MiR-101 promotes nasopharyngeal carcinoma cell apoptosis through inhibiting Ras/Raf/MEK/ERK signaling pathway


Abstract. – OBJECTIVE: Extra-cellular signal regulated kinase (ERK)/mitogen activated protein kinase (MAPK) signaling pathway is widely involved in cell proliferation and apoptosis. MAPK kinase 1 (MEK1) is the upstream protein kinase of ERK that can activate ERK/MAPK signaling pathway. microRNA-101 (MiR-101) down-regulation is found to be associated with nasopharyngeal carcinoma (NPC) pathogenesis. Bioinformatics analysis shows the complementary targeted relationship between miR-101 and the 3’-UTR of MEK1 mRNA. This study explores the role of miR-101 in regulating MEK1 expression, ERK/MAPK signaling pathway activation, and NPC pathogenesis.

MATERIALS AND METHODS: Dual luciferase assay confirmed the targeted relationship between miR-101 and MEK1. MiR-101 and MEK1 expressions were compared in inflammatory nasopharynx tissue and NPC tissue. MiR-101, MEK1, phosphorylated ERK 1/2 (p-ERK1/2), survivin expressions in NP69, CNE-1, HONE1, and C666-2 cell lines were detected. NPC cell line C666-1 was cultured in vitro and divided into four groups, including miR-NC, miR-101, si-NC and si-MEK1. Cell apoptosis was determined by flow cytometry. Cell proliferation was evaluated by EdU staining.

RESULTS: MiR-101 targeted inhibited MEK1 expression. MiR-101 was significantly down-regulated, while MEK1 was significantly elevated in NPC tissue compared with inflammatory nasopharynx tissue. MiR-101 was markedly declined, whereas MEK1, p-ERK1/2, and survivin were apparently increased in CNE-1, HONE1, and C666-1 cells compared with NP69 cells. MiR-101 mimic and/or si-MEK1 transfection significantly reduced MEK1, p-ERK1/2, and survivin levels, attenuated cell proliferation, and enhanced cell apoptosis.

CONCLUSIONS: Down-regulation of miR-101 was related to NPC pathogenesis. MiR-101 elevation suppressed NPC cell proliferation and promoted apoptosis through targeted inhibiting MEK1 expression to alleviate ERK/MAPK signaling pathway and survivin expression.

Key Words: miR-101, MEK1, Ras/Raf/MEK/ERK, Apoptosis, Proliferation, Nasopharyngeal carcinoma.

Introduction

Nasopharyngeal carcinoma (NPC) is a kind of epithelial malignant tumor occurred in the top and side wall of nasopharynges. It is a common type of malignancy in South-East Asia and South China with an incidence at 40-60/100,000. The incidence of NPC has been top in head and neck malignant tumors. NPC may cause various symptoms, including nasal congestion, nose blood, diplopia, headache, and cranial nerve compression based on different severity degree, leading to serious threat to the quality of life and health. Therefore, in-depth study of NPC pathogenesis and exploration of the abnormal change of molecule expression are of great significance for NPC early diagnosis, therapeutic efficacy, and prognosis. Extra-cellular signal regulated kinase (ERK)/mitogen activated protein kinase (MAPK) signaling pathway that widely expresses in various tissues and cells, regulates multiple biological processes, including cell proliferation, cycle, apoptosis, migration, and invasion. ERK/MAPK signaling pathway excessive activation induces cell abnormal proliferation, apoptosis, and differentiation, which are closely related to tumor pathogenesis, progression, and metastasis. MAPK kinase 1 (MEK1) phosphorylates the residue of Tyr/Thr on ERK protein to activate ERK/MAPK signaling pathway. MEK1 elevation plays a crucial promoting role in a variety of tumor occurrence and development. It was showed that MEK1 expression and function enhancement are related to NPC cell proliferation, apoptosis, and drug resistance. microRNA (MiRNA) is a type of...
endogenous single stranded non-coding RNA at the length of 22-25 nt discovered from eukaryote. It plays a degrading or inhibiting role on mRNA by binding with the 3'-UTR, thus participating in cell proliferation, differentiation, and migration. The role of miRNA in tumorigenesis receives more and more attention. Numerous studies revealed that miR-101 significantly reduced in NPC tissue and cells, suggesting that miR-101 may play a tumor suppressor role in NPC occurrence and development. Bioinformatics analysis shows the complementary targeted relationship between miR-101 and the 3'-UTR of MEK1 mRNA. This study explores the role of miR-101 in regulating MEK1 expression, ERK/MAPK signaling pathway activation, and NPC pathogenesis.

