MicroRNA-21 induces cisplatin resistance in head and neck squamous cell carcinoma

Shuyan Sheng1☯, Wenzhuo Su2☯, Deshen Mao1, Conghan Li1, Xinyang Hu1, Wanyu Deng1, Yong Yao3, Yongsheng Ji4* ☯ These authors contributed equally to this work.

1 First Clinical Medical College, Anhui Medical University, Hefei, P. R China, 2 Second Clinical Medical College, Anhui Medical University, Hefei, P. R China, 3 College of Life Sciences, Anhui Medical University, Hefei, P. R China, 4 Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, Anhui, P.R China

Abstract

Drug resistance, either intrinsic or acquired, can impair treatment effects and result in increased cell motility and death. MicroRNA-21 (miR-21), a proto-oncogene, may facilitate the development or maintenance of drug resistance in cancer cells. Restoring drug sensitivity can improve therapeutic strategies, a possibility that requires functional evaluation and mechanistic exploration. For miR-21 detection, matched tissue samples from 30 head and neck squamous cell carcinoma (HNSCC) patients and 8 head and neck cancer (HNC) cell lines were obtained. Reverse transcription-PCR to detect expression, MTT and clonogenic assays to evaluate cell proliferation, apoptosis assays, resazurin cell viability assays, western blot and luciferase reporter assays to detect protein expression, and flow cytometry to analyse the cell cycle were adopted. Compared to the corresponding normal control (NC) tissues, 25 cancer tissues had miR-21 upregulation among the 30 matched pair tissues (25/30, 83.8%); furthermore, among the 8 HNC cell lines, miR-21 expression that was notably upregulated in three: UPCI-4B, UMSCC-1, and UPCI-15B. In both the UMSCC-1 and UPCI-4B cell lines, the miR-21 mimic enhanced cell proliferation with reduced apoptosis and increased viability, whereas the miR-21 inhibitor resulted in the opposite effects (**P < 0.001**); additionally, miR-21 directly targeted the tumour suppressor phosphatase and tensin homologue (PTEN) and inhibited PTEN expression. Furthermore, the miR-21 mimic induced cisplatin resistance, while the miR-21 inhibitor restored cisplatin sensitivity. Overexpression of miR-21 can enhance cell proliferation, reduce apoptosis, and induce drug resistance by inhibiting PTEN expression. Targeting miR-21 may facilitate cancer diagnosis, restore drug sensitivity, and improve therapeutic effects.

Introduction

Head and neck cancer (HNC) ranks as the 6th most common malignancy worldwide, with over 650,000 new cases diagnosed and 330,000 deaths reported annually [1]. In the United
States, there are approximately 53,000 new cases annually and 10,800 deaths from the disease, accounting for 3% of deaths from all malignancies [2]. Aggravating risk factors most frequently related to this disease include tobacco product (cigarettes, cigars, pipes) smoking, alcohol consumption, betel nut chewing, human papillomavirus (HPV) infection (especially for oropharyngeal cancers), and Epstein-Barr virus (EBV) infection (especially for nasopharyngeal cancers in Asia) [3]. Chronic exposure of the upper aerodigestive tract to these carcinogenic factors leads to dysplastic or premalignant lesions in the oropharyngeal mucosa and ultimately results in HNC. The relative prevalence of these risk factors contributes to the variations in the observed distribution of HNCs in different regions of the world.

Despite the decrease in the overall incidence of HNC in the United States over the past 30 years, there has been a drastic increase in the incidence of head and neck squamous cell carcinoma (HNSCC) of the base of the tongue and tonsils, especially in young to middle-aged populations due to the rising incidence of HPV-associated HNSCC [4]. Although HNC treatments include surgery, radiotherapy, chemotherapy, targeted therapy, or a combination of treatments, drug resistance results in a low survival rate in locoregionally advanced HNSCC [5]. However, the resistance mechanism remains unclear.

MicroRNAs (miRNAs), a class of small, single-stranded, ~19–23 nt RNA molecules, play pivotal roles in modulating neoplastic processes in cancers, including HNC [6] by regulating pathogenesis by inhibiting target genes [7]. The expression patterns of miRNAs may become robust biomarkers for the diagnosis and prognosis of HNC. In addition, miRNA therapy could be a novel strategy for HNC prevention and therapy [8]. Therefore, understanding how miRNAs are involved in HNC pathogenesis will help validate potential clinical applications to target these entities. Previous study identified that miR-21 was dramatically upregulated in HNC tissues compared with the corresponding matched NC tissues [9]. Due to the capability of increasing cell proliferation and invasion and reducing apoptosis by targeting the 3’-UTR of the genes tropomyosin 1 (TPM1), phosphatase and tensin homolog (PTEN), cyclin dependent kinase 2 associated protein 1 (CDK2AP1), reversion inducing cysteine rich protein with kazal motifs (RECK), and Clusterin (CLU) [9–12], upregulation of miR-21 has been associated with resistance to the favoring HNSCC, ovarian cancer, oral squamous cell cancer, gastric malignancy and non-small cell lung cancer (NSCLC) development and patients’ poor prognosis [13]. However, the function of miR-21 in in drug resistance in HNSCC tissues and cell lines remains unclear.

