Shiraz E-Med J. 2019 February; 20(2):e69057. doi: 10.5812/semj.69057.

Published online 2018 August 18. **Research Article**

Potential Effect of Simvastatin as an Anti-Cancer Agent on *SOX7* and *SOX9* Expression in Prostate Cancer Cell Lines

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Received 2018 April 03; **Revised** 2018 June 28; **Accepted** 2018 June 30.

Abstract

Background: Prostate cancer (PCa) is the second leading cause of cancer death in men, worldwide. Geranylgeranylation and farnesylation have a main role in the carcinogenic process, which can be prevented via statins as HMGCOA reductase enzyme inhibitors in cholesterol biosynthesis. These effects might be controlled by several transcription factors such as *SOX7* and *SOX9*, which have been involved in PCa initiation and progression. To the best of our knowledge, no study has demonstrated the association of simvastatin and *SOX* status in PCa. Therefore, this study is an attempt to evaluate whether simvastatin induces anti-neoplastic effects via the *SOX9* and *SOX7* transcription factors.
Methods: Prostate cancer cell lines LNCaP and PC3 were used to evaluate the expression of *SOX7* and *SOX9* using quantitative RT-PCR.
Results: Our data was analyzed by applying one-way ANOVA and Tukey's test and determined that 0.07 μM of simvastatin after 24 h was sufficient to upregulate *SOX7* mRNA expression ratio by 3.58 fold in LNCaP. In addition, the level of *SOX9* mRNA expression was increased by 12.18 fold at 0.07 μM after 24 h, 8.67 fold at 0.001 μM after 24 h, and 6.33 fold at 0.07 μM after 12 h in LNCaP and in PC3 cell line. The level of *SOX9* mRNA expression was increased by 2.64 fold at 0.5 μM after 24 h and 2.78 fold at 0.1 μM after 12 h, however, it decreased by 0.67 fold at 0.1 μM after 24 h.
Conclusions: Our findings suggest that simvastatin can induce the anti-cancer properties via manipulating the expression of *SOX7* in LNCaP, as the androgen-dependent cell.

Keywords: Simvastatin, LNCaP, PC3, *SOX7*, *SOX9*

1. Background

Prostate cancer (PCa) is the second leading cause of cancer death in men and the most common non-skin cancer detected in the United States (1) with 29720 deaths and 238590 new cases in 2013 (2).

Moreover, PCa is the most common cancer after stomach cancer in Iranian males and the second malignancy after bladder cancer, among genitourinary cancers in Iran (3).

Various factors such as genetic/ethnical origin, diet, life style, and environmental factors have been proposed to play a role in development of PCa (4). An alteration in lipid metabolic enzymes and the associated pathways has been detected in several diseases including metabolic, immune, central nervous system dysfunction, as well as cancer.

Lipids play a fundamental role in membrane homeostasis of healthy cells (5). Moreover, accumulation of cholesterol has been associated with various diseases such as atherosclerosis and cancers. Cholesterol, as a vital molecule, plays a crucial role in the organization and structure of the cell membrane. Recently, several studies have demonstrated the association between PCa with cholesterol disequilibrium. Therefore, initiation and development of PCa can be prevented by targeting cholesterol metabolism (6). HMG-CoA reductase transforms Acetyl-CoA to mevalonate and its inhibition leads to reduction in cholesterol synthesis; this step is a rate-limiting step (7). Geranylgeranylation and farnesylation, occurring through cholesterol biosynthesis intermediates, also play an important role in the carcinogenic process, which can

be prevented via HMGCOA reductase inhibition (8). Cholesterol also activates the phosphatidylinositol 3-kinase/Akt pathway by accumulation in the lipid rafts (9).

On the other hand, cholesterol is a precursor for production of androgen hormones byproducts such as testosterone and dihydrotestosterone (9). Recently, the conflicting relationship between testosterone level and PCa has attracted attention among researchers (10). However, more studies show high level of circulating testosterone and consequently, the dihydrotestosterone, as a strong ligand for the androgen receptor, will increase PCa prevalence (9-11).

