RESEARCH ARTICLE

miR-10b Promotes Migration and Invasion in Nasopharyngeal Carcinoma Cells

Xiao-Jin Sun[1], Hao Liu[1]*, Pei Zhang[1], Xu-Dong Zhang[1,2], Zhi-Wen Jiang[1], Chen-Chen Jiang[1,2]*

Abstract

MicroRNA-10b (miR-10b) has been reported to play an important role in some types of cancer, but the effects and possible mechanisms of action of miR-10b in the metastasis of nasopharyngeal carcinoma cells (NPC) have not been explored. The aim of the present study was to investigate the function of miR-10b in nasopharyngeal carcinoma and to determine the molecular mechanisms underlying its action. The MTT assay was used to assess proliferation of CNE-2Z cells. Wound healing and transwell migration assays were applied to assess cell migration and invasion, while and expression of E-cadherin and MMP-9 were detected using Western blot analysis. Real-time PCR was employed to detect the expression of genes related to migration and invasion and the $2^{-\Delta\Delta Ct}$ method was used to calculate the degree of expression. MTT assay showed the expression of miR-10b to have no effect on the proliferation of NPC cell lines. The wound healing assay showed that miR-10b mimics promoted the mobility and invasion of NPC cell lines. Inhibitors of miR-10b reduced the ability of NPC cell lines to migrate and invade. In addition, the expression of genes related to migration and invasion, such as E-cadherin, vimentin, and MMP-9, were confirmed to be different in the CNE-2Z NPC cell line transfected with miR-10b mimics and with miR-10b inhibitors. In the present study, miR-10b was found to upregulate the expression of MMP-9 and knockdown of miR-10b was found to significantly downregulate the expression of E-cadherin. On the whole, these results showed that miR-10b plays an important role in the invasion and metastasis of NPC cells.

Keywords: miR-10b - NPC - invasion - metastasis

Introduction

Nasopharyngeal carcinoma is a prevalent head and neck cancer which is very common in southern China and southeastern Asia. High transfer of nasopharyngeal carcinoma is the main cause of treatment failure (McDermott et al., 2001; Li et al., 2004; Yu et al., 2009). As diagnostic and treatment technology have improved, certain early-stage patients have come to be cured. However, the 5-year survival rate of most middle-late- and late-stage nasopharyngeal carcinoma patients is only about 50%. For this reason, exploring the molecular mechanisms underlying the metastasis of nasopharyngeal carcinoma, searching for the new therapeutic targets, and reducing the transfer rate are all of considerable significance.

MicroRNAs (miRNAs) are an abundant class of endogenous, highly conserved, non-coding RNAs. They are about 18–25 nucleotides in length and are expressed in a wide range of organisms (Griffiths-Jones 2004; Davis-Dusenbery et al., 2010; Dawood 2010). This is primarily accomplished by binding to the 3' UTRs of the mRNAs that target the transcripts for degradation or by blocking translation. miRNAs are estimated to regulate the translation of more than 60% of protein-coding genes (Baffa et al., 2009; Edmonds et al., 2009; Tie et al., 2011). miRNAs are involved in a series of normal biological mechanisms, such as embryonic development, cell growth, proliferation, apoptosis, and differentiation, where it acts by preventing the expression of target genes (Bartel 2004; Baffa et al., 2009; Iorio et al., 2009). Abnormal expression of miRNAs figures in many diseases, including cancer (Lu et al., 2005). miR-10b has been reported to be dysregulated in some types of cancer and to play an important role in invasion and metastasis. miR-10b has been shown to be highly expressed in metastatic breast cancer cell lines and in metastatic breast tumors from patients. The present study focuses on the expression and roles of miR-10b in NPC. It has been found that miR-10b is up-regulated in metastatic NPC cell lines. This study may provide new ideas and methods suitable for the treatment of nasopharyngeal carcinoma.