The purpose of this study was to address the functional importance and molecular mechanisms of miR-21 in regulating HNC cell growth and proliferation and the association of this miRNA with drug resistance.

Materials and methods

Patients and samples

Paired HNC tissue samples from thirty patients were obtained from the First Affiliated Hospital of Anhui Medical University with informed consent and agreement, and the characteristics of those patients had been described in the Table 1. All specimens were snap frozen in liquid nitrogen immediately after surgical resection and stored at -80˚C until use. According to federal and institutional guidelines, written informed consent was obtained for each participant.

Reagents and antibodies

The RT-PCR primer pairs for miR-21 (AM30102) and 5S rRNA (AM30302) were procured from Ambion Inc. (Austin, TX). The miRNA-21 mimics (MCH01533) were obtained from Applied Biological Materials Inc. (Richmond, BC, USA). The miRNA-21 inhibitors
Table 1. Characteristics of the LC patients (n = 30).

| Characteristic                        | n (%) |
|---------------------------------------|-------|
| No. of patients                       | 30    |
| Age, y                                |       |
| Median                                | 69    |
| Range                                 | 50–86 |
| Sex                                   |       |
| Male                                  | 24 (80)|
| Female                                | 6 (20) |
| Tobacco smoking                       |       |
| Ever                                  | 26 (87)|
| Never                                 | 4 (13) |
| Primary tumor location                |       |
| Tongue base                           | 13 (43)|
| Larynx                                | 15 (50)|
| Hypopharynx                           | 2 (7)  |
| TNM stage                             |       |
| T category                            |       |
| 1–2                                   | 2 (7)  |
| 3–4                                   | 28 (93)|
| N category                            |       |
| 0                                     | 10 (33)|
| 1–3                                   | 20 (67)|
| M category                            |       |
| 0                                     | 29 (97)|
| 1                                     | 1 (3)  |
| Tumor stage                           |       |
| I-II                                  | 0 (0)  |
| III-IV                                | 30 (100)|
| Histological differentiation          |       |
| WD                                    | 12 (40)|
| Moderately differentiated             | 14 (47)|
| Poorly differentiated                 | 4 (13) |
| HPV status                            |       |
| HPV positivity                        | 2 (7)  |
| HPV negativity                        | 28 (93)|
| Treatment                             |       |
| Surgery only                          | 18 (60)|
| Surgery + adjuvant treatment *        | 12 (40)|
| *radiotherapy and/or concurrent chemotherapy |     |

(MH10206) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The rabbit polyclonal anti-PTEN antibody (9552) was obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture and transfection
The human HNC cell lines UMSCC-1, UMSCC-10A, UMSCC-22B, Cal33, UPCI-4B, UPCI-15B, 1483, and 686LN were authenticated and maintained as described previously [14]. Most of these cell lines were obtained from ATCC, and some were provided by Dr. Grandis.
The in vitro experiments in cancer cell lines were approved by the medical ethics committees of Anhui Medical University and the University of Pittsburgh School of Medicine. UMSCC-1 is a unique human HNSCC cell line, and UPCI-4B (SCC090) is a cell line derived from squamous cell carcinoma of the base of the tongue. The miRNA-21 mimics, inhibitors, and negative controls were introduced into cells by transfection using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA) according to the instructions of the manual as described previously [15]. Cells were incubated for 24 hours at 37°C in a humidified incubator containing 5% CO2 before testing and further experiments.

Reverse transcription-PCR

As previously described [16], total RNA was extracted using a mirVana miRNA Isolation Kit (AM1560, Ambion Inc., Austin, TX). We used 15% denaturing polyacrylamide gel electrophoresis and spectrophotometry (Eppendorf BioPhotometer, Eppendorf, Hamburg, Germany) to assess the integrity of the extracted RNA. The absorbance at 260 nm (A260) was used to determine the RNA concentration, and the A260/A280 ratio was used to indicate the RNA purity. Moreover, the A260/A280 ratio was used to indicate the RNA purity. Reverse transcription-PCR (RT-PCR) was performed according to the instructions of the mirVana qRT-PCR miRNA Detection Kit (1558, Ambion, Austin, TX). The constructed primers of miR-21 and PTEN were shown in Table 2.

| Primer   | forward                        | reverse                        |
|----------|--------------------------------|--------------------------------|
| hu miR-21| 5’-GCCAGGCATAGCTTATCAGACTG-3’  | 5’-CCACTGTCTAGACGACACTAA-3’   |
| hu PTEN  | 5’-AAAGGGAGCAACTGTGTAATG-3’   | 5’-TGGTCCTTTATTTCCCATAGAA-3’  |
| hu β-actin| 5’-GCAAGGCGAGAGGATCCTC-3’     | 5’-TCGCCCCAGTTGGTACGAT-3’     |
| hu 5sRNA | 5’-GTCTACGCGCATACCACCTG-3’    | 5’-AAAGCCTACAGCAGCCGAT-3’     |

Cell proliferation assay

Cells transiently transfected with the miR-21 mimic, inhibitor, and negative controls were digested with trypsin and inoculated in 96-well plates at a concentration of 1×10^4 cells/well after counting. Cell proliferation was monitored by an MTS assay using a CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay Kit (G5421, Promega, Madison, WI).

