

Study of miR-10b regulatory mechanism for epithelial-mesenchymal transition, invasion and migration in nasopharyngeal carcinoma cells

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Abstract. The aim of the study was to investigate the miR-10b regulatory mechanism for epithelial-mesenchymal transition (EMT) and its effect on the proliferation and migration of nasopharyngeal carcinoma cells. RT-qPCR was used to detect the expression of miR-10b in CNE1 nasopharyngeal carcinoma cell line. The NP69 nasopharyngeal mucosal cell line was used to determine the expression of miR-10b after infection with lentivirus. The effect of miR-10b on the proliferation of NP69 was examined using cell counting kit-8. The effect of miR-10b on NP69 migration was examined using scratch assay. Western blot analysis was used to detect the effects of miR-10b on the expression of epithelial cell markers E-cadherin and β -catenin and mesenchymal cell markers fibronectin, N-cadherin, vimentin and matrix metalloproteinase-9 (MMP-9). The present study showed that miR-10b was highly expressed in CNE1 cells. The stable expression of miR-10b promoted the proliferation and migration of NP69 cells, downregulated the expression of epithelial cell markers E-cadherin and β -catenin, and upregulated the expression of mesenchymal cell markers fibronectin, N-cadherin, vimentin and MMP-9 resulting in cell EMT. In conclusion, miR-10b promotes the proliferation and migration of nasopharyngeal carcinoma cells, and induces EMT in nasopharyngeal carcinoma cells, thereby having the potential to become a new target for the treatment of nasopharyngeal carcinoma.

Introduction

Nasopharyngeal carcinoma is a malignant tumor of nasopharyngeal mucosa with high incidence in Guangdong, Guangxi and other regions in China (1). Epstein-Barr virus infection is closely associated with the carcinogenesis of nasopharyngeal

carcinoma. Nasopharyngeal cancer is highly malignant, has distant metastasis in the early stages, and is mainly located in cervical lymph nodes (2). Epithelial-mesenchymal transition (EMT) refers to the transformation of epithelial cells into motile mesenchymal cells, which is an important biological process for epithelial cell-derived malignant tumor cells to obtain migration and invasion capabilities. After EMT, cell morphology is altered, with increased and thickened cell surface fibers and increased pseudopodia. The expression of epithelial cell markers E-cadherin and β -catenin are decreased, whereas the expression of mesenchymal cell markers fibronectin, N-cadherin and vimentin are increased, resulting in the increase of cell migration capacity and tumor metastasis (3-5). Following EMT, epithelial cells in a static state change into mesenchymal cells with a strong migration ability. Moreover, proteolytic enzymes, such as matrix metalloproteinase-9 (MMP-9), can degrade the basement membrane, thereby facilitating cells to invade the extracellular matrix (6). During the process of EMT in nasopharyngeal carcinoma cells, these markers have similar changes, but the mechanism leading to these changes remains unclear.

miRNAs affect the cell apoptosis, proliferation and differentiation processes by regulating the expression of target genes, and they are probably associated with tumor metastasis (7). Studies have reported that miRNAs are involved in the carcinogenesis and development of nasopharyngeal carcinoma (8-10). At present, changes of 35 kinds of miRNA expression levels have been found in nasopharyngeal carcinoma tissue (11). The mutual effect of miR141 and tumor-associated genes c-myc and PTEN promotes the carcinogenesis and development of tumors (12). MicroRNA microarray analysis has shown there is a significant difference between miR-10b expression in nasopharyngeal carcinoma cells and normal nasopharyngeal epithelial cells (13). To evaluate the role of miR-10b in the carcinogenesis and development of nasopharyngeal carcinoma, we used lentivirus to infect normal nasopharyngeal epithelial cells aiming to observe cell proliferation and migration changes, and to analyze the difference of expression levels in epithelial cell and stromal cell markers.

