miR-185-3p regulates nasopharyngeal carcinoma radioresistance by targeting WNT2B in vitro

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As nasopharyngeal carcinoma (NPC) is a squamous cell carcinoma with a high incidence in South China and Southeast Asia.1,2 The primary treatment for this malignant tumor is radiotherapy. Although NPC tends to be more sensitive to radiation compared with some other cancers, the overall survival is not improved in advanced NPC.1,2 Radioresistance mainly contributes to the disappointing prognosis in NPC treatment.3,4 However, the precise molecular mechanism responsible for the radioresistance of NPC still remains largely a mystery.

MicroRNA (miRNA) belong to a class of conserved endogenous non-coding small RNA, which negatively regulate gene expression at the post-transcriptional level by matching to the 3′-untranslated region (UTR), 5′-UTR or coding region of mRNA.5,6 They are important regulators related to tumor malignant biobehaviors, including proliferation,7 invasion,8 metastasis,9 angiogenesis10 and chemoresistance.11 Recently, several groups have also reported aberrant expression of miRNA, such as miR-7, miR-21 and miR-210 in the radioresistance of multiple cancers.12–14 With regard to NPC, Qu et al. found that miR-205 determined the radioresistance of NPC by directly targeting PTEN.15 Moreover, our previous study also revealed that miR-324-3p could affect NPC radioresistance.16 Altogether, these data demonstrate that miRNA provide a new perspective for the study of NPC radioresistance.

In our previous study, we identified miR-185-3p as a candidate in the aberrant profile of radioresistant miRNA.17 Here, we confirmed that miR-185-3p can affect the radiosensitivity of NPC cells and directly target the coding region of WNT2B. Their reciprocal relationship was also confirmed in NPC cells and tissues. Furthermore, downstream proteins were detected in order to find the potential mechanisms of NPC radioresistance. Our findings suggest that miR-185-3p and WNT2B might act as valuable targets for the management of NPC radioresistance.

Materials and Methods

Cell lines and culture conditions. Poorly differentiated NPC cell lines were all purchased from the Cell Center of Central South University, Changsha, China. The cells were propagated in RPMI medium 1640 (HyClone, Logan, UT, USA) containing 10% FBS (Gibco BRL, Gaithersburg, MD, USA) and 1% antibiotics (Gibco BRL) and were cultured in an incubator at 37°C with saturated
humidity and 5% CO₂. Cells in an exponentially growing state were used for all of the following experiments.

**Irradiation.** Irradiation was delivered at room temperature using a 6 MeV electron beam generated by a linear accelerator (2100EX, Varian Medical, Inc., Palo Alto, CA, USA) at a dose rate of 300 cGy/min. A compensation glue with 1.5 cm thickness covered the cell culture containers. The source-to-skin distance was 100 cm.

**RNA sample preparation.** Total RNA was extracted using TRIzol reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer’s recommended protocol. The yield and purity of the RNA was determined by measuring the absorbance (Abs) at 260 and 280 nm. The RNA samples were only used when the ratio of the Abs260/Ab280 nm was ≥1.8. The integrity of the RNA samples was confirmed using a 1% agarose gel using the RNA 6000 Nano Assay Kit and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The extracted total RNA was stored at −80°C for subsequent use.

**Transfection of miR-185-3p mimic or inhibitor.** CNE-2 and 5-8F cells were transfected with miR-185-3p mimic/inhibitor (Gene-Pharma Co, Shanghai, China) according to the manufacturer’s protocol. To determine the efficiency of miRNA mimic/inhibitor, transfected cells were observed using the fluorescence image system directly and the expression of miRNA was assessed by using the qRT-PCR detection System (Bio-Rad, Hercules, CA, USA).