Clonogenic assay

Cells transiently transfected with the miR-21 mimic, inhibitor, and negative controls were digested with trypsin and plated in 10-cm dishes at a concentration of 500 cells/well after counting. The experiments were performed as previously described [17].

Flow cytometry assay

Flow cytometry assays were performed with BrdU and propidium iodide (PI) double staining (MP Biomedicals, Inc., Santa Ana, CA), as described previously [18]. Cisplatin was added at 5 ng/ml to 1×10^6 cancer cells seeded in 10-cm dishes, and cells were harvested for flow cytometric detection after 24 hours of culture.
Apoptosis detection assay

Cells transiently transfected with the miR-21 mimic, inhibitor, and negative controls were digested with trypsin and plated in 96-well plates at a concentration of $1 \times 10^5$ cells/well after counting. A Cell Apoptosis PI Detection Kit (Cat. # L00311, Genscript, Piscataway, NJ) was used to evaluate apoptosis according to the instructions of the manual. Briefly, cells were incubated at 37˚C in a humidified incubator containing 5% CO$_2$. After incubation for the designated durations, cells were harvested by centrifugation at 2,000 rpm for 5 minutes (min). Then, the cells were resuspended and adjusted to $1 \times 10^6$ cells/ml after washing. Five microlitres of PI was added to 95 μl of the cell suspension prepared as described above, and the cells were incubated in the dark at room temperature for 5 min. After incubation, fluorescence was recorded using a H1 Synergy Hybrid Reader (BioTek, Winooski, VT). Experiments were carried out in quadruplicate, and the data were processed with GraphPad Prism. The results are shown as the mean±SD of three independent experiments. We adopted relative fluorescence units (RFUs) to express fluorescence values, as previously described [19].

Western blotting

Cells transiently transfected with the miR-21 mimic, inhibitor, and negative controls were collected, and total cell lysates were prepared. After quantitation using a Bicinchoninic Acid Protein Assay Kit (Pierce, Rockford, IL), equal amounts of protein samples which was incubated with the primary antibody at 1: 2000 dilution in PBST, were subjected to western blotting for the measurement of PTEN expression levels.

Luciferase reporter assay

pGL3-PTEN-3'UTR vectors, which contained the putative binding site for miR-21 downstream of the stop codon in the pGL3 firefly luciferase reporter, were constructed. UMSCC-1 and UPCI-4B cells were plated at $1 \times 10^6$ cells/well, and transfection was performed with one microgram of the pGL3-PTEN-3'UTR vector and one microgram of the URL-TK Renilla luciferase expression vector (Promega, Beijing). Luciferase assays were performed 48 hours after transfection using a dual-luciferase reporter assay system (Promega, Beijing). Firefly luciferase activity was normalized to Renilla luciferase activity.

Database selection and miRNA target prediction

During the process of identification, bioinformatic predictions were performed according to the mature miRNA sequence (5'-uagcuuacagacaguguaguu-3') using TargetScan Release 7.2 (http://www.targetscan.org/vert_72/).

Statistical analysis

Results are shown as the mean ± SD values. Student’s $t$ test was used to evaluate comparisons unless another test is specified. (For all analyses, statistical significance was set at $P<0.05$, and all tests were two-sided. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$; ns, nonsignificant.)

Results

Prevalence of miR-21 overexpression in HNSCC tissues and cell lines

The discovery of tumour-specific miRNA expression profiles with widespread dysregulation and differential expression of miRNA molecules can enhance the understanding of the diverse
characteristics of cancers and their underlying mechanisms. In our previous study on miRNA expression profiles, based on matched cancer and adjacent NC tissues from five HNSCC patients, a total of 471 miRNA transcripts were identified (S1 Fig; S1 Table). Among these miRNAs, miR-21 was one of the most abundantly expressed miRNAs in tumour tissues compared to their NC counterparts. However, the underlying mechanisms of miR-21 remain unclear.

In this study, we assessed the expression levels of miRNA-21 in matched tissues from 30 different individuals with HNSCC, among which 25 cancer tissues had miR-21 upregulation compared to the corresponding matched adjacent NC tissues, respectively. \( P < 0.0001 \). Among 8 head and neck SCC cell lines, miR-21 was significantly overexpressed in UMSCC-1, UPCI-4B, and UPCI-15B cells. CT, cancer tissue; NC, normal control, referring to the adjacent normal tissue. \(^{***}, P < 0.0001\).

