**Effect of MiR-10b on cervical cancer rats through mTOR/P70S6K signaling pathway**

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**ABSTRACT**

The purpose of this experiment was to observe the biological effect and mechanism of miR-10b on cervical cancer (CC) rats. For this purpose, the rat model of CC was established and divided into three groups (Inhibitors/ Mics/Mimics/Control). The miR-10b transfection efficiency was analyzed via RT-PCR in cervical tissues in each group. The content of CD3+, CD4+, and CD8+ was detected. The levels of IL-8, TNF-β, IL-6, (CAT, SOD, and MDA were determined via ELISA, and the apoptosis of cervical tissues was detected using TUNEL assay. The expressions of Caspase-3, Bel-2, and the mTOR/P70S6K pathway genes and proteins were detected by qRT-PCR and Western blotting. Results showed that miR-10b was significantly increased in the Mics group and decreased in the Inhibitors group. The content of IL-8, TNF-β, IL-6, CAT and MDA was raised, while that of SOD notably declined in the Inhibitors group. There were remarkably more apoptotic cells in the Mics group, dominated by gliocytes, and fewer apoptotic cells in the Inhibitors group, with increased content of CD3+, CD4+ and CD8+. The Bel-2, mTOR, and P70S6K mRNA expressions in the Inhibitors group were up-regulated than those in the other two groups, and the Caspase-3 gene in the Mics group was increased and close to that in the control group. In the Mics group, the mTOR and P70S6K protein were remarkably lower than those in the Inhibitors group. In conclusion, miR-10b can inhibit the occurrence and development of CC in rats by suppressing mTOR/P70S6K signaling, reducing the level of inflammation and oxidative stress, and increasing the level of immune factors.

**Introduction**

Cervical cancer (CC) is the most common gynecological tumor, only next to breast cancer. About 500,000 CC cases are diagnosed each year, and 45% of them are fatal (1-2). The morbidity rate of CC gradually increases with increasing age, seriously threatening women's health and life including in China (3). The development of CC is extremely complex and has not yet been fully elucidated (4). Early detection of cancer is crucial for the therapeutic effect. The number of new cases is gradually increasing and the death cases also increase sharply every year, and the operation, radiation therapy, or chemotherapy will eventually fail in approximately 30% of CC patients (5). Therefore, the discovery and identification of new biomarkers for early CC are urgently needed for the early detection and individualized treatment of CC. High-risk human papillomavirus detection will be an important detection means and individualized treatment of CC. High-risk human papillomavirus detection will be an important detection means for CC (6). Furthermore, cisplatin-based radiotherapy and chemotherapy regimens can improve the disease condition, which is also considered to improve the tumor-free and progression-free survival of female patients, but there are serious side effects, and the long-term chemotherapy seriously threatens patients' lives and health, at a high cost, increasing the living burden of living for patients and their family (7-8). Therefore, searching for new therapeutic methods for CC has become the focus of scientists.

In recent years, with the progress in gene study, researchers have paid attention to microribonucleic acids (miRNAs) and have begun to study their specific biological roles (9-10). Increasingly more studies have confirmed that 1/3 of genes could be regulated by miRNAs in human (11-12). MiRNAs have become important regulators of gene expression in many diseases including cancer (13). It is reported that miR-10b is down-regulated in advanced small cell carcinoma of the cervix (SCCC) compared with that in patients with early SCC (14). However, the role of miR-10b in CC remains unclear currently.

The mammalian target of rapamycin (mTOR) belongs to the protein kinase in the phosphokinase-associated kinase family, and it mainly regulates p70S6 kinase (P70S6K) in mammals (15). mTOR -mediated P70S6K phosphorylation is believed to improve the mRNA translation of cell growth-related proteins and the mTOR abnormal activation has been found in a variety of cancers (16-18), but the mTOR signaling pathway function in CC has not been well revealed.

MiR-10b could exert a therapeutic effect on CC in rats through the mTOR/P70S6K signaling pathway. Our results revealed the therapeutic effect of miR-10b on rats CC, and provide an experimental basis for the follow-up research of new drugs.
Materials and Methods

Commonly used reagents and instruments
SIHA cell lines (ATCC, USA), chloral hydrate (Sigma), tissue homogenizer (FLUKO), microplate reader (Thermo, USA), qPCR instrument (Applied Biosystems), SPT rats (Biological Sciences, Shanghai), SuperScript III RT kit, SYBR qPCR Mix (ABI), IL-1 and IL-6 ELISA kits (Wuhan Hanbio Co., Ltd.), radio RIPA lysis buffer (Beyotime), TUNEL fluorescence staining kit (Roche, Germany), BCA protein concentration assay kit (Biosharp), and β-actin, secondary, and primary antibodies (CST).

Animal grouping and modeling
After the concentration was adjusted, 150 μL of SIHA cell suspension (2.0×106/L) was taken and subcutaneously inoculated at the axilla to establish the CC rat model. MiR-10b was transferred into rats. Rats were divided into the miR-10b inhibitors group (Inhibitors group), miR-10b mimics group (Mimics group) and normal group (Control group), and each group has 15 rats. All operations were performed according to the regulations in the NIH Laboratory Animal Guide. The serum of the rat was collected and stored at -80°C, and the biochemical indexes detached. The rats were anesthetized with pentobarbital sodium. Two samples of cervical tissues were taken, one for TUNEL staining and the other stored at -80°C for detecting the genes and proteins expression levels.