Materials and Methods

Cell lines

CNE-2Z is one of nasopharyngeal carcinoma cell lines

[1]Faculty of Pharmacy, Bengbu Medical College, Bengbu, Anhui, China, [2]Immunology and Oncology Unit, Room 443, Calvary Mater Newcastle Hospital, NSW, Australia *For correspondence: liuhao6886@foxmail.com, chenchen_jiang@yahoo.com.cn

with low differentiation and high transfer which could be conductive to the study in migration and invasion in nasopharyngeal carcinomca cells. Poorly differentiated human CNE-2Z cell lines were obtained from Zhongshan University and cultured in the current laboratory. Cells were cultured in RPMI-1640 supplemented with 5% FBS and 100 U of penicillin-streptomycin with 5% CO2 in a humidified incubator at 37°C.

Reagents and antibodies

RPMI-1640 medium, fetal bovine serum (FBS), and phosphate buffered saline (PBS) were purchased from Gibco (Grand Island, NY). Matrigel was purchased from BD Biosciences (Bedford, MA, U.S.). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) from Sigma Chemical Co (Castle Hill, Australia). Primary antibodies for E-cadherin, MMP-9 (1:500), and β-actin (1:2000) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.).

Quantitative reverse transcription and real-time PCR analysis of miRNA

Total RNA was extracted using TRIzol (Invitrogen, USA) from CNE-2Z transfected with miR-10b inhibitors or miR-10b mimics. The primers for RT–PCR to detect miRNA were designed based on the miRNA sequences provided and synthesized by the Takara (Kyoto, Japan.). The quantitative PCR was performed according to the indications of miR-10 qPCR Quantitation Kit (Takara, Kyoto, Japan). RT reactions were performed by means of the iScript cDNA synthesis kit (Takara, Kyoto, Japan) in an ABI StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, U.S.). U6 RNA was used as an endogenous control for miRNA detection.

Cell proliferation assay

Cell viability was detected by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) assay. Cells were seeded at a density of 5×10^3 cells/well in 96 well plates 24h with growth medium after 48h transfection. Then, MTT solution (5 mg/mL, 20 μL) was added to each well. After incubation at 37°C for additional 4 h, 150 ml DMSO (Sigma, U.S.) was added to each well to dissolve the crystals. The plates were shaken for 10 min to dissolve the MTT formazan crystals. The optical density (OD) of the wells were determined using a scanning multi-well spectrophotometer at 490 nm wavelength. Experiments were repeated in triplicate and 4 parallel samples were measured each time.

qPCR analysis of copy number variations

Quantitative real-time RT-PCR was used to evaluate the mRNA levels of genes related to migration and invasion expressed by the CNE-2Z cells. Total RNA was extracted with TRIzol Reagent (Invitrogen). Aliquots (1 μg) of RNA were reverse transcribed to cDNA (20 μL) with oligo (dT) and M-MuLV reverse transcriptase (Fermentas, Glen Burnie, MD, U.S.) in accordance with the manufacturer's instructions. One-fifth of the cDNA was used as a template for PCR using the SYBR Green PCR kit (Takara, Kyoto, Japan) in an ABI StepOne™

Real-Time PCR System (Applied Biosystems, Foster City, CA, U.S.). The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was selected as an internal control for each experiment. The primer sequences for target genes were as follows: E-cadherin (forward, 5'-TCGACACCCGATTCAAAGTGG-3'; reverse, 5'-TT CCAGAAACGGAGGCCTGAT-3'); GAPDH (forward, 5'-ACGGGAAGCTCACTGGCATGG-3'; reverse, 5'-G GTCCACCACCCTGTTGCTGTA-3'), vimentin (forward, 5'-AGATGGCCCTTGACATTGAG-3'; reverse, 5'- TGGAAGAGGCAGAGAAATTC-3'); and MMP-9 (forward, 5'-CGGAGTGAGTTGAACCAG-3'; reverse, 5'-GTCCCAGTGGGGATTTAC-3'). Cycling conditions were as follows: An initial 10 min of predenaturation at 95°C, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 15 s. The specificity of the amplification products was confirmed by melting curve analysis. All of the assays were performed in triplicate. The $2^{-\Delta\Delta Ct}$ method was used for relative quantification of gene expression and to determine the levels of E-cadherin, vimentin, or MMP-9 mRNA expression.