**MiR-21 enhances cell proliferation and reduces cell apoptosis**

Since miR-21 upregulation in human head and neck SCC tissues and cell lines is a prevalent phenomenon, particularly in the cell lines UMSCC-1 and UPCI-4B, we both overexpressed and downregulated miR-21 and tested UMSCC-1 and UPCI-4B cell proliferation.

Upon cell proliferation assessment, the MTT assay showed that the miR-21 mimic significantly enhanced cancer cell growth and proliferation, while the miR-21 inhibitor decreased cell growth and proliferation in both the UMSCC-1 and UPCI-4B cell lines (Fig 2C and 2D). Furthermore, we detected miR-21 expression in 8 HNC cell lines and found that it was notably upregulated in UPCI-4B, UMSCC-1, and UPCI-15B cells (Fig 1C). These results demonstrated that miR-21 overexpression was prevalent among HNSCC tissues and HNSCC cell lines.

In the apoptosis detection assay, PI staining showed that the miR-21 mimic significantly reduced apoptosis, while the miR-21 inhibitor significantly increased apoptosis in both the
miR-21 can promote cell cycle progression and induce cisplatin resistance

Since miR-21 had a strong promotive effect on cell growth and proliferation, we adopted a flow cytometry assay to test the impact of this miRNA on the cell cycle distribution via BrdU and PI co-staining. In both the UMSCC-1 and UPCI-4B cell lines, the miR-21 mimic enhanced cell entry into S (synthesis) phase and reduced cisplatin sensitivity, while the miR-21 inhibitor arrested cells in G2 and M phases and enhanced cisplatin sensitivity (Fig 5A, 5B and 5C, S2 and S3 Figs). This result implied that miR-21 can promote cell cycle progression to S phase and induce cisplatin resistance.

miR-21 can directly target the 3’-UTR of PTEN and suppress PTEN expression

To investigate the underlying mechanism by which miR-21 enhances cell proliferation, reduces apoptosis, and induces cisplatin resistance, we adopted bioinformatic analysis to screen potential candidates. The screened candidates, as the targets of miR-21 and miR-21*, included a large number of oncogenic proteins and tumour suppressors, such as Ras, GRHL3, CHL1, PDCD4, PTEN, RECK and HNRPK (S2 Table).

Structural analysis indicated that miR-21 can directly target the 3’-UTR of PTEN with the lowest free energy and that its binding to this site in PTEN may inhibit PTEN transcription (Fig 6A). We further assessed the protein level of PTEN with an immunoblot assay and found that the miR-21 mimic significantly inhibited PTEN expression, while the miR-21 inhibitor exerted the opposite effects in both cell lines (Fig 6C and 6E). In addition, the luciferase activity...
Fig 3. The clonogenic assay showed that miR-21 can enhance cell proliferation. (A) and (B) The miR-21 transfection efficiency was assessed by PCR in UMSCC-1 and UPCI-4B cells, respectively. (C) and (D) Clonogenic assays were performed to examine the effects of miR-21 in both cell lines. *, P < 0.05; **, P < 0.01; ***, P < 0.001. ns, nonsignificant.

https://doi.org/10.1371/journal.pone.0267017.g003

Fig 4. miR-21 can reduce apoptosis and enhance cell viability. (A) and (B) Apoptosis evaluation with Cell Apoptosis PI Detection assays showed that the miR-21 mimic significantly decreased apoptosis, while the miR-21 inhibitor increased cell apoptosis in both the UMSCC-1 and UPCI-4B cell lines, respectively. (C) and (D) Cell viability assessment with resazurin cell viability assays showed that the miR-21 mimic group had significantly more viable cells than the miR-21 inhibitor group in both the UMSCC-1 and UPCI-4B cell lines. **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

https://doi.org/10.1371/journal.pone.0267017.g004
assay with the pGL3-PTEN-3’-UTR plasmid validated miR-21’s suppressive effect on PTEN expression (Fig 6F and 6G).

**Discussion**

Numerous studies have demonstrated that miRNAs participate in tumorigenesis and that during this process, miRNAs play dual roles in either promoting or inhibiting tumour progression [20]. Based on sequence complementarity, these regulatory miRNAs act as guides to recognize specific mRNA sequences, resulting in site-specific cleavage or translational inhibition [21]. Among these miRNAs, miR-21 is a proto-oncogene, and its upregulation is often associated with ineffective treatment and unfavourable prognosis [22]. However, in HNC, the mechanisms of miR-21 remain unclear.

Cellular transcription driven by independent promoter elements can directly regulate miR-21 expression; however, members of the transforming growth factor β (TGF-β) family can also manipulate this regulation. Genome mapping and decoding indicate that pre-miR-21 is located on chromosome 17q23.2 [20]. Although pre-miR-21’s chromosomal location overlaps with that of TMEM49, pre-miR-21 has distinct promoter regions that contain specific binding
sequences for transcriptional activators (activator protein 1, AP1) and suppressors (nuclear factor I, NFI). These binding structures indicate that miR-21 has independent promoters [23]. As an additional supplemental regulator, TGF-β can upregulate pre-miR-21 expression, while BMP6 (a member of the TGF-β family) can downregulate miR-21 expression [24]. These contrasting functions suggest that the members of the TGF-β family may establish an equilibrium state, which is disrupted within the tumour microenvironment because tumour cells can excessively express autocrine TGF-β1 [25, 26], which can lead to further production of an abundance of miR-21 and generate a feed-forward loop in cancer progression.

