Exosomal miR-655-3p inhibits growth, and invasion and macrophage M2 polarization through targeting CXCR4 in papillary thyroid carcinoma

Lei Qiao, Chao Dong, Wenlei Jia and Binlin Ma

Papillary thyroid cancer (PTC) is an endocrine malignancy whose incidence has increased rapidly worldwide. Exosomal miR-655-3p was down-regulated in patients with PTC. However, the effect and molecular mechanism of exosome-miR-655-3p in PTC was indistinct until now. Our study found that exosome-miR-655-3p was decreased in serum of PTC patients. Overexpression of miR-655-3p with mimics significantly shrunk the cell viability, reduced the number of chemotactic and invasive PTC cells. Besides, the proportion of CD163 positive cells and the expression of markers of M2 subtype macrophages was markedly decreased when mononuclear macrophage THP-1 was cultured with exosomes of miR-655-3p mimics. Oppositely, the inhibitor of miR-655-3p exacerbated growth, chemotaxis and invasion of PTC cells, and enhanced the M2 subtype macrophages. Structurally, miR-655-3p could target the 3’ untranslated region (3’UTR) of CXCR4 and restrict the expression of CXCR4. In Xenograft tumor experiment, upregulated exosome-miR-655-3p effectively inhibited the growth of tumor and reduced the expression of CXCR4, Ki67 and CD163 in vivo. In summary, exosomal miR-655-3p inhibited growth, invasion and macrophage M2 polarization through targeting CXCR4 in papillary thyroid carcinoma. Regulating exosome-miR-655-3p/CXCR4 may be a potential treatment strategy for PTC.

Keywords: Papillary thyroid cancer, exosome, miR-655-3p, macrophages, CXCR4

INTRODUCTION

Papillary thyroid cancer (PTC) is the most common endocrine malignancy. Increasing incidence of PTC arouses widespread public concern for several decades (Lin et al., 2019). PTC is well differentiated and a relatively indolent tumor. Most tumors are inert tumors, progressing slowly, and have a good prognosis (McLeod et al., 2019). However, a few of them showed increased aggressiveness. In the aggressiveness PTC lymph node metastasis and other distant metastases can occur even at the early stage or post operation (Cho et al., 2019). Although there are some advances in the means of detection and treatment, the diagnostic accuracy still needs to be improved (Riesco-Eizaguirre et al., 2016). Therefore, the development of alternative diagnostic therapeutic markers remains an urgent clinical challenge.

Exosomes are small membrane vesicles produced by multivesicular endosomes and secrete from multiple types of cells. Exosomes participate in intercellular communication and change the physiological state of the recipient cells through delivering intracellular substances (Yáñez-Mó et al., 2015). All exosomes have surface molecules that help them target recipient cells. When they attach to the recipient cells, exosomes will fuse with the cell membrane and release their substances into the target cells (Dai et al., 2020). It has been reported that exosome-carried microRNAs are more stable than that in circulation (Cheng et al., 2014). More and more evidences indicate that exosomal microRNAs are diagnostic and prognostic biomarkers in cancers, such as prostate cancer (Huang et al., 2015), colorectal cancer (Liu et al., 2018), and ovarian cancer (Kanlikilicer et al., 2016). The aberrant expression of exosomal microRNAs in the plasma of PTC patients was found, suggesting that exosomal microRNAs are related to progression and prognosis of PTC (Dai et al., 2020).

Recent study has shown that exosome-carried miR-655-3p is down-regulated in plasma of patients with PTC compared with the healthy group (Liang et al., 2020). MiR-655-3p, an important component for the regulatory network of microRNAs, is identified as a tumor suppressor in multiple cancers, including lung cancer (Wang et al., 2019a), hepatocellular carcinoma (Wu et al., 2016), and oral squamous cell carcinoma (Yu et al., 2020). However, the function and molecular mechanism of exosomal miR-655-3p in PTC have not been reported. In the present research, we found that the decreased level of exosomal miR-655-3p was related with function of PTC cells. Elevated expression of miR-655-3p could remarkably inhibit M2 polarization of macrophages and restrain cell growth, chemotaxis and invasion through targeting CXCR4. Therefore, our work reveals a potential diagnostic and prognostic biomarker of PTC.

