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# Lovastatin inhibits the proliferation of human cervical cancer hela cells through the regulation of tp53 pathway by mir-92a-1-5p

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**Abstract**: To study the effects of lovastatin on the proliferation, migration, apoptosis and cell cycle of human cervical cancer cell line HeLa *in vitro* and to investigate the underlying molecular mechanism. The effect of lovastatin on the expression of miRNA in HeLa cells was analyzed using an Affymetrix hybridization chip. The most significantly differentially expressed miRNA and its potential target genes were identified by Ingenuity Pathway Analysis(IPA) and verified by real-time qPCR. Lovastatin could inhibit the proliferation of HeLa cells with the IC50 of 14μmol/L. The proportion of cells in $G_1$ phase increased, while the proportion of cells in S phase and $G_2$/M phase decreased upon treatment with lovastatin. Real-time qPCR found that the expression of miR-192-5p was significantly upregulated ($p<0.001$) and miR-92a-1-5p was significantly downregulated ($p<0.001$). Using Ingenuity IPA analysis, MDM2 and TP53 were identified as the potential target genes of miR-192-5p and miR-92a-1-5p, respectively. The expression of TP53 ($p<0.01$) was significantly up regulated by Lovastatin through miR-92a-1-5p. Lovastatin can inhibit HeLa cell proliferation, promote apoptosis, inhibit migration and block the cell cycle and it may play an anti-tumor effect by downregulating the expression of miR-92a-1-5p and up regulating the expression of TP53.

**Keywords**: Lovastatin, HeLa cells, cell apoptosis, cell cycle, miRNA, TP53.

## INTRODUCTION

Cervical cancer (CC) is one of the most common malignant tumors affecting women's health. Its mortality rate ranks only after breast cancer among gynecological diseases and it has recently become increasingly prevalent in youth (WHO, 2014). More than half a million women around the world are still diagnosed with cervical cancer every year, and 300000 die as a result (Allanson and Schmeler, 2021). Current treatments options for cervical cancer include surgery, chemotherapy and radiotherapy (Hill, 2020). Chemical drugs with significant anti-tumor activity have deleterious effects on normal cells and the cancer cells often develop resistance against them. These factors undermine the efficacy and clinical application of chemotherapeutic agents (Hangauer *et al*., 2017; Stone and DeAngelis, 2016). Recent studies (El-Far *et al*., 2018; Kim and Ku, 2018; Kurokawa *et al*., 2017) showed that traditional Chinese medicine (TCM) has potential for the clinical treatment of tumors. TCM could act at multiple targets to affect different stages of tumor initiation and development. Its multitude of anti-tumor effects and few adverse side effects make TCM a popular topic of research. MicroRNAs (miRNA) are a group of single-stranded non-coding RNAs in eukaryotic organisms with a length of about 18-25 bp. miRNA sequences pair either perfectly or imperfectly with the 3'UTR of target mRNA molecules and can induce the degradation of the mRNAs or the inhibition of translation, thus modulating the expression of target genes post-transcriptionally (Bartel, 2009; Lu and Rothenberg, 2018; Rupaimoole and Slack, 2017). It has been shown that miRNA is an important synergistic or antagonistic factor in the pathogenic process of cervical cancer caused by human papillomavirus HPV (Yang *et al*., 2014). Mounting evidence suggests that miRNAs are an important pathogenic factor of cervical cancer that regulate cell proliferation, apoptosis and migration and are functionally related to the oncogenic HPV E6/E7 proteins (Sharma *et al*., 2014). Therefore, in the present study, we aimed to investigate the molecular mechanism for the oncogenic/anti-tumor effects of lovastatin and its target miRNAs, providing new clues for the prevention and treatment of cervical cancer.

Monascus has been produced and used in China for more than 1000years, Its fermentation product, 'red koji rice,' is the most widely used of its products, which has a high nutritional and medicinal value. Lovastatin is one of the main functional ingredients of red koji rice. Owing to the inhibitory effects of lovastatin on cholesterol, there has been considerable interest in evaluating the potential anti-tumor effects of lovastatin (Anscher *et al*., 2016; Kato *et al*., 2010; Liu *et al*., 2015; Walther *et al*., 2016; Zhang *et al*., 2015). Increasing evidence suggests that lovastatin has anti-tumor effects on a variety of cancers (Xie *et al*., 2021), such as brain cancer (Amadasu *et al*., 2022), colon cancer (Xiao *et al*., 2022), anaplastic thyroid cancer (Zhong *et al*., 2018) and breast cancer (El-Ashmawy *et al*., 2020; Huang *et al*., 2019; Vasquez-Bochm *et al*., 2019). The present study investigated the effects of lovastatin on the proliferation, migration, apoptosis and cell cycle of cervical cancer HeLa cells. This study provides scientific

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basis for studying the potential application of lovastatin in the prevention and treatment of cervical cancer and for developing food-grade anticancer medicine for this disease.