Upregulation of miR-21 can promote tumour progression because the majority of targets of miR-21 are tumour-suppressing genes. Studies of miR-21 upregulation and its tumour-promoting roles have been reported in various cancers, for example, in lung cancer [27], gastrointestinal cancer [28], colorectal cancer [29], breast cancer [30], glioblastoma [31], oral cancer, and HNSCC [32, 33]. Moreover, the target genes of miR-21 are generally PDCD4, RECK, p53 and PTEN, as well as their associated signalling pathways [33]. In the study of The Cancer Genome Atlas Network for HNSCC, the percentages (%) of PTEN genetic alterations in HPV (+) and HPV (-) tumours were 12% and 6%, respectively [34], and either PTEN mutation or inhibition could result in PIK3CA/CCND1/CDK6 pathway activation and thereby enhance cell proliferation [34]. In conclusion, these target genes and pathways perform tumour-suppressing functions, and their functional loss may result in tumour progression and distant metastasis. Consistent with previous studies, our results validated the upregulation of miR-21 and its tumour-promoting role in both the UMSCC-1 and UPCI-4B cell lines.

Inhibited PTEN expression resulted in the cisplatin resistance in HNSCC and recovery the PTEN function had high potential for better functional preservation and survival improvement. Previous studies had revealed that miR-21 could directly bind PTEN mRNA and thereby disrupt PTEN mRNA stability and inhibit PTEN protein expression. Specifically, PTEN is a lipid phosphatase that antagonizes phosphatidylinositol 3 kinase (PI3K) signaling by

![Fig 6. miR-21 can directly target the 3'-UTR of PTEN and suppress PTEN expression.](https://doi.org/10.1371/journal.pone.0267017.g006)
converting phosphatidylinositol trisphosphate (PIP3) to phosphatidylinositol bisphosphate (PIP2). This converting suppressed PIP3-dependent kinases (i.e., AKT, PDK1) that enhanced cell growth, protein synthesis, and cell cycling and migration and thereby inhibited tumor progression [35]. Importantly, cisplatin is widely used in cancer chemotherapy since its cellular toxicity function with DNA damage and inhibition of DNA synthesis, which further activates tyrosine kinase and AKT/PI3K signaling, while AKT/PI3K signaling can enhance cellular proliferation and cisplatin resistance [36]. Therefore, PTEN can inhibit AKT/PI3K signaling and enlarge the effects of cisplatin chemotherapy, while loss of PTEN resulted in chemotherapy resistance in various tumors [37]. Our finding had validated miR-21 can inhibit PTEN, and targeting miR-21 may have the potential to improve the effects of cisplatin chemotherapy.

MiR-21 overexpression can induce drug resistance, and this resistance mechanism, based primarily on miR-21’s target genes and pathways, has potential therapeutic relevance. In breast cancer, miR-21 can decrease PTEN and PDCD4 expression, whereas treatment with a miR-21 inhibitor combined with trastuzumab [38] or doxorubicin [39] can restore drug sensitivity. Similarly, in leukaemia, miR-21 upregulation leads to daunorubicin (DNR) resistance via PTEN suppression [40]. Moreover, miR-21 in glioblastoma cells can target LRRFIP1 and thereby facilitate NFκB pathway activation [41], while silencing miR-21 can strengthen the anti-tumour effects of sunitinib (a tyrosine kinase inhibitor) in U87 human glioblastoma cells [42]. Similar to these previous findings, our study on HNSCC cancer cell lines established that miR-21 overexpression can induce chemotherapeutic resistance to cisplatin via PTEN suppression, while miR-21 inhibition can restore drug sensitivity. In laryngeal cancer, concurrent chemo- and radiotherapy help to preserve laryngeal function [43], and restoration of drug sensitivity may contribute to reducing the motility of HNSCC cells.

Many studies have focused on tumour-associated miRNAs and their roles in carcinogenesis; however, quantitative studies of miR-21 inhibitors, from cellular mechanistic studies in vitro and tumour-bearing mouse models in vivo to clinical trials with full evaluation, have rarely been reported. Furthermore, miR-21 is essential for effective CD8+ T cell activation [44] and type 1 macrophage (M1) polarization and maintenance [45], which plays a crucial role in antitumour immunity. Similar to the side effects of chemotherapy on the immune system, miR-21 inhibitors may also inhibit these tumour-killing immune cells while suppressing tumour progression. Therefore, further observation of the effects of miR-21 inhibitors on tumour-infiltrating lymphocytes (TILs) in vitro and in vivo is suggested.

