Tumor Suppressor Functions of miRNA-375 in Nasopharyngeal Carcinoma through Inhibition of Ubiquitin-Specific Protease 1 Expression

Xu Jiayuan

The Third Affiliated Hospital of Wenzhou Medical University

Li Bangliang

The Third Affiliated Hospital of Wenzhou Medical University

Song Wei

the third affiliated hospital of Wenzhou Medical University

Cao Longhe

the third affiliated hospital of Wenzhou Medical University

Zhu Chuansai

the third affiliated hospital of Wenzhou Medical University

Lin Sen (✉ lnns@163.com)

the third Affiliated Hospital of Wenzhou Medical University <https://orcid.org/0000-0002-1175-0232>

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Abstract

Background: Nasopharyngeal carcinoma (NPC) development involves many genetic alterations. This study profiled differentially expressed miRNAs and selected miR-375 for further study.

Methods: Differentially expressed miRNAs (DE-miRNAs) were screened using online databases and subjected to various analyses. miR-375 mimics with negative control cDNA, and ubiquitin-specific protease 1 (USP1) as well as a vector-only control were transfected into NPC cells for analysis by quantitative PCR, western blotting, wound healing, Transwell, cell viability, flow cytometry, and luciferase gene reporter assays.

Results: A total of 308 NPC and 23 normal tissues were analyzed, and 67 DE-miRNAs were identified. Among these, miR-375 was downregulated and miR-21-5p was upregulated. Bioinformatical analysis identified USP1 as a potential target gene of miR-375. Furthermore, miR-375 expression was decreased, whereas USP1 expression was increased in NPC. Increased USP1 expression was associated with poor survival of head and neck cancer patients. The luciferase assay confirmed miR-375 binding to the *USP1* 3'-untranslated region (UTR), while the transfection experiment confirmed miR-375 expression reduced USP1 expression, and USP1 overexpression reversed the anti-tumor activity of miR-375 in NPC cells, determined by tumor cell migration, invasion, apoptosis, and gene expression. In addition, USP1 overexpression activated phosphoinositide 3-kinase (PI3K) signaling, whereas a selective PI3K inhibitor (S2793) could reverse the effects of USP1 on NPC cells *in vitro*.

Conclusions: This study confirmed decreased miR-375 expression and increased miR-21 expression in NPC tissues. Downregulated miR-375 expression led to USP1 upregulation, which in turn activated PI3K/Akt signaling and promoted NPC cell migration and invasion, but inhibited NPC cell apoptosis.

Introduction

Nasopharyngeal carcinoma (NPC), the most common cancer originating in the nasopharynx, differs significantly from other head and neck cancers in terms of risk factors, pathogenesis, clinicopathological characteristics, and treatment options [1]. Global cancer statistics have estimated that more than 133,000 new NPC cases and more than 80,000 NPC cancer-related deaths occurred in 2020 across the globe, accounting for 0.7% and 0.8% of all cancer incidence and death, respectively [2]. The majority of NPC patients are geographically localized to Southeast Asia, while China has the highest incidence and mortality rates[3; 4]. NPC risk factors include viral infections, environmental influences, and genetic factors, but infection with Epstein-Barr virus (EBV) is one of the primary risk factors for the development of NPC[5]. Activation of nuclear factor-kappa B (NF- κ B) signaling has been shown in approximately 50% of NPC cases [6; 7]. Clinically, NPC patients can be treated with surgery, chemotherapy, radiotherapy, and immune-based therapy[8]. However, the development of more effective treatments for NPC is urgently needed.

microRNA (miRNA) is a class of small non-coding RNA molecules in the human genome that can regulate gene expression by directly binding to the 3'-untranslated region (UTR) of target genes to exert a biological effect on a cell [9]. miRNAs have been reported to play an important role in various human cancers[10]. In NPC, recent studies have shown that exosomal miRNA-301a-3p expression was able to promote NPC cell proliferation and invasion [11], miRNAs derived from circulating small extracellular vesicles could be used to diagnose early NPC [12], and EBV-encoded miRNA BART8-3p was able to enhance NPC radioresistance-associated metastasis[13]. In this regard, further bioinformatical analysis into the involvement of miRNAs in NPC could allow for an in-depth investigation of NPC genetic data using various online databases. Thus, miRNAs that are related to NPC were screened and assessed

using data from the Gene Expression Omnibus (GEO) database and further bioinformatical analyses were performed including miRNA-gene targeting, gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and protein-protein interaction (PPI) network analyses. The NPC-related differentially expressed miRNAs (DE-miRNAs) and the underlying mechanisms by which these DE-miRNAs could play a role in the development and progression of NPC were explored. Additionally, a series of experiments were performed to verify the data obtained from the above bioinformatics analysis.