**Quantitative reverse transcription–polymerase chain reaction analysis.** Small RNA was extracted using the miRNEasy Mini Kit (Qiagen, Germantown, MD, USA). The All-in-One miRNA qRT-PCR Detection Kit (GeneCopoeia Inc., Rockville, MD, USA) was used for the quantitative detection of mature miRNA. Reverse transcription of miRNA was performed according to the manufacturer’s recommended protocol. Primers for miR-185-3p were purchased from the GeneCopoeia Company (Guangzhou, China).

Total RNA were isolated from NPC cells and tissues and the cDNA was subsequently synthesised from total RNA using a PrimeScript RT reagent kit with a DNA Eraser (TaKaRa, Kyoto, Japan). Primers (Jinsirui Biotechnology Company, Jiangsu, China) used in the present study are as follows: HLA-F forward primer 5′-agc gct tct atg agc cag ag-3′ and reverse primer 5′-cag cag tcc aac gag aac aa-3′; ICAM1 forward primer 5′-cag agg ttg aac ccc aca gt-3′ and reverse primer 5′-cct ggc tct atg agg cag ag-3′; GAPDH forward primer 5′-tcc aat atc aag aag gaa-3′ and reverse primer 5′-agt gcc ggg cgg gcg gga gct ggc cag ctt cct cgc cac cac ccc gtc gtc gaa gct ggc cag ctt cgt ctc cag ggt ggc-3′, and the luciferase activity was measured using the Dual Luciferase Reporter Assay system (Promega, Madison, WI, USA).

**Patients and tissue specimens.** A total of 15 fresh, undifferentiated NPC (World Health Organization type III) tissues and six samples from different cases of non-carcinoma epithelial tissues (NCET) from the nasopharynx were obtained from January 2013 to September 2013 at the Department of Otolaryngology Head and Neck Surgery, Xiangya Hospital, Central South University, Changsha, China. All patients had no history of previous malignancies and no history of radiotherapy or other therapy. All specimens were snap-frozen immediately and stored in liquid nitrogen prior to total RNA and small RNA extraction. The present study was approved by the Research Ethics Committee of the Central South University, Changsha, China. Informed consent was obtained from all patients. All specimens were handled and made anonymous according to ethical and legal standards.

**Statistical analysis.** The statistical significance of the differences between two groups was analysed using two-sided Student’s t-tests (for equal variances) or with Welch’s corrected t-test (unequal variances) using SPSS 18.0 software (IBM Corporation, Somers, NY, USA). Results were considered to be statistically significant at *P < 0.05.*

**Results**

**miR-185-3p expression in NPC cells.** To investigate the expression of miR-185-3p in NPC, qRT-PCR was applied to detect the prediction. The prediction values were calculated to estimate the binding affinities of the miRNA and their predictive target genes. The rules used for target prediction are based on those suggested by Allen et al. and Schwab et al.[19,20]
its expression level in four NPC cells, including CNE-2, HNE-1, 5-8F and 6-10B (Fig. 1). The results showed CNE-2 had the lowest expression and 5-8F had the highest expression of miR-185-3p, which were then selected for subsequent experiments.

Ectopic expression of miR-185-3p in CNE-2 cells increases their sensitivity to irradiation. Based on the differential expression of miR-185-3p in NPC cells, we aimed to examine the potential role of miR-185-3p on NPC radioresistance by overexpressing miR-185-3p in CNE-2 cells. A miR-185-3p-expressing vector and a control vector were used to transfect CNE-2 cells in vitro. A transfection efficiency of 91.6 ± 5.4% was observed under a fluorescence microscope and qRT-PCR assays demonstrated that miR-185-3p was successfully upregulated 101.4 ± 20.9 times in CNE-2 cells ($P < 0.01$; Fig. 2a,b). The CCK-8 assay revealed that following 2, 4 and 6 Gy irradiation stimulation, the survival rates of CNE-2 cells with miR-185-3p overexpression were decreased ($P < 0.05$; Fig. 2c). When exposed to irradiation, less clones were stained by crystal violet and the survival fraction decreased compared with control CNE-2 cells (0.17 ± 0.08 vs 0.39 ± 0.12; $P < 0.01$; Fig. 2d). These results demonstrate that overexpression of miR-185-3p could significantly inhibit the radioresistance of NPC cells.