MATERIAL AND METHODS

Clinical Samples

All experiments were reviewed and approved by the Ethics Committee of Xinjiang Medical University affiliated Tumor Hospital and conformed to the principles outlined in the Declaration of Helsinki (No. XJMU2018-CT31). Peripheral blood was collected from PTC patients before undergoing thyroidectomy in Xinjiang
Medical University affiliated Tumor Hospital. Peripheral blood from patients with benign thyroid nodules was used as control. All patients provided written informed consent.

Serum preparation and Exosome isolation

Blood samples with a minimum of 10 mL were collected and centrifuged at 3000 rpm for 15 min at 4°C to obtain serum. Then, serum was placed into a new tube and stored at −80°C for standby application. A combination method of centrifugation and ultracentrifugation was used to isolated the exosomes according to the previously published study (Dai et al., 2020). Briefly, serum samples were first centrifuged to remove cell debris and collect supernatant, then exosomes were separated by the Optima XPN-100 ultracentrifuge (Beckman Coulter, USA) at 120000×g for 2 h under 4°C and washed with deionized water.

Cell culture

PTC cell line (TPC-1), normal human thyroid cell line Nthy-ori3-1 and Human monocye cell line THP-1 were purchased from the Cell Bank of the Shanghai Chinese Academy of Sciences (Shanghai, China). TPC-1 and Nthy-ori3-1 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Beyotime, Shanghai, China). THP-1 cells were cultivated in RPMI-1640 (Beyotime). The DMEM and RPMI-1640 medium contained 10% FBS and 1% penicillin and were incubated at 37°C with 5% CO2. Cells used for exosome isolation were cultured in DMEM supplemented with 10% exosome-depleted FBS. Exosome-depleted FBS was prepared by ultracentrifugation.

MTT assay

TPC-1 cells at a density of 4×10^4 cells/well were coated on the 96-well plates and cultured overnight at 37°C with 5% CO2. Cells were incubated with 10μM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml in PBS) at 37°C for 3 h. After removing the media, 200 μl of isopropanol was added to dissolve the crystals. Optical density (OD) for each well was determined at 490 nm using microplate reader (Infinite® 200 PRO, Tecan).

Western blotting

Protein concentration was determined by BCA assay after lysing cells. 20 μg of each sample was separated by SDS-PAGE, then transferred to PVDF membranes. The membranes were probed at 4°C with primary antibodies CXCR4 (1:1000, Abcam, Cambridge, UK), TSG101 (1:1500, Abcam), CD63 (1:1000, Abcam), and β-actin (1:1000, Abcam) overnight, then washed and incubated with appropriate secondary antibody (1:3000, Abcam) at room temperature for 1 h. Finally, the blots were analyzed with an ECL kit (Pierce Biotechnology, USA).

Cell transfection

MiR-655-3p mimics, miR-655-3p inhibitor, NC mimics and NC inhibitor were bought from GenePharma (Shanghai, China). According to the manufacturer’s instructions, cells were transfected undergoing Lipofectamine 2000 (Invitrogen), then harvested at 48 hours after transfection.

RT-qPCR

Trizol reagent (Invitrogen) was applied to extract total RNAs from cell lines. MiRNAs were separated utilizing miRcute miRNA isolation kit (Tiangen, Beijing, China). Then, reverse translation experiment was performed using miScript Reverse Transcription kit (Qiagen, Hilden, Germany) to transcribe the obtained RNAs into cDNAs. Then amplifying cDNAs used SYBR1 Premix Ex Taq™ II (Takara, Shiga, Japan). GAPDH and U6 were used as the internal reference and endogenous controls, respectively. The primer sequences were showed as follows: miR-655-3p forward, 5'-ATAATACATGTTAACAACCCCTT-3', and reverse 5'-AGAGGTTAACCATGTATTATTT-3'; U6 forward, 5'-CTCCTTCCGAGGACACA-3' and reverse, 5'-AACGCCTCCAGGATTTGCGT-3'; CXCR4 forward, 5'-GGCCAAAGTTCTTAGTTGAGTGTA-3' and reverse, 5'-ACGTTCACCGGGAATGGAGG-3'; CD206 forward, 5'-GGGCGGTACCTCAAGATAT-3' and reverse, 5'-ACGAAAGCCATTGTGAAAC-3'; Arg1 forward, 5'-GGGCTGCTGCTGTGGAAAC-3' and reverse, 5'-ATTGCCAAAAGCTGTGTCCTC-3'; IL-10 forward, 5'-TTACCTGGAGGAGGTGATGC-3', and reverse, 5'-GGGGCTCTGTGTGTCTGAC-3'; IL-1ß forward, 5'-AAGCTGAGGAAGATGCGTGT-3' and reverse, 5'-ATCTCAGTCCTCCAGCTG-3' ; GAPDH forward, 5'-TCCTCTCTGTGGCA-AGTGAC-3' and reverse 5'-CCCAATACGACCAAATCCCGTT-3'.

