miR-211-3p enhances induction chemotherapy insensitivity by upregulating CSF2/CCL20/TNF signaling in hypopharyngeal squamous cell carcinoma

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Research Article

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Abstract

Purpose To investigate the potential mechanisms of miR-211-3p on induction chemotherapy (IC) sensitivity in hypopharyngeal squamous cell carcinoma (HSCC).

Methods qRT-PCR was assessed to compare the miR-211-3p expression between IC sensitive and insensitive tumor tissues. The MTT assay was performed to analyze cell proliferation and viability to paclitaxel after alteration of miR-211-3p. Flow cytometry assay was conducted to explore cell apoptosis. Transwell assay was used to explore the effect of miR-211-3p on cell migration. Transcriptome sequencing was then performed to select differentially expressed genes (DEGs) after over-expression of miR-211-3p. GO and KEGG enrichment analyses were conducted to annotate DEGs. PPI analysis was conducted to screen candidate genes. The differential expression and survival status of candidate genes were further validated in TCGA-HNSCC data. The single sample GSEA method was used to investigate the association between downstream genes and immune cell infiltration.

Results MiR-211-3p was up-regulated in IC insensitive larynx-hypopharyngeal tumor tissues. Over-expression of miR-211-3p promoted cell proliferation and migration, and inhibited apoptosis.

The IC50 value of miR-211-3p overexpression (OE) group was significantly higher than negative control (NC) group treated with paclitaxel, suggesting miR-211-3p enhanced IC insensitivity in HSCC. We found 778 DEG after over-expression of miR-211-3p and 11 significant genes were then identified. Finally, CSF2 and CCL20 were validated to be significantly high expressed and associated with poorer overall survival in head and neck squamous cell carcinoma, which were involved in TNF signaling pathway and then regulated immune cell infiltration.

Conclusion The miR-211-3p could promote HSCC progression and upregulates CSF2/CCL20/TNF signaling to promote IC insensitivity in HSCC, which may provide new ideas for HSCC therapy.

Introduction

Head and neck cancer is the sixth most common malignant tumor worldwide in 2018, with 890,000 new cases and 450,000 death cases [1]. Hypopharyngeal squamous cell carcinoma (HSCC) is one of the classical head and neck squamous cell carcinoma (HNSCC), which is highly aggressive and invasive, prone to local recurrence and metastasis. The 5-year overall survival rate of HSCC patients is approximately 40% [2]. About 60% of patients presented with advanced (stage III or IV) disease at clinical diagnosis [3], which would greatly affect patients' quality of life after the treatment. In recent years, induction chemotherapy (IC) followed by surgery and/or concurrent chemoradiotherapy (CCR) is a strong option for inoperable diseases. The application of IC can effectively decrease the tumor volume, increase larynx preservation rates and overall survival rates, yet, some hypopharyngeal cancer patients exhibited no response to IC [4].

MicroRNAs (miRNAs) are small endogenous RNAs (19-25 nucleotides long) that regulate gene-expression post-transcriptionally [5]. The abnormal expression of some miRNAs can alter tumor cells chemosensitivity and reverse multi-drug resistance. For instance, the miR-155/TP53 negative feedback loop is involved in resistance to multiple chemotherapeutic drugs used in lung cancer [6]. MiR-10b-mediated PPARγ inhibition enhances cisplatin resistance by activating AKT/mTOR/P70S6K signaling in esophageal cancer [7]. MiR-621 enhances the sensitivity to paclitaxel plus carboplatin chemotherapy by suppressing FBXO11 and enhancing P53 activity in breast cancer [8]. MiR-211 is localized on intron 6 of the Trpm1 gene at 15q13-q14, a locus that is frequently lost in cancers. In the previous high throughput sequencing analysis, we found miR-211-3p was high expressed in IC insensitive hypopharyngeal tumor tissues. Nevertheless, the underlying molecular mechanisms of miR-211-3p involved in IC remain poorly understood.

In our study, we demonstrated that miR-211-3p was up-regulated in insensitive larynx-hypopharyngeal tumor tissues to IC by qRT-PCR. In HSCC tumor cells the over-expression of miR-211-3p promoted cell proliferation and migration, and inhibited apoptosis. The IC50 value of miR-211-3p OE group was significantly higher than miR-211-3p NC group treated with paclitaxel. Furthermore, we identified that the high expression of miR-211-3p lead to high CSF2 and CCL20 expression which correlated with poor prognosis in HNSCC.