Cell invasion assay

Before cell seeding, a 24-well cell culture plate was coated with 8.0 μm Matrigel (Bedford, MA, U.S.). Cells were seeded in the inserts after transfections which were resuspended in 200 μl serum-free medium and placed in the upper chambers at 5×10^4 cells and normal growth medium were placed in underneath chambers. Then, 24 h after treatment, the cells on the upper surface of the membrane were removed with cotton swabs, and the cells on the lower chamber were incubated with 4% paraformaldehyde in PBS buffer and stained with 0.1% crystal violet. Finally, migratory and invasive cells were counted at 200× magnification in 5 different fields per filter. The experiments were performed in triplicate.

Cell migration assay

A cell migration assay was performed with uncoated transwell cell culture chambers. Cells were seeded in the inserts after transfections, which were resuspended in 200 μl serum-free medium and placed in the upper chambers at 5×10^4 cells and 600 μl RPMI-1640 medium containing 5% FBS were placed in underneath chambers. Then, 24 h after treatment, the cells on the upper surface of the membrane were removed with cotton swabs, and the cells on the lower chamber were incubated with 4% paraformaldehyde in PBS buffer and stained with 0.1% crystal violet. Five visual fields were randomly selected for each membrane and photographed under a light microscope at 200× magnification. Experiments were performed in triplicate.

Wound healing assay

After transfection, cells were seeded on six-well plates at 5×10^5 cells/well. After 24 h, the cells were washed with PBS and wounds were created using a sterile 200 μL pipette tip. The cells were then washed 3 times with PBS and incubated in RPMI-1640 medium containing 5% FBS. Migration at the wound site was observed under an inverted microscope and imaged at the appropriate
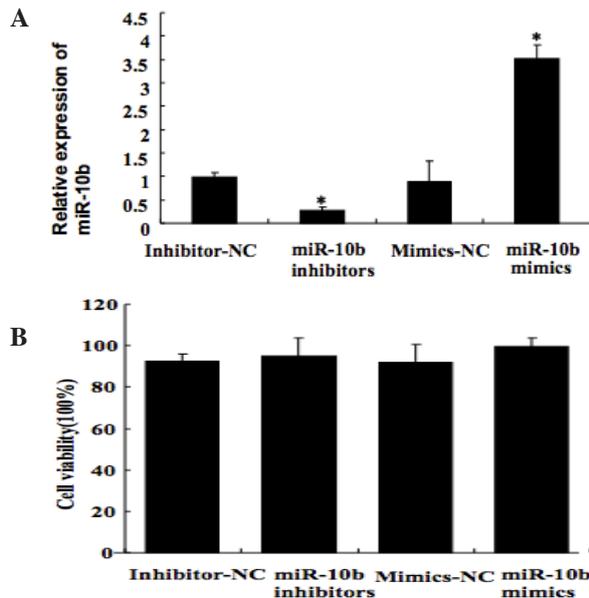
Figure 1. Silencing and Overexpression of miR-10b and Cell Proliferation. A) Real-time PCR assay was used to detect the level of miR-10b in CNE-2Z cells which tansfected with miR-10b inhibitors and miR-10b mimics. It showed that the miR-10b inhibitors and miR-10b mimics have been successfully transfected. B)Viability of CNE-2Z cells treated as detailed above was measured by MTT assay. Data showed the expression of miR-10b to have no effect on cell proliferation

fields to calculate healing percentages. Experiments were performed in triplicate.