**Materials and Methods**

**Main Reagents and Materials**

Human NPC cell line CNE-1, HONE1, and C666-1, and human nasopharynges epithelial cell line NP69 were purchased from Xinyu Biotech co., Ltd (Shanghai, China). Dulbecco’s Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute 1640 (RPMI-1640) medium were got from Lonza Inc. (Allendale, NJ, USA). Fetal bovine serum (FBS), penicillin, and streptomycin were bought from Gibco BRL, Co., Ltd., (Grand Island, NY, USA). MiniBEST Universal RNA Extraction Kit, PrimeScript™ RT reagent Kit, and SYBR Green were obtained from TaKaRa (Dalian, China). Horse radish peroxidase (HRP) conjugated secondary antibody was derived from NeoBioscience (Shenzhen, China). Lipofectamine 2000 was provided by Invitrogen Life Technologies (Carlsbad, CA, USA). MiR-101 mimic, miR-101 inhibitor, and miR-NC were bought from Ribobio (Guangzhou, China). Mouse anti-human MEK1, p-ERK1/2, survivin, and β-actin primary antibodies were obtained from Abcam Biotech. (Cambridge, MA, USA). Mouse anti-human MEK1, p-ERK1/2, survivin, and β-actin primary antibodies were obtained from Abcam Biotech. (Cambridge, MA, USA). Horse radish peroxidase (HRP) conjugated secondary antibody was derived from NeoBioscience (Shenzhen, China). Luciferase reporter gene vector pLUC was bought from Ambion Inc. (Austin, TX, USA). Dual luciferase activity detection kit was purchased from Promega (Madison, WI, USA). RIPA and bicinchoninic (BCA) protein quantification kit, and BeyoECL Plus enhanced chemiluminescent (ECL) reagent were obtained from Beyotime (Haimen, China). Annexin V-FITC/PI cell apoptosis detection kit was derived from BD Biosciences (Franklin Lakes, NJ, USA). EdU cell proliferation detection kit was purchased from Molecular Probes (Eugene, OR, USA).

**Clinical Information**

A total of 65 NPC patients who received treatment in the 2nd Affiliated Hospital of Fujian Medical University between March 2016 and December 2016 were enrolled, including 29 males and 36 females with mean age at 52.3 ± 11.6 years old. All the subjects received surgery for the first time without preoperative radiotherapy or chemotherapy. Tumor tissue was resected and stored at -80°C. Another 40 cases of patients suffered from chronic nasopharyngitis were selected as control, including 18 males and 22 females with average age at 50.6 ± 10.8 years old (Table I). No statistical difference was observed on age and gender between two groups (Table I, p>0.05). The experimental protocol has been pre-approved by the Ethical Committee of the 2nd Affiliated Hospital of Fujian Medical University and written consents have been obtained from all patients and healthy volunteers.

**Cell Culture**

CNE-1, HONE1, C666-1, and NP69 cells were cultured in RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin. The cells were passaged at 1:4.

**Dual-luciferase Reporter Gene Assay**

The PCR products containing the full-length of MEK1 gene 3'-UTR segment were cloned to pLUC after endonuclease SacI and XbaI cloning. Next, it was named as pLUC-MEK1-wt (or pLUC-MEK1-UTR-mut) and co-transfected to HEK293T cells using Lipofectamine 2000 together with miR-101 mimic (miR-101 inhibitor or miR-NC). The luciferase activity was detected according to the Stop&Glo Luciferase Assay manual after cultured for 48 h.

