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Original Article

Tumor suppressor miR-124 involved in stromal cells growth and apoptosis by targeting RANKL in giant cell tumor of bone

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Abstract: Giant cell tumor of bone (GCTB) is an aggressive skeletal neoplasm and exhibits malignant bone lytic behavior. However, little is known regarding the molecular mechanisms of GCTB at present. Accumulating evidences demonstrate that microRNAs (miRNAs) play essential roles in various tumors through regulating their target genes. MicroRNA-124 (miR-124) is reported as a tumor suppressor in many human cancers, but little is known about its function in GCTB. In this study, we showed that miR-124 was more frequently down-regulated in GCTB tissues. Down-expression of miR-124 was positively associated with tumor extension (P = 0.000) and grade (P = 0.000). Further *in vitro* researches proved that restoration of miR-124 suppressed giant-cell tumor stromal cells (GCTSCs) growth and induced apoptosis, whereas exogenous knock-down of miR-124 promoted GCTSCs proliferation and inhibited apoptosis. Moreover, mechanistic studies identified that miR-124 could regulate the expression of RANKL by directly binding to its 3'-UTR region. Thus, our results indicated that miR-124 could directly target RANKL and participate in growth and apoptosis in the GCTSCs, which suggested that miR-124 may be a new and potential therapeutic target for the GCTB treatment.

Keywords: GCTB, miRNAs, miR-124, GCTSCs, RANKL

Introduction

Giant cell tumor of bone (GCTB) is characterized by extensive bone resorption and usually occurs in the metaphyseal position of long bones, which accounts for about 6% of all primary skeletal tumors [1, 2]. The osteoclast-like multinucleate giant cells, spindle-like stromal cells (GCTSCs) and monocytic round cells are the mainly three components in GCTB [3]. Although regarded as a non-cancerous tumor, GCTB is known for potential recurrence following clinical treatment with a statistical incidence rate between 20% and 50% [4, 5]. Previous studies have proved that proliferating GCTSCs are believed to be the neoplastic composition of GCTB [6]. Thus, further understanding of the molecular mechanisms leading to GCTSCs growth is useful for developing new treatment methods for GCTB.

MicroRNAs (miRNAs) are a class of small and abundant, the length of 21 to 25 nucleotides non-coding RNAs that mediate target genes by binding to its mRNA 3'-untranslated region (3'-UTR) [7]. MiRNAs usually regulate gene expression by repressing DNA translation or accelerating mRNA degradation [8, 9]. Aberrant expressions of miRNAs play key roles in biological processes of human cancers, such as cells differentiation, development, proliferation and apoptosis [10, 11]. For instance, miR-188 is down-regulated in oral squamous cell carcinoma and inhibits cells proliferation and invasion by directly targeting SIX1 [12]. MiR-320b represses cells proliferation by targeting c-Myc in human colorectal cancer cells [13]. MiR-451a is associated with cells proliferation, migration and apoptosis in renal cell carcinoma [14].

Previous researches have demonstrated that microRNA-124 (miR-124) has been classified as a tumor suppressor in many human cancers, including bladder and prostate carcinoma, colorectal cancer, breast cancer and non-small cell lung cancer [15-19]. However, the specific

Table 1. The relationship of miR-124 expression with clinicopathological features in 50 cases of GCTB patients

Group	Cases (%)	miR-18b expression		*p* value	χ^2
		Low (%)	High (%)		
Age (years)				0.912	0.012[a]
< 30	37 (74)	25 (50)	12 (24)		
≥ 30	13 (26)	9 (18)	4 (8)		
Sex				0.386	0.751[a]
Male	30 (60)	19 (38)	11 (22)		
Female	20 (40)	15 (30)	5 (10)		
Pathologic fracture				0.716	0.132[a]
No	42 (84)	29 (58)	13 (26)		
Yes	8 (16)	5 (10)	3 (6)		
Campanacci grade				0.000*	34.059[a]
Grade I/II	18 (36)	3 (6)	15 (30)		
Grade III	32 (64)	31 (62)	1 (2)		
Tumor extension				0.000*	19.997[a]
T1	21 (42)	7 (14)	14 (28)		
T2	29 (58)	27 (54)	2 (4)		
Recurrence				0.305	1.053[a]
No	36 (72)	26 (52)	10 (20)		
Yes	14 (28)	8 (16)	6 (12)		

GCTB: giant cell tumor of bone; [a]: Pearson Chi-Square; *P < 0.05.

roles and molecular mechanisms of miR-124 in GCTB remain unclear. Here, we found miR-124 was significantly down-regulated in GCTB tissues, compared to normal cancellous bone tissues. MiR-124 over-expression markedly decreased GCTSCs proliferation and induced apoptosis, while knock-down its expression can lead to the opposite effects. Furthermore, we demonstrated that miR-124 could directly target RANKL expression in GCTSCs. Our study shows a new mechanism for RANKL regulation, and that miR-124 is a tumor suppressor for GCTB.

Materials and methods

Clinical samples and ethical approval

All 50 cases of clinical specimens were collected from surgical resections from GCTB patients registered at the People's Hospital of Xinjiang Uygur Autonomous Region, between January 2008 and January 2013. The paired adjacent normal bone tissues were used as the control specimens. All specimens were immediately snap-frozen in liquid nitrogen following surgical resection. The clinical features of all of the patients with GCTB were summarized in **Table 1**. The progression of the GCTB in each patient was assessed according to the Campanacci grading and Enneking staging systems [20].

All patients (or patients' parents on behalf of the children) approved to participate in this study and gave written informed consent. Both this clinical research and consent were complied with the Declaration of Helsinki and authorized by the Ethics Committee of People's Hospital of Xinjiang Uygur Autonomous Region.