Transwell assay

Chemotaxis and invasion were determined by transwell assay. TPC-1 cells were serum-starved for 24 h, and then inoculated into upper chambers coated with or without Matrigel (BD Biosciences, Bedford, MA, USA) at a density of 2×10^4. The lower chambers were filled with 500 μl of 1640 RPMI containing 10% FBS, followed by incubation at 37°C for 24 h. Then, the cells on the dorsal side of the lower chamber were fixed with 100% methanol and stained with 0.1% crystal violet, photographed and counted fewer than five random 200× microscopic fields per well using a Nikon Inverted Research Microscope Eclipse Ti microscope.

Dual luciferase reporter assay

The fragmented sequences of CXCR4 3’UTR were amplified and cloned into psi-CHECK™-2 luciferase plasmid (Promega, Madison, WI, USA). These fragment-seqenced contained the wild-type or mutant-type binding sites for miR-655-3p. The plasmids were named as wild-type CXCR4 3’UTR (CXCR4 wt) and mutant-type CXCR4 3’UTR (CXCR4 mut). Cells were then co-transfected with the above plasmids and miR-655-3p mimics (80 nM; GenePharma) for 48 hours. Then luciferase activities were analyzed by Dual Luciferase Reporter Assay System (Promega).

Flow Cytometry Analysis

Flow Cytometry was used to analyzed M2 subtype macrophage marker CD163. In brief, cells (2×10^6 cells/mL) were suspended in a staining buffer and incubated using fluorescent labeled-human anti-CD163 antibody (Abcam) for 30 min, then washed twice with staining buffer. FACScalibur (BD Biosciences, CA, USA) was used for evaluating the fluorescence intensity.
Tumor xenograft

BALB/c nude SPF mice (6 weeks, 18–22 g in weight) were purchased from the National Laboratory Animal Center (Beijing, China). Animal experiment was approved by the Institutional Animal Care and Use Committee of Xinjiang Medical University affiliated Tumor Hospital (No. XJMU2018-AM31). TPC-1 cells were cultured in vitro and transfected with miR-655-3p mimics. Then the transfected TPC-1 cells (3.5×10⁶ cells per mouse) were subcutaneously injected into BALB/c nude mice. The tumor size was measured every 3 days during the experiment. Twenty-eight days after injection, the mice were executed by cervical dislocation. Tumors were removed, the weight was measured, and immunohistochemical analysis was performed.

Immunohistochemistry

The expression of Ki67, CXCR4 and CD163 in tumor tissues was determined using immunohistochemical staining as previously described (Wang et al., 2017). In brief, tissue sections were dewaxed with xylene and hydrated at different concentration gradients. The sections were then immersed in a pH 6.0 sodium citrate buffer and boiled for 30 min. Sections were incubated at 37°C with anti-Ki67 (1:200, Abcam), anti-CXCR4 (1:200, Abcam) and anti-CD163 (1:100, Abcam), followed by observation under light microscope (Olympus Corporation, Japan).

Statistical analysis

At least 3 independent experiments were repeated in all the experiments. Results were presented as mean ± S.E.M. GraphPad Prism software (GraphPad Prism Software Inc., San Diego, USA) was utilized to determine the statistical analyses. Student’s t-test or One-way analysis of variance (ANOVA) was performed to determine the statistical significance of the differences. Statistical significance was chosen as p<0.05.

RESULTS

Exosome-carried miR-655-3p was down-regulated in serum of patients with PTC

We isolated exosomes from serum of patients with PTC or patients with benign thyroid nodules, then the expression of exosome-specific markers (TSG101 and CD63) was also characterized by western blot analysis. The results indicated that the exosomes were isolated successfully (Fig. 1A). Then we determined the expression of exosome-carried miR-655-3p using RT-qPCR.

