvastatin is a class of statins that can effectively lower blood cholesterol levels. Currently, statins have been widely studied internationally and domestically in the prevention and treatment of tumors, such as melanoma[20], uterine fibroids[21], and thyroid cancer[22]. This suggests simvastatin may also have anti-cancer effects. In this study, CNE1 and HK1 cells were treated with simvastatin, and CCK-8 assay showed that simvastatin suppressed cell proliferation time-dependently and dose-dependently. Colony formation assay revealed that simvastatin could inhibit the colony formation ability of NPC cells. Furthermore, we found that simvastatin remarkably inhibited the growth of NPC *in vivo*.

Since cell cycle is often dys-regulated in most common malignancies[23,24], controlling cell cycle progression is regarded as an effective approach for controlling tumor growth[25,26]. In this study, flow cytometry results demonstrated that simvastatin could regulate the cell cycle distribution of CNE1 and HK1 cells. It significantly increased the ratio of G0/G1 cells, while reduced the percentage of S cells. Meanwhile, it could affect the expression of cell cycle related genes, including p21, Bim, and cyclin D1. This suggested that one of the mechanisms by which simvastatin attenuated the proliferative ability of NPC cells was through cell cycle inhibition.

Cell apoptosis is an active cell death mechanism that plays a key role in some biological processes[27]. Bcl-2 and Bax activate the caspase cascade, thereby of great importance in regulating the internal pathway of apoptosis[28]. Caspase-3 is the main actor and terminal shear enzyme in the process of cell apoptosis. Activated caspase-3 can shear the adp-ribose polymerase PARP, making it unable to perform the normal functions of DNA repair and gene integrity monitor-