Moreover, statins as HMGCOA inhibitors not only decrease the level of cholesterol but are also able to inhibit the cancer cell growth (12). There are special reasons for anti-neoplastic properties of statins, however, as noted earlier, it might be related to the cholesterol as precursor of androgen hormone, the true target of statins (9). Most information about statins and decreased risk of PCa comes from clinical trials studies. For example, 28% lower risk of advanced PCa was reported in men taking statin medication for five years or longer. However, much more research is needed to find the putative anti-cancer mechanism of statin, before being recommended as a proper treatment to reduce risk of PCa (13). Recently, some reports showed that statins could increase apoptosis or reduce proliferation of prostatic epithelium and stroma (14). Among different types of statins, simvastatin could regulate prostate cancer cell proliferation, migration, invasion *in vitro* as well as *in vivo*. Treatment with simvastatin also inhibited Aktactivity in prostate cancer cells (15). Akt/PKB is a serine/threonine protein kinase that acts as a critical regulator of cell survival and proliferation (16, 17).

Furthermore, experimental evidence supports a role for *SOX9* in prostate function. *SOX9* regulates the proliferation of epithelial cells in the prostate, contributing to neoplastic transformation (18, 19).

In addition, *SOX7*, as the other *SOX* (*SRY*-related HMG-box) family of transcription factors, is able to regulate multiple biological processes (20). One study showed that ectopic *SOX7* expression represses migration, invasion, and proliferation of breast cancer. Moreover, the low level of *SOX7* was detected in many human cancers and in several studies *SOX7* was introduced as a tumor suppressive molecule in the lung, breast, prostate, and colon cancers (21).

Owing to the anti-cancer effect of statins and the important role of the *SOX* transcription factor members including *SOX7* and *SOX9* in PCa initiation and progression, we aimed to evaluate the role of simvastatin on the aforementioned transcription factors.

2. Methods

2.1. Cell Culture

Prostate cancer cell lines LNCaP (androgen-dependent) and PC3 (androgen-independent), purchased from National Cell Bank of Iran Pasture Institute, were cultured in a humid incubator at 37°C, under 5% CO_2 and in 10% fetal calf serum (Cinagen, Iran) containing RPMI 1640 media (Biosera, UK).

2.2. Cell Viability Assay

LNCaP and PC3 cells with 70% confluency were harvested and seeded in 96-well plates at a density of 10000 and 9000 cells/mL, respectively.

Following 24 hours (h) incubation time, monolayer cells were incubated in the presence of different simvastatin (Sigma-Aldrich) concentrations for 12 and 24 h. Then, 100 μL MTT solution in PBS (0.5 mg/mL) was added per well, and incubated for 1 to 4 h at 37°C. The absorbance was determined at 570 nm using ELISA reader (Mikura Ltd., Horsham, UK).

2.3. RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted according to the manufacture's protocol (RNA Extraction Kit, Yekta Tajhiz, Iran), after proper time of treatment and incubation with simvastatin. Subsequently, RNA quantity were assessed by Nano drop (Termo). The integrity of RNA was verified by the presence of two rRNA bands, using formaldehyde gel electrophoresis (18s, 28s). Reverse transcription was performed with 5 μg of total RNA and random primers using the First Strand cDNA Synthesis Kit (Fermentas, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using a 7500 Real-Time PCR System (Applied Biosystems, USA) with syber Green® PCR Master Mix (Yekta Tajhiz, Iran), according to the manufacturer's instructions. The PCR reaction mixture contain 5 μL of cDNA (approximately 150 ng), 0.5 μL of 10 pM solutions of each of the forward and reverse primers, and appropriate Master Mix (2X) syber Green in a total volume of 25 μL. Specific primers were designed using the allele ID software. The relative expression level (fold changes) of abovementioned genes in prostate cancer cells were calculated by the 2-$\Delta\Delta$CT method. The housekeeping gene, GAPDH, was used to normalize the results.

2.4. Statistical Analysis

Data were presented as mean $\pm$ SD of three independent experiments and were analyzed by applying SPSS 20.

One way analysis of variance and Tukey's test were performed to compare the control and treated groups. To indicate a statistically significant difference, $P < 0.001$ was used.