Materials and methods

Cells. The nasopharyngeal carcinoma cell line CNE1, was stored in our laboratory and cultured in RPMI-1640 medium

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containing 10% calf serum (100 U/ml penicillin and 100 μ g/ml streptomycin). The immortalized nasopharyngeal epithelial cell line NP69, was cultured with the same RPMI-1640 medium to which growth factors were added. The cells were cultured at 37°C in a 5% CO₂ incubator.

Quantitative PCR. Total RNA was extracted using the TRIzol kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). RT-PCR was performed according to the manufacturer's instructions. The reverse transcription conditions were as follows: 25°C for 5 min, 42°C for 30 min, 85°C for 5 min to inactivate the RNA enzyme. qPCR was performed with U6 snRNA as the internal reference, and the reaction conditions were: Pre-denaturation at 95°C for 30 sec, denaturation at 95°C for 5 sec, followed by 40 cycles of annealing at 60°C for 30 sec and extension at 72°C for 30 sec. The C_q values of miR-10b and U6 were calculated according to the amplification curves. C_q was calculated using the formula: $Cq = Cq_{miR-10b} - Cq_{U6}$. The expression level of miR-10b in CNE1 and NP69 cells was compared using the 2^{-C_q} method.

NP69 cells infected with lentivirus-miR-10b. The lentivirus system was constructed to express miR-10b. The sequence of miR-10b was inserted into the lentiviral plasmid pLP-VSVG. PLP-VSVG-miR-10b, pLP1 and pLP2 were co-transfected into 293 T cells, and after 48 h, lentivirus-containing supernatant was collected. NP69 cells (1×10⁶) were seeded in 6-well plates, cultured overnight into monolayer cells, and then incubated with lentivirus supernatant for 24 h to allow the lentivirus to infect cells. Lentivirus-infected cells were collected on the same day and days 2-6 after infection and the expression levels of miR-10b were detected by RT-qPCR. NP69 cells infected with blank pLP-VSVG served as the control.

CCK-8 assay. Lentivirus-infected NP69 cells were seeded in 96-well plates with 5×10³ cells/well, and cultured overnight at 37°C in a 5% CO₂ incubator. Then, 10 μ l of the cell counting kit-8 (CCK-8) solution was added to each well, and incubated for 24 h. The absorbance at a wavelength of 450 nm was measured. NP69 cells infected with the blank lentivirus served as the control. Cell viability was calculated using the formula: $\text{Cell viability} = (\text{the test well OD value} - \text{the blank well OD value}) / (\text{the control well OD value} - \text{the blank well OD value}) \times 100\%$.

Cell scratch assay. Lentiviral-infected NP69 cells were cultured in 24-well plates with 5×10⁴ cells/well, and incubated at 37°C in a 5% CO₂ incubator until the cell density reached 90%. The cell monolayer was scraped in a straight line to create a scratch with a 10 μ l pipette tip, and any debris was removed by washing the cells once with PBS and the cells were cultured continuously. Scratches were observed under a microscope (Olympus BX53; Olympus Corporation, Tokyo, Japan) at 24, 48 and 72 h, the size of the scratches was measured, and the results represented the mean of the three experiments. CNE1 and NP69 cells without lentivirus infection served as controls.

Western blot analysis. NP69 cells were collected at 72 h after lentiviral infection and lysed with lysate. The lysate was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene

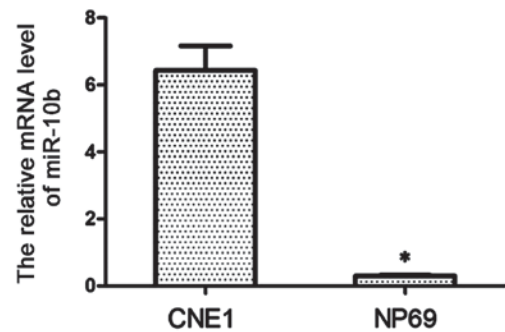


Figure 1. The expression levels of miR-10b in cells. The expression levels of miR-10b in CNE1 and NP69 cells determined by RT-qPCR. *P<0.01.