Methods And Materials

qRT-PCR

The relative expression of miR-211-3p in 20 IC sensitive larynx-hypopharyngeal tumor tissues and 13 IC insensitive tissues was assessed by qRT-PCR. Total RNA was extracted from tissues using Trizol reagent (Invitrogen) and then reverse-transcribed to cDNA using miR-211-3p specific primers. Quantitative PCR analysis and data collection were carried out in triplicate for each sample and performed on the ABI 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) using the primer pairs (miR-211-3p-F: TTGTACGCAGGGACAGCAAA, miR-211-3p-R: TATGGTTGTTCACGACTCCTTCAC). Relative quantification was achieved according to the $2^{-\Delta\Delta Ct}$ relative quantification method.

Cell culture and transfection

Fadu hypopharyngeal carcinoma cell line weas obtained from Beijing Institute of Otorhinolaryngology. The cells were maintained in DMEM (Gibco) with 10% FBS (Hyclone) at a 37°C humidified atmosphere containing 5% CO2 for a maximum of 8 weeks. miR-211-3p overexpression (OE) and negative control (NC) lentivirus vectors, harboring green fluorescent protein (GFP), were constructed by GeneChem (Shanghai, China). After 16 hours post-transfection, cell culture media was replaced. After 72 hours, the mean percentage of Fadu cells expressing GFP was calculated under a fluorescence microscope (IX70, Olympus, Japan), and successful overexpression was confirmed by qRT-PCR.

MTT cell proliferation assay

For the MTT assay, cell proliferation was evaluated by adding 20 μL 5 mg/mL MTT to the culture medium for 5 consecutive days. The absorbance was determined at 490/570 nm by a microplate reader (Tecan infinite, Tecan Group Ltd, Switzerland). The same method was used to detect the tumor cell viability after paclitaxel treatment.

Cell apoptosis assay

The apoptotic cells were detected using Annexin V-allophycocyanin (Annexin V-APC) apoptosis detection kit (eBioscience, San Diego, CA, USA). The process of culturing infected cells in growth medium is the same as before. After trypsinization and PBS washes, the cells were washed with 1× binding buffer and centrifuged at 1300 rpm for 3 min. Then, the cell pellet was resuspended in 200 μL of 1× binding buffer, and Fadu cells were stained with 10 μL Annexin V-APC in the dark for 15 min and detected by flow cytometry using the FACS system.

Transwell migration assay

The migration potential of Fadu cells was assessed in 24-well Transwell plates. Briefly, cells ($1x10^5$, $2x10^5$, $4x10^5$, $8x10^5$ cells) were suspended in 200 μl serum-free medium and added into the upper chamber, 500 μL medium was added to the lower chamber. After 20 hours culture, cells on the upper surface were removed and migrant cells on the lower surface were fixed with 500 μL methanol for 10 min, and stained with 500 μL crystal violet for 20 min. And then the migrated cells were visualized using a microscope (400x magnification).

Transcriptome sequencing and differential expression analysis

Total RNA was extracted using Trizol reagent according to manufacturer's recommendations, and then RNA quantification and qualification analyses were performed. Transcriptome sequencing was performed on an Illumina Novaseq platform. Differential expression analysis of two groups (over-expression of miR-211-3p vs control) was performed using the DESeq2 R package [9]. Significant differentially expressed genes (DEGs) were selected for further analyses with setting the $|log2(FC)|>1$ & $P.adj <0.05$ (FC, fold change).

GO and KEGG enrichment analyses of differentially expressed genes

Gene Ontology (GO) and KEGG ((Kyoto Encyclopedia of Genes and Genomes) functional enrichment analyses were performed based on the hypergeometric test. The clusterProfiler R package was used to test the statistical enrichment of DEGs in GO terms and KEGG pathways [10, 11]. $P.adj < 0.05$ & $qvalue < 0.2$ was considered significance.

PPI analysis of differentially expressed genes

DEGs were uploaded into the STRING online database to further screen significant gene module [12]. Then PPIs with a combined score > 0.7 (high confidence) were chosen. And then, PPI networks were constructed by the Cytoscape software [13]. The plug-in Molecular Complex Detection (MCODE) was applied to pick out the significant gene modules.