3. Results

3.1. MTT Assay

MTT assay was performed by incubating monolayers cells in the presence of different concentration of simvastatin for 12 and 24 h. At the end of the treatment period, the number of viable cells was assessed using the colorimetric method. The optimum simvastatin concentration, for the PC3 cell line at 0.1, 0.01, and 0.5 μM and for LNCaP cell line at 0.07 and 0.001 μM, were determined as nontoxic concentrations.

3.2. Expression of SOX7 and SOX9 mRNA in Prostate Cancer Cell Lines

Using quantitative RT-PCR, we determined the effect of simvastatin treatment on the mRNA expression of *SOX7*, and *SOX9* relative to GAPDH in prostate cancer cell lines, PC3 and LNCaP, using different concentrations and different treatment times compared to control. As shown in Figure 1A, the level of *SOX9* mRNA expression was increased by 12.18 fold at 0.07 μM after 24 h, 8.67 fold at 0.001 μM after 24 h, and 6.33 fold at 0.07 μM after 12 h in LNCaP cell line. However, in PC3 cell line, the level of *SOX9* mRNA expression was increased by 2.64 fold at 0.5 μM after 24 h and 2.78 fold at 0.1 μM after 12 h, however, it decreased by 0.67 fold at 0.1 μM after 24 h (Figure 1B).

As shown in Figure 2A, in LNCaP cells, the level of *SOX7* mRNA expression was increased by 3.58 fold after 24 h treatment with 0.07μM of simvastatin compared to the control, however, the results were not significant at any other condition. Additionally, the mRNA expression level of *SOX7* was not significantly changed in PC3 cell line (Figure 2B).

4. Discussion

The current study was the first attempt to show the effect of simvastatin via transcription factors including *SOX9* and *SOX7* as the important signaling pathways in prostate cancer.

Wang et al. showed that *SOX* proteins, such *SRY*, *SOX7*, and *SOX9*, have an important role in prostate (22). Our data show that in LNCaP cells, in presence of 0.07 μM of simvastatin, the level of *SOX7* mRNA expression was increased after 24h treatment compared to the control; however, the results were not significant at any other condition. Additionally, the mRNA level of *SOX7* was not significantly changed in PC3 cell line.

Our data also showed that the level of *SOX9* mRNA has significantly increased at several dosages/times of experiment in LNCaP cells; however, we saw diverse behavior at different concentrations in PC3 cells.

To the best of our knowledge, no study has been found to investigate the potential effects of simvastatin as an anticancer agent, on the levels of *SOX7* and *SOX9* mRNA expression.

However, several studies demonstrated that *SOX7* mRNA expression, as a tumor suppressor, is downregulated in lung, colon, and prostate tumor tissues and cell lines (21). Guo et al. showed that *SOX7* protein expression is decreased in 47% (15 of 32) of prostate adenocarcinomas. Furthermore, *SOX7* mRNA was decreased in 60% of snap-frozen prostate tumors (23). We speculate that simvastatin might be a potential drug with the anti-cancer effect at proper time course and concentration in LNCaP family tumors (0.07 μM after 24 h), affecting the role of *SOX7* as tumor suppressor.

Recently, Stovall et al. has found that *SOX7* is elevated during silencing of DNA methyltransferase 1 (*DNMT1*), the important enzyme, which maintains DNA methylation patterns in MDA-MB-231 and MCF-7 breast cancer cells (21). Therefore, promoter methylation could be one of the mechanisms regulating the *SOX7* expression.

Another study demonstrated that statin can inhibit the signaling pathway Ras/Raf/MAPK/JNK, leading to *DNMT1* down-regulation. Moreover, they suggested that statins may downregulate the *DNMT1* through its association with HDACs (24). Therefore, simvastatin, at low concentration, might affect LNCaP family tumors via epigenetic pathways such as promoter hypermethylation. However, further studies are needed to prove this hypothesis.