Materials And Methods

Bioinformatical analysis

In this study, miRNA data from NPC samples were first screened and downloaded from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/index.cgi?qb=dat>). GEO 2R data were selected due to the differentially expressed miRNAs in NPC, and the adjusted *p*-value (0.05) and fold change (2) cutoffs were set to obtain expression levels using the University of Alabama Cancer Database (UALCAN)[14]. A volcano map was plotted, and survival analysis was performed using GEO 2R and OncomiR, respectively. Next, the target genes of the miRNAs were analyzed using the miRtarBase[15] and TargetScan [16] tools. In addition, GO enrichment, pathway enrichment, PPI network analysis, and hub gene analyses were also performed using Metascape, the String database, and Cytoscape (version 3.8).

Clinical samples

A total of 25 NPC patients and age-matched healthy controls were recruited from The Third Affiliated Hospital of Wenzhou Medical University (Wenzhou, China) between March 2001 and March 2020. The patients were histologically diagnosed with NPC using the Head and Neck Tumor (HNT) classification published by the World Health Organization (WHO) in 2017[17]. All patients had not received any radiotherapy or chemotherapy before surgery. Tumor tissues and normal nasal mucosa tissues from healthy controls were collected and stored at -80°C until subsequent use for polymerase chain reaction (PCR)-based detection of gene expression.

This study was approved by the Ethics Committee of Wenzhou Medical University and all participants provided written informed consent prior to study enrollment.

Cell culture and transfection

Cell lines including CNE-1 (highly differentiated nasopharyngeal squamous cell carcinoma), CNE-2 and HNE-1 (poorly differentiated nasopharyngeal squamous cell carcinoma), as well as human nasal mucosal epithelial NP69 cells were originally obtained from Runxin Bio. (Suzhou, China) and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mL glutamine, 0.1 mL non-essential amino acids, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin in a humidified incubator with 5% CO₂ at 37°C. When the cells were approximately 80% confluent, they were collected for western blot analysis.

The pcDNA3.1 plasmid carrying the USP1 coding sequencing (CDS; pcDNA3.1-USP1) was obtained from Zhejiang Gloucester Biotechnology Company (Hangzhou, China) and the miR-375 mimics and negative control (NC) were from Thermo Fisher Scientific (Waltham, MA, USA). For gene transfection, cells were grown in a 96-well plate overnight and then transfected for 24 h with pcDNA3.1-USP1, vector-only control, miR-375 mimics, or a NC using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. After the transfection, cells were harvested for further experiments.

Transwell invasion assay

According to the manufacturer's instructions, Transwell chambers pre-coated with Matrigel (pore size of 8 μm ; Corning Inc., Corning, NY, USA) were set to include post-transfection CNE1 cells (5×10^4) suspended in serum-free medium in the upper chamber, whereas the lower chamber was filled with FBS-containing medium. After 48 h in culture, the cells remaining on the top surface of the membrane were removed and the cells that had invaded onto the bottom of the membrane were stained with crystal violet and counted using a DMI8 optical microscope (Leica; Weztlar, Germany).

Wound healing assay

After the 24 h transfection, CNE1 cells were inoculated into six-well plates (4×10^5 cells/well) and cultured for 24 h to reach 100% confluency. Using a sterile 200- μL pipette, the cell monolayer was wounded, and cells were further cultured for 48 h. The wounds were measured using a DMI8 optical microscope and the cell migration rate was calculated using the following equation: (width at 0 h - width at 48 h) / width at 0 h.

Flow cytometry

After the transfection, cells were subjected to an Annexin V-fluorescein isothiocyanate (FITC) apoptosis assay using the Annexin V-FITC Apoptosis Detection Kit from Thermo Fisher Scientific. In brief, CNE1 cells were grown and transfected with pcDNA3.1-USP1, vector-only control, miR-375 mimics, or the NC using Lipofectamine 2000 (Thermo Fisher Scientific) for 48 h. After the transfection, cells were harvested and subjected to Annexin V-FITC and propidium iodide (PI) staining according to the manufacturer's instructions. Finally, after a 5 min incubation at room temperature in the dark, the apoptosis rate of cells was analyzed using a flow cytometer (Becton Dickinson FACS Vantage SE, USA).