Inhibition of miR-185-3p in 5-8F cells decreases their sensitivity to irradiation. Following overexpression of miR-185-3p in CNE-2 cells, we then suppressed the expression of miR-185-3p in 5-8F cells. Our data revealed that the transfection efficiency was 95.1 ± 4.0% and miR-185-3p was successfully inhibited in 5-8F cells (13.6 ± 2.6%; $P < 0.01$; Fig. 3a, b). The 5-8F cells with less miR-185-3p had a higher survival capacity following 6 Gy irradiation ($P < 0.05$; Fig. 3c). At the same time, the number of surviving clones was significantly increased and the size of colonies was larger compared with the control 5-8F cells (72.1 ± 15.2% vs 40.5 ± 6.7%; $P < 0.05$; Fig. 3d). Taken together, these data confirmed that miR-185-3p could increase the radioresistance of NPC cells to irradiation.

Prediction of miR-185-3p using target genes. Our previous study established radioresistance mRNA profiles and KEGG pathway analysis showed upregulated mRNA might function via the human T-cell leukemia virus type I (HTLV-I) infection pathway (which contains the most upregulated genes). (17) MiR-
NA target prediction program RNAhybrid indicated three up-regulated genes (i.e. WNT2B, ICAM1 and HLA-F) in the HTLV-I infection pathway were targeted by miR-185-3p (Fig. 4a). Herein, mRNA expression of WNT2B, ICAM1 and HLA-F were detected in NPC cells transfected with the miR-185-3p mimic/inhibitor. Interestingly, only WNT2B mRNA demonstrated converse alterations (Fig. 4b). Thus, further validation on the relationship between WNT2B and miR-185-3p was performed. Western blotting analyses showed that in miR-185-3p-mimic transfected cells, WNT2B decreased and downstream β-catenin and p-GSK-3β were also altered (Fig. 4c). In addition, we detected the expression of miR-185-3p and WNT2B in 15 NPC tissues and six NCET samples via qRT-PCR. Our results confirmed that the average expression level of miR-185-3p was significantly lower, but WNT2B was obviously upregulated in NPC specimens compared with NCET tissue samples (Fig. 4d). A negative correlation between miR-185-3p and WNT2B was also observed (R = −0.631; P = 0.002; Fig. 4e). These results revealed that WNT2B might be the target gene of miR-185-3p.

miR-185-3p directly targets the coding region of WNT2B. As we predicted, WNT2B is most likely the target gene of miR-185-3p. A series of experiments was performed to confirm our prediction. Hybridization of miR-185-3p and WNT2B mRNA can be predicted using RNAhybrid software and the minimum free energy required for this hybridization is −35.2 kcal/mol (Fig. 5a). Thus, specific targeting of WNT2B by miR-185-3p was examined using luciferase reporter assays. A mutant WNT2B reporter gene was constructed by deleting the seed sequence GGT GCG GAG GAA GCU GCG CAG CUC CC and mutating this sequence to GCT GGG CTC CTT TCT CGG GTC GGG GC (Fig. 5b). Our data revealed that disruption of the binding sites between miR-185-3p and the coding region of WNT2B mRNA abolished the miR-185-3p-mediated inhibition of WNT2B luciferase activity (P < 0.01; Fig. 5b). Taken together, these data indicate that miR-185-3p inhibits the expression of WNT2B protein via specific binding to the coding region of its mRNA.