In case of *SOX9*, several studies showed that *SOX9* have involved in PCa, however, its precise role has not been clearly understood. Several studies have shown that it can increase invasion and proliferation of prostate cell lines in a xenograft model via Akt signaling pathway. In contrary, other studies showed that it can suppress the tumor growth (18, 25). Overexpression of *SOX9* has been detected in early prostate cancer and LNCaP, CWR22, PC3, and DU145 cell lines. It was found that Wnt/β-catenine pathway regulate the *SOX9* expression level in PCa (22, 26). Therefore, *SOX9*, as a transcription factor, can be an important target for cancer therapy.

To our knowledge, the anti-cancer effect of simvastatin on *SOX9* has not yet been evaluated. In one study, Kochuparambil et al. reported that simvastatin could inhibit the Akt activity in prostate cancer cell lines, in a dose and time-dependent manner (15). Our data suggest that simvastatin at 0.1 μM concentration causes the downregulation of *SOX9* expression in PC3 cell line after 24 h.

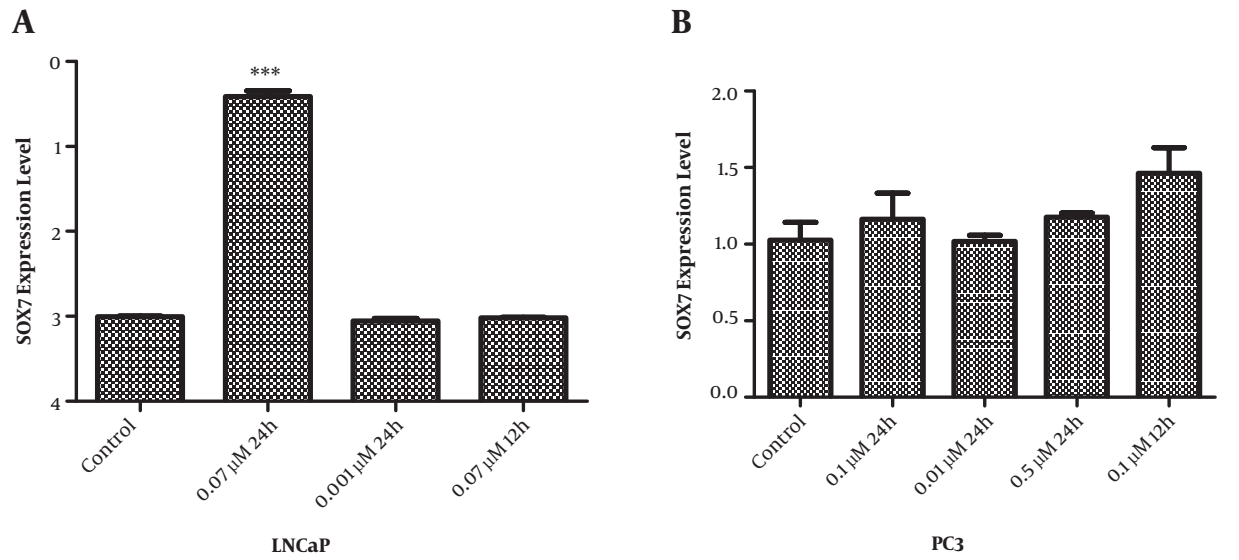

Figure 1. Quantification of mRNA expression of *SOX9* by quantitative reverse transcriptasepolymerase chain reaction (QRT-PCR) in A, LNCaP and B, PC3 cell lines. Data are the relative expression levels of *SOX9* mRNA to GAPDH in treated groups compared to control cells. Data are presented as mean ± SD of three independent experiments ($P < 0.001$).