## MATERIALS AND METHODS

*Cell culture*

HeLa cells (Chinese Academy of Science) were cultured in high glucose DMEM (HyClone, Logan, UT, USA) supplemented with 10% FBS (HyClone) at 37°C, 5% $CO_2$ in a 95% humidity control incubator (Thermo Fisher, Waltham, MA, USA) and the medium was changed every two or three days.

***Cell proliferation assay***

HeLa cells growing at the log phase were seeded at a density of 3000 cells/well in a 98-well plate (Corning, NY, USA) and allowed to grow overnight. The next day, cells were subjected to different working concentrations of lovastatin (Abcam, Cambridge, UK) (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 and 18 μmol/L) for 48 hrs and each concentration was tested in triplicates. The cell viability was measured using a celltiter-gloluminescent cell viability assay (Promega, Madison, WI, USA) according to the manufacturer's protocol. Viable cells% = (mean value of the experimental group-mean value of the blank)/ (mean value of the control group-mean value of the blank) × 100%.

***Cell migration assay***

Cells of appropriate number growing in log phase were seeded into a 6-well plate (Corning) to achieve 100% confluence in 24 hrs. The next day, 200 μL pipette tips were used to create a constant wound across the diameter of each well (pipette tips were changed between each well) before touching a different well; although wounds did not need necessarily to be exactly uniform, even lines could be drawn on the back of the plate to assist the scratching process. Cells in each well were gently washed using 1×PBS to remove floating cells. Cells in the experimental group were then maintained in medium containing lovastatin (0, 7, 14 and 28 μmol/L) and were photographed at the time points of 0 h, 24 hrs and 48 hrs. Migration rate% = (wound width at time 0-wound width at subsequent timepoint)/wound width at time 0 ×100%.

***Cell apoptosis assay***

HeLa cells growing at the log phase were seeded in a 6-well plate ($2 \times 10^5$ cells/ well) and maintained in complete growth medium for 24 hrs. Cells in the experimental group were then cultured in culture medium containing lovastatin (0, 7, 14 and 28 μmol/L) for another24, 48 and 72 hrs. Each concentration and time point was tested in triplicates. Cells in each well were digested, collected and centrifuged at 800 g for 5 min. Supernatants were discarded and the pellets were washed twice in 1× PBS. Cells in the positive control group were heated in a water bath (Thermo Fisher) to 80 °C for 10 min and then centrifuged at 800 g for 5 min. The cells were resuspended in 100 μl of Binding Buffer and incubated with 5 μl Annexin-V-FITC (BioLegend, San Diego, CA, USA) and 10 μl PI (BioLegend) at room temperature in the dark for 15 min. Annexin-V Binding Buffer (BioLegend) preheated to room temperature was used to dilute the cell suspension to $4 \times 10^5$ cells/mL. The cell apoptosis rate was assessed in a Cellometer K2 Cell Viability Counter machine (Nexcelom, Boston, MA, USA) following the manufacturer's instructions.

***Cell cycle assay***

HeLa cells growing at the log phase were seeded in a 6-well plate ($2 \times 10^5$ cells/well) and maintained in complete growth medium for 24 hrs. Cells in the experimental group were then cultured in medium containing lovastatin (0, 7, 14 and 28 μmol/L) for another 24 hrs and 48 hrs. Each concentration and time point was tested in triplicates. Cells in each well were digested, collected and centrifuged at 800 g for 5 min.

Supernatants were discarded and the pellets were washed in 1ml cold 1× PBS; 500 μl ethanol (anhydrous) was then used to resuspend each pellet and the suspension was incubated at 20 °C for 15 min and then centrifuged at 800 g for 5 min. Cold PBS was used to wash the pellets twice. The cells were resuspended in 200 μl cold 1×PBS containing 2 μl of 4 mg/mL RNase A Solution (Qiagen, Germany) and 20 μl of 500 μg/mL PI (BioLegend). The cells were incubated at room temperature in the dark for 5min and then their cell cycles were assessed using a 20 μl suspension in the Cellometer K2 machine.