Transfection efficiency of miR-10b
To deeply explore the miR-10b function in CC, our team transfected miR-10b into rats using the adenovirus and detected the miR-10b transfection efficiency in cervical tissues was via RT-PCR. Chloral hydrate was injected into the abdominal cavity of rats for anesthesia, and an appropriate number of cervical tissues were carefully taken and mashed with tissue homogenizer to analyze miR-10b expression, to prepare for the in-depth study of the miR-10b regulatory mechanism in CC.

Inflammatory factors detection
The expression of inflammatory factors in serum was detected by ELISA. According to the actual conditions and instructions, use the kit to detect the changes in each index. Finally, the inflammatory factors absorbance in rats was detected by the microplate reader.

Statistical analysis
SPSS 20.0 software was used for the processing of raw experimental data, and multiple comparisons were performed for the data. The results were expressed as mean ± standard deviation (χ±SD). p<0.05 suggested the statistically significant difference. Each experiment was performed at least 3 times.

### Table 1. Primer sequences of indexes in RT-PCR.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>F: 5’-TGACTTCAACAGCGACACCCA-3’ R: 5’-CACCCTGTGTGCTGTAAGCCAA-3’</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>F: 5’-CTACCGCACCCGGTACTAT-3’ R: 5’-TCCCAGTTAACAACAGAATGAG-3’</td>
</tr>
<tr>
<td>B-cell lymphoma-2 (Bel-2)</td>
<td>F: 5’-GGTGCTCTTGAGATCTCTGG-3’ R: 5’-CCATCGATCTTCAGAAGTCTC-3’</td>
</tr>
<tr>
<td>mTOR</td>
<td>F: 5’-CTG GGA CTC AAA TGT GTG CAG TTC-3’ R: 5’-GAAA CAA TAG GGT GAA TGA TCC GGG-3’</td>
</tr>
<tr>
<td>P70S6K</td>
<td>F: 5’-TACTTCCGGTACTTGGTAA-3’ R: 5’-GATGAAAGGTGATCTCTTTC-3’</td>
</tr>
<tr>
<td>miR-10b</td>
<td>F: 5’-AGCTGTTTCTTGCACACCAAG-3’ R: 5’-GTGCTACCCCTGTGAAAC-3’</td>
</tr>
</tbody>
</table>
Results

miR-10b expression in each group
To detach the miR-10b transfection efficiency in each group, its expression level was detected. MiR-10b was upregulated in the Mimics group (p<0.05) and decreased in the Inhibitors group (p<0.05), indicating that the transfection effect is obvious and subsequent experiments can be performed (Figure 1).

Levels of immune indexes CD3+, CD4+, and CD8+
CD3+, CD4+ and CD8+ levels in the peripheral blood obviously decreased in the Inhibitors group, while they were increased in the mimic group (p<0.05) (Table 2).

Serum TNF-β, IL-8 and IL-6 content
The level of IL-8, IL-6 and TNF-β was increased in the Inhibitors group (p<0.05), while it declined in the Mimics group (p<0.05) (Table 3).

TUNEL apoptosis assay
In the Mimics group, the TUNEL-positive cells number was larger than in the Control group (p<0.05), and there were fewer positive cells in the Inhibitors group (Figure 2), suggesting that the abnormal cell proliferation occurs and the apoptosis declines in CC and the miR-10b can promote the apoptosis of CC cells.

Expressions of related genes
The inhibitor group had significantly higher Bcl-2, mTOR and P70S6K (p<0.05), and a remarkably lower expression of Caspase-3 than the control group (p<0.05), while the expressions in the Mimics group displayed the opposite trends (p>0.05) (Figure 3).

Table 2. Levels of CD3+, CD4+ and CD8+ (%).

<table>
<thead>
<tr>
<th>Group</th>
<th>CD3+</th>
<th>CD8+</th>
<th>CD4+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>70.5±2.1</td>
<td>38.4±1.1</td>
<td>45.7±2.1</td>
</tr>
<tr>
<td>Inhibitors</td>
<td>31.4±3.4*</td>
<td>15.4±1.9*</td>
<td>14.4±1.8*</td>
</tr>
<tr>
<td>Mimics</td>
<td>68.8±2.5*</td>
<td>32.5±1.1*</td>
<td>40.6±2.1*</td>
</tr>
</tbody>
</table>

Note: The levels of CD3+, CD4+ and CD8+ in the peripheral blood decline in the Inhibitors group, while they are increased in the Mimics group (p<0.05). *p<0.05 vs. Control group, #p<0.05 vs. Inhibitors group.

Table 3. Content of serum TNF-β, IL-6 and IL-8.