Western blot

After the 24 h transfection, CNE1 cells were harvested for extraction of total cellular protein using radioimmunoprecipitation assay (RIPA) lysis buffer. The cell lysate was then centrifuged, supernatant was collected and mixed with the sample buffer, and the mixture was boiled for 5 min. Equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Thermo Fisher Scientific). For protein blotting, the membranes were first blocked in 5% skim milk in phosphate buffered saline (PBS)-Tween 20 (PBS-T) for 1 h at the room temperature, then incubated with diluted primary antibodies of anti-USP1 (Fine biotech, Wuhan, China), c-Myc (Huabio, Hangzhou, China), Bad (Biyotime Technology, Shanghai, China), Bax (Biolab Technology, Xian, China), Bcl-2 (Hengyuan Biological, Shanghai, China), phosphoinositide 3-kinase (PI3K; Biolab Technology, Xian, China), phospho-PI3K (p-PI3K; Bioworld Technology, Bloomington, USA), caspase-3 (Biolab Technology, Xian, China), E-cadherin (Huabio, Hangzhou, China), Akt (Huabio, Hangzhou, China), or p-Akt (Bioworld Technology, Bloomington, USA) at 4°C overnight. The following day, membranes were washed 3x with PBS-T and then incubated with a horseradish peroxidase (HRP)-conjugated second antibody (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature. After washing 3x with PBS-T, the membranes were subjected to an incubation with enhanced chemiluminescence (ECL) reagents to detect the protein signal. Blots were observed on an ImageQuant LAS4000 biomolecular imager (General Electric Medical, Shanghai, China) to visualize protein expression. Experiments were repeated at least three times.

Cell viability CCK-8 assay

After the 24 h transfection, CNE1 cells (1×10^4) were inoculated into each well of a 96-well plate and then incubated for 48 h. Next, the CCK-8 reagent (10 μL ; MedChemExpress [MCE], Shanghai, China) was added to each well, and cells were incubated for 4 h according to the manufacturer's instructions. The cells were then used immediately for

measurement of the optical density using a microplate reader at an absorbance of 490 nm. The experimental conditions were performed in triplicate and repeated at least three times.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Following the 24 h transfection, CNE1 cells and tissue specimens were subjected to RNA isolation using TRIzol (Thermo Fisher Scientific) and the RNA was reverse transcribed using M-MuLV reverse transcriptase (New England Biolabs, Beijing, China) using the ABI StepOne Plus system according to the manufacturers' instructions. Next, the cDNA samples were subjected to qPCR amplification using the maxima SYBR Green/ROX qPCR Master Mix according to the manufacturer's instructions. The primer sequences of genes were: 5'-TTT GTT CGT TCG GCT CGC-3'(miR-375), 5'-ACU GGA CUU GGA GUC AGA AGG-3'(miR-375 mimics), and 5'-CTC CCG GGA TGT AGT TGG TG-3'(USP1). The amplified gene of interest was normalized to the relative level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA.

Prediction of miRNA targets and dual-luciferase reporter assay

miRNA targets were predicted using the miRtarBase [18] and TargetScan[16] tools. The luciferase reporter gene detection system was used to verify potential miRNA targets. Specifically, the putative 3'-UTR miRNA binding sequences were synthesized and cloned into the SpeI and HindIII cloning sites of the pMIR-REPORT vector (Biolab Technology, Xian, China), while the *USP1* 3'-UTR containing miR-375 mutant sequences was synthesized by Eurofins genomics (Vienna, Austria) and the miR-375 binding site was mutated to construct mutant *USP1*, and the mutant (MUT)/wild type (WT) were cloned into the pMIR-REPORT vector between the SpeI and HindIII sites. After DNA sequence confirmation, these constructs were co-transfected with the target miRNA and luciferase control plasmid into HEK293 reporter cells in a 48-well plate using Lipofectamine 2000. Plasmid DNA (0.2 µg) and miRNA (100 nmol/L) were used for the transfection experiments, and subsequently the luciferase activity was measured 24 h after gene incubation using the luciferase reporter gene detection system (Biolab Technology) according to the manufacturer's instructions.

Statistical analysis

All experimental data were analyzed using GraphPad Prism (version 8.0; GraphPad Software, La Jolla, CA, USA) and SPSS (version 19.0; SPSS, Chicago, IL, USA). All data were collected from at least three independent experiments and expressed as the mean $\pm$ standard deviation (SD). The difference between two groups was analyzed using the Student's *t*-test, while the Pearson correlation coefficient was used to analyze the correlation of gene expression. A *p*-value less than 0.05 indicated statistical significance.

