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ORIGINAL ARTICLE

MicroRNA-205 targets HER3 and suppresses the growth, chemosensitivity and metastasis of human nasopharyngeal carcinoma cells

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Summary

Purpose: *Nasopharyngeal carcinoma is one of the lethal cancers prevalent in Southeast Asia and Southern China. The frequent relapses, development of drug resistance, the adverse effects of chemotherapy and lack of therapeutic targets form the major hurdles in nasopharyngeal carcinoma treatment. This study was undertaken to investigate the role and therapeutic potential of miR-205 in human nasopharyngeal carcinoma cells.*

Methods: *Expression analysis was performed by qRT-PCR. The WST-1 and colony formation assays were used for the assessment of the cell viability. Autophagy was detected by electron microscopy and apoptosis was detected by DAPI staining. Protein expression was determined by western blot analysis.*

Results: *The expression of miR-205 was significantly downregulated in human nasopharyngeal carcinoma cells. Overexpression of miR-205 caused significant inhibition in the proliferation of CNE1 nasopharyngeal carcinoma cells. The miR-205-triggered growth inhibition was found to be mainly due to the induction of autophagy which was associated with increase in LC3B II and decrease in p62 expression. The miR-205 overexpression also caused apoptotic cell death of CNE1 cells which was concomitant with increase in the Bax/Bcl-2 ratio. Additionally, miR-205 enhanced the chemosensitivity of the nasopharyngeal carcinoma cells to cisplatin and suppressed their migration and invasiveness. In silico analysis showed that miR-205 exerts its effects by inhibiting human epidermal growth factor receptor 3 (HER3). The expression of HER3 was found to be significantly upregulated in nasopharyngeal carcinoma cells and overexpression of HER3 could nullify the effects of miR-205 on the proliferation of nasopharyngeal carcinoma cells*

Conclusion: *miR-205 may exhibit therapeutic implications in the treatment of nasopharyngeal carcinoma.*

Key words: *nasopharyngeal carcinoma, microRNA, cell cycle arrest, HER3, migration, invasion*

Introduction

Being one of the prevalent malignant tumors in Southeast Asia and Southern China, nasopharyngeal carcinoma is responsible for significant human mortality [1]. The early-stage metastasis of nasopharyngeal carcinoma makes it one of lethal cancers [2]. The 5-year survival rate under combined treatment with adjuvant cisplatin chemotherapy and radiotherapy is 50-60% [3]. The constant relapses and distant metastasis of nasopharyngeal cancer makes it difficult to manage with the current treatment strategies [4]. Generally, surgical removal, systemic chemotherapy or radiotherapy are employed for this malignancy, however, owing to the severe adverse effects, the patient quality of life is drastically impaired [5]. Therefore, there is need for the identification of biomarkers for early detection and exploration of novel therapeutic targets for efficient treatment of nasopharyngeal

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carcinoma [6]. Over the last few decades, research endeavours have been directed to explore the roles of microRNAs (miRs) in human cells. MiRs control the majority of human genes and are thus involved in vital cellular functions [7]. They have been found to regulate the expression of target genes via post transcriptional regulation [8]. Dysregulation of miRs expression has been shown to be responsible for the development of deadly diseases such as cancer. Thus, miRs exhibit therapeutic implications in treating human diseases such as cancer [9]. The miR-205 has been shown to be dysregulated in several types of cancers including non-small cell lung and prostate cancer [10,11]. In prostate cancer, miR-205 has been shown to deactivate several tumor suppressor genes [12]. In renal cancer, miR-205 suppresses the proliferation by suppressing src-mediated oncogenic pathways [13]. The growth of glioblastoma cells is also suppressed by miR-205 via inhibition of VEGF-A expression [14]. The miR-205 has also been reported to regulate the growth of laryngeal squamous cell carcinoma [15]. However, the role and therapeutic potential of miR-205 has not been thoroughly explored in nasopharyngeal carcinoma.

Therefore, this study investigated the therapeutic potential of miR-205 in nasopharyngeal cancer.