**Conclusion**

In our studies, we confirmed that miR-21 overexpression is prevalent across HNSCC tissues and cell lines, and we further identified that miR-21 can enhance cell proliferation, reduce apoptosis and induce cisplatin resistance by inhibiting PTEN expression. Taken together, these findings indicate that miR-21 performs crucial functions in HNSCC cell proliferation and cisplatin resistance, while targeting miR-21 may facilitate the discovery of drugs with greater effectiveness and fewer side effects than current drugs, which may further improve the therapeutic strategies for HNSCC.

**Supporting information**

**S1 Fig.** Representative chip images demonstrate regions of miRNA transcripts. Five patient-matched snap-frozen head and neck tissue samples were employed for expression analysis of microRNAs by LC Sciences (http://www.lcsciences.com/; Houston, TX). Each chip included multiple control probes and 471 miRNA transcripts as listed in Sanger miRBase Release 9.2 (http://www.sanger.ac.uk/Software/Rfam/mirna/). T, tumor tissue; C, patient-
matched adjacent normal control tissue. When Cy3 level was higher than Cy5 level, the color is green; when Cy3 level is equal to Cy5 level, the color is yellow; and when Cy5 level is higher than Cy3 level the color is red.

(TIF)

S2 Fig. Flow cytometry analysis demonstrates that miR-21 can enhance cisplatin resistance (three independent experiments).

(TIF)

S3 Fig. The gating strategy divides cell cycling into three phases.

(TIF)

S1 Table. Differentially expressed miRNAs in five patient-matched samples.

(DOCX)

S2 Table. Predicted protein targets of miR-21 in human by bioinformatics analysis.

(DOCX)

S1 Raw images.

(PDF)

Acknowledgments

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. All figures submitted have been created by the authors who confirm that the images are original with no duplication and have not been previously published in whole or in part. The eight authors worked together to complete the paper. Yongsheng Ji, as the total corresponding author, guided the design of the entire experiment and the writing of the paper. Shuyan Sheng, and Wenzhuo Su are responsible for the collection of specimens, the writing of papers, design of the experiment, and other scattered work, they contributed equally to this work as first authors. Conghan Li and Deshen Mao were responsible for the collection of data. Wanyu Deng, Yong Yao and Xinyang Hu are responsible for statistical analysis of the data. All the authors agreed to publish the article.

Author Contributions

Conceptualization: Yongsheng Ji.

Data curation: Shuyan Sheng, Xinyang Hu, Wanyu Deng, Yong Yao.

Formal analysis: Wenzhuo Su, Yong Yao.

Funding acquisition: Yongsheng Ji.

Investigation: Wenzhuo Su, Deshen Mao, Conghan Li.

Methodology: Shuyan Sheng, Deshen Mao, Conghan Li.

Software: Xinyang Hu, Wanyu Deng.

Supervision: Yongsheng Ji.

Validation: Deshen Mao.

Writing – original draft: Shuyan Sheng, Wenzhuo Su, Yongsheng Ji.

Writing – review & editing: Deshen Mao, Conghan Li, Xinyang Hu, Yong Yao, Yongsheng Ji.
References

1. Vigneswaran N, Williams M. Epidemiologic trends in head and neck cancer and aids in diagnosis. Oral and maxillofacial surgery clinics of North America. 2014; 26(2):123–41. https://doi.org/10.1016/j.coms.2014.01.001 PMID: 24794262.

2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA Cancer J Clin. 2019; 69(1):7–34. Epub 2019/01/09. https://doi.org/10.3322/caac.21551 PMID: 30620402.

3. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin. 2005; 55(2):74–108. Epub 2005/03/12. https://doi.org/10.3322/canjclin.55.2.74 PMID: 15761078.

4. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011; 61(2):69–90. Epub 2011/02/08. https://doi.org/10.3322/caac.20107 PMID: 21296855.

5. Posner MR, Hershock DM, Blajman CR, Mickiewicz E, Winquist E, Gorbounova V, et al. Cisplatin and fluorouracil alone or with docetaxel in head and neck cancer. N Engl J Med. 2007; 357(17):1705–15. Epub 2007/10/26. https://doi.org/10.1056/NEJMoa070956 PMID: 17960013.

6. Sankaranarayanan R, Masuyer E, Swaminathan R, Ferlay J, Whelan S. Head and neck cancer: a global perspective on epidemiology and prognosis. Anticancer Res. 1998; 18(6b):4779–86. Epub 1999/01/19. PMID: 9891557.

7. Curado MP, Hashibe M. Recent changes in the epidemiology of head and neck cancer. Curr Opin Oncol. 2009; 21(3):194–200. Epub 2009/04/14. https://doi.org/10.1097/CCO.0b013e32832a8ca PMID: 1936341.

8. Shiboski CH, Schmidt BL, Jordan RC. Tongue and tonsil carcinoma: increasing trends in the U.S. population ages 20–44 years. Cancer. 2005; 103(9):1843–9. Epub 2005/03/18. https://doi.org/10.1002/cncr.20998 PMID: 15772957.

