Original Article

Effect of simvastatin on c-myc, cyclin D1 and p53 expression in DMBA-induced breast cancer in mice

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Abstract

Introduction: Recently, the therapeutic and antioxidant effects of simvastatin on 7,12-dimethylbenz[a]anthracene (DMBA) induced breast cancer have been studied. To gain further understanding of the molecular mechanisms of simvastatin, this study investigated its effects on the expression of c-myc, cyclin D1 and p53 in normal mammary glands and tumors.

Methods: Female albino mice were divided into two groups: 1) N group, healthy mice without DMBA and 2) D group, mice with DMBA administration. After the appearance of tumors, D group mice are subdivided into 3 groups, as control (C), simvastatin-treated group (S) which received 80 mg/kg/day, orally and tamoxifen-treated group (T) with 50 mg/kg/day, orally. After 4 weeks, animals were sacrificed. Also, the tumors and normal mammary glands were removed for histopathological evaluations and analysis of gene expression by qRT-PCR.

Results: The results showed the up-regulation of c-myc and cyclin D1 in tumors of the control group compared with mammary glands of the N group. Similar to tamoxifen, the simvastatin treatment could normalize the expression of c-myc and cyclin D1; however, the expression of p53 did not change in the treated groups.

Conclusion: Down-regulation of c-myc and cyclin D1 in treated tumors with simvastatin could be a possible molecular mechanism for its therapeutic effects in DMBA-induced breast cancer in mice.

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Introduction

One of the most common invasive cancers diagnosed among women is the breast cancer. It was estimated that 14.9 million new cases of breast cancer were identified in 2012 which are expected to reach up to 22 million within the next two decades (Ghoncheh et al., 2016). Breast cancer must be considered as a multifactorial disease with gene-environment interactions (Martin and Weber, 2000). A better understanding of molecular mechanisms involved in cancers is essential for improving of our knowledge and developing new therapeutic protocols against cancers (Ashrafi et al., 2012). Mutations in the proto-oncogenes and tumor suppressor genes are termed as gain-of-function and loss-of-function mutations which overall lead to uncontrolled cell proliferation (de Leon, 1994).
One of the most well-known tumor suppressor genes is p53. The classic role of p53 is known as cell cycle arrest and apoptosis. The p53 mutations are frequent events in more than 50% of many different cancers (Ozaki and Nakagawara, 2011). On the other hand, c-myc, as a cellular proto-oncogene, regulates the expression of gene families involved in cell growth, migration, metastasis and apoptosis (Takwi et al., 2012; Miller et al., 2012). Also cyclin D1, as another oncogene, plays a crucial role for G1/S transition in cell cycle by activation of cyclin-dependent kinases (CDKs) 4 and 6 which lead to phosphorylation of retinoblastoma (Kim and Diehl, 2009). This ability proves its oncogenic potential for developing neoplastic transformation (Musgrove et al., 2011). The 7,12-dimethylbenz[a]anthracene (DMBA), one of the polycyclic aromatic hydrocarbons, is used widely for the induction of mammary tumors in rodents (de Oliveira et al., 2015). Reactive metabolites of DMBA could form DNA adducts and induce DNA mutation. Moreover, elevated expression of cyclin D1 and c-myc has been found in DMBA-induced mammary tumors (Currier et al., 2005). Simvastatin belongs to the first-line agents for hypercholesterolemia, commonly called statins. The lipid-lowering effect of statins is based on the inhibition of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Stancu and Sima, 2001). Studies have also reported that statins possess anticancer activity through anti-proliferative, pro-apoptotic, anti-metastatic and anti-angiogenic properties (Chan et al., 2003; Dulak and Józkowicz, 2005; Campbell et al., 2006; Shen et al., 2015). Some clinical evidences have shown that statin therapy has protective effects against breast cancer and also reduces breast cancer mortality (Graaf et al., 2004; Beckwitt et al., 2018). Despite these findings, little is known about the molecular alterations underlying efficacy of statins on breast cancer. In this study, the effects of simvastatin on gene expression of cyclin D1, c-myc and p53, in DMBA-induced mammary tumors of mice was investigated and compared with tamoxifen as a commonly used drug in the treatment of breast cancer.