Western blot analysis

After transfection, the cells were plated in 6-well culture dishes at a density of 5×10^5 cells/well. Cells were washed 3 times with cold PBS 1 ml/well and digested using trypsin. The protein concentrations were detected using a bicinchoninic acid (BCA) assay. A total of 50 μg of protein was separated using 10% SDS-PAGE and electro-blotted onto PVDF membranes using a semi-dry blotting apparatus. After blocking in 5% non-fat milk, the membranes were incubated overnight at 4°C with primary antibodies. The membranes were then incubated in the secondary anti-bodies for 2 h at room temperature on a shaker. The bands were visualized using Western Lightning ECL Pro with horseradish peroxidase (HRP). β-actin was used as a loading control.

*Statistical analysis*

All experiments were repeated at least three times. Data are presented as the mean ± SEM. The differences between means were analyzed using the two-tailed Student's t-test. All statistical analyses were performed using SPSS 13.0 software (Chicago, IL, U.S.). Differences with *P*<0.05 were considered statistically significant.

Results

Overexpression vs. silencing of miR-10b and cell proliferation

The real-time RT-PCR assay showed that the miR-10b
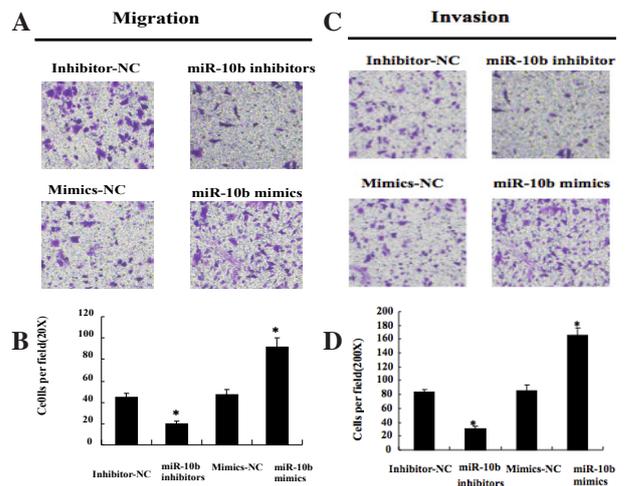
Figure 2. miR-10b and human NPC cell migration and invasion. (A, B) Transwell migration assay of CNE-2Z cells transfected with miR-10b inhibitors and miR-10b mimics or NC. (C, D) Transwell Matrigel invasion assay of CNE-2Z cells transfected with miR-10b inhibitors and miR-10b mimics or NC. The cells passing through the 8 μm pore insert to the underneath well were stained. The number of cells transfected with miR-10b mimics passing through to the underneath well was significantly greater than the number of cells transfected with control factor**s**
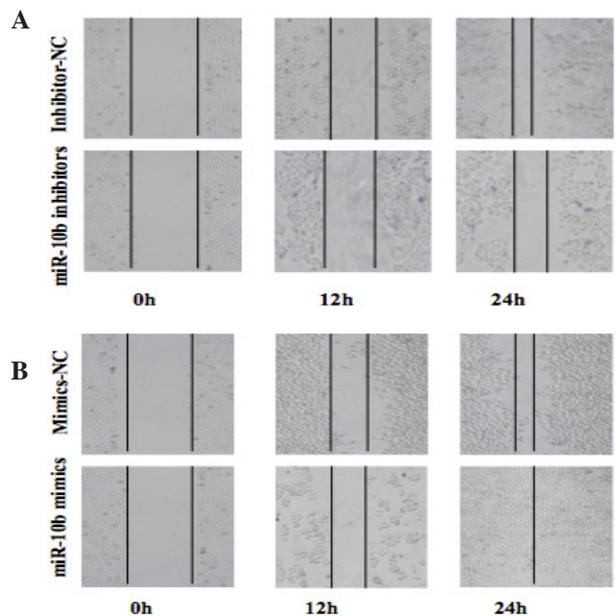

Figure 3. miR-10b and wounding-induced migration. (A, B) Overexpression of miR-10b using mimics markedly promoted the wound gap closure and did so in a time-dependent manner. Knocking down miR-10b using chemically synthesized miR-10b inhibitors delayed the closure of the wound gap

inhibitors and miR-10b mimics have been successfully transfected (Figure 1A, *P*<0.05).An MTT assay was used to observe the effects of miR-10b on the growth of NPC cell lines. Neither silencing nor overexpression of miR-10b had any effect on cell proliferation (Figure 1B, *P*<0.05).