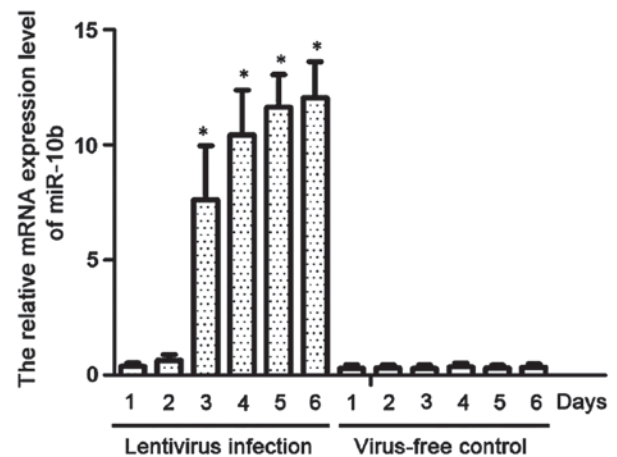


Figure 2. The expression levels of miR-10b after lentivirus infection in NP69 cells. The expression levels of miR-10b were detected by RT-qPCR at day 1, 2, 3, 4, 5 and 6 after infecting NP69 cells with lentivirus. The blank pLP-VSVG was a virus-free control. *P<0.01.

fluoride membrane and blocked with 5% skim milk at room temperature for 2 h. The membrane was incubated, using the following mouse anti human primary antibodies: Anti-E-cadherin, anti- β -catenin, anti-fibronectin, anti-vimentin and anti-MMP9 (dilution, 1:1,500; cat. nos. ab1416; ab22656; ab6328; ab8978; ab58803 respectively, purchased from Abcam plc., Burlingame, CA, USA) at room temperature for 1 h. The membrane was then incubated with HRP-conjugated rabbit anti-mouse secondary polyclonal antibody (dilution, 1:2,500; cat. no. ab6728; Abcam plc.) for 1 h. The HRP enzyme substrate was added and incubated for 5 min to develop color, and the immunoblotting result was recorded using a fully automated western blot WEs analysis system (ProteinSimple, San Jose, CA, USA). The density of each band was determined by ImageJ software, and β -actin was used as the internal reference. CNE1 and NP69 cells infected with blank lentivirus were used as controls. The relative expression level was calculated as the ratio of the assessed protein and β -actin and expressed as the mean \pm standard deviation (n=3).

Statistical analysis. Data were expressed as mean \pm standard deviation, the difference between two groups was compared with the Student's t-test, and multiple group comparison was performed using Fisher's LSD test. P<0.05 was considered to indicate a statistically significant difference.

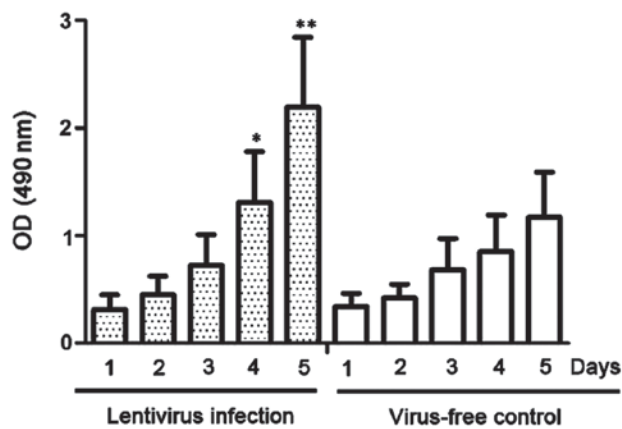


Figure 3. The proliferation of NP69 cells after infection with lentivirus. The proliferation of NP69 cells was detected by CCK-8 assay at day 1, 2, 3, 4 and 5 after infection with lentivirus. NP69 cells infected with miR-10b lentivirus, and the blank pLP-VSVG was a virus-free control. * $P<0.05$; ** $P<0.01$; CCK-8, cell counting kit-8.