**Cell Grouping and Transfection**

C666-1 cells were cultured in vitro and divided into four groups, including miR-NC, miR-101 mimic, si-NC and si-MEK1 groups. MiR-NC or miR-101 mimic at 30 nmol/L and si-NC or si-MEK1 at 10 nmol/L were incubated with Lipofectamine at 10 μL at room temperature for 15-20 min. Then, they were added to the cells and incubated for 6 h. After changing the medium and incubation for 72 h, the cells were collected for the following experiments.

**Quantitative Real-time PCR (qRT-PCR)**

Total RNA was extracted using MiniBEST Universal RNA Extraction Kit and adopted for PCR reaction by PrimeScript™ RT reagent Kit.
The PCR reaction was composed of 95°C pre-denaturation for 15 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Real-time PCR was performed on Applied Biosystems QuantStudio 3 connect (PE Applied Biosystems, Foster City, CA, USA) to test the relative expression.

**Western Blot**

Total protein was extracted by RIPA from cells. A total of 40 μg protein was separated by sodium dodecyl sulfonate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membrane. Next, the membrane was blocked by 5% skim milk and incubated in primary antibody at 4°C overnight (MEK1, p-ERK1/2, survivin, and β-actin at 1:3000, 1:1000, 1:2000, and 1:10000, respectively). Then, the membrane was incubated in HRP labeled secondary antibody (1:30000) for 1 h after washed by phosphate buffer solution-tween 20 (PBST) for three times. At last, the protein expression was detected by BeyoECL Plus (Beyotime Biotech., Shanghai, China).

**Cell Apoptosis Detection**

The cells were digested by trypsin and resuspended in binding buffer. Next, the cells were incubated in 5 μl Annexin V-FITC and 5 μl propidium iodide (PI). At last, the cells were tested on Beckman Coulter CytoFLEX flow cytometry to evaluate cell apoptosis.

**EdU Staining**

Cell proliferation was assessed by Click-iT® EdU Alexa Fluor 488 Flow Cytometry Assay Kits. The cells were added with 10 μM EdU solution for 2 h. After they were incubated for 48 h, the cells were digested and collected. After washed by PBS, fixed, and penetrated, the cells were incubated in reaction liquid containing Alexa Fluor 488 at room temperature avoiding light for 30 min. The cells were washed and tested by Beckman Coulter CytoFLEX flow cytometry (Beckman Coulter Inc., Brea, CA, USA).

**Statistical Analysis**

All data analyses were performed on SPSS 18.0 (SPSS Inc., Chicago, IL, USA) software. The measurement data were depicted as mean ± standard deviation and compared by t-test. p<0.05 was considered as statistical significance.

**Results**

**MiR-101 Targeted Inhibited MEK1 Expression**

Bioinformatics analysis showed the targeted binding site between miR-101 and 3’-UTR of MEK1 mRNA (Figure 1A). Dual luciferase assay revealed that miR-101 mimic transfection significantly declined, while miR-101 inhibitor transfection significantly elevated the relative luciferase activity of HEK293 cells, indicating the regulatory relationship between miR-101 and MEK1 mRNA (Figure 1B).

**MiR-101 Down-regulated, While MEK1 Increased in NPC Tissue**

qRT-PCR revealed that miR-101 significantly declined, whereas MEK1 mRNA obviously up-regulated in NPC tissue compared with inflammatory nasopharyngeal tissue (Figure 2A). MiR-101 was markedly correlated with tumor size and clinical staging. Western blot demonstrated that MEK1 protein apparently enhanced in NPC tissue compared with inflammatory nasopharyngeal tissue (Figure 2B).
MiR-101 Reduced, Whereas MEK1 Enhanced in NPC Cells

qRT-PCR revealed that miR-101 significantly declined, whereas MEK1 mRNA obviously up-regulated in NPC cell lines CNE-1, HONE1, and C666-1 compared with NP69 cells (Figure 3A and B). Western blot demonstrated that MEK1 protein markedly increased in NPC cell lines CNE-1, HONE1, and C666-1 compared with NP69 cells (Figure 3C).