9. Li J, Huang H, Sun L, Yang M, Pan C, Chen W, et al. MiR-21 indicates poor prognosis in tongue squamous cell carcinomas as an apoptosis inhibitor. Clin Cancer Res. 2009; 15(12):3998–4008. Epub 20090609. https://doi.org/10.1158/1078-0432.CCR-08-3053 PMID: 19509158.

10. Jung HM, Phillips BL, Patel RS, Cohen DM, Jakymiw A, Kong WW, et al. Keratinization-associated miR-7 and miR-21 regulate tumor suppressor reversion-inducing cysteine-rich protein with kazal motifs (RECK) in oral cancer. J Biol Chem. 2012; 287(35):29261–72. Epub 20120702. https://doi.org/10.1074/jbc.M112.366518 PMID: 22761427; PubMed Central PMCID: PMC3436145.

11. Mydlarz W, Uemura M, Ahn S, Hennessy P, Chang S, Demokan S, et al. Clusterin is a gene-specific target of microRNA-21 in head and neck squamous cell carcinoma. Clin Cancer Res. 2014; 20(4):868–77. Epub 20131210. https://doi.org/10.1158/1078-0432.CCR-13-2675 PMID: 24327270; PubMed Central PMCID: PMC3970211.

12. Zheng J, Xue H, Wang T, Jiang Y, Liu B, Li J, et al. miR-21 downregulates the tumor suppressor P12CDK2AP1 and stimulates cell proliferation and invasion. J Cell Biochem. 2011; 112(3):872–80. https://doi.org/10.1002/jcb.22995 PMID: 21328460.

13. Taheri M, Shoorei H, Tondro Anamag F, Ghafouri-Fard S, Dinger ME. LncRNAs and miRNAs participate in determination of sensitivity of cancer cells to cisplatin. Exp Mol Pathol. 2021; 123:104602. Epub 20210108. https://doi.org/10.1016/j.yexmp.2021.104602 PMID: 34322487.

14. Cramm M, Schmitz M, Karch A, Mitrova E, Kuhn F, Schroeder B, et al. Stability and Reproducibility Underscore Utility of RT-QuIC for Diagnosis of Creutzfeldt-Jakob Disease. Mol Neurobiol. 2016; 53(3):1896–904. Epub 2015/04/01. https://doi.org/10.1007/s12035-015-9133-2 PMID: 25823511; PubMed Central PMCID: PMC4789202.
MicroRNA-21 induces cisplatin resistance in head and neck squamous cell carcinoma

20. Kumarswamy R, Volkman I, Thum T. Regulation and function of miRNA-21 in health and disease. RNA Biol. 2011; 8(5):706–13. Epub 2011/06/30. https://doi.org/10.4161/rna.8.5.16154 PMID: 21712654; PubMed Central PMCID: PMC3256347.

21. Franco-Zorrilla JM, Valli A, Todesco M, Mateos I, Puga MI, Rubio-Somoza I, et al. Target mimicry provides a new mechanism for regulation of microRNA activity. Nat Genet. 2007; 39(8):1033–7. Epub 2007/07/24. https://doi.org/10.1038/ng2079 PMID: 17643101.

22. Huang Y, Yang YB, Zhang XH, Yu XL, Wang ZB, Cheng XC. MicroRNA-21 gene and cancer. Med Oncol. 2013; 30(1):376. Epub 2013/01/02. https://doi.org/10.1007/s12032-012-0376-8 PMID: 23277281.

23. Cai X, Hagedorn CH, Cullen BR. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. Rna. 2004; 10(12):1957–66. Epub 2004/11/05. https://doi.org/10.1261/rna.7135204 PMID: 15525708; PubMed Central PMCID: PMC1370684.

24. Du J, Yang S, An D, Hu F, Yuan W, Zhai C, et al. BMP-6 inhibits microRNA-21 expression in breast cancer. J Mol Med. 2014; 92(4):453–62. Epub 2014/03/11. https://doi.org/10.1007/s00109-014-1402-1 PMID: 24605967; PubMed Central PMCID: PMC4016602.

25. Toiyama Y, Takahashi M, Hur K, Nagasaki T, Tanaka K, Inoue Y, et al. Serum miR-21 as a diagnostic and prognostic biomarker in colorectal cancer. J Natl Cancer Inst. 2013; 105(12):849–59. Epub 2013/05/25. https://doi.org/10.1093/jnci/djt101 PMID: 23704278; PubMed Central PMCID: PMC3687369.

26. Asangani IA, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S, et al. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene. 2008; 27(15):2128–36. Epub 2007/10/31. https://doi.org/10.1038/sj.onc.1210856 PMID: 17968323.

27. Frankel LB, Christoffersen NR, Jacobsen A, Lindow M, Krogh A, Lund AH. Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. J Biol Chem. 2008; 283(2):1026–33. Epub 2007/11/10. https://doi.org/10.1074/jbc.M707224200 PMID: 17991735.