<table>
<thead>
<tr>
<th>Group</th>
<th>TNF-β (fmol/mL)</th>
<th>IL-6 (mg/L)</th>
<th>IL-8 (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.2±3.0</td>
<td>19.0±1.1</td>
<td>21.0±4.1</td>
</tr>
<tr>
<td>Inhibitors</td>
<td>48.1±2.0*</td>
<td>84.4±1.4*</td>
<td>96.8±2.4*</td>
</tr>
<tr>
<td>Mimics</td>
<td>19.7±3.5*</td>
<td>26.1±3.0*</td>
<td>29.5±3.1*</td>
</tr>
</tbody>
</table>

Note: The content of IL-8, IL-6 and TNF-β is increased in the Inhibitors group (p<0.05), while it declines in the Mimics group (p<0.05). *p<0.05 vs. Control group, #p<0.05 vs. Inhibitors group.

Table 4. Levels of CAT, SOD and MDA in cervical tissues.

<table>
<thead>
<tr>
<th>Group</th>
<th>CAT (IU/mL)</th>
<th>SOD (μ/mg)</th>
<th>MDA (μ/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.7±2.3</td>
<td>290.8±1.9</td>
<td>5.0±0.4</td>
</tr>
<tr>
<td>Inhibitors</td>
<td>38.9±1.0*</td>
<td>109.7±2.1*</td>
<td>30.4±2.1*</td>
</tr>
<tr>
<td>Mimics</td>
<td>8.3±1.2*</td>
<td>268.5±1.1*</td>
<td>9.3±1.1*</td>
</tr>
</tbody>
</table>

Note: The content of CAT and MDA is increased in the Inhibitors group (p<0.05) and declines in the Mimics group (p<0.05), while that of SOD shows the opposite trend (p<0.05). *p<0.05 vs. Control group, #p<0.05 vs. Inhibitors group.
better outcomes for immunotherapy (23-24). In our study, national therapy of advanced malignant tumors can provide Monitoring the T-cells general functions during conventional therapy involves the abnormal expression of cancer-related genes. Currently, the therapeutic methods for CC include operation, radiotherapy, and chemotherapy, but these methods will eventually fail in about 30% of CC patients. Moreover, there are great side effects, and long-term chemotherapy seriously threatens patients’ lives, with a high cost, increasing the living burden of patients and their families (19-20). Therefore, searching for new therapeutic methods for CC has become the top priority.

In this study, the CC rat model was established. Our results confirmed that miR-10b was up-regulated in rat CC. Its role and function in CC rats needed further research. CD8+ cells can control tumor growth and are usually as potential targets for cancer immune monitoring. High levels of CD3+, CD4+ and CD8+ with a better efficacy in ovarian patients (21-22). During and after chemotherapy, there is a strong correlation between clinical tumor response and CD8+/CD4+ cell functions. In addition, Monitoring the T-cells general functions during conventional therapy of advanced malignant tumors can provide better outcomes for immunotherapy (23-24). In our study, the CD3+, CD4+ and CD8+ levels in the peripheral blood declined in the Inhibitors group, while they were increased in the Mics group, consistent with the above studies.

IL-6 can accelerate the other inflammatory mediators’ production (25). Excessive responses to inflammatory and oxidative stress are found in our mouse models, including neutrophils, IL-6, and TNF. The content of IL-8, IL-6 and TNF-β was up-regulated in the inhibitors group, while it was reduced in the mimics group. In addition, oxygen-free radicals participate in CC and affect subsequent resuscitation. SOD exists widely, and MDA could resist the function of SOD, with cytotoxicity. Antioxidant therapy can alleviate organ oxidative stress and achieve better results (26-27). It was found that the content of CAT and MDA was raised in the Inhibitors group and decreased in the Mics group, while that of SOD showed opposite trends, similar to those of the above studies.

MTOR mainly regulates P70S6K in mammals. The mTOR signaling pathway involves many cancers occurrence and development (28), but its role of it in human CC remains unclear. MiRNAs have attracted much attention in recent years. However, the role of miR-10b in CC remains unclear. In this study, no positive cells were observed in the Control group, the TUNEL positive cells number in the Mics group was larger than in the Control group, and there were fewer positive cells in the Inhibitors group, indicating that abnormal cell proliferation occurs and the apoptosis declines in CC, and miR-10b overexpression of miR-10b can enhance apoptosis of CC cells. Furthermore, at the Inhibitors group, higher mRNA expressions of Bcl-2, mTOR and P70S6K and a significantly lower expression of Caspase-3 than the Control group, while the expressions in the Mics group showed opposite trends. The mTOR and P70S6K protein expressions were significantly up-regulated in the Inhibitors group, while they significantly declined in the Mics group. All these results are similar to previous research (29-30). In summary, miR-10b could block CC occurrence and development by suppressing the mTOR/P70S6K signaling pathway activation. Nextly, such an effect will be further verified through cell experiments.

In conclusion, a series of pathological changes will occur in CC, such as cellular oxidative stress, apoptosis, and inflammation, but miR-10b can block the occurrence of disease by restraining the mTOR/P70S6K signaling pathway. Its effect can be further explored using more molecular means in the future. This study not only provides a solid theoretical basis for CC prevention but also provides experimental bases for further research.

Declaration of interest
The authors declare that they have no conflict of interest.

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