Overexpression vs. silencing of miR-10b and human NPC cell migration and invasion

The transwell migration and invasion assay revealed that cells transfected with miR-10b mimics led migrated more than 50% further and the cells transfected with
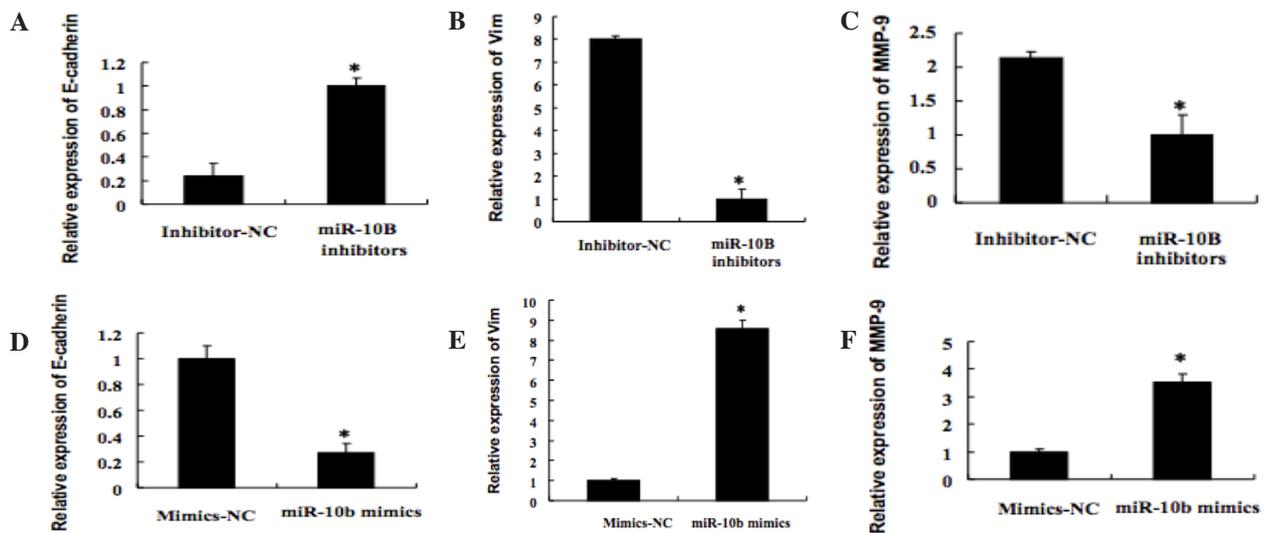
Figure 4. miR-10b and Levels of Endogenous Proteins Related to Migration and Invasion. (A, B, C) CNE-2Z cells were transfected with miR-10b inhibitors or Inhibitors-NC. D, E, F) CNE-2Z cells were transfected with miR-10b mimics or Mimics-NC. The levels of E-cadherin, Vim, MMP-9 treated as described above were measured using qRT-PCR
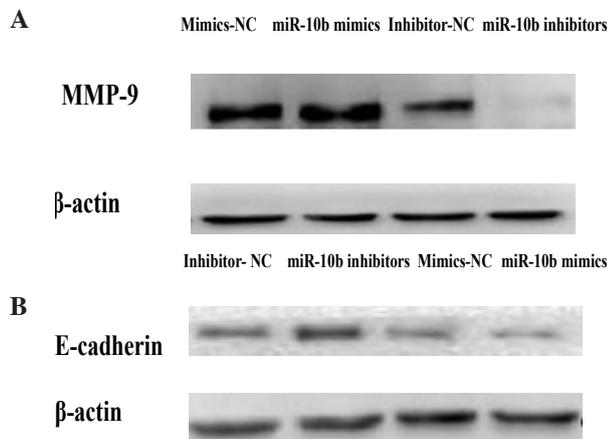
Figure 5. miR-10b and Proteins Related to Migration and Invasion. (A) CNE-2Z cells were transfected with miR-10b mimics or miR-10b inhibitors. The levels of MMP-9 protein were analyzed by Western blot 48 h later. (B) CNE-2Z cells were transfected with miR-10b mimics or miR-10b inhibitors. The levels of E-cadherin protein were analyzed by western blot 48 h later