28. Gaul AB, Holbeck SL, Colburn NH, Israel MA. Downregulation of Pdcd4 by miR-21 facilitates glioblastoma proliferation in vivo. Neuro Oncol. 2011; 13(6):580–90. Epub 2011/06/04. https://doi.org/10.1093/neuonc/noq133 PMID: 21636706; PubMed Central PMCID: PMC3107097.

29. Kawakita A, Yanamoto S, Yamada S, Naruse T, Takahashi H, Kawasaki G, et al. MicroRNA-21 promotes oral cancer invasion via the Wnt/β-catenin pathway by targeting DKK2. Pathol Oncol Res. 2014; 20(2):253–61. Epub 2013/09/04. https://doi.org/10.1007/s12253-013-9689-y PMID: 23999978.

30. John K, Wu J, Lee B-W, Farah CS. MicroRNAs in head and neck cancer. International journal of dentistry. 2013;2013. https://doi.org/10.1155/2013/650218 PMID: 24260035

31. CGA N. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature. 2015; 517(7536):576–82. Epub 2015/01/30. https://doi.org/10.1038/nature14125 PMID: 25631445; PubMed Central PMCID: PMC4311405.

32. Hopkins BD, Hodakoski C, Barrows D, Mense SM, Parsons RE, PTEN function: the long and the short of it. Trends Biochem Sci. 2014; 39(4):183–90. Epub 2014/03/25. https://doi.org/10.1016/j.tibs.2014.02.006 PMID: 24656806; PubMed Central PMCID: PMC4043120.

33. Basu A, Krishnamurthy S. Cellular responses to Cisplatin-induced DNA damage. J Nucleic Acids. 2010;2010. Epub 2010/09/03. https://doi.org/10.4061/2010/2010367 PMID: 20811617; PubMed Central PMCID: PMC2929606.

34. Lee S, Choi EJ, Jin C, Kim DH. Activation of PI3K/Akt pathway by PTEN reduction and PIK3CA mRNA amplification contributes to cisplatin resistance in an ovarian cancer cell line. Gynecol Oncol. 2005; 97(1):26–34. Epub 2005/03/26. https://doi.org/10.1016/j.ygyno.2004.11.051 PMID: 15790433.

35. Gong C, Yao Y, Wang Y, Liu B, Wu W, Chen J, et al. Up-regulation of miR-21 mediates resistance to trastuzumab therapy for breast cancer. J Biol Chem. 2011; 286(21):19127–37. Epub 2011/04/08. https://doi.org/10.1074/jbc.M110.216887 PMID: 21471222; PubMed Central PMCID: PMC3099726.
39. Wang ZX, Lu BB, Wang H, Cheng ZX, Yin YM. MicroRNA-21 modulates chemosensitivity of breast cancer cells to doxorubicin by targeting PTEN. Arch Med Res. 2011; 42(4):281–90. Epub 2011/08/09. https://doi.org/10.1016/j.arcmed.2011.06.008 PMID: 21820606.

40. Bai H, Xu R, Cao Z, Wei D, Wang C. Involvement of miR-21 in resistance to daunorubicin by regulating PTEN expression in the leukaemia K562 cell line. FEBS Lett. 2011; 585(2):402–8. Epub 2010/12/29. https://doi.org/10.1016/j.febslet.2010.12.027 PMID: 21187093.

41. Li Y, Li W, Yang Y, Lu Y, He C, Hu G, et al. MicroRNA-21 targets LRRFIP1 and contributes to VM-26 resistance in glioblastoma multiforme. Brain Res. 2009; 1286:13–8. Epub 20090624. https://doi.org/10.1016/j.brainres.2009.06.053 PMID: 19559015.

42. Costa PM, Cardoso AL, Nóbrega C, Pereira de Almeida LF, Bruce JN, Canoll P, et al. MicroRNA-21 silencing enhances the cytotoxic effect of the antiangiogenic drug sunitinib in glioblastoma. Hum Mol Genet. 2013; 22(5):904–18. Epub 2012/12/04. https://doi.org/10.1093/hmg/dds496 PMID: 23201752; PubMed Central PMCID: PMC3561912.

43. Bhattacharyya T, Kainickal CT. Current Status of Organ Preservation in Carcinoma Larynx. World J Oncol. 2018; 9(2):39–45. Epub 2018/05/16. https://doi.org/10.14740/wjon1105w PMID: 29760831; PubMed Central PMCID: PMC5942206.

44. Carissimi C, Carucci N, Colombo T, Piconese S, Azzalin G, Cipolletta E, et al. miR-21 is a negative modulator of T-cell activation. Biochimie. 2014; 107 Pt B:319–26. Epub 2014/10/12. https://doi.org/10.1016/j.biochi.2014.09.021 PMID: 25304039.

45. Wang Z, Brandt S, Medeiros A, Wang S, Wu H, Dent A, et al. MicroRNA 21 is a homeostatic regulator of macrophage polarization and prevents prostaglandin E2-mediated M2 generation. PLoS One. 2015; 10(2):e0115855. Epub 2015/02/24. https://doi.org/10.1371/journal.pone.0115855 PMID: 25706647; PubMed Central PMCID: PMC4338261.