Methods

Cell lines and culture conditions

The normal nasopharyngeal cell line (NP460) and nasopharyngeal cancer cell lines (CNE1, CNE2, SUNE1, HK1) were purchased from American Type Culture Collection (Manassas, VA, USA). The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc., Waltham, MA, USA), antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), and 2 mM glutamine. The cells were cultured in an incubator (Thermo Fisher Scientific, Inc.) at 37˚C with 98% humidity and 5% CO_2. All transfections were carried out by Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, California, USA) as per the manufacturer's protocol.

qRT-PCR analysis

The total RNA from the normal and the nasopharyngeal cancer cell lines was isolated by TRIzol Reagent (Invitrogen) following the manufacturer's instruction. The cDNA was synthesized using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and amplified with Platinum SYBR Green qPCR SuperMix-UDG reagents (Invitrogen) using the CFX96 sequence detection system (Bio-Rad, Hercules, CA, USA). The expression was estimated by $2^{-\Delta\Delta Ct}$ method and actin was used as an internal control.

Analysis of cell proliferation

The CNE1 cell line showed the lowest expression of miR-205, hence only this cell line was selected for further experimentation.

The proliferation rate of CNE1 cells was monitored by WST-1 assay. In brief, CNE1 cells were cultured in 96-well plates at a density of 2×10^5 cells / well. The cells were then transfected with miR-negative control (NC) or miR-205 mimics and again incubated for 24 h at 37°C. This was followed by incubation of the cells with WST-1 at 37°C for 4 h. The absorbance was then measured at 450 nm using a victor 3 microplate reader to determine the proliferation rate at 0, 12, 24, 48 and 96 h time intervals.

Cell transfection

The miR-205 mimics and NC were synthesized by RiboBio (Guangzhou, China). The transfection was then carried out by the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. As the CNE1 cells reached 80% confluence, the appropriate concentrations of miR-205 mimics or NC were transfected into these cells.

Analysis of cell death

After transfection with miR-NC or miR-205 mimics, the CNE1 cells were cultured in 24 well-plates for 24 h at 37°C. The cells were then collected by centrifugation and washed with phosphate buffered saline (PBS). After this, the cells were stained with 1.2 mM Hoechst 33342. The CNE1 cells were then washed with PBS and then observed both by fluorescence and phase contrast microscopy. For annexin V/propidium iodide (PI) assay, the miR-NC or miR-205 mimics-transfected CNE1 cells (5×10^5 cells per well) were incubated for 24 h. This was followed by staining these cells with annexin V-FITC/PI. The percentage of apoptotic CNE1 cells was determined by flow cytometry.

Cell migration and invasion assay

The effects of miR-205 overexpression on the invasion ability of CNE1 cells was determined by transwell chambers with Matrigel. The CNE1 cells were transfected with miR-205 mimics and around 200 ml cell cultures were placed onto the upper chamber and only medium was placed in the bottom chamber. After 24 h of incubation, the cells were removed from the upper chamber and the cells that invaded via the chambers were fixed with methyl alcohol and subsequently stained with crystal violet. Inverted microscope was used to count the number of invaded cells at 200x magnification. The same procedure was used for cell invasion, however, in case of migration assay, the inserts were not coated with 50 µl extracellular matrix gel.

Dual-luciferase reporter assay

The miR-205 target was identified by TargetScan online software (http://www.targetscan.org). The miR-205 mimics or NC were co-transfected with Plasmid pGL3-HER3´-UTR-WT or pGL3-HER3´-UTR-MUT into CNE1

cells. Dual-luciferase reporter assay (Promega, Madison, Wisconsin, USA) was carried out 48 h after transfection. *Renilla* luciferase was used for normalization.

Western blotting

The normal and the nasopharyngeal carcinoma cell lines were cultured at 37°C for 24 h and then centrifuged at 12000 rpm. The cell pellet was washed with PBS and then suspended again in RIPA lysis buffer. Thereafter, the concentrations of the proteins were determined and equal concentrations of the proteins were loaded on SDS-PAGE gel (15%). The samples were transferred to polyvinylidene fluoride membranes and blocking was done using 5% skimmed milk powder. This was followed by membrane incubation with primary antibodies at 4˚C for 24 h. Next, the membranes were incubated with horseradish peroxidase-linked biotinylated secondary antibodies for 2 h. The membranes were washed and immunoreactive bands were observed by ECL-PLUS/Kit as per the manufacturer's instructions.