Materials and methods

Animals and experimental design
A total of 51 female albino mice with the average body weight of 23±0.9g and aged 21 days were used in this study. The mice were bred in the laboratory breeding house of the Comparative and Experimental Medical Center Institute of Preventive Medicine, Shiraz, Iran. The animals were maintained under controlled room temperature (24±4°C) with relative humidity (60%), a 12h light/dark cycle and were given commercial pelleted and water ad libitum. The protocol for this study was approved by our Institutional Animal Ethical Committee and was in accordance with the guidelines for the care and use of laboratory animals prepared by Shiraz University, Shiraz, Iran (IACUC no: 4687/63). They were acclimated for one week and were divided into two groups as follows: N group, healthy animals without DMBA administration (n=7) and D group, DMBA-treated group (n=44) that received DMBA (50mg/kg) dissolved in sesame oil, orally once a week for the next 4 weeks, beginning at 5 weeks of age. The mice were weighed and palpated weekly for the appearance of mammary tumors 4 weeks after DMBA administration till to the end of the experiment. When tumors size reached 0.5cm in the largest dimension, animals in group D were divided into three groups, 7 in each group (Karimi et al., 2018). Groups were labeled as follows: C, tumoric mice without treatment which were represented as the control group (n=7); S, tumoric mice with simvastatin treatment that received it by gavage with a dose of 80 mg/kg/day for 4 consecutive weeks (n=7) and T: tumoric mice with tamoxifen treatment that received it by gavage with a dose of 50 mg/kg/day for 4 consecutive weeks (n=7).

Tissue sample preparation
At the end of the experiment, all animals were sacrificed under deep anesthesia and the normal mammary glands and tumor tissues were removed, frozen in liquid nitrogen and kept at -80°C until use.

RNA Isolation and cDNA Synthesis
All mammary glands and tumor tissues were used to extract total RNA using RNX-Plus Reagent (Cinnagen, Iran) and DNase treatment according to the manufacture's instructions. The quality and quantity of the extracted RNAs were evaluated by agarose gel electrophoresis and spectrophotometry. Concentrations of all extracts were normalized before cDNA synthesis. All RNA extracts were reverse...
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Table 1: Oligonucleotide primers used for qRT-PCR analysis

| Gene     | Primers sequences | Annealing Temperature (°C) | Product size (bp) |
|----------|-------------------|---------------------------|-------------------|
| GAPDH    | F: 5ʹ-GGCAAATTCAACGGGCAGT-3ʹ R: 5ʹ-CCTTTTGGCTCCACCCTTTCA-3ʹ | 57             | 125               |
| C-myc    | F: 5ʹ-CTCCACTCACCAGCACAATC-3ʹ R: 5ʹ-GTTTCCCTCTGACGTTCC-3ʹ | 59             | 199               |
| Cyclin D1| F: 5ʹ-CACGGACTACAGGGGAGTTTTG-3ʹ R: 5ʹ-GGTGTTCCATGGCAGCGG-3ʹ | 57.5           | 197               |
| p53      | F: 5ʹ-CACAGGTGTTGTACCTTA-3ʹ R: 5ʹ-AACCTCAAGCTGTCGCCGT-3ʹ | 59             | 177               |

The primer sequence, annealing temperature and expected size of PCR products. GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Table 2: Primer optimization data. The best performing concentrations for each primer sets

| Primer set | Optimum concentration (nM) | Efficiency |
|------------|-----------------------------|------------|
| GAPDH      | F:600/R:600                 | 100 %      |
| C-myc      | F:300/R:600                 | 108.4%     |
| Cyclin D1  | F:400/R:400                 | 108.75%    |
| p53        | F:400/R:400                 | 109.6%     |

Data were reported as mean±SD and were analysed using one-way ANOVA followed by Tukey’s test as a post hoc. The statistical level of P<0.05 was considered significant.