Validation downstream genes in TCGA-HNSCC data

These candidate genes were further validated at gene expression level and OS (overall survival) time based on TCGA-HNSCC data. Gene expression validation involved in noncorresponding normal samples (502 tumor samples; 44 normal samples) and corresponding normal controls (43 tumor samples and matched normal samples). For the survival analysis, all patients with available OS time data were divided into low and high expression groups by the median gene expression level. $P < 0.05$ is regarded as statistically significant.

Association of downstream genes and immune cell infiltration in HNSCC

The single-sample gene set enrichment analysis (ssGSEA) method from GSVA R package was used to investigate the association between downstream genes and immune cell infiltration [14, 15]. A total of 24 immune cell types were available to analysis. These immune cells included activated DC (aDC), B cells, CD8 T cells, Cytotoxic cells, DC, Eosinophils, immature DC (iDC) Macrophages, Mast cells, Neutrophils, NK CD56bright cells, NK CD56dim cells, NK cells, Plasmacytoid DC (pDC), T cells, T helper cells, T central memory (Tcm), T effector memory (Tem), T follicular helper (Tfh), T gamma delta (Tgd), Th1 cells, Th17 cells, Th2 cells and Treg

The association between downstream gene expression and enrichment of immune cells was evaluated by Spearman's analysis. Then, the levels of immune cell infiltration were compared for high or low mRNA expression of downstream genes by Wilcoxon rank sum test. $P < 0.05$ is considered as statistically significant.

Results

MiR-211-3p promoted cell proliferation and insensitivity to IC in HSCC

Firstly, the expression of miR-211-3p in the insensitive tissues of IC were significantly higher than that of sensitive tissues by qRT-PCR (Fig. 1a). Subsequently, we constructed miR-211-3p OE lentivirus to upregulate the expression of miR-211-3p in Fadu cells. The miR-211-3p OE lentivirus increased miR-211-3p expression by 2171.144 times, indicating the high efficiency and stability of the transfection (Fig. 1b). Next, MTT assay showed that the over-expression of miR-211-3p significantly promoted tumor cell growth (Fig. 1c). The IC50 value of paclitaxel in miR-211-3p OE group was significantly higher than NC group, suggesting over-expression of miR-211-3p in Fadu cells induced resistance to IC drugs (Fig. 1d).

These results illustrated that miR-211-3p played an oncogene role and promoted insensitivity to IC in HSCC.

MiR-211-3p inhibited cell apoptosis and promoted migration in HSCC

Annexin V-APC-FACS assay demonstrated the number of apoptotic cells was decreased in the miR-211-3p OE group compared to NC group (Fig. 2a). Transwell assay indicated the number of migrated cells was increased in the miR-211-3p OE group compared to NC group (Fig. 2b). These data indicated that over-expression of miR-211-3p could inhibit HSCC cell apoptosis and promote migration.

Prediction and analysis of downstream genes of miR-211-3p

RNA sequence was performed to screen downstream genes after overexpression of miR-211-3p. A total of 778 genes were identified as DEGs, including 499 up-regulated mRNAs and 279 down-regulated mRNAs (Fig. 3a, b). According to GO enrichment analysis, the up-regulated genes were mainly enriched in peptidyl-tyrosine phosphorylation, collagen-containing extracellular matrix and receptor ligand activity, whereas the down-regulated genes were enriched in cornification and intermediate filament. According to KEGG pathway analysis, the up-regulated genes were mainly enriched in TNF signaling pathway and MAPK signaling pathway, whereas the down-regulated genes were enriched in estrogen signaling pathway (Fig. 3c, d).

CSF2 and CCL20 were validated as downstream genes of miR-211-3P

A total of 228 mRNAs were picked out from 499 up-regulated mRNAs to construct the PPI networks (score > 0.7, high confidence) (Fig. 4a). Eleven candidate genes were chosen by MCODE and further validated in TCGA-HNSCC data (Fig. 4b). CSF2 and CCL20 expression were statistically increased in tumor tissues compared with noncorresponding normal samples and corresponding normal controls (Fig. 4c, d, f, g). In addition, Kaplan–Meier analysis showed significantly better OS rates in patients with lower CSF2 or CCL20 expression than in patients with higher CSF2 or CCL20 expression (Fig. 4e, h, $p < 0.05$). CSF2 and CCL20 genes involved in TNF signaling pathway were finally identified as downstream genes of miR-211-3P.