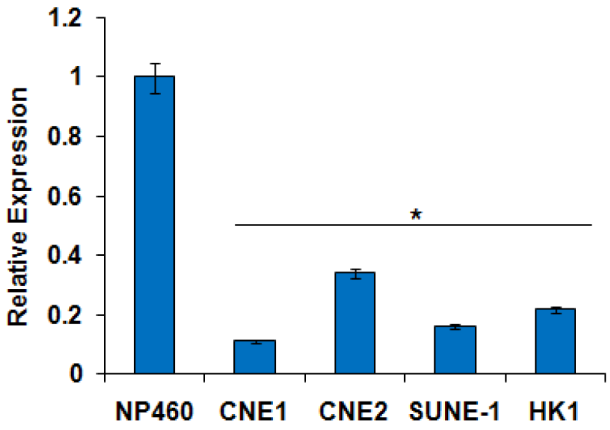

Figure 1. Expression of miR-205 in different nasopharyngeal carcinoma cell lines and the normal cell line NP460, as determined by qRT-PCR. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

Statistics

The experiments were done in triplicate and the values are shown as mean ± standard deviation (SD). P<0.05 was considered as significant difference. Student's t-test using Graph Pad prism 7 software was used for the statistical analysis.

Results

miR-205 suppresses the proliferation of nasopharyngeal carcinoma cells

To unveil the role of miR-205 in nasopharyngeal carcinoma, the expression profile of miR-205 was examined in four different nasopharyngeal carcinoma cell lines (see Methods) as well as in the normal cell line ND460 by qRT-PCR. The results showed that miR-205 was significantly suppressed in the nasopharyngeal carcinoma cells relative to its expression in normal NP460 cells (Figure 1A). The expression of miR-205 was observed to be 9-fold lower in the nasopharyngeal carcinoma cells. Additionally, the expression of miR-205 was found to be highly downregulated in the CNE1 cells. To ascertain the role of miR-205 in the proliferation of the nasopharyngeal carcinoma CNE1 cells, the cells were transfected with miR-205 mimics. The overexpression of miR-205 in CNE1 cells was validated by qRT-PCR which showed 5.5-fold increase in the miR-205 expression (Figure 2A). Next, the proliferation rate of miR-205 overexpressing CNE1 cells was monitored at different time periods and the results showed that miR-205 overexpression resulted in significant decrease in the proliferation rate of the CNE1 nasopharyngeal carcinoma cells (Figure 2B).