Results

Identification of differentially expressed miRNAs (DE-miRNAs)

In this study, both the GSE 70970 dataset, which contained 246 NPC and 17 normal tissues, and the GSE 36682 dataset, which contained 62 NPC and six normal tissues, were used. After an initial analysis, 67 DE-miRNAs were obtained from these two datasets by applying the GEO 2R online tool for analysis ($|\log FC| \geq 1$; *p*-value < 0.05). Two common DE-miRNAs were then assessed further: down-regulated miR-375 ($|\log FC| < 0$) and up-regulated miR-21-5p ($|\log FC| > 0$). The volcano plots are shown in Fig. 1A and B, while Fig. 1C and D show the expression of miR-21 and miR-375 in head and neck squamous cell carcinoma (HNSCC) tissues vs. normal tissues using data from the

UALCAN database. Figure 1E and F show the survival analyses stratified by miR-375 and miR-21 expression in NPC patients from the OncomiR database. Next, the detailed differential expression of miR-375 and miR-21 as well as the statistical data from GSE 36682 and GSE 70970 are shown in Table 1.

Table 1
Differential expression of miR-21 and miR-375 in nasopharyngeal carcinoma (NPC) vs. normal tissues using data from two different datasets.

	miRNA	Adj. <i>p</i> -value	Log fold change
GSE 36682	miR-21	8.31e-31	-1.643277
	miR-375	1.97e-04	1.172213
GSE 70970	miR-21	2.61e-08	-2.889119
	miR-375	2.32e-09	4.0474

Bioinformatical data of miRNA targets, functions, and gene-gene interactions

The StarBase and TargetScan Human 7.2 were used to predict potential target genes, and a total of 4491 potential genes were found. A Venn diagram was used to consider the accuracy of the biological analysis and evaluate genes closely related to the miRNAs and mRNAs, which identified 436 target genes. To investigate the effects of these genes at the molecular level, GO terms and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were used to predict biological functions and related pathways among distinct differentially expressed genes (DEGs). Figure 2A shows the functions of genes related to cellular processes, the regulation of biological processes, and cellular component organization or biogenesis. These target genes were significantly enriched in vasculature development, response to growth factor, mesenchyme development, sensory system development, negative regulation of cell differentiation, mesodermal commitment pathway, mitogen-activated protein kinase signaling pathway and signaling by receptor tyrosine kinases (Fig. 2A). Figure 2B and C show the result of the enrichment analysis and each term is represented by a circle node, where the size is proportional to the number of input genes that fall into that term, and the color represents the cluster identity (nodes of the same color belong to the same cluster). Furthermore, a PPI network analysis of these target genes was performed to confirm the connection of the network modules using MCODE. The enrichment analysis of each MCODE module pathway and process was applied independently and the data are shown in Fig. 2D and E.

PPI network and miRNA-hub gene network data

Cytoscape was used to draw the PPI network of the miR-375 and miR-21 target genes. The matching nodes of these target genes were constructed using the String database and the PPI network showed the importance of a gene that is positively correlated with the number of genes it may affect. Overall, 250 hub genes were identified according to node degree (Fig. 3A and B). Specifically, miR-375 could modulate 78 hub genes, while miR-21 could regulate 168 hub genes. This PPI network suggested that miR-375 and miR-21 might be the critical potential regulators in NPC.

Next, the top 10 target genes for both miR-21 and miR-375 were selected (ranked by degree value, data shown in Table 2). Among these, genes with insufficient data were excluded e.g., tripartite motif containing 33 (TRIM33) and genes with insignificant differences were excluded e.g., neurogenic locus notch homolog (NOTCH2). As such, genes with insignificant differences in survival curves were also excluded (i.e., G protein subunit gamma 12 (GNG12), GATA zinc finger domain containing 2B (GATAD2B), YOD1 deubiquitinase (YOD1), Protein tyrosine phosphatase non-receptor type 14 (PTPN14), Fas ligand (FASLG), Heterogeneous nuclear (HNRNPU), SKI proto-oncogene (SKI), DnaJ heat shock protein family (DNAJC16), Eukaryotic translation initiation factor 4 gamma 3 (EIF4G3), SERPINE1 mRNA binding protein 1 (SERBPI), ETS transcription factor ELK4 (ELK4), ENAH actin regulator (ENAH), and Y-box binding protein 1 (YBX1). Additionally, there was negative regulation between most miRNAs and their downstream target genes; therefore, genes with an expression not negatively correlated to miRNA levels were also excluded, such as S100 calcium binding protein A10 (S100A10) and Nuclear factor I A (NFIA), resulting in the identification of USP1 with a potential tumor-inhibition role in NPC. Therefore, it was hypothesized that miR-375 might exert a tumor-suppressive effect by directly targeting USP1 expression. In this regard, USP1 expression was assessed in various cancer tissues using data from ONCOMINE and Gene Expression Profiling Interactive Analysis (GEPIA; Fig. 4A and B), and USP1 expression was associated with the survival of HNSC patients using UALCAN data (Fig. 4C-G). More specifically, Fig. 4A shows USP1 expression in association with the prognosis of 16 types of cancer (GEPIA database), while Fig. 4B reveals USP1 expression in association with the prognosis of 15 types of cancer (ONCOMINE database). Figures C through G reveal data regarding the highest USP1 expression in stage IV, N4, and male NPC patients.