Figure 1. Exosome-carried miR-655-3p was down-regulated in serum of patients with PTC. Exosomes were isolated from the serum of patients with PTC or with benign thyroid nodules. (A) The Exosome surface markers TSG101 and CD63 was determined by western blotting assay. (B) The expression of exosomal miR-655-3p was measured utilizing RT-qPCR. *p<0.01.

Figure 2. Expression of miR-655-3p in exosomes of PTC cells. The exosomes were isolated from PTC cells TPC-1 and normal human normal thyroid cell Nthy-ori3-1. Then the exosome markers and miR-655-3p were analyzed. (A) Western blotting assay tested the exosome markers TSG101 and CD63. (B) RT-qPCR assay analyzed the expression of miR-655-3p in exosome of PTC and human normal thyroid cells. **p<0.01.

Expression of miR-655-3p in exosomes of PTC cells

For analyzing the expression of exosome-carried miR-655-3p in PTC cells, exosomes were isolated from PTC cell TPC-1 and normal human normal thyroid cell Nthy-ori3-1. The exosome marker protein was also assessed by western blotting (Fig. 2A). The level of miR-655-3p was measured by RT-qPCR. As expected, miR-655-3p was down-regulated in exosomes from TPC-1 compared with that in Nthy-ori3-1 exosomes (Fig. 2B). In addition, miR-655-3p level in normal Nthy-ori3-1 cells was elevated when compared with that in TPC-1 cells. The results suggested that expression of miR-655-3p in TPC-1 cells and exosomes of TPC-1 cells was decreased in PTC.

Exosome-carried miR-655-3p inhibited the growth and invasion of PTC cells

To confirm the function of miR-655-3p in PTC cells, we applied mimics and inhibitor to upregulate or down-regulate the expression of miR-655-3p in TPC-1 cells (Fig. 3A). MTT assay was performed to test the cell proliferation, and the results have shown that miR-655-3p mimics remarkably decreased the cell viability while miR-655-3p inhibitor deregulated the cell viability, indicating that miR-655-3p restrained TPC-1 cells proliferation. (Fig. 3B). Consistently, the number of chemo static cells and invasive cells was significantly reduced by miR-655-3p mimics measured by transwell assay (Fig. 3C). On the contrary, suppression of miR-655-3p by inhibitor could enhance the chemotaxis and invasion of TPC-1 cells (Fig. 3C).

Exosome-carried miR-655-3p inhibited M2 polarization of macrophages

It has been reported that the enrichment of M2 subtype macrophages promoted the proliferation, chemotaxis and invasion in PTC cells, and inhibition of M2 polarization attenuated malignant phenotype of PTC cells (Lv et al., 2020). In order to explore the effect of exosome-carried miR-655-3p on M2 polarization of macrophages, THP-1 was cultured with exosomes from TPC-1 cells with miR-655-3p mimics/inhibitor. Then the proportion of CD163 positive cells was analyzed by flow cytometry and the expression of M2 subtype markers was determined by RT-qPCR. The results have shown that miR-655-3p mimics obviously reduced the proportion of CD163 positive cells (Fig. 4A) and effectively dwindled...
CD206, Arg1, IL-10 and IL-1β (Fig. 4B). Evidently, miR-655-3p inhibitor exhibited the opposite effect on the rate of CD163 positive cells and expression of M2 markers (Fig. 4A and 4B). The results indicated that exosome-carried miR-655-3p inhibited M2 polarization of macrophages.

CXCR4 was a target of miR-655-3p

In order to explore the targets of miR-655-3p, TargetScan ([http://www.targetscan.org](http://www.targetscan.org)) was applied to predict suitable binding sites. Figure 5A showed the putative binding sites between miR-655-3p and the 3'UTR of CXCR4. As shown in the dual luciferase reporter assay, miR-655-3p overexpression significantly reduced the luciferase activity of CXCR4-wt 3'UTR, whereas this effect was abolished by the mutated binding sites (Fig. 5B). In addition, the RT-qPCR and western blotting results showed that miR-655-3p mimics could negatively regulate CXCR4 expression in TPC-1 cells, while miR-655-3p inhibitor elevated CXCR4 level (Fig. 5D and 5C). The above data suggested that CXCR4 was a direct target of miR-655-3p and miR-655-3p negatively regulated CXCR4 expression.