MiR-101 Over-expression or MEK1 Down-regulation Suppressed NPC Cell Proliferation and Induced Cell Apoptosis

MiR-101 mimic and/or si-MEK1 transfection significantly reduced MEK1, p-ERK1/2, and survivin levels in C666-1 cells (Figure 4A and B), attenuated cell proliferation (Figure 4C), and enhanced cell apoptosis (Figure 4D).

Discussion

Globally, there are more than 100,000 patients diagnosed as NPC each year. More than 80% new cases were from South East Asia, China, and other Asian countries. The morbidity peak is between 40-50 years old. It may cause nasal congestion, nose blood, aural fullness, hearing loss, diplopia, and headache, and may induce cranial nerve because of peripheral tissue infiltration, which seriously threatens to life and health.

MAPK signaling pathway is an important signal transduction system that widely exists in eukaryotes. It regulates various target genes expression and activation mediated by intracellular receptor tyrosine kinase, G-protein coupled receptor, and cytokine receptor under the effect of cytokines, growth factors, neurotransmitter, and G-protein coupled receptor, thus participating in
affecting cell survival, proliferation, migration, apoptosis, angiogenesis, and immune response. ERK/MAPK signaling pathway excessive activation leads to cell abnormal proliferation, apoptosis, and differentiation, and promote malignant transformation. It is closely associated with various tumors pathogenesis, progression, and metastasis, such as oral cancer, lung cancer, and esophageal cancer. ERK/MAPK signaling pathway mainly includes small G protein Ras, Raf kinase, MEK, and ERK. ERK/MAPK signaling pathway conforms to the classic three-step enzymatic cascade, and presents the same activation mode under different stimulus. As a member of MAP2K family, MEK1 phosphorylates the Tyr/Thr residue of ERK protein, thus activating ERK/MAPK signaling pathway. MEK1 expression and functional activity are related to a variety of cancers pathogenesis, progression, metastasis, and drug resistance, including pancreatic cancer, bile duct cancer, and hepatic cancer. It was showed that MEK1 over-expression is related to NPC cell abnormal proliferation, apoptosis reduction, and drug resistance. Multiple studies revealed that miR-101 expression reduced in NPC tissue and cell line, suggesting that miR-101 may play a tumor suppressive role in NPC development.

**Figure 4.** MiR-101 over-expression or MEK1 down-regulation suppressed NPC cell proliferation and induced cell apoptosis. (A) qRT-PCR detection of gene expression. (B) Western blot detection of protein expression. (C) EdU staining detection of cell proliferation. (D) Flow cytometry detection of cell apoptosis. *p* < 0.05, compared with miR-NC; *p* < 0.05, compared with si-NC.
MiR-101 restrains NPC proliferation

Bioinformatics analysis shows the complementary targeted relationship between miR-101 and the 3'-UTR of MEK1 mRNA. This study explores the role of miR-101 in regulating MEK1 expression, ERK/MAPK signaling pathway activation, and NPC pathogenesis.