CSF2 and CCL20 were correlated with immune cell infiltration in HNSCC patients

The association between the expression level of CSF2 and CCL20 and 24 immune cell types were evaluated by Spearman's analysis (Fig. 5a, b). Scatter diagrams showed that the expression level of CSF2

were positively correlated with infiltrating levels of Tgd, Th1 cells, neutrophils, DC and Th2 cells, while negatively correlated with cytotoxic cells, B cells, T cells and CD8 T cells ($P<0.05$, Fig. 5c). CCL20 was positively correlated with CD8 T cells, while negatively correlated with mast cells, iDC, NK cells and DC ($P<0.05$, Fig. 5d). More specifically, we evaluated the infiltration levels of relevant immune cells in distinct CSF2 or CCL20 groups to further validate their correlation (Fig. 5e, f). These data indicated that CSF2 and CCL20 might play oncogenic role by regulating the level of immune cell infiltration in HNSCC.

Discussion

IC followed by surgery and/or CCR is a comprehensive treatment modality for locally advanced hypopharyngeal cancer patients. However, some patients do not benefit from this treatment due to resistance to IC. So, it is urgently needed to investigate the mechanism of IC resistance. In our study, we provide evidences for the role of miR-211-3p in promoting HSCC progression and identify that miR-211-3p upregulates CSF2/CCL20/TNF signaling to promote IC insensitivity in HSCC.

In HNSCC, previous studies have reported miRNAs take part in its chemoresistance. High level of plasma exosome miR-196a is correlated with poor overall survival and confer cisplatin resistance by downregulating CDKN1B and ING5 in head and neck cancer [16]. MiR-24-3p promotes cell proliferation and regulates chemosensitivity by targeting CHD5 in HNSCC [17]. In tongue squamous cell carcinoma (TSCC), miR-23a promotes chemoresistance and protects cisplatin-induced apoptosis through inducing Twist expression by a JNK-dependent mechanism [18]. miR-211 is reported to be involved in the development and progression of HNSCC. High expression of miR-211 is associated with poor prognosis in oral carcinoma patients, and significantly promotes tumor cell proliferation, migration, and anchorage-independent colony formation [19]. In oral squamous cell carcinoma (OSCC), miR-211 directly targets TCF12, which leads to FAM213A over-expression and plays oncogenic role [20]. In HNSCC, miR-211 regulates TGFβRII and c-Myc promoting cancer progression [21]. However, studies on the molecular mechanism of miR-211-3p involved in IC resistance are still in a developing stage, a comprehensive exploration is needed urgently.

In our present study, we demonstrated that miR-211-3p was up-regulated in IC insensitive larynx-hypopharyngeal tumor tissues, which was consistent with our previous microarray data [22]. We also found that overexpression of miR-211-3p promoted cell proliferation and migration, and inhibited cell apoptosis. The overexpression of miR-211-3p enhanced the IC insensitivity in HSCC cancer cells.

The transcriptome sequencing data of our results showed CSF2 and CCL20 were downstream genes of miR-211-3p. Colony stimulating factor 2 (CSF2), also known as granulocyte macrophage-colony stimulating factor (GM-CSF), acts as a tumor-stimulating factor which promotes immune-independent tumor progression [23]. In gliomas, CSF2 can trigger and drive the alternative activation of tumour-infiltrating microglia/macrophages and shape the tumor immune microenvironment [24]. High CSF2 expression, which is caused by DNA demethylation, affects the immune response to tumor cells and tumor microenvironment. CSF2 plays hub gene role in chemoresistance and correlates worse prognosis

in colorectal Cancer [25]. CSF2 is a novel pro-inflammatory factor in human gastric cancer, which effectively induces PD-L1 expression on neutrophils via activation of JAK-STAT3 pathway [26]. Besides, C-C motif chemokine ligand 20 (CCL20) promotes chemoresistance and regulate tumor immune microenvironment in many cancers. CCL20 can recruit regulatory T cells to promote chemoresistance via FOXO1/CEBPB/NF-κB signaling in colorectal cancer [27]. Chemotherapy-induced CCL20 can activate NF-κB pathway and then increase ABCB1 expression, leading to chemoresistance in triple-negative breast cancer patients. The activated NF-κB pathway can also regulate CCL20 expression to further enhance the effect of CCL20 on chemoresistance by a positive feedback loop [28]. Tumor cell-derived CCL20 can promote hepatocellular carcinoma (HCC) progression by interacting with CCR6 highly expressed CD19+CD5+B cells [29]. In lung adenocarcinoma, RUNX3 can upregulate the expression of CCL3 and CCL20 to attract CD8+ T cells into tumor immune microenvironment [30].