***Gene Chip analysis***

Cells were cultured in 6-well plates to a 70% confluence. Cells in the experimental group were then cultured in medium containing lovastatin (0 and 14 μmol/L) for another 48 hrs. Total RNA was isolated using the QIAGEN miRNeasy-Mini Kit and end-labeled with the FlashTag™ Biotin HSR RNA Labeling Kit (Thermo Fisher). Hybridization was performed with the GeneChip™ miRNA 4.0 (Thermo Fisher) and the subsequent washing and scanning steps were conducted following the manufacturer's instructions (Thermo Fisher). For data analysis, Transcriptome Analysis Console software (Thermo Fisher) was used and the miRNAs or mRNAs with ANOVA p-value < 0.05 and |fold change (linear)| >2 were considered to be differentially expressed RNAs.

***RT-qPCR***

Cells were cultured in 6-well plates to a 70% confluence. Cells in the experimental group were then cultured in medium containing lovastatin (0 and 14 μmol/L) for another 48h. Total RNA was isolated using the QIAGEN

miRNeasy-Mini Kit. Reverse transcription was performed using the mi Script II RT Kit (Qiagen). U6 was used as the internal reference for miR-34c and the qPCR assay was conducted using the mi Script SYBR Green PCR Kit (Qiagen) following the manufacturer's instructions. GAPDH was the internal reference for TP53 and MDM2 and the qPCR assay was conducted using the Trans Start Top Green qPCR Super Mix following the manufacturer's instructions. qPCR assays were performed in triplicates for each gene in the Roche Cycler480 machine and the $2^{-\Delta\Delta CT}$ method was used to calculate the expression level of each gene relative to the control.

## STATISTICAL ANALYSIS

Quantitative data are shown in mean ± standard deviation (x±SD). All statistical analysis was performed using GraphPad Prism 8 software. The independent sample *t*-test was used to compare the difference of mean values between two groups and $p<0.05$ was considered statistically significant.

## RESULTS

### *Effects of lovastatin on HeLa cell proliferation*

Cell proliferation assays showed that the half maximal inhibitory concentration of lovastatin was about 14 µmol/L and that the inhibitory effect of lovastatin on HeLa cell proliferation was dose-dependent (fig. 1).

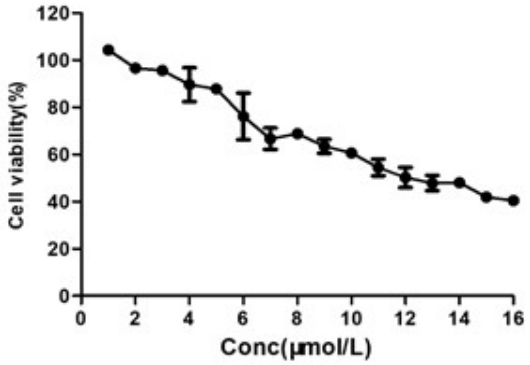


**Fig. 1**: The inhibition rate of HeLa cell proliferation after 48 h treatment with different concentrations of lovastatin.

### *Effects of lovastatin on HeLa cell migration*

Cell migration assay showed that lovastatin had an inhibitory effect on HeLa cell migration and this effect was time-and concentration-dependent. The wound width was smaller in cells treated with higher concentrations of lovastatin (fig. 2A). Cells in the control group showed clear migration, whereas in the cells treated with higher concentrations of lovastatin less prominent migration and more floating cells were observed. The migration rates of cells treated with 14µmol/L lovastatin for 24h and 48h were 7.24% ($p<0.001$) and 7.70% ($p<0.01$), respectively (fig. 2B).

### *Effects of lovastatin on HeLa cell apoptosis*

The cell apoptosis assay showed that lovastatin led to HeLa cell apoptosis. The apoptosis rate of cells treated with 7 µmol/L, 14 µmol/L and 28 µmol/L lovastatin for 48 hrs was 22.99% ($p<0.01$), 35.03% ($p<0.001$) and 45.7% ($p<0.001$), respectively (fig. 3B).