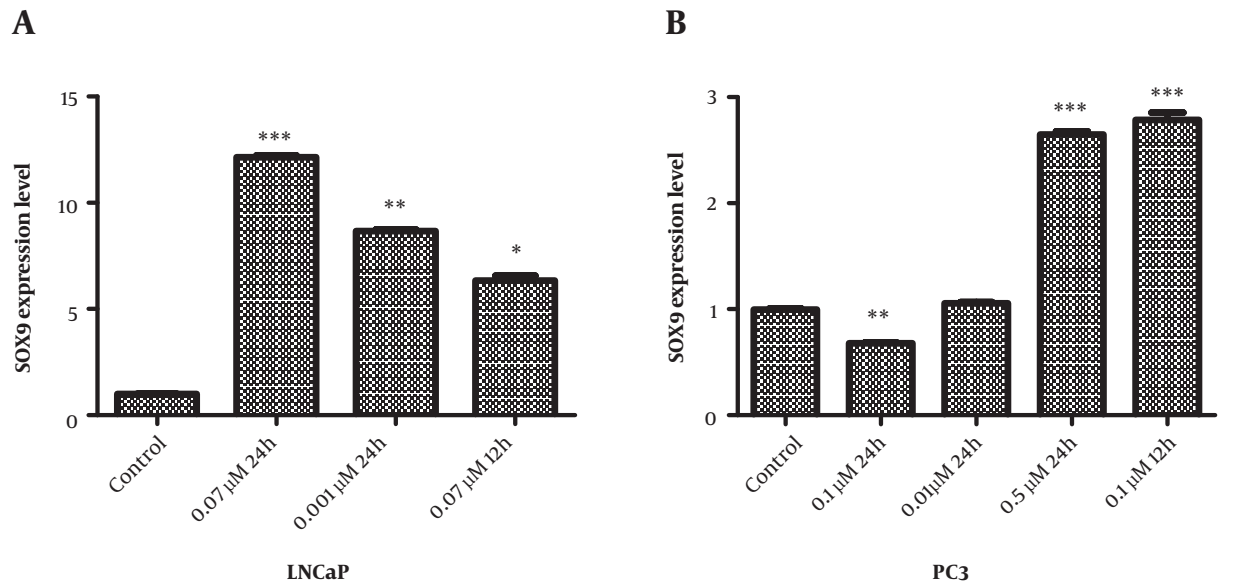

Figure 2. Quantification of mRNA expression of *SOX7* by QRT-PCR in A, LNCaP and B, PC3 cell lines. Data are the relative expression levels of *SOX7* mRNA to GAPDH in treated groups compared to control cells. Data are presented as mean ± SD of three independent experiments ($P < 0.001$).

Our results also showed that *SOX9* expression is upregulated at all dosages and times of incubation with simvastatin in LNCaP cells and its expression is upregulated in PC3 cells at 0.5 μM and 0.1 μM after 24 h and 12 h, respectively.

Due to limited information regarding the effect of simvastatin on *SOX9*, it is concluded that if overexpression of *SOX9* causes proliferation in tumors, *SOX9* cannot be a target for cancer therapy in LNCaP family tumors; however, in PC3 special dose and time might be effective. Nonetheless, if overexpression of *SOX9* has a suppressive effect on tumor growth, it seems that simvastatin could have induced the best action on *SOX9* in all dosages and times in LNCaP and

PC3 except at 0.1 after 24 h treatments in PC3.

Regarding the suppressive effect, researcher have shown that insulin-like growth factor binding protein-related protein (ILGFBPRP), having high expression in senescent prostate epithelial cell line (M12 cells), could increase *SOX9* expression (22). On the other hand, it has been demonstrated that at the low concentration, lovastatin induced G1 cell cycle arrest and senescence in human prostate cancer cells (27). Therefore, our data suggest that simvastatin might increase ILGFBPRP and induce *SOX9* expression.

4.1. Conclusion

In this study, an attempt has been made to provide new insight into the effect of statins on the prevention of PCa via regulation of expression of transcription factors *SOX7* and *SOX9*. Our findings suggest that simvastatin can induce the anti-cancer properties via manipulating the *SOX7* expression levels in prostate cancer cell lines, especially in LNCaP, as androgen-dependent cell line having cross talk with cholesterol metabolism. However, there is ambiguity regarding the effect of simvastatin on prostate cancer via *SOX9* expression and future investigations on Akt activity, GSK3B/β-catenin expression, and genes involved in apoptosis and epigenetic can further clarify the mechanism of action of statins.

Acknowledgments

The present article was extracted from the MSC thesis written by Elham Arabizadeh and was financially supported by Shiraz University of Medical Sciences, grants number 94-01-01-9800.

Footnote

Authors' Contribution: Pooneh Mokarram and Saeid Ghavami have the same contribution.

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