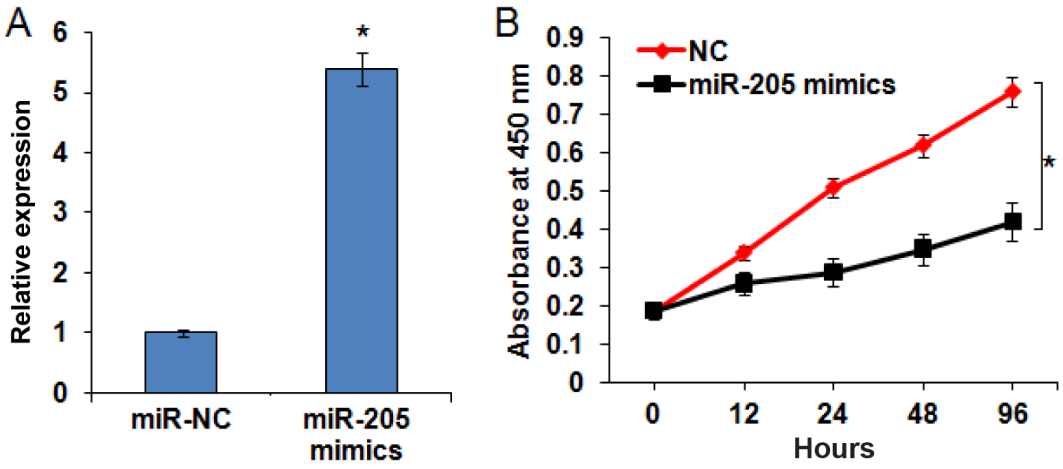

Figure 2. A: Expression of miR-205 in miR-NC and miR-205 mimics transfected CNE1 nasopharyngeal carcinoma cells as determined by qRT-PCR. **B:** The cell viability of the miR-NC and miR-205 mimics transfected CNE1 nasopharyngeal carcinoma as determined by the WST-1 assay. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

miR-205 induces autophagy and apoptosis in nasopharyngeal carcinoma cells

Electron microscopic analysis was carried out to find out the underlying mechanism for inhibition of CNE1 cell proliferation upon miR-205 overexpression. The results showed that miR-205 overexpression led to the development of autophagosomes in the CNE1 cells (Figure 3A). This was also accompanied by increase in the expression of LC3B II and decrease in the expression of p62, suggestive of autophagy (Figure 3B). DAPI staining showed that miR-118 triggered remarkable changes in the morphology of the CNE1 cells such as nuclear fragmentation, indicative of apoptosis (Figure 4A). The annexin V/PI staining also showed increase in the percentage of the CNE1 apoptotic cells upon miR-205 overexpression (Figure 4B). Western blot analysis showed that miR-205 caused upregulation of Bax and downregulation of Bcl-2 expression in CNE1 cells, further confirming the apoptotic cell death (Figure 4C).

miR-205 enhances the drug sensitivity of nasopharyngeal carcinoma cells

The effects of miR-205 were examined on the sensitivity to cisplatin of the nasopharyngeal carcinoma CNE1 cells. The nasopharyngeal carcinoma CNE1 cells were transfected with miR-205 mimics or treated with 5 μM cisplatin or transfected with miR-205 mimics plus treated with 50 μM cisplatin and then subjected to WST-1 assay. The results showed that the effects of miR-205 mimics and cisplatin treatment were more profound on the

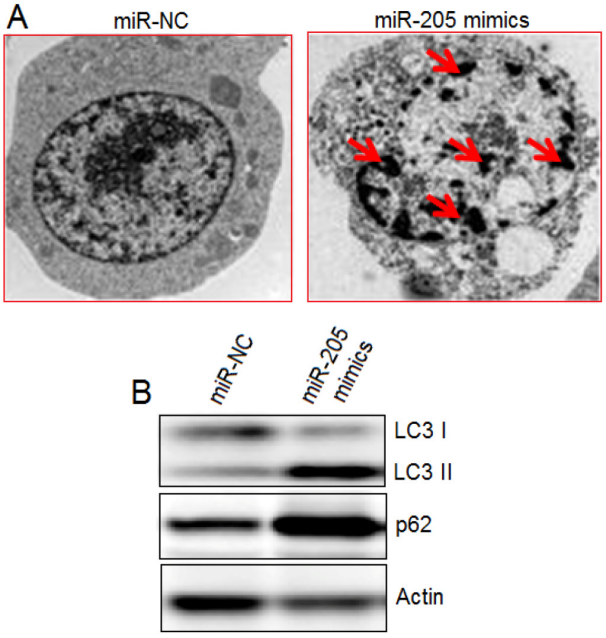

Figure 3. A: Electron microscopic analysis of miR-NC and miR-205 mimics transfected CNE1 nasopharyngeal carcinoma cells showing induction of autophagy (arrows show autophagosomes). **B:** Western blot analysis of the miR-NC and miR-205 mimics transfected CNE1 nasopharyngeal carcinoma cells showing the expression of LC3B I, II and p62. The experiments were performed in triplicate.