Table 2
Top ten hub genes of miR-21 and miR-375 target genes.

miR-21	Gene name	Normal vs. Primary (p -value)	Survival (p -value)	Expression (tumor)	miR-375	Gene name	Normal vs. Primary (p -value)	Survival (p -value)	Expression (tumor)
	GNG12	1.14E-03 ($p < 0.05$)	0.640	Up-regulated		EIF4G3	8.65E-07 ($p < 0.05$)	0.510	Up-regulated
	TRIM33	NA	NA	NA		SERBP1	1.55E-06 ($p < 0.05$)	0.170	Up-regulated
	S100A10	1.80E-03 ($p < 0.05$)	0.015	Up-regulated		NOTCH2	6.79E-01 ($p > 0.05$)	0.360	Down-regulated
	GATAD2B	1.66E-12 ($p < 0.05$)	0.180	Up-regulated		ELK4	1.62E-12 ($p < 0.05$)	0.350	Up-regulated
	YOD1	4.78E-02 ($p < 0.05$)	0.390	Down-regulated		ENAH	< 1E-12 ($p < 0.05$)	0.940	Up-regulated
	PTPN14	1.49E-12 ($p < 0.05$)	0.710	Up-regulated		SKI	3.66E-05 ($p < 0.05$)	0.790	Up-regulated
	FASLG	1.84E-10 ($p < 0.05$)	0.110	Up-regulated		DNAJC16	1.14E-02 ($p < 0.05$)	0.063	Up-regulated
	HNRNPU	4.63E-14 ($p < 0.05$)	0.640	Up-regulated		YBX1	< 1E-12 ($p < 0.05$)	0.510	Up-regulated
	SKI	3.66E-05 ($p < 0.05$)	0.790	Up-regulated		NFIA	6.43E-09 ($p < 0.05$)	0.015	Down-regulated

miR-21	Gene name	Normal vs. Primary (p -value)	Survival (p -value)	Expression (tumor)	miR-375	Gene name	Normal vs. Primary (p -value)	Survival (p -value)	Expression (tumor)
	DNAJC16	1.14E-02 ($p < 0.05$)	0.063	Up-regulated		USP1	< 1E-12 ($p < 0.05$)	0.049	Up-regulated

Downregulation of miR-375 but upregulation of USP1 in NPC cells

To confirm the role of miR-375 and USP1 in NPC development, their expression profiles were assessed using the ONCOMINE data, and USP1 was found to be overexpressed in various human cancers (Fig. 4C), whereas miR-375 expression was shown to be reduced in these cancers, indicating that miR-375 may possess anti-tumor activity in NPC (Fig. 1D). Next, qRT-PCR analysis revealed that compared to the normal cell line, miR-375 expression was downregulated in NPC cell lines, whereas USP1 expression was up-regulated in NPC cell lines (Fig. 5). Figures 5C through F also show that expression of miR-375, USP1, and the pathway proteins in CNE1 cells were significantly different. Therefore, CNE1 cells were chosen for the following experiments.

miR-375 directly targets USP1 in NPC cells

To confirm USP1 as a target gene of miR-375, a series of experiments were conducted. Expression of *USP1* mRNA in CNE1 cells was remarkably upregulated, whereas miR-375 was downregulated, according to western blot and qRT-PCR experiments, respectively. Moreover, a dual-luciferase reporter assay was performed, and luciferase activity was markedly lower in CNE1 cells co-transfected with the wild-type USP1 and miR-375 mimics compared to the control group, indicating that miR-375 negatively regulated USP1 expression in NPC by directly binding to the 3'-UTR of USP1.

miR-375-mediated inhibition of NPC cell migration and invasion, and induction of NPC cell apoptosis in vitro

To assess the effects of changes in miR-375 expression in NPC cells, various assays were performed. The data revealed that transfection of miR-375 mimics into CNE1 cells induced miR-375 expression compared to NC cells. The Transwell invasion and wound healing assays showed that upregulation of miR-375 expression enhanced NPC cell migration and invasion (Fig. 7A). The western blot data showed that invasion-related proteins, such as N-cadherin, Snail and vimentin, were significantly decreased, but E-cadherin expression was upregulated following miR-375 expression in NPC cells (Fig. 7G). In contrast, NPC cell apoptosis was significantly increased after miR-375 mimic transfection compared to the NC and control groups (Fig. 7C). Western blot data showed that after transfection with miR-375 mimics, the level of cleaved caspase-3 (CCS-3), Bad, and Bax proteins was significantly increased, whereas the expression of c-Myc and Bcl-2 was significantly reduced (Fig. 7G).