Dual luciferase assay revealed that miR-101 mimic transfection significantly declined, while miR-101 inhibitor transfection elevated the relative luciferase activity of HEK293 cells, indicating the regulatory relationship between miR-101 and MEK1 mRNA. MiR-101 significantly declined in NPC tissue compared with inflammatory nasopharyngeal tissue. MiR-101 was markedly correlated with tumor size and clinical staging. It indicated that miR-101 down-regulation may play a role in increasing MEK1 expression and facilitating NPC tumorigenesis. Tang et al. reported that miR-101 level declined in NPC tissue compared with normal nasopharynx epithelium, following lymph node metastasis. In this study, miR-101 expression reduced in NPC tissue, which was in accordance with Tang et al. MiR-101 markedly declined, while MEK1, p-ERK1/2, and survivin apparently enhanced in NPC cell lines CNE-1, HONE1, and C666-1 compared with NP69 cells, revealing that MEK1 elevation mediates ERK/MAPK signaling pathway and survivin expression, while miR-101 decrease is its promoting factor. Sun et al. showed that miR-101 level significantly declined in NPC cell lines CNE-1, CNE-2, 5-8F, and 6-10B compared with NP69 cells. Tang et al. demonstrated that miR-101 down-regulated in NPC cell lines CNE-2, 5-8F, and C666-1 compared with NP69 cells. This study found that miR-101 abnormally reduced in NPC cells, which was similar to Sun et al. and Tang et al. Further analysis revealed that miR-101 mimic and/or si-MEK1 transfection significantly reduced MEK1, p-ERK1/2, and survivin levels, attenuated cell proliferation, and enhanced cell apoptosis. Alajez reported that pre-miR-101 transfection upregulated miR-101 expression, attenuated C666-1 cell proliferation and survival, and enhanced radiotherapy sensitivity through targeting EZH2. Tang observed that miR-101 mimic transfection weakened NPC cell migration and invasion in vitro, and inhibited NPC cell pulmonary metastasis in mice model via targeted suppressing ITGA3 expression. MiR-101 inhibitor transfection enhanced NPC cell migration and invasion. Sun et al. presented that miR-101 over-expression markedly alleviated NPC cell proliferation and survival, and reduced radiotherapy resistance in CNE-2 and 5-8F cells. It confirmed that miR-101 declined malignant characteristic of NPC cells, which supported our results. Wong et al. showed that NPC cell proliferation attenuated, while cell apoptosis enhanced after treated by AZD6244 to suppress MEK1 function and ERK/MAPK signaling pathway activity, indicating that MEK1 mediated ERK/MAPK signaling pathway is related to NPC pathogenesis. Yang et al. presented that MEK1 suppression inhibited ERK1/2 phosphorylation and ERK/MAPK signaling pathway, arrested cell cycle, and attenuated cell proliferation in NPC cells. Cheung et al. exhibited that application of PD098059 blocked MEK1 activity, induced NPC cell apoptosis, and enhanced sensitivity to taxol. Wang et al. demonstrated that MEK1 siRNA or U0126 treatment markedly attenuated NPC cell migration and motility, whereas MEK1 over-expression enhanced cell invasion. It suggested that MEK1 mediated ERK/MAPK signaling pathway enhancement is a promoting factor of NPC pathogenesis, while reducing MEK1 expression may inhibit NPC. This study revealed that down-regulation of miR-101 increased MEK1, enhanced ERK/MAPK signaling pathway, elevated survivin expression, and promoted NPC pathogenesis. This study only used C666-1 cells to investigate the regulatory role of miR-101 on MEK1, ERK/MAPK signaling pathway, NPC cell proliferation and apoptosis. However, we did

### Table 1. MiR-101 expression in NPC tissue with different characteristics.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cases</th>
<th>miR-101 expression</th>
<th>p-value</th>
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<td>Age</td>
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<tr>
<td>≤45 years</td>
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<td>1.62±0.25</td>
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<td>&gt;45 years</td>
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<td>1.53±0.22</td>
<td>0.182</td>
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<tr>
<td>Male</td>
<td>29</td>
<td>1.49±0.24</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>36</td>
<td>1.55±0.28</td>
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<tr>
<td>T stage</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
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<tr>
<td>T1-T2</td>
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<td>1.87±0.26</td>
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<tr>
<td>T3-T4</td>
<td>24</td>
<td>1.23±0.21</td>
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<td>N stage</td>
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<td>0.173</td>
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<tr>
<td>N0-N1</td>
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<td>1.57±0.27</td>
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<tr>
<td>N2-N3</td>
<td>34</td>
<td>1.63±0.24</td>
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<tr>
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<tr>
<td>M1</td>
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<td>1.55±0.26</td>
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<td>Clinical stage</td>
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<tr>
<td>III-IV</td>
<td>37</td>
<td>1.18±0.25</td>
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</table>
ANGPTL3 is a novel protein that promotes survivin expression. It alleviates ERK/MAPK signaling pathway and promotes NPC cell proliferation and apoptosis through targeted inhibition of MEK1 expression.

Conclusions

Down-regulation of miR-101 was related to NPC pathogenesis. MiR-101 elevation suppressed NPC cell proliferation and promoted apoptosis through targeted inhibiting MEK1 expression to alleviate ERK/MAPK signaling pathway and survivin expression.

Acknowledgments

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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