Exosome-carried miR-655-3p inhibited tumor growth in vivo

For exploring the effect of exosome-carried miR-655-3p on tumor growth in vivo, TPC-1 cells with miR-655-3p mimics were injected subcutaneously into BALB/c nude mice to generate xenograft models, then tumor volume and weight were measured. As shown in Fig. 6A–C, miR-655-3p mimics significantly lessened tumor weight.

Figure 3. Exosome-carried miR-655-3p inhibited the growth and migration of PTC cells.

TPC-1 cells were transfected with miR-655-3p mimics or inhibitor, and the exosomes carried with miR-655-3p mimics/inhibitor were isolated from the transfected TPC-1 cells. Then miR-655-3p-positive/negative exosomes were used to culture the non-transfected TPC-1 cells. (A) After TPC-1 cells incubated with exosomes, the level of miR-655-3p was measured by RT-qPCR. (B) MTT assay was performed to test the cell viability. (C) Cell migration and invasion was measured by Transwell assay. *p<0.05, **p<0.01.

Figure 4. Exosome-carried miR-655-3p inhibited M2 polarization of macrophages.

TPC-1 cells were transfected with miR-655-3p mimics or inhibitor, and the exosomes carried with miR-655-3p mimics/inhibitor were isolated from the transfected TPC-1 cells. THP-1 cells were incubated with exosomes isolated from TPC-1 cells transfected with miR-655-3p mimics or inhibitor. (A) Flow Cytometry was used to analyze M2 subtype macrophage marker CD163. (B) The mRNA levels of CD206, Arg1, IL-10, and IL-1β were analyzed using RT-qPCR. *p<0.05, **p<0.01.

Figure 5. CXCR4 was a target of miR-655-3p.

(A) The binding sites between CXCR4 3'UTR and miR-655-3p was analyzed by bioinformatics website TargetScan. (B) Luciferase reporter analysis was performed to examine the binding ability between miR-655-3p and CXCR4 3'UTR. Reporter constructs containing either CXCR4 wt or CXCR4 mut at the predicted miR-655-3p target sequences were co-transfected into TPC-1 cells, along with miR-655-3p mimics or NC mimics. (C) Western blotting assay was performed to analyze the expression of CXCR4 protein in TPC-1 cells with miR-655-3p mimics or inhibitor. (D) The mRNA expression of CXCR4 was detected by RT-qPCR. *p<0.05, **p<0.01.
In the present study, we found that CXCR4 expression was positively associated with tumor microenvironment, M1 polarization of macrophages produces type I pro-inflammatory factors and exhibits antitumor effects, and M1 polarization of macrophages produces anti-inflammatory factors and exhibits antitumor effects (Wang et al., 2020a). In our research, we probed the function of exosome-carried miR-655-3p by transfecting miR-655-3p mimics or inhibitor into PTC cells. Consistent with the results of literatures, we found that miR-655-3p could effectively inhibit cell growth, chemotaxis and invasion in PTC cells.

Tumor-associated macrophages (TAMs), are the most plentiful immune associated stromal cells that regulate genesis and distant metastasis of tumors (Wang et al., 2020a, Doak et al., 2018). In the tumor microenvironment, M1 polarization of macrophages produces type I pro-inflammatory factors and exhibits antitumor effects, and M1 polarization of macrophages produces anti-inflammatory factors and exhibits antitumor effects (Wang et al., 2020a). Contrary to the M1 polarization of macrophages, a more M2-polarized state caused extracellular matrix remodeling, immune evasion, and malignant phenotype in tumor (Wang et al., 2020a, Brown et al., 2017). It had been reported that inhibiting the M2 polarization of macrophages reversed the proliferation, invasion, cancer stem cells, and angiogenesis in PTC (Cho et al., 2015, Mazzoni et al., 2019). In the present study, our data testified that exosome-carried miR-655-3p visibly hindered the M2 polarization of macrophages, suggesting that miR-655-3p was correlated with tumor microenvironment in PTC. Also, based on previous studies and our present research, it might be speculated that miR-655-3p inhibits PTC cell growth and invasion by inhibiting macrophages M2 polarization.