USP1 promotion of NPC cell proliferation and invasion, and suppression of NPC cell apoptosis in vitro

Next, the role of USP1 in NPC was determined by overexpression of USP1 protein in CNE1 cells. Compared to the NC and control groups, USP1 protein was upregulated in CNE1 cells (Fig. 7). Next, the Transwell and wound healing assays revealed that USP1 overexpression enhanced the capacity of NPC cells to invade and migrate compared to the vector-only-transfected NC cells (Fig. 7B and H). Compared with the NC and control cells, the rate of apoptosis in CNE1 cells was significantly reduced after USP1 transfection (Fig. 7D). Western blot data showed that cell invasion-related proteins (N-cadherin, Snail, and vimentin), as well as apoptosis-related proteins (c-Myc and Bcl-2) were significantly increased. Additionally, E-cadherin expression was significantly decreased and the levels of CCS-3, Bad, and Bax proteins were significantly reduced (Fig. 7G).

Furthermore, a rescue assay was performed by co-transfection of miR-375 mimics with USP1 cDNA into CNE1 cells. Compared to the NC and control cells, the CCK-8 and wound-healing assays showed that the reversed regulation of USP1 could partially abolish the effect of miR-375 on tumor proliferation. Western blot analysis revealed that after USP1 overexpression, the levels of CCS-3, c-Myc, Bad, Bcl-2, and Bax were significantly reversed compared to cells that were only transfected with miR-375 mimics. Meanwhile, the levels of E-cadherin, N-cadherin, Snail, and vimentin were also significantly reversed compared to cells that were only transfected with miR-375 mimics. These experimental data demonstrated that USP1 overexpression could reverse the miR-375-mediated effects on NPC cells *in vitro*.

USP1 regulation of NPC cell viability and invasion by activation of the PI3K/AKT signaling

USP1 has been shown to de-ubiquitinate Akt and subsequently inhibit PI3K signaling in muscle during prolonged starvation[19]. Therefore, the effects of USP1 expression on regulation of the PI3K signaling were evaluated using a PI3K inhibitor (S2793). USP1 cDNA was first transfected into NCNE1 cells, which were then treated with or without S2793. Compared to the control group, USP1 overexpression significantly promoted tumor cell migration and invasion, but inhibited tumor cell apoptosis, whereas treatment with S2739 was able to reverse these events, even after USP1 overexpression (Fig. 9A-C). Western blot data also showed that the expression of USP1, Akt, c-Myc, and PI3K in cells with overexpressed USP1 was significantly higher compared to the control group, although S2793 treatment alone was not able to significantly reverse their expression in NPC cells (Fig. 9E). In contrast, the protein levels of PI3K and Akt were significantly reduced following S2793 treatment of USP1 cDNA-transfected cells, indicating that the activation of the PI3K/Akt pathway induced by USP1 was significantly inhibited by S2793 treatment. Overall, these data suggest that USP1 was able to upregulate PI3K/AKT signaling (Fig. 9D).

Discussion

In the current study, bioinformatical analysis was performed on DE-miRNAs in NPC tissues vs. normal mucosae, which led to the identification of potential miRNA target genes. There were numerous DE-miRNAs in NPC tissues and focus was placed on down-regulated miR-375 and up-regulated miR-21 in NPC cells. miR-375 regulates 78 central genes, while miR-21 regulates 168 central genes. Indeed, reduced miR-375 was previously shown to be associated with the development of hepatocellular carcinoma [20], hepatomas[21], oral squamous cell carcinoma [22], esophageal cancer[23], non-small cell lung cancer[24], and NPC tumorigenesis [3; 25]. Next, *in vitro* experiments were

performed to confirm the anti-NPC activity and the potential target gene USP1. miR-375 was found to play an essential role in NPC tumorigenesis by targeting USP1 expression and PI3K signaling.