Results

Tumor parameters and the histopathological study of tumors were reported in our previous work (Karimi et al., 2018). In this study, the gene expression of c-myc, cyclin D1 and p53 in mammary glands and tumor tissues in different experimental groups were measured using real-time RT-PCR technique.

Effect of simvastatin and tamoxifen on c-myc expression

As shown in Figure 1, the results indicate a significant increase of c-myc expression in the control group in comparison to the normal group (P<0.05). While simvastatin and tamoxifen-treated groups showed eduction in c-myc expression, which was significant in both groups compared to control group (P<0.05), there was no significant difference between simvastatin and tamoxifen groups (P>0.05).

Effect of simvastatin and tamoxifen on cyclin D1 expression

As shown in Figure 2, an increase in cyclin D1

Data were transcribed into cDNA in a total volume of 20µl using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Inc., USA) according to the manufacturer’s instructions.

Primer design and qRT-PCR experiment

In this study, all primer sets were designed using Primer3 software based on standard rules of quantitative PCR primer design (Untergasser et al., 2012) and all sets were checked in database of SNP-NCBI (Table 1). All primer sets were ordered from Cinnagen, Iran. Also, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene was used as a reference gene. For the best optimized and specified products in our qRT-PCR assay, primer optimization, efficiency test and melting curve analysis were performed (Table 2). The qRT-PCR was run on a MiniOpticon instrument (BioRad, USA), using the SYBR Green PCR master kit (Parstous, Mashhad, Iran) according to the instructions provided by the manufacturer. The amplification cycles consisted of an initial denaturation step of 2 min at 95°C, 40 cycles and a two-step amplification with 30s at 95°C and 20s at 59°C. According to the efficiency results, relative quantitations were calculated by 2−(ΔΔCt) (Pfaffl, 2001).
expression was found in the control group relative to normal group, but statistically was no significant (P>0.05). The messenger RNA expression levels of cyclin D1 in simvastatin and tamoxifen-treated groups were significantly lower than those in the control group (P<0.05). Also, there was no significant difference between simvastatin and tamoxifen groups (P>0.05).

**Effect of simvastatin and tamoxifen on p53 expression**

In Figure 3, there was no significant change in the expression of p53 among groups (P>0.05). However, its expression decreased in control and simvastatin groups compared with other groups (P>0.05).

**Discussion**

Statins are common medications for treatment of hypercholesterolemia. Although many studies have shown the anticancer efficacy of statins (Riemsma...
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et al., 2010; Shen et al., 2015), their molecular mechanisms yet remain unknown. This study is the first research which investigated the effects of simvastatin on the gene expression of cyclin D1, c-myc and p53 in DMBA-induced mammary tumors of mice.