Results

miR-10b expression in cells. The expression of miR-10b in CNE1 cells was detected by RT-qPCR. As shown in Fig. 1, the expression level of miR-10b in CNE1 cells was significantly higher than that in NP69 cells ($P<0.01$), and miR-10b was almost absent in NP69 cells.

miR-10b expression in lentivirus-infected NP69 cells. We established a cell line stably expressing miR-10b by infecting NP69 cells with lentivirus. RT-qPCR was used to detect the expression levels of miR-10b. As shown in Fig. 2, miR-10b was highly expressed on day 3 after lentiviral infection, and its level on day 5 was significantly higher than that of NP69 cells with blank lentiviral infection ($P<0.01$).

miR-10b promotes NP69 cell proliferation. A CCK-8 assay was used to detect the proliferative ability of lentivirus-infected NP69 cells. As shown in Fig. 3, the proliferation of NP69 cells stably expressing miR-10b was significantly more rapid than that of NP69 cells with no lentivirus infection at day 4 after infection ($P<0.05$).

miR-10b promotes the migration of NP69 cells. The effect of miR-10b on the migration of NP69 cells was examined using a cell scratch assay. As shown in Fig. 4 and Table I, following cell scratching, CNE1 and NP69 cells expressing miR-10b grew and covered half of the scratches at 48 h, and almost all the scratches after 72 h. By contrast, NP69 cells in the control group did not cover half of the scratches after 72 h. miR-10b promoted the migration of NP69 cells in a time-dependent manner. The difference in expression levels of miR-10b between CNE1 and NP69 cells led to a difference in the cell migration capacity, thereby further resulting in cancer cell metastases.

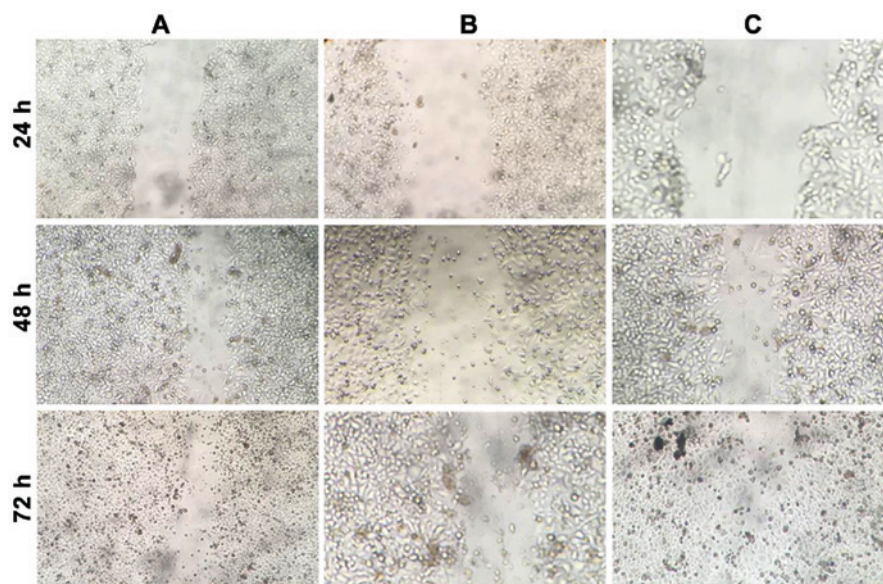


Figure 4. Scratch testing of NP69 cells infected with miR-10b lentivirus. The effect of miR-10b on cell migration ability detected by cell scratch assay at day 1, 2 and 3 after NP69 cells were infected with lentivirus. (A) NP69 cells infected with lentivirus-miR-10b, (B) NP69 cells infected with blank lentivirus, and (C) nonlentivirus-infected CNE1 cells.