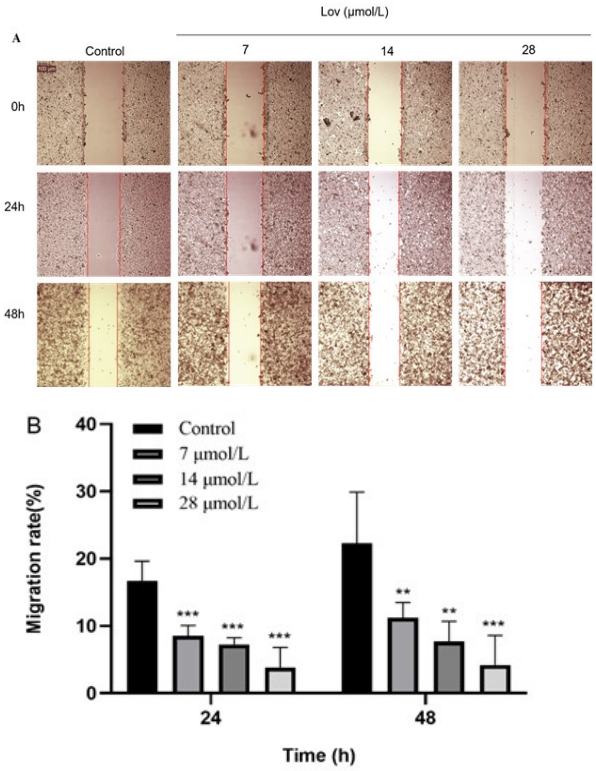


**Fig. 2**: Results of the scratch wound assay (A); Migration rates of HeLa cells analyzed after treatment with different concentrations of lovastatin at different time points (B). Scale bar = 100 µm.* $p<0.05$, ** $p<0.01$, *** $p<0.001$.

### *Effects of lovastatin on cell cycle of HeLa cells*

Cell cycle assay showed that lovastatin led to cell cycle arrest (fig. 4). The percentages of cells at the G0/G1 stage after 14 µmol/L or 28 µmol/L lovastatin treatment for both 24 hrs and 48 hrs were significantly higher than the control and the percentages of cells at S phase were significantly lower than the control.

The percentages of cells at G2/M stage after 28 µmol/L lovastatin treatment for 24 hrs was significantly lower than the control ($p<0.05$). The above results suggest that lovastatin prevents HeLa cells from passing through the G1/S checkpoint and thus inhibits mitosis.

### *Effects of lovastatin on the expression of miR-192-5p and miR-92a-1-5p*

Gene transcription analysis on the Affymetrix Gene Chip platform identified 37 differentially expressed miRNAs upon treatment with 14 µmol/L lovastatin for 48h (fold change ≥2, $p<00.01$, fig. 5A). The expression level of

miR-192-5p showed a 4.87-fold increase from 2.43±0.04 (control) to 4.64±0.17 (lovastatin treated) and the difference was statistically significant ($p$<0.001, fig. 5B). The expression of miR-92a-1-5p displayed a 2.98-fold decrease from 3.38±0.42 (control) to 1.90±0.26 (lovastatin treated) ($p$<0.001, fig. 5C).

***Validation of the differential expression of miR-192-5p and miR-92a-1-5p by RT-qPCR***

RT-qPCR results showed that 14 µmol/L lovastatin treatment for 48 hrs significantly increased the expression of miR-192-5p with a fold change of 2.11 ($p$<0.001, fig. 6A) and decreased expression of miR-92a-1-5p with a fold change of 0.47 ($p$<0.001, fig. 6B).

***Validation of the expression of the target genes MDM2 and TP53 by RT-qPCR***

Using the IPA software (QIAGEN) MDM2 was prioritized as the prime target gene of miR-192-5p and TP53 as the prime target gene of miR-92a-1-5p. Treatment with 14 µmol/L lovastatin for 48h led to an increase in the expression level of MDM2, however the difference was not statistically significant (fig. 7A). Lovastatin treatment significantly increased TP53 expression ($p$<0.01, fig. 7B).

## DISCUSSION

Recent research has found that lovastatin has a great variety of benefits to human health. It decreases cholesterol levels in the blood (Xiong *et al*., 2019) and therefore has been applied in producing hypolipidemic drugs. Additionally, it has protective effects on diverse diseases, such as orokeratosis (Stephens *et al*., 2021), Parkinson's disease (Lin *et al.,* 2021), cardiolaminopathy (Sayed *et al*., 2020), multiple sclerosis (Singh *et al*., 2018) and Angelman syndrome (Chung *et al*., 2018). TP53 is a tumor suppressor gene encoding a widely expressed transcription factor p53, which plays a critical role in protecting against cytotoxicity and in regulating the cell cycle, apoptosis, DNA repair, cell metabolism and aging. p53 monitors the integrity of DNA during G phase of cell cycle and inhibits mitosis until the damaged DNA has been repaired.