Cell cycle progression is one of the important changes associated with proliferating tumoric cells and understanding this provides rational targets for cancer therapy (Meeran and Katiyar, 2008). Cyclin D1 is one of the cell-cycle related cyclins whose activity is required for the G1/S transition phase and regulation of cell proliferation (Kim and Diehl, 2009). More than 50% of human breast cancers showed over-expression of cyclin D1 (Roy, 2006). It is reported that the over-expression of cyclin D1 in the tumors of DMBA-induced breast cancer, is associated with the activation of NF-κB and Wnt pathway as up-stream regulators of cyclin D1 expression (Currier et al., 2005). Furthermore, activation of the Ras signaling pathway can stimulate the expression of cyclin D1 (Feldt et al., 2015). In the present study, the levels of cyclin D1 mRNA increased in tumors of the control group in comparison to normal group which is in line with previous studies (Currier et al., 2005; Papaconstantinou et al., 2006). It is evidenced that H-ras point mutations are common events detected in DMBA-induced mammary tumorigenesis (Currier et al., 2005). Therefore, over-expression of cyclin D1 in the present study can be related to Ras signalling activation. The results showed that in tumors of simvastatin and tamoxifen-treated groups, cyclin D1 expression reduced in comparison to tumors of control group which may be a mechanism for anti-proliferative effects of simvastatin against breast cancer. Consistent with the results of this study, Shen et al. (2015) demonstrated the inhibitory effect of simvastatin on the proliferation of breast cancer cell lines by affecting cell cycle and down-regulation of cyclin D1 and CDKs expression. On the other hand, the anti-proliferative effects derived from statins may be attributed to the inhibition of isoprenoid synthesis which is important in the prenylation of proteins implicated in intracellular signalling pathway, particularly in Ras/Rho superfamily. Therefore, inhibiting isoprenylation of the Ras protein by statins may be suggested as a possible mechanism that involves in the prevention of the cyclin D1 expression (Feldt et al., 2015).

C-myc is another direct target for transactivation by the Wnt signaling pathway (de Oliveira et al., 2015). In the present study, higher expression of c-myc has been detected in tumors of control group in comparison to the normal mammary glands of normal group and its expression is modulated in treated groups. In 2012, Takwi and colleagues indicated that lovastatin reduces c-myc expression and up-regulates miR-33b expression in medulloblastoma cells. They explained that miR-33b acts as the potent
tumor suppressor which reduces c-myc expression and negatively affects the cell cycle progression and proliferation. It is important to note that c-myc participates in many signaling pathways including Ras, Bcl-2 and factors that inactivate p53 and repress the induction of several p53 targets such as p21 and gadd45 and it leads to reduction of p53-induced apoptosis (Hult et al., 2001).

The p53 is an important factor in many intracellular pathways. It serves an important roles in DNA repair, cell growth arrest, induction of apoptosis and inhibition of angiogenesis (Wawryk-Gawda et al., 2014). In immunohistochemical evaluation of the previous study on mammary tumors induced by DMBA in mice, no over-expression of p53 was observed. However, the sequencing of its gene confirmed the existence of wild type of p53 in DMBA-induced tumors (Jerry et al., 1994). Our results show that there is no significant change in the expression of p53 between the control and normal groups and it is in line with the study by Jerry’s et al. (1994). Similarly, loss of expression of wild-type of p53 has been reported in DMBA-induced oral carcinogenesis in the hamster buccal pouch model. In the present study, tamoxifen-treated group showed a non-significant increase in the expression of p53 compared with the control group. However, in simvastatin group, its expression is similar to control group. The result is in agreement with the study by Koyturk et al. (2007). They indicated that the treatment of ER-positive MCF-7 cells (with wild-type of p53) and MDA-MB 231 cells (with mutant p53) with simvastatin for 24 hours did not alter in the expression of p53 and induced apoptosis via involvement of JNK pathway. However, Li et al. (2015) showed that fluvastatin inhibited proliferation of hypoxic human umbilical vein endothelial cells by elevating of p27 and p53 expression and reduces levels of cyclin B1, cyclin D1 and VEGF expression. Pääjärvi et al. (2005) explained that the effects of statins on Mdm2/p53 regulation in DNA-damaged HepG2 cells. They concluded pravastatin induces phosphorylation of Mdm2 on Ser166 and thereby, facilitates degradation of Mdm2/p53 complex in cells stressed by DNA damaging agents.

Conclusion

In conclusion, the significant decrease in the expression of cyclin D1 and c-myc oncogenes in the simvastatin-treated group in comparison to control group may be suggested as a possible mechanism for anti-tumor effects of simvastatin in DMBA-induced breast cancer. Further efforts are needed to elucidate other mechanisms of simvastatin involved in the anti-breast cancer effect.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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