Furthermore, the analysis using TargetScan and Starbase tools confirmed that USP1 is likely a target of miR-375. Experimental data confirmed that USP1 expression was reduced following miR-375 overexpression in NPC cells. Meanwhile, the luciferase reporter assay verified that miR-375 was able to bind to the *USP1* 3'-UTR. miR-375 overexpression downregulated USP1 expression and negatively regulated USP1-mediated NPC cell migration, invasion, and apoptosis *in vitro*. In addition, the rescue experiments by co-transfection of miR-375 mimics with USP1 cDNA into CNE1 cells confirmed that USP1 overexpression was able to reverse the effect of miR-375 on NPC cell migration, invasion, apoptosis, and viability.

Indeed, previous studies showed that different miRNAs could target USP proteins as a potential strategy in cancer therapy[26; 27; 28]. USP1, a member of the ubiquitin-specific processing (UBP) family of proteases that is a deubiquitinating enzyme (DUB) with His and Cys domains, is known to play a role in protein ubiquitination and is believed to be essential for maintenance of cellular protein levels, apoptosis, DNA repair, and cell motility [29; 30; 31; 32; 33; 34]. Importantly, the effects of ubiquitination lead to the suppression or progression of tumorigenic pathways in certain contexts of cell states[35]. For example, previous studies have shown that USP1 was an underlying target in hepatocellular carcinoma therapy [36; 37] and played a pivotal role in the development of non-small-cell lung cancer [38] and ovarian cancer[39]. Nevertheless, the underlying molecular progression by which USP1 suppressed NPC progression remains unknown[40]. In the current study, the analysis using data from the Oncomine Research Edition database[41] provided evidence showing that the level of USP1 mRNA was significantly upregulated in several tumor types[40], while the analysis of the data presented in this study indicates that USP1 may promote NPC development and progression (such as enhancing tumor cell migration and invasion).

In addition, the Akt pathway is known to be involved in cell survival and apoptosis [42; 43]; however, PI3K/Akt signaling was reported to be altered by miR-375[25]. Moreover, USP1 has been shown to induce tumor progression by regulation of Akt activity in lung cancer [19] and lymphoblastic leukemia [24]. In the current study, USP1 upregulation could influence the deubiquitination level of Akt, which in turn affects PI3K activity. When NPC cells were treated with S2793 to inhibit the PI3K pathway, the effects of USP1 overexpression in NPC cells were reversed. However, additional studies are needed to clarify their exact link and precise signaling in the regulation of NPC cell behaviors.

Indeed, the current study does have some limitations. For example, the influence of other non-coding RNAs (lncRNAs and circRNAs) on NPC tumorigenesis was not explored. Moreover, these data regarding miR-375 were not verified *in vivo*. The novelty of the current study involves discovering the complex mechanism of USP1 regulation by miR-375 and their interactions potentially affecting NPC development and metastasis. Meanwhile, it was further demonstrated that an interaction between miR-375 and USP1 affected PI3K-Akt signaling in NPC, which is also novel.

Conclusions

Numerous DE-miRNAs were found during NPC development. Among them, miR-375 was downregulated and miR-21 was upregulated. Downregulated miR-375 expression led to USP1 upregulation in NPC, which in turn activated PI3K/Akt signaling and promoted NPC cell migration and invasion, but inhibited NPC cell apoptosis. Thus, future study of induced miR-375 expression may represent a novel therapeutic approach for the treatment of NPC patients.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethical Review Committee of the Third Affiliated Hospital of Wenzhou Medical University (202010211603000390417)

Availability of data and materials

All data can generated or used during the study appear in the submitted article.

Consent for publication

Written informed consent for publication was obtained from all participants.

Conflict of interest statement

The authors declared that there is no conflict of interest in this work.

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Author contribution

Xu Jiayuan conceived and designed the experiments; Xu Jiayuan and Li Bangliang prepared the manuscript; Song Wei, Cao Longhe and Zhu Chansai performed the experiments; and Lin Sen analyzed the data.

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Figures

Figure 1

Identification of differentially expressed microRNAs (DE-miRNAs) using data from the Gene Expression Omnibus (GEO) database using NPC-DEM analysis. (A) Volcano plot of GEO Series (GSE) 36682 dataset. (B) Volcano plot of GSE 70970 dataset. The blue dots represent downregulated miRNAs, while the red dots represent upregulated miRNAs in nasopharyngeal carcinoma (NPC) vs. normal tissue samples. (C and D) Expression of miR-375 and miR-21 in tumor tissue vs. normal tissue. (E and F) Kaplan-Meier survival curves of NPC patients stratified by miR-375 and miR-21 expression.