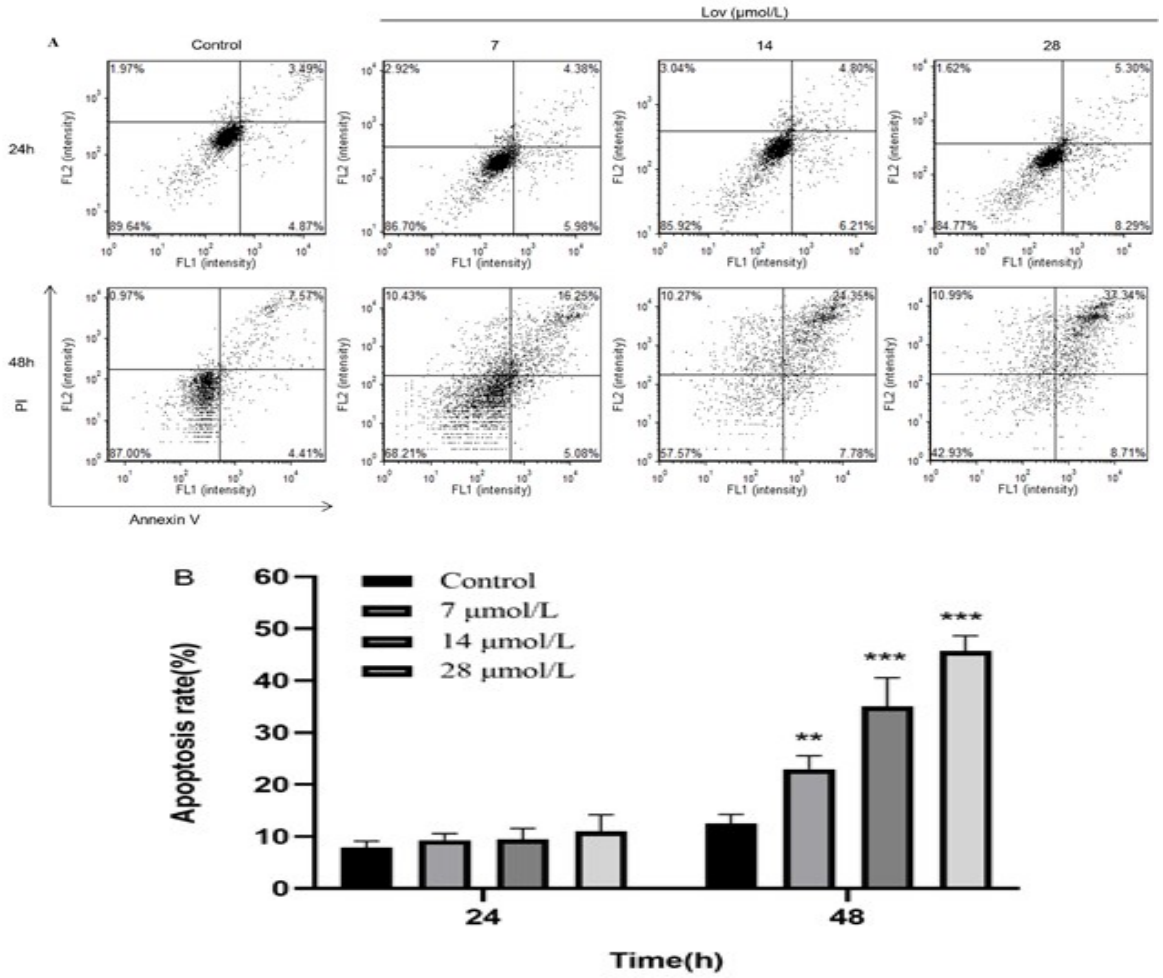


**Fig. 3**: Effects of lovastatin on HeLa cell apoptosis. ** $p$<0.01,*** $p$<0.001.