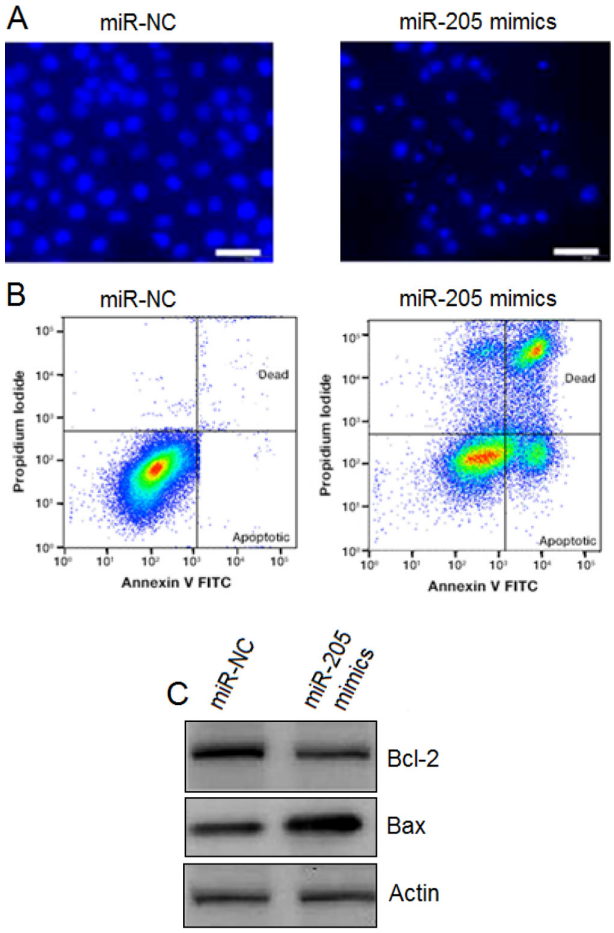

Figure 4. A: DAPI staining and **B:** annexin V/PI staining of the miR-NC and miR-205 mimics transfected CNE1 nasopharyngeal carcinoma cells showing induction of apoptosis. **C:** Western blot analysis of miR-NC and miR-205 mimics transfected CNE1 nasopharyngeal carcinoma cells showing the expression of Bax and Bcl-2. The experiments were performed in triplicate.

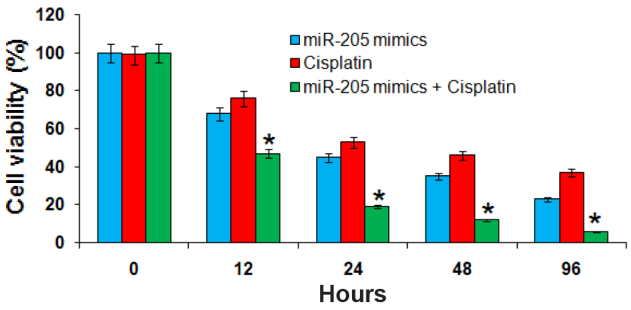

Figure 5. Overexpression of miR-205 in CNE1 cells enhances their chemosensitivity to cisplatin as determined by the WST-1 assay. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

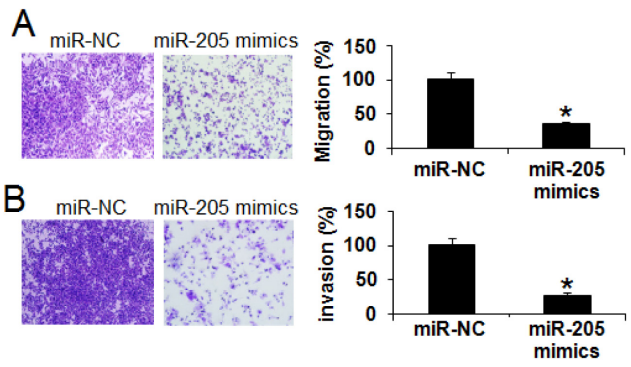

Figure 6. Transwell assays showing A: migration and B: invasion of the miR-NC and miR-205 mimics transfected CNE1 nasopharyngeal carcinoma cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

CNE1 cell proliferation than in miR-205 mimics or cisplatin individually (Figure 5), suggesting miR-205 overexpression enhances the drug sensitivity of nasopharyngeal carcinoma cells.

miR-205 suppresses the metastasis of nasopharyngeal carcinoma cells

The effects of miR-205 on the metastasis of CNE1 cells were determined by transwell assay which revealed that miR-205 caused significant decrease in the migration and invasion of the CNE1 cells. The migration of the CNE1 cells was suppressed by 67% (Figure 6A), while invasion was inhibited by 75% (Figure 6B) upon miR-205 overexpression.