WNT2B effects on the radioresistance of NPC cells. To confirm the precise functions of WNT2B, we silenced its expression in NPC cells using stable RNA interference plasmid. A WNT2B silencing vector and a control vector were used to transfect 5-8F cells. A transfection efficiency of 52.6±6.3% was observed using a fluorescence microscope and western blotting analyses demonstrated that WNT2B protein was successfully downregulated (Fig. 6a,b). The CCK-8 assay revealed that the survival rate of 5-8F cells with WNT2B loss was decreased after 4 Gy irradiation stimulation (P < 0.01; Fig. 6c) and fewer clones formed (0.11±0.03 vs 0.53±0.15; P < 0.01; Fig. 6d). These results revealed that silencing of WNT2B could significantly inhibit the radioresistance of NPC cells. Western blotting showed downstream β-catenin and p-GSK-3β were also altered (Fig. 6e).

miR-185-3p and WNT2B effect epithelial-mesenchymal transition (EMT)-related proteins. Our previous study showed EMT participated in NPC radioresistance. (21) Classic EMT biomar-
kers vimentin and E-cadherin were detected in the cells. In miR-185-3p-mimic transfected cells and WNT2B-silenced cells, downregulated vimentin and upregulated E-cadherin were spotted. In miR-185-3p-inhibitor transfected cells, vimentin increased and E-cadherin decreased (Fig. 7). These results suggest that WNT2B and miR-185-3p might be
upstream regulators of vimentin and E-cadherin and induce the genesis of the EMT phenotype.

Discussion

Radioresistance is a major restriction for the management of NPC. Elucidation of the molecular mechanisms underlying radioresistance is crucial to enhance the efficacy of treatment and to improve the survival rates for NPC, which at present remain poor. Our preliminary study has shown that miR-185-3p may be a radioresistance-associated miRNA in NPC, but limited data are available regarding its functions. In the present study, we found that miR-185-3p can regulate the radioresistance of NPC by targeting WNT2B in vitro. Our results indicate that miR-185-3p, as a novel radioresistance-related miRNA, might be a novel treatment strategy for NPC patients.

MiR-185-3p is defined as the mature miRNA of the precursor miR-185, which is processed from the 3′ side. To date, few studies have examined the function of miR-185-3p, let alone its malignant tumor behaviors. A literature review showed that miR-185-3p targeted the 3′ amino acid-coding of c-Myc mRNA in response to growth signals, resulting in cell cycle arrest and a block in cell proliferation. In vitro, functional analyses revealed that miR-185-3p could affect mental disorders via binding to sequences in the 3′UTR of TrkB-T1. Additional studies have focused on miR-185-5p, which, as a tumor suppressor, affected the proliferation, metastasis, cell cycles and overall survival in various cancers. Wang et al. also reported that miR-185-5p increases the radiosensitivity of renal cell carcinoma via repression of the ATR (ataxia telangiectasia and Rad-3-related) pathway. Because miRNA in the same family demonstrate a very similar expression pattern and biofunction, miR-185-3p might also function as a tumor suppressor. Indeed, our results showed that miR-185-3p was downregulated in NPC tissues and further experiments then validated its negative effect on radioresistance in vitro.

Fig. 5. MiR-185-3p directly targets the coding region of WNT2B. (a) The potential second structure of WNT2B and miR-185-3p and the minimum free energy required for this hybridization. (b) A mutation was generated in the WNT2B coding region. In particular, the mutation was located in the complementary site for the seed region of miR-185-3p as indicated. The wild-type WNT2B coding region and mutant WNT2B coding region were subcloned into a luciferase reporter construct, as shown. Relative luciferase activity in 5-8F cells was determined after the WNT2B coding region or mutant plasmids were co-transfected with miR-185-3p mimics or a negative control (**P < 0.01).
miRNA exert their biological functions mainly via the regulation of target genes. The target genes of miRNA can help to better understand the function of miRNA. Based on our previous prediction results, three potential target genes (i.e., \textit{WNT2B}, \textit{ICAM1} and \textit{HLA-F}) were detected in miR-185-3p transfected cells; however, only \textit{WNT2B} mRNA changed accordingly. \textit{WNT2B}, as an important member of the WNT signalling pathway, was upregulated in a spectrum of human malignancies, including colorectal, gastric and breast cancer.\cite{29-31} Silencing \textit{WNT2B} can inhibit metastasis and enhance chemotherapy sensitivity in ovarian cancer via caspase-9/BCL2/BCL-xL and EMT/p-AKT pathways \textit{in vitro} \cite{32} and Cox multivariate analysis demonstrated that \textit{WNT2B} was a significant prognostic factor in malignant pleural mesothelioma patients.\cite{32,33} Taken together, these studies highlight the importance of \textit{WNT2B} in pathological cancer processes. Nevertheless, the function of \textit{WNT2B} in radioresistance has been not been studied in detail. Our team first reported that \textit{WNT2B} mRNA and protein were upregulated in NPC tissues and \textit{WNT2B} expression was correlated with NPC clinical stages.\cite{16} The present study also reported \textit{WNT2B} plays a significant role in NPC radioresistance.