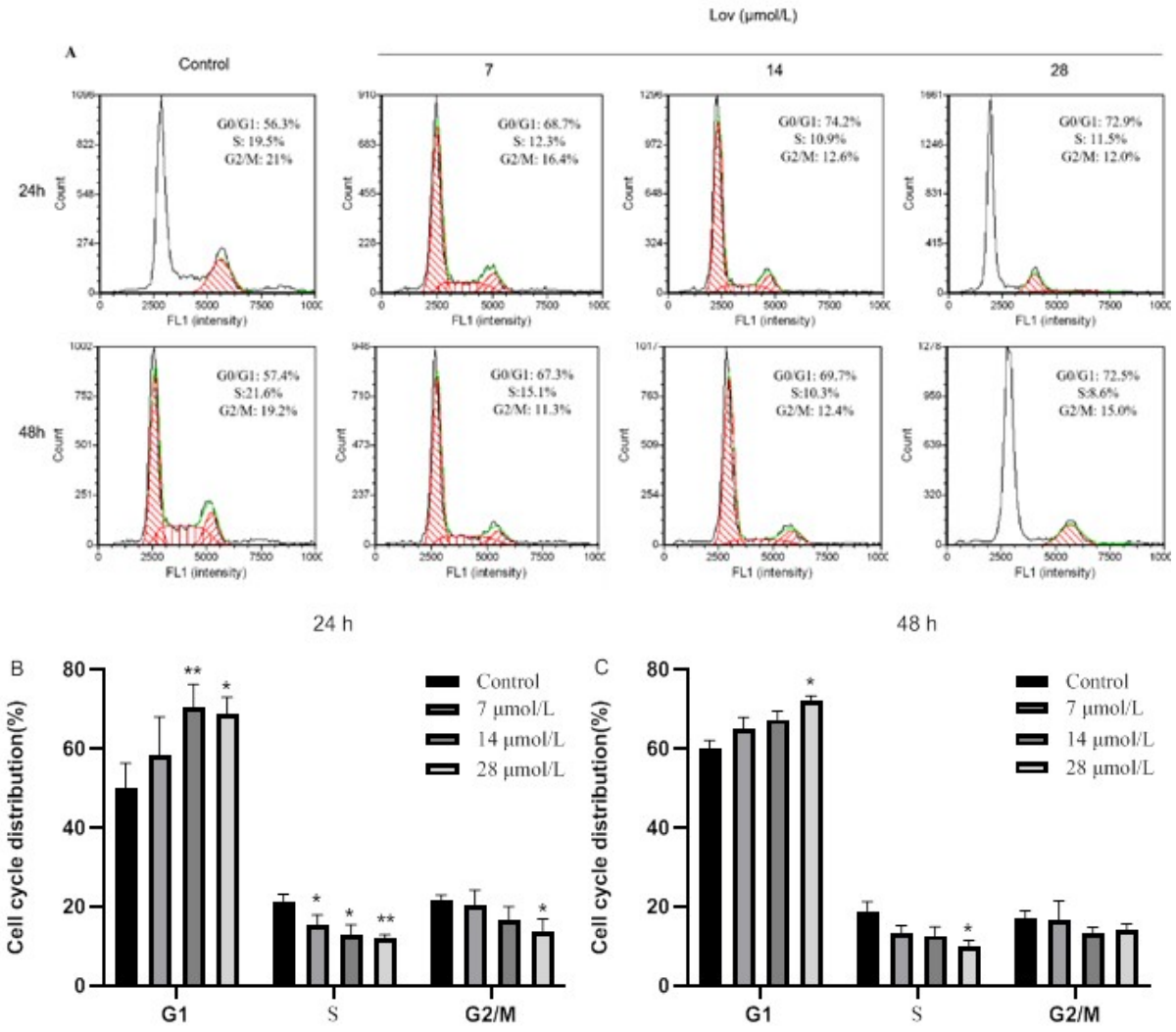


**Fig. 4**: Lovastatin treatment of HeLa cells for 24 h and 48h leads to cell cycle arrest (A,B,C). * $p<0.05$,** $p<0.01$.