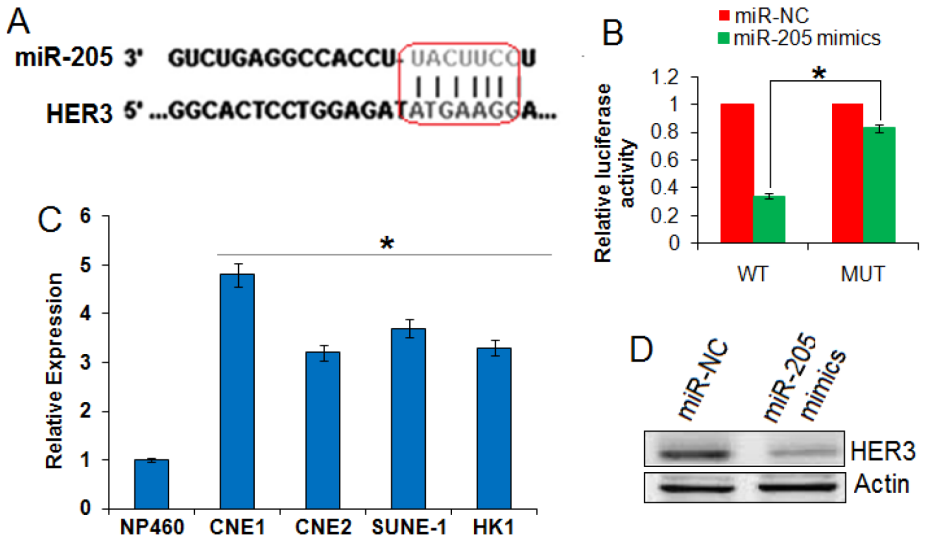

Figure 7. A: TargetScan analysis showing HER3 as the target of miR-205. **B:** Dual luciferase assay. **C:** Expression of HER3 in different nasopharyngeal carcinoma and normal NP460 cells. **D:** Western blot analysis showing the expression of HER-3 in miR-NC and miR-205 mimics transfected CNE1 nasopharyngeal carcinoma cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

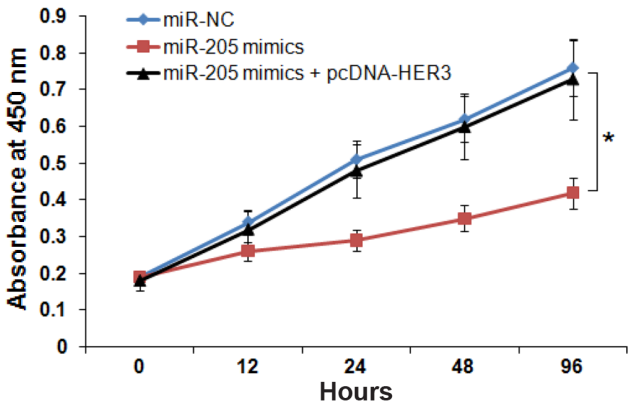

Figure 8. Cell viability of the miR-NC, miR-205 mimics or miR-205 mimics plus pcDNA-HER3 transfected CNE1 cells showing miR-205 overexpression abolishes the effects of miR-205 mimics on the proliferation of the CNE1 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

miR-205 targets HER3 in nasopharyngeal carcinoma cells

The TargetScan revealed that HER3 acts as the target of miR-205 in nasopharyngeal carcinoma cells (Figure 7A). HER3 was further confirmed as the target of miR-205 by dual luciferase assay (Figure 7B). The western blotting further revealed that the HER3 was significantly overexpressed in all the nasopharyngeal carcinoma cells as compared to the normal NP460 cells (Figure 7C). Nonetheless, overexpression of miR-205 resulted in suppression of HER3 expression in CNE1 cells (Figure 7D), confirming HER3 as the target of miR-205.