In accordance with the literature, we found CSF2 and CCL20 were involved in TNF signaling pathway and correlated with poor prognosis in HNSCC. Meanwhile, the TNF signaling pathway, a typical inflammatory pathway, was activated in HSCC cells after overexpression of miR-211-3p. Furthermore, our results also indicated that CSF2 and CCL2 were correlated with immune cell infiltration. These results suggest that miR-211-3p may promote IC insensitivity via CSF2/CCL20/TNF signaling to regulate tumor immune cell infiltration in HSCC. However, further molecular and cellular experiments are required in the future.

Conclusions

In summary, as we know, this is the first study to demonstrate that miR-211-3p enhances IC insensitivity and exerts an oncogenic role by upregulating CSF2/CCL20/TNF signaling in HSCC, which may provide new insight in multi-target therapy.

Declarations

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Competing Interests

Financial interests: All authors declare they have no financial interests. The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Lingwa Wang, Ru Wang and Tianqiao Huang. The first draft of the manuscript was written by Lingwa Wang, Ru Wang and Jugao Fang, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are not publicly available due to the raw data also forms part of an ongoing study, but are available from the corresponding author on reasonable request.

Ethics approval

No ethical approval is required.

Consent to participate

Informed consent is not required.

Consent to publish

Informed consent is not required.

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Figures

Figure 1

MiR-211-3p promoted cell proliferation and insensitivity to IC in HSCC.

a. qRT-PCR tested the gene expression of miR-211-3p insensitive tumor tissues and insensitive tumor tissues to IC.

b. qRT-PCR analysis of miR-211-3p expression after miR-211-3p OE lentivirus transfection.

c. Cell proliferation was detected through MTT assay.

d. Drug sensitivity testing was determined by MTT assay.

Figure 2

MiR-211-3p inhibited cell apoptosis and promoted migration in HSCC.

a. Tumor cell apoptosis was evaluated byAnnexin V-APC-FACS assay.

b. Cell migration was assessed by transwell assay.

Figure 3

Prediction and analysis of downstream genes of miR-211-3p.

a. Volcano plot of DEGs. Red: up-regulated DEGs; Blue: down-regulated DEGs.

b. Heatmap of DEGs. Red: up-regulated DEGs; Blue: down-regulated DEGs.

c. Significantly enriched signal terms of up-regulated DEGs in GO and KEGG terms.

d. Significantly enriched signal terms of down-regulated DEGs in GO and KEGG terms.

Figure 4

CSF2 and CCL20 were identified as downstream genes of miR-211-3P.

a. The PPI networks of all up-regulated mRNAs were constructed by STRING database.

b. The most significant gene module was chosen by MCODE.

c, f. The expression of CSF2 and CCL20 in tumor tissues and non-corresponding normal samples.

d, g. The expression of CSF2 and CCL20 in tumor tissues and corresponding normal samples.

e, h. Kaplan-Meier survival analysis of HNSCC patients using the log-rank test. Kaplan–Meier analysis demonstrated that CSF2 and CCL20 overexpression were prognostic factor for LSCC patients.

Figure 5

CSF2 and CCL20 were correlated with immune cell infiltration in HNSCC patients

a. Relationships among infiltration levels of 24 immune cell types and CSF2 expression.

b. Relationships among infiltration levels of 24 immune cell types and CCL20 expression.

c. Scatter diagrams of the correlation of CSF2 expression level with enrichment of immune cells.

d. Scatter diagrams of the correlation of CCL20 expression level with enrichment of immune cells.

e. The comparison of infiltration levels of most correlated immune cells under high or low CSF2 expression subgroup.

f. The comparison of infiltration levels of most correlated immune cells under high or low CCL20 expression subgroup.