Furthermore, we demonstrated that CXCR4 was a target of miR-655-3p. CXCR4 (C-X-C chemokine receptor type 4) is an alpha chemokine receptor and plays a key role in several pathological processes, including growth, invasion and metastasis of cancers (Tian et al., 2019). CXCR4 expression was positively associated with CD206+ TAMs in colorectal cancer with liver metastasis, high expression of CXCR4 recruited more macrophages to the colonic tissue and induced macrophage M2 polarization, leading to angiogenesis and tumor metastasis (Wang et al., 2020a). The expression of CXCR4 was related to tumor size, lymph node metastasis and distal attack in PTC (Wagner et al., 2008, Wang et al., 2013, Torregrossa et al., 2012). Enhanced CXCR4 promotes motility and invasiveness in PTC (Lin et al., 2018). In our study, we ascertained that miR-655-3p could target CXCR4 in PTC cells, upregulated miR-655-3p inhibited and downregulated miR-655-3p enhanced the level of CXCR4. According to the literature and the present study, it might be speculated that miR-655-3p impeded macrophage M2 polarization, inhibited tumor growth and reeded invasiveness of PTC cells by hindering CXCR4 expression.

Conclusively, we identified the function of exosomal miR-655-3p in PTC. The down-regulated expression of exosomal miR-655-3p was correlated with PTC progression. For the molecular mechanism, CXCR4 was a downstream target of miR-655-3p. By inhibiting CXCR4 expression, miR-655-3p alleviated the macrophages M2 polarization and repressed PTC development. In general, this research may provide a novel biomarker and potential therapeutic strategy for PTC.

**DISCUSSION**

In recent years, although the incidence of PTC has increased globally and the mortality rate was decreasing, the metastasis and recurrence are the difficulties of PTC therapy (Kim et al., 2020). Therefore, novel molecular markers are essential for risk stratification and personalized management of PTC (Iñiguez-Ariza et al., 2018). Due to the high stability and low complexity, cargos carried by circulating exosomes can be promising biomarkers in cancers (Von Schulze et al., 2020). Previous studies have revealed that the exosomal miRNAs were potential PTC markers (Wang et al., 2019b). For instance, decreased expression of serum exosomal miR-29a and increased expression of plasma exosomal miR-146b-5p and miR-222-3p predicted poor prognosis of PTC patients (Wen et al., 2021, Jiang et al., 2020). In the present study, we found that serum exosome-carried miR-655-3p was downregulated in PTC patients, suggesting that miR-655-3p may be involved in the progression of PTC.

Given that the expression of miR-655-3p was declined in multiple cancers and played a role in restricting the tumor progression. In hepatocellular carcinoma, miR-655-3p was low-expressed in tissues and cell lines, upregulated miR-655-3p inhibited cell proliferation, chemotaxis and invasion through inhibiting ADAM10 and β-catenin pathway (Wu et al., 2016). Inhibition of miR-655-3p by NFIA-AS2 remarkably conducted to cell proliferation, motility, and impeded apoptosis in glioma via enhancing the expression of ZFX (Xin et al., 2020). Through targeting LSD1 to activate BMP-2/Smad pathway, miR-655-3p hindered the progression of osteoporosis (Wang et al., 2020b).

In our research, we probed the function of exosome-carried miR-655-3p by transfecting miR-655-3p mimics or inhibitor into PTC cells. Consistent with the results of literatures, we found that miR-655-3p could effectively inhibit cell growth, chemotaxis and invasion in PTC cells.
Declarations

Authors’ contributions. Binlin Ma designed the experiments. Chao Dong performed the experiments. Wenlei Jia analyzed and interpreted the data. Lei Qiao was the major contributors in writing the manuscript. All authors read and approved the final manuscript.

Competing interests. The authors declare no potential conflicts of interest.

Availability of materials. All the dataset and materials generated and/or analyzed during the current study are available.

Ethics approval and consent to participate. All experiments in this study were approved by the Animal Care and Use Committee of Xijing Medical University affiliated Tumor Hospital and performed in accordance with the Guide for the Care and Use of Laboratory Animals.

References


The role of exosomal miR-655-3p in PTC