Next, it was found that overexpression of HER3 in CNE1 cells could promote the growth of the CNE1 cells and nullified the growth inhibitory effects of miR-205 overexpression on CNE1 cell proliferation (Figure 8).

Discussion

Nasopharyngeal carcinoma is one of the common types of head and neck malignancies [16]. The early metastasis of nasopharyngeal carcinoma, late diagnosis, unavailability of therapeutic targets and the adverse effects of the treatment strategies used are the main obstacles that limit its treatment [17]. The limited availability of reliable and efficient therapeutic targets/agents hurdles the treatment of nasopharyngeal carcinoma [4]. The wide array of roles that miRs carry out in humans by controlling the expression of genes suggests that miRs may prove useful therapeutic targets for treating human diseases, including cancer [18]. In this study, we investigated the role of miR-205 in nasopharyngeal carcinoma and found the expression of miR-205 is significantly downregulated in nasopharyngeal carcinoma cells. Previous studies have also shown that the expression of miR-205 is dysregulated in cancer cells. For example, the expression of miR-205 has been shown to be downregulated in lung and prostate cancer cells [10,11]. miR-205 was overexpressed in CNE1 nasopharyngeal carcinoma cells. It was observed that miR-205 overexpression resulted in significant decline in the proliferation rate of CNE1 cells. Electron microscopic analysis revealed that miR-205 overexpression promoted autophagy in the CNE1 cells by increasing the expression of LC3B II and decreasing the expression of p62. Moreover, DAPI staining revealed that miR-205 overexpression resulted in nuclear fragmentation of the CNE1 cells. This was also accompanied with increase in Bax and decrease in the Bcl-2 expression. These results are in concordance with a study, wherein two previous studies, wherein miR-205 has been shown to promote the apoptosis of breast cancer cells and has also been shown to suppress the expression of Bcl-2 in adrenocortical carcinoma [19,20]. The frequent relapses and the development of the drug resistance, such as cisplatin, in nasopharyngeal carcinoma cells often makes it difficult to manage [21]. Herein, we also investigated the effects of miR-205 overexpression on the sensitivity of the nasopharyngeal carcinoma CNE1 cells to cisplatin and the results showed that miR-205 enhanced the chemosensitivity of the CNE1 cells to this drug. These results are in concordance with a study wherin miR-205 has been shown to increase the chemosensitivity of pancreatic cancer cells [22]. The effects of miR-205 were also examined on the CNE1 cell migration and invasion and it was found that this miR suppressed the migration and invasion other CNE1 nasopharyngeal carcinoma cells, indicating the implications of miR-205 in the management of metastatic cancers. These studies are in agreement with previous reports wherein miR-205 has been reported to inhibit the migration and invasion of the breast and prostate cancers [23,24]. miRs exert their effects by suppressing the expression of the target genes and each miR has several targets [25]. Herein, bioinformatic analysis together with dual luciferase assay showed that miR-205 exerts its effects by targeting HER3. Additionally, the expression of HER3 was considerably increased in all the nasopharyngeal carcinoma cells and overexpression of miR-205 could inhibit the expression of HER3. Moreover, the overexpression of HER3 could nullify the effects of the miR-205 overexpression on the proliferation of the CNE1 nasopharyngeal carcinoma cells. This is in agreement with a previous study wherein miR-205 has been shown to regulate the expression of HER3 in breast cancer [26].

Conclusion

Taken together, the findings of the present study revealed that miR-205 is downregulated in the nasopharyngeal carcinoma cells. Overexpression of miR-205 in CNE1 nasopharyngeal carcinoma cells inhibited their proliferation by inducing autophagy and apoptosis. Overexpression of miR-205 also suppressed the migration and invasion and enhanced the chemosensitivity of CNE1 nasopharyngeal carcinoma cells, indicative of the therapeutic implications of miR-205 in nasopharyngeal carcinoma treatment.

Conflict of interests

The authors declare no conflict of interests.

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