miR-10b inhibitors showed a 20% reduction in distance migrated (Figure 2; $P<0.05$). These results indicate that miR-10b plays a positive role in migration and invasion of human NPC cell lines and that this effect had nothing to do with any changes in cell viability. A wound healing assay was used to detect the mobility of NPC cell lines. Results showed that overexpression of miR-10b using mimics promoted closure of the wound gap in a time-dependent manner (Figure 3B, $P<0.05$). Silencing miR-10b using chemically synthesized miR-10b inhibitors delayed closure of the wound gap (Figure 3A, $P<0.05$).

Relative expression of migration and invasion genes in human NPC cells

Quantitative real-time RT-PCR was used to detect the levels of mRNA expression of genes related to migration and invasion. The level of expression E-cadherin was lower in cells transfected with miR-10b mimics than in cells transfected with miR-10b inhibitors (Figure 4A, B,

C; $P<0.05$). However, the levels of expression of vimentin and MMP-9 were up-regulated in cells transfected with miR-10b mimics while the expression levels of vimentin and MMP-9 were lower in cells transfected with miR-10b inhibitors than in other cells (Figure 4 D, E, F; $P<0.05$).

miR-10b and expression of E-cadherin and MMP-9

Although the precise mechanisms underlying NPC metastasis are not well understood, the expression of genes related to migration and invasion may contribute to specific stages of the metastatic cascade. In order to elucidate the molecular mechanisms of the effect of miR-10b on the metastasis of NPC, E-cadherin and MMP-9 expression levels were assessed using Western blot analysis. Transfection with miR-10b mimics reduced the rate of E-cadherin expression and increased the rate of MMP-9 expression, but transfection with miR-10b inhibitor increased the rate of E-cadherin expression and reduced the rate of MMP-9 expression (Figure 5, $P<0.05$).

Discussion

Since miRNA was first discovered in 1993 (Iorio et al., 2009), over 700 miRNAs have been identified in humans, and many of them are involved in tumor generation and development (Sossey-Alaoui et al., 2009). Recently, miRNAs have received a great deal of attention because they have been reported to inhibit the epithelial-mesenchymal transition (Luo et al., 2011). The roles of miRs in tumorigenesis have been thoroughly studied. In human cancer, miRNA expression profiles differ between normal tissues and the tumors that are derived from them. They also and between different types of tumors. miRNAs can act as oncogenes or as tumor suppressors and they have key functions in tumorigenesis (Wang et al., 2012; Wei et al., 2013; Wu et al., 2013).

Although previous studies have shown that treatment with anti-miR-10b effectively inhibits the invasiveness of breast cancer cells (Ma et al., 2007) and that inhibition of miR-10b expression may be a new preventative measure against the spread of deadly tumors (Schuldt 2007; Steeg

2007), targeting miR-10b for the disruption of cancer metastasis remains a matter of debate (Gee et al., 2008). At the same time, our data indicated that high levels of miR-10b expression promoted the metastasis of NPC cells.

The MTT and wound healing assays demonstrated that miR-10b mimics had no effect on growth but did increase the movability of NPC cells. However, miR-10b inhibitors obviously restricted the mobility of NPC cells. In the present study, overexpression of miR-10b downregulated the expression of E-cadherin and upregulated that of MMP-9, but miR-10b inhibitor markedly upregulated the expression of E-cadherin and reduced the expression of MMP-9. This suggested that miR-10b may be involved in NPC metastasis.