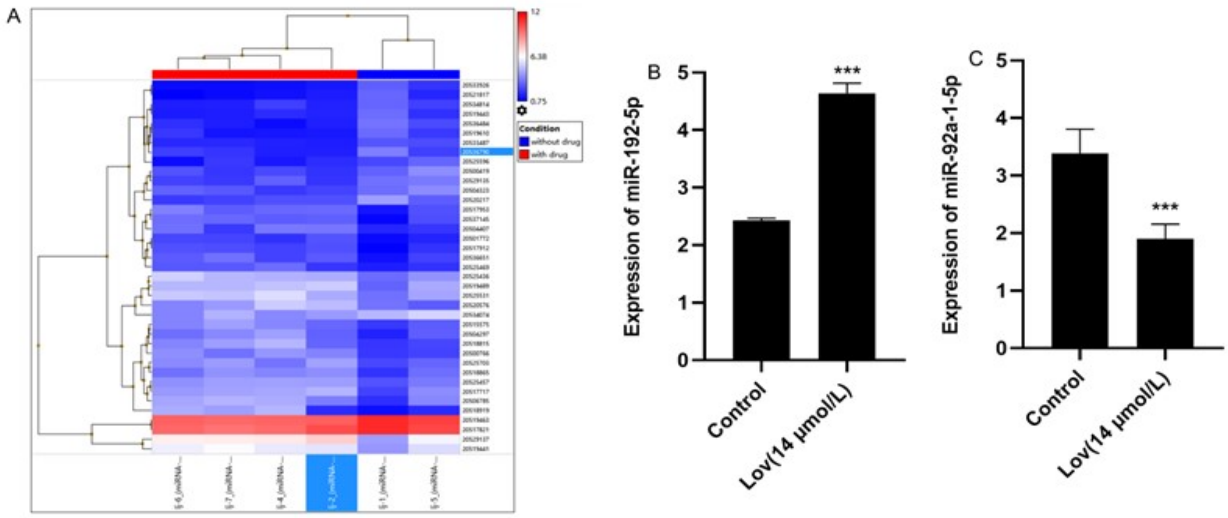


**Fig. 5**: GeneChip results of miRNAs expression levels. A. a heat map of miRNAs expression levels ≥2 fold change; B. miR-192-5p; C. miR-92a-1-5p. ** $p<0.01$,*** $p<0.001$.

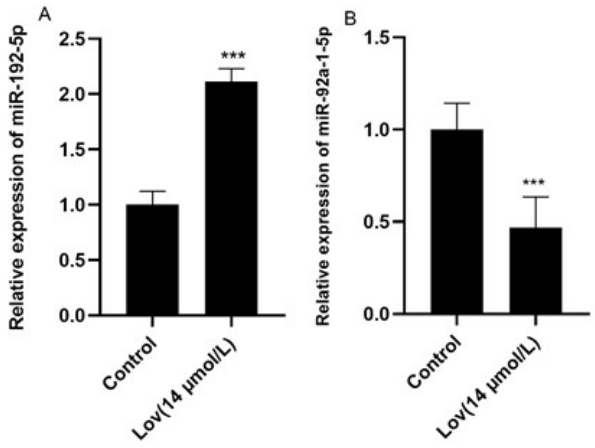


**Fig. 6**: Effects of 14 μmol/L 48h lovastatin treatment on the expression of miR-192-5p and miR-92a-1-5p in Hela cells. A. miR-192-5p; B. miR-92a-1-5p.*** $p<0.001$.

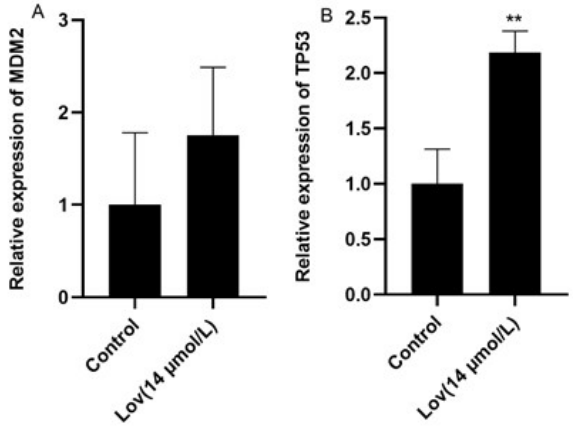


**Fig. 7**: Effects of 48h 14 μmol/L lovastatin treatment on the expression of MDM2 and TP53 in Hela cells. A. MDM2; B. TP53. **$p<0.01$.

Mechanisms of the p53 regulation include up regulating the expression of cyclin-dependent kinase inhibitor (CDKI) p21 to induce G1 phase arrest (Barr *et al*., 2017; Maddocks *et al*., 2013). Alternatively, p53 triggers apoptosis if DNA cannot be completely repaired (Zhang *et al*., 2019). p53 inactivation leads to aberrant cell proliferation. p53 and one of its target genes, MDM2, compose a negative feedback loop in which p53 activates *MDM2* transcription and MDM2 inhibits p53 activity through the ubiquitin-mediated degradation of p53.