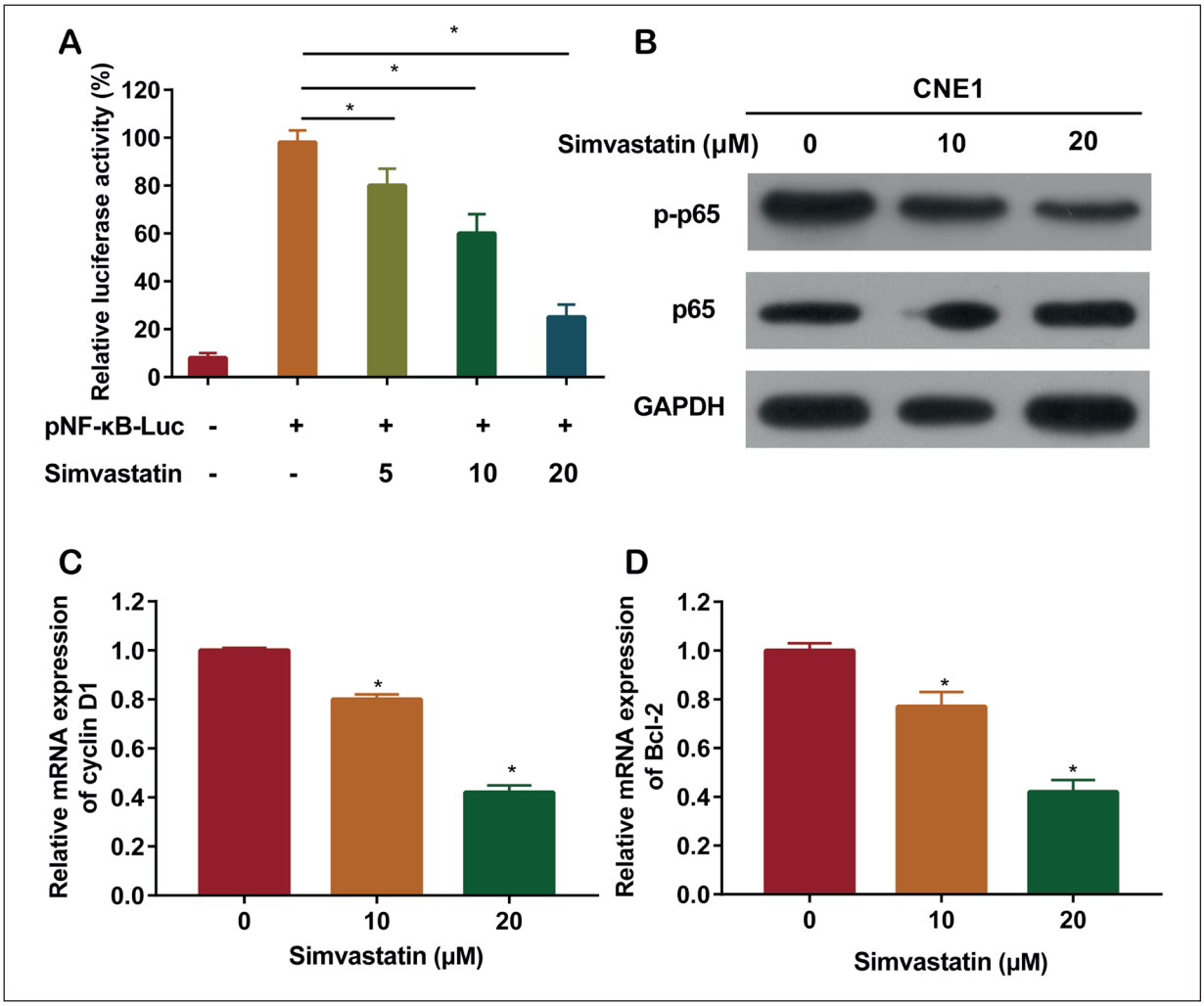

Figure 4. Simvastatin affects the NF-κB signaling pathway. A, Dual-Luciferase reporter gene assay revealed that simvastatin inhibited NF-κB signaling in a dose-dependent manner. B, CNE1 cells were treated with increasing concentrations of simvastatin for 24 hours, and the protein expression of p-p65, p65, and GAPDH was detected. Western blot indicated that simvastatin could inhibit phosphorylation of p65 in a dose-dependent manner. C-D, QRT-PCR was used to detect the mRNA expression levels of cyclin D1 and Bcl-2 in CEN1 cells treated with different concentrations of simvastatin for 24 hours.

ing. This may eventually increase the activity of endonuclease, lysis DNA between nucleosomes, and induce apoptosis[29]. In this study, we found that after simvastatin treatment, the Luciferase activity of caspase-3 increased significantly in CNE1 cells. The results of Western blot showed that simvastatin could upregulate the protein expression level of pro-apoptotic proteins (Bax and caspase-3) and downregulate the expression of anti-apoptotic protein Bcl-2. These results suggested that simvastatin could promote cell apoptosis in NPC.

Nuclear factor-kappa B widely exists in eukaryotic cells, which can regulate many of the genes involved in immune and inflammatory reaction[30]. NF-κB has been confirmed to be aberrantly expressed in a variety of tumors. Meanwhile, it is engaged in the regulation of apoptosis and transformation of cells[31]. NF-κB also participates in the chemotherapy-related resistance of multiple cancers[32]. Therefore, NF-κB has been considered as an effective target for anti-tumor drugs. Bcl-2 is a cell intima protein that plays a pivotal role in the mitochondrial mediated APA-F1/caspase-9 apoptosis pathway. When Bcl-2 is highly expressed, BAX-Bcl-2 heterodimer can be formed to inhibit cell apoptosis[33]. It is generally believed that NF-κB activates the transcription of apoptotic inhibitory proteins in the Bcl-2 family[34]. In this

study, simvastatin was shown to reduce the Luciferase activity of NF-κB. Moreover, it could downregulate p65 phosphorylation, suggesting that simvastatin might regulate NPC cells through the NF-κB pathway. Subsequently, we detected the expressions of NF-κB signaling pathway related genes cyclin D1 and Bcl-2. The results found that the mRNA levels of cyclin D1 and Bcl-2 in CNE1 cells remarkably decreased with increased concentration of simvastatin treatment. In conclusion, simvastatin could dampen the activation of NF-κB, inhibit the proliferative activity of NPC cells, and lead to cell apoptosis.

Conclusions

In summary, we first found that simvastatin can induce apoptosis of NPC cells *via* inhibiting the NF-κB signaling pathway. All our findings suggest that simvastatin is a potential chemotherapy drug for NPC treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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