Table I. Cell scratch size (mean \pm standard deviation, $n=3$).

Scratch size (μm)	24 h	48 h	72 h	P-value
NP69 cells stably expressin miR-10b	21.7 \pm 1.4	10.7 \pm 2.3	4.2 \pm 1.9	<0.01
NP69 cells infected with blank lentivirus	25.5 \pm 3.2	19.7 \pm 4.3	15.1 \pm 3.7	<0.05
CNE1 cells	32.6 \pm 2.5	11.8 \pm 3.1	1.3 \pm 0.4	<0.01

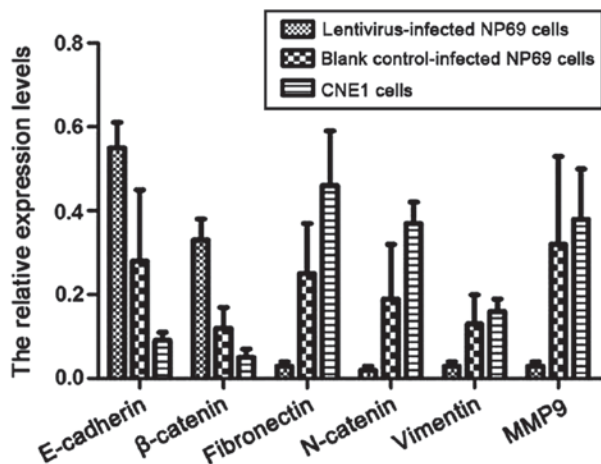


Figure 5. The expression levels of epithelial cell and stromal cell markers. The expression levels of epithelial cell and stromal cell markers were detected by western blot analysis, and CNE1 cells served as a control.

The expression levels of epithelial cells and stromal cell markers. Western blot analysis was used to detect the expression levels of epithelial cells and interstitial cell markers in CNE1 cells and lentivirus-infected NP69 cells expressing miR-10b. As shown in Fig. 5, the expression levels of E-cadherin and β -catenin in CNE1 and NP69 cells stably expressing miR-10b were significantly decreased compared to NP69 cells with no miR-10b expression ($P < 0.05$). By contrast, the expression levels of stromal cell markers, fibronectin, N-catenin, vimentin and MMP-9 were significantly increased ($P < 0.05$).

Discussion

MicroRNAs regulate gene expression at transcriptional levels, and increasing research has focused on the role of microRNAs in the carcinogenesis and development of tumors. Previous findings have shown there is a difference in terms of the expression of miR-10b between nasopharyngeal carcinoma and normal nasopharyngeal mucosa tissue (12). The present findings showed that miR-10b expression in the CNE1 nasopharyngeal carcinoma cell line was higher than that in normal nasopharyngeal mucosa. The expression of epithelial cell markers E-cadherin and β -catenin, decreased after NP69 cells were infected with lentivirus and expressed miR-10b. E-cadherin enhances cell-cell adhesion, and its downregulation can decrease cell adhesion and increase cell motility through the basement membrane (14,15). The expression of stromal cell markers fibronectin, N-cadherin, vimentin and MMP-9, was increased and the cell migration ability was enhanced, indicating that miR-10b promoted the EMT of nasopharyngeal carcinoma cells. miR-10b promoted tumor cell invasion and migration by downregulating the expression of KLF4 (16) and as a direct target of miR-10b, KLF4 was capable of inhibiting tumor cell invasion and migration (17), which could be used for target for further study.

In summary, the present findings show that miR-10b is involved in the EMT of nasopharyngeal carcinoma cells, promotes cell proliferation and migration and is closely associated with the metastasis of nasopharyngeal carcinoma. Further research is needed to elucidate the molecular

mechanism of miR-10b regulating the EMT of nasopharyngeal carcinoma cells. miR-10b is expected to be a key target in the treatment of nasopharyngeal carcinoma, providing new opportunities for the development of new targeted therapies.

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