Maintaining a proper MDM2/p53 ratio is crucial for normal cell proliferation. Excess of p53 leads to cell apoptosis and lack of p53 leads to malignant transformation of normal cells (Fujita, 2019). MDM2 could also induce G1 phase arrest (Levav-Cohen *et al*., 2014). The function of p53 is affected by its expression levels, post-translational modifications, target genes and other coordinators. Low levels of p53 expression inhibit cell cycle progression and a high level of its expression induces apoptosis. The phosphorylation and acetylation of p53 induce apoptosis. For instance, phosphorylated p53 induces the expression of the pro-apoptotic factor p53AIP1, which in turn releases cytochrome c and induces apoptosis. p53 can induce apoptosis through mitochondrial membrane permeabilization by interacting with and antagonizing the anti-apoptotic proteins BCL-xL and BCL-2 (Wei *et al*., 2021). P53 levels are induced by cellular stress, such as that following DNA damage or viral infection. After p53 activation, the transcription of p21/CDKN1A is strongly induced as a direct target of p53. Then, cyclin-dependent kinase inhibitor p21 blocks the activity of several cyclin-CDK complexes. This leads to low phosphorylation of RB, which promotes the formation of RB-E2F complexes and their binding to E2F sites in the target promoter. Because of this indirectly dependent transcriptional inhibition mechanism of p53, many target genes are down regulated. Since most of the suppressed genes are involved in the cell cycle process, their down regulation leads to cell cycle arrest (Engeland, 2022). Concomitant p53 activation and Bcl-2 inhibition in acute myeloid leukemia cells switch the cellular response from G1 arrest to apoptosis and thus overcome the apoptosis resistance of cancer cells (Pan *et al*., 2017).

In the present study, we found that lovastatin was cytotoxic to HeLa cells and that it could induce apoptosis, G0/G1 cell-cycle arrest, upregulation of miR-192-5p expression and down regulation of miR-92a-1-5p expression. The expression level of TP53, the target gene of miR-92a-1-5p, significantly increased after lovastatin treatment. The expression level of MDM2, the target gene of miR-192-5p, also showed an increase after lovastatin treatment but the increase was not statistically significant. miRNAs degrade or inhibit the translation of the target mRNAs through base pairing with the seed sequence of the mRNA target molecules (Bartel, 2009). Mounting evidence shows that microRNAs (miRNAs) and their target genes are aberrantly expressed in many cancers and are linked to tumor occurrence and progression, such as in esophageal cancer (Zhao *et al*., 2021), prostate cancer (Saliminejad *et al*., 2019; Sun *et al*., 2021) and breast cancer (Ali Ahmed *et al*., 2022). The genetic deletion or amplification and epigenetic methylation of miRNA genomic loci and the transcription factor-mediated regulation of primary miRNA often alter the landscape of miRNA expression in cancer. Dysregulation of the multiple processing steps in mature miRNA biogenesis can also cause alterations in miRNA expression in cancer (Ali Syeda *et al*., 2020). In the performance of distinguishing cancer types, the m6A-miRNAs signature showed superior sensitivity in each cancer type and presented a satisfactory AUC in identifying lung cancer, gastric cancer, and hepatocellular carcinoma (Zhang *et al*., 2021). Cancer is a generic term for a large group of diseases that can affect any part of the body. Other common terms used for it are malignant tumors and

neoplasms. One defining feature of cancer is the rapid creation of abnormal cells that grow beyond their usual boundaries, which can then invade adjoining parts of the body and spread to other organs. Cancer arises from the transformation of normal cells into tumor cells in a multi-stage process that generally progresses from a pre-cancerous lesion to a malignant tumor. Mutations in the TP53 gene are the most common genetic variations in cancer and lead to the occurrence, development, and metastasis of cancer cells, as well as resistance to therapies (Sabapathy and Lane, 2018). Over half of the previously reported cases with tumors were caused by *TP53* mutations and the other half were due to inactivation of TP53 pathways (Cheok *et al*., 2011). Cell cycle regulation is executed through a regulatory network composed of a series of check points. Lovastatin arrests HeLa cells at the G0/G1 phase and inhibits mitosis. We deduce that lovastatin exerts anti-tumor effects through the down regulation of miR-92a-1-5p expression and thereby the upregulation of TP53 expression, arresting cells at the G1/S checkpoint. Altogether, lovastatin may be a promising therapeutic target in the treatment of cervical cancer, and miR-92a-1-5p and TP53 likely contribute to its anticancer function.

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