In summary, the data collected here indicated high levels of miR-10b expression increased the mobility of NPC cells and promoted invasion of NPC cells in vitro. In conclusion, these results support the possibility that miR-10b may be involved in the NPC progression, especially in the metastasis of NPC. Further studies are needed to evaluate the function of miR-10b and its target genes. It has been suggested that miR-10b expression may be a prognostic factor for NPC patients.

Acknowledgements

References

Baffa R, Fassan M, Volinia S, et al (2009). MicroRNA expression profiling of human metastatic cancers identifies cancer gene targets. *J Pathol,* **219**, 214-21.

Bartel DP (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, **116**, 281-97.

Davis-Dusenbery BN, Hata A (2010). MicroRNA in Cancer: The Involvement of Aberrant MicroRNA Biogenesis Regulatory Pathways. *Genes Cancer*, **1**, 1100-14.

Dawood S (2010). Novel biomarkers of metastatic cancer. *Expert Rev Mol Diagn*, **10**, 581-90.

Edmonds MD, Hurst DR, Vaidya KS, et al (2009). Breast cancer metastasis suppressor 1 coordinately regulates metastasis-associated microRNA expression. *Int J Cancer*, **125**, 1778-85.

Gee HE, Camps C, Buffa FM, et al (2008). MicroRNA-10b and breast cancer metastasis. *Nature*, **455**, E8-9; author reply E9.

Griffiths-Jones S (2004). The microRNA Registry. *Nucleic Acids Res*, **32**, D109-11.

Iorio MV, Croce CM (2009). MicroRNAs in cancer: small molecules with a huge impact. *J Clin Oncol*, **27**, 5848-56.

Li XP, Peng Y, Kung HF, Lin MC (2004). Suppression of Epstein-Barr virus-encoded latent membrane protein-1 by RNA interference inhibits the metastatic potential of nasopharyngeal carcinoma cells. *Biochem Biophys Res Commun*, **315**, 212-18.

Lu J, Getz G (2005). MicroRNA expression profiles classify human cancers. *Nature*, **435**, 834-8.

Luo Z, Zhang L, Jiang C, et al (2011). miR-149 promotes epithelial-mesenchymal transition and invasion in nasopharyngeal carcinoma cells. *Zhong Nan Da Xue Xue Bao Yi Xue Ban*, **36**, 604-9.

Ma L, Teruya-Feldstein J, Weinberg RA, et al (2007). Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature*, **449**, 682-8.

McDermott AL, Dutt SN, Watkinson JC, et al (2001). The aetiology of nasopharyngeal carcinoma. *Clin Otolaryngol Allied Sci*, **26**, 82-92.

Schuldt A (2007). Micromanaging metastasis. *Nat Cell Biol*, **9**, 1121.

Sossey-Alaoui K, Bialkowska K, Plow EF (2009). The miR200 family of microRNAs regulates WAVE3-dependent cancer cell invasion. *J Biol Chem*, **284**, 33019-29.

Steeg PS (2007). Cancer: micromanagement of metastasis. *Nature*, **449**, 671-3.

Tie J, Fan D (2011). Big roles of microRNAs in tumorigenesis and tumor development. *Histol Histopathol*, **26**, 1353-61.

Wang YS, Wang YH, Xia HP, et al (2012). MicroRNA-214 regulates the acquired resistance to gefitinib via the PTEN/AKT pathway in EGFR-mutant cell lines. *Asian Pac J Cancer Prev*, **13**, 255-60.

Wei J, Wang F, Kong LY, et al (2013). miR-124 inhibits STAT3 signaling to enhance T cell-mediated immune clearance of glioma. *Cancer Res*, **73**, 3913-26.

Wu W, Yang J, Feng X, et al (2013). MicroRNA-32 (miR-32) regulates phosphatase and tensin homologue (PTEN) expression and promotes growth, migration, and invasion in colorectal carcinoma cells. *Mol Cancer*, **12**, 30.

Yu WM, Hussain SS (2009). Incidence of nasopharyngeal carcinoma in Chinese immigrants, compared with Chinese in China and South East Asia: review. *J Laryngol Otol*, **123**, 1067-74.