A limited number of studies has focused on the pathways and target genes of miR-185-3p. The present study confirmed miR-185-3p targeting \textit{WNT2B}, which involved three predicted signalling pathways, including the HTLV-1 infection pathway, basal cell carcinoma pathway and Hedgehog signalling pathway. HTLV-1 infection, following activation of WNT signalling, induced genesis of leukemia and lymphoma.\cite{34} Hedgehog signalling could also cross-talk with WNT signalling to regulate stem cell renewal and affect the radioresistance of oesophageal adenocarcinoma.\cite{35} The present studies
revealed that β-catenin and GSK-3β, as key molecules of WNT signalling pathways, alternated accordingly when miR-185-3p increased. These studies provided several clues for exploring the potential mechanisms of miRNA-185-3p and WNT2B involved in NPC radioresistance.

Epithelial–mesenchymal transition is recognized as a vitally important mechanism resulting in tumor migration and invasion. Increasing evidence has demonstrated that EMT is also involved in other malignant behaviors, such as irradiation resistance, drug resistance and cancer stem cells. Alterations of cellular surface markers indicate the genesis or changes of EMT, such as epithelial marker E-cadherin and mesenchymal cellular surface markers indicate the genesis or changes of EMT during exposure to irradiation. Thus, we detected E-cadherin and vimentin in the transacted cells. Our results revealed that miR-185-3p and WNT2B could influence the expression of EMT biomolecules, indicating that miR-185-3p and WNT2B might induce NPC radioresistance via EMT. Furthermore, recent literature has also reported EMT participated in activation of the WNT/β-catenin pathway in various tumors, such as colorectal, hepatocellular and head and neck squamous cell carcinoma. Here, we first observed that activation of WNT pathways accompanied EMT change in NPC. The underlying mechanism deserves further exploration.

In addition, most human miRNA function as onco-miRNA or anti-onco-miRNA depending on their potential target mRNA genes by binding to sequences in 3′-UTR. The other binding regions between mRNA and miRNA, such as 5′-UTR and coding sequences, were usually observed in plants with a very high sequence complementarity. Recently, several studies have uncovered that these plant-binding ways are also involved in animals, such as miR-324-3p targeting 5′-UTR of WNT2B mRNA and the miR-148 family targeting the Dnmt3B coding sequence, but with an imperfect complementarity. We also found that the coding sequence of WNT2B directly mediates regulation by miR-185-3p. Thus, we provided evidence that coding regions of human genes can be targeted by miRNA and such a mechanism might play a role in determining relative malignant biobehaviors.

In summary, we revealed that miR-185-3p has tumor suppressor functions in NPC. The newly identified miR-185-3p/WNT2B axis sheds light on the molecular mechanism of NPC cell radioresistance, indicating that it is a valuable NPC-associated biomarker and a promising therapeutic target in the management of NPC. Given that the clinical correlation results were based on limited tissue samples and no prognostic information was provided during the current investigation, the prognostic value of miR-185-3p requires further confirmation in a larger cohort of NPC patients with more complete clinical information.

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Disclosure Statement

The authors have no conflict of interest.
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