Figure 2

The gene ontology (GO) terms and gene pathway enrichment analysis of the predicted miR-375 and miR-21 target genes. (A) The GO terms. The data show the enriched biological process, cellular component, and molecular function of the target genes. (B and C) Pathway enrichment analysis of the target genes. (D and E) MCODE network. Each MCODE network is assigned a unique color.

Figure 3

The protein-protein interaction (PPI) network. The data show the top 250 hub genes network and top 10 hub genes of miR-21 and miR-375 target genes (ranked by degree value). (A) PPI network of the target genes. (B) Hub genes identified in the PPI interaction (top 250). More important genes are represented by darker colors.

Figure 4

Ubiquitin-specific protease 1 (USP1) expression and association with cancer prognosis. (A and B) Level of USP1 expression in various cancers. (C-F) USP1 expression in head and neck squamous cell carcinoma (HNSC; samples, tumor grade, metastasis and gender). (G) Kaplan-Meier curves of HNSC patients stratified by USP1 expression.

Figure 5

Expression of miR-375 and ubiquitin-specific protease 1 (USP1) in nasopharyngeal carcinoma (NPC) tissues and cell lines. (A) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of miR-375 levels in 25 pairs of primary NPC and adjacent normal tissues. (B) qRT-PCR analysis of USP1 mRNA in 25 pairs of primary NPC and adjacent normal tissues. (C) qRT-PCR analysis of miR-375 levels in three NPC cell lines and normal RBE cells. (D) Western blot analysis of USP1 protein in cell lines. (E) Western blot analysis of various proteins. * $p < 0.05$ and ** $p < 0.01$.

Figure 6

miR-375 targets ubiquitin-specific protease 1 (USP1) expression. (A and B) Luciferase assay. The relative luciferase activity in CNE1 and HNE1 cells after co-transfection with wild type (wt) or mutant (mut) USP1 3'-untanslated region (UTR) reporter constructs together with miR-375 mimics and negative control (NC). (C) Bioinformatic prediction of the miR-375 binding sites to the USP1 3'-UTR. *p < 0.05 and **p < 0.01.

Figure 7

Effects of miR-375 and ubiquitin-specific protease 1 (USP1) expression in nasopharyngeal carcinoma (NPC) cells. (A) Transwell assay. miR-375 mimics inhibit NPC cell invasion. (B) Transwell assay. USP1 expression induces NPC cell invasion. (C) Flow cytometric assay. miR-375 mimics accelerated NPC cell apoptosis. (D) Flow cytometric assay. USP1 expression suppresses NPC cell apoptosis. (E) Cell viability cell counting kit-8 (CCK-8) assay. miR-375 reduced NPC cell viability. (F) Cell viability CCK-8 assay. USP1 promotes NPC cell viability. (G and H) Wound-healing assay. miR-375 inhibits NPC cell migration, whereas USP1 expression promotes NPC cell migration. (I) Western blot analysis of proteins in miR-375 mimics and negative control (NC) group-transfected NPC cells. The graphs are quantified data. *p < 0.05 and **p < 0.01.

Figure 8

Effects of ubiquitin-specific protease 1 (USP1) overexpression to evaluate miR-375-mediated effects on nasopharyngeal carcinoma (NPC) cells. (A) Flow cytometric assay. Transfection of USP1-OE into miR-375-transfected NPC cells inhibits NPC cell apoptosis. (B) Transwell assay. Transfection of USP1-OE into miR-375-transfected NPC cells promotes NPC cell invasion capacity. (C) Cell viability cell counting kit-8 (CCK-8) assay. Transfection of USP1-OE into miR-375-transfected NPC cells reverses the effect of miR-375 mimics on cell viability. (D) Wound-healing assay. Transfection of USP1-OE into miR-375-transfected NPC cells promotes NPC cell migration. (E) Western-blot. The graphs are quantified data. * $p < 0.05$ and ** $p < 0.01$.

Figure 9

Inhibition of phosphoinositide 3-kinase (PI3K)/Akt signaling by ubiquitin-specific protease 1 (USP1), a target of miR-375 in nasopharyngeal carcinoma (NPC) cells. (A) Flow cytometric assay. NPC cells were transfected with USP1 cDNA and then treated with or without S2739 and subjected to flow cytometric apoptosis assay. (B) Transwell assay. The duplicated cells were subjected to a Transwell invasion assay. (C) Transfection of USP1-OE reversed the effect of S2739 on cell growth. (D) Cell viability cell counting kit-8 (CCK-8) assay. The same treatment cells were subject to CCK-8 assay. (E) Western blot. NPC cells were transfected with USP1 cDNA and then treated with or without S2739 and subjected to western blot assay. The graphs were quantified data. *p < 0.05 and **p < 0.01.