Isolation and primary culture of giant-cell tumor stromal cells (GCTSCs)

Primary of GCTSCs was established from fresh GCTB tissues with razor blades, according to the mature methods founded by Mak et al [21, 22]. GCTSCs were cultured in DMEM medium (Invitrogen, CA, USA), supplemented with 10% FBS (Invitrogen, CA, USA) and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin). The culture was maintained at 37°C in a humidified atmosphere with 5% CO_2.

Cell transfection

MiR-124 mimics, mimics control, miR-124 inhibitor and inhibitor control were chemically synthesized by Shanghai Genechem Co., Ltd (Shanghai, China). The specific sequences were as follows: 5'-UGUCCAUACAAUUAAGGCACG-3' (mimics); 5'-AUCGGUCUACUCACCCGAGCU-3' (mi-mics control); 5'-UCCGGAGAGAGAGGCACAAGU-3' (inhibitor); 5'-AGUCAUGCAGGACUACUGCCA-3' (inhibitor control). For transfection, miR-124 mimics or mimics control and miR-124 inhibitor or inhibitor control were transfected in GCTSCs using Lipofetamine 2000 reagent (Invitrogen, CA, USA) following the manufacturer's protocols. In brief, GCTSCs were cultured on six-well plates at about 5×10^5 cells per well and transfected at 55%-75% confluency. RNA oligonucleotides were diluted in a

transfection medium containing transfection reagent to obtain a concentration of 200 pmol and incubated for 30 min at 37°C. The fold changes of miR-124 in GCTSCs cells were calculated by Real-time PCR at 48 h after transfection.

Real-time PCR (RT-qPCR)

Total RNA of GCTB tissues and GCTSCs was isolated with the TRIzol® reagent (Invitrogen, CA, USA), according to the manufacturer's instructions. After that, 1 μg RNA of each sample was reverse transcribed to cDNA using specific stem-loop RT primers or random primers (Qiagen, Germany). RT-qPCR was conducted using SYBR® Premix Ex Taq™ II (Takara Bio Inc., Otsu, Japan) on an Applied Biosystems 7500 Real-time PCR System (Applied Biosystems, USA). The assay conditions were as follows: 98°C, 5 min; 95°C, 15 s; and 60°C, 30 s for 45 cycles. Small nuclear U6 snRNA and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) were used as reference genes. The specific primers used for miR-124 and U6 were as follows: miR-124: 5'-GCGGTGAATGCCAAGAATGGGGCTG-3' (forward), miScript SYBR Green PCR kit Universal Primer (reverse); U6: 5'-CTCGCTTCGGCAGCACA-3' (forward), 5'-ACGCTTCACGAATTTGCGT-3' (reverse). The RANKL and GAPDH amplification primers were: RANKL: 5'-TGTATGCCAACATTTGCTTTCG-3' (sense), 5'-TGCTTCCTCCTTTCATCAGGGT-3' (antisense); GAPDH: 5'-AGAAGGCTGGGGCTCATTTG-3' (sense), 5'-AGGGGCCATCCACAGTCTTC-3' (antisense). The relative expression levels of miR-124 and RANKL in GCTB tissues and GCTSCs were quantified using the 2-ΔΔCT method [23].

Cell proliferation assay

The ability of GCTSCs proliferation was evaluated through MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide) assay. In brief, GCTSCs were seeded into 96-well culture plates and transfected as above description. At 0, 24, 48 and 72 h after transfection, 30 μL of 5 mg/ml MTT solution was added to each well and incubated for another 3 h. After that, compounded mediums were discarded and added 150 μL DMSO (dimethyl sulfoxide) to each well to dissolve formazan crystals. Absorbance was measured at 490 nm by using a Multiskan Spectrum plate reader (ThermoElectron Corporation, Vantaa, Finland).

Cell apoptosis assay

Cell apoptosis assay was used to assess cells apoptosis ability of miR-124. After 48 h transfection, GCTSCs cells were collected and washed with ice-cold PBS for three times, then adjusted to a concentration of 1×10^6 cells/mL. Whereafter, 200 μL of GCTSCs suspension were labeled with 10 μL annexin V-FITC and 5 μL propidium iodide (PI), according to the apoptosis detecting kit protocols (Invitrogen, CA, USA). Finally, the samples were detected using flow cytometer (Beckman Coulter, Inc., Brea, CA, USA), and data were analyzed with Cell Quest software (Beckman Coulter, Inc., Brea, CA, USA).

Western blot

Total proteins of GCTSCs were isolated with RIPA lysis (Invitrogen, CA, USA). After that, equal amount of total proteins was separated by 12% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and transferred onto PVDF (Millipore, MA, USA). The membranes were incubated with the primary anti-RANKL antibody (Santa Cruz Biotechnology, Inc., USA) overnight after blocking with 5% non-fat milk for 2 h at 37°C, then followed by corresponding HRP-linked secondary antibody (Beyotime Biotechnology, Inc., China). The results were observed using Imaging System and quantified with Odyssey v1.2 software (LI-COR Biosciences, NE, USA).

Dual-luciferase reporter assay

MiRanda (http://www.targetscan.org/) and PicTar (http://pictar.mdc-berlin.de/) were used to predict the potential target genes of miR-124. The 3'-UTR of RANKL mRNA containing the putative binding sites of miR-124 was amplified and sub-cloned into psiCHECK-2 luciferase promoter vector (Promega, Madison, WI, USA). RANKL 3'-UTRs containing sequences with mutations in the putative binding site of miR-124 was also obtained by Shanghai Genechem Co., Ltd (Shanghai, China). The wide type (WT) or mutant (MUT) recombinant vector were co-transfected with miR-124 mimics or mimics control into GCTSCs for 48 h. The Renilla and Firefly luciferase activities were detected using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA), according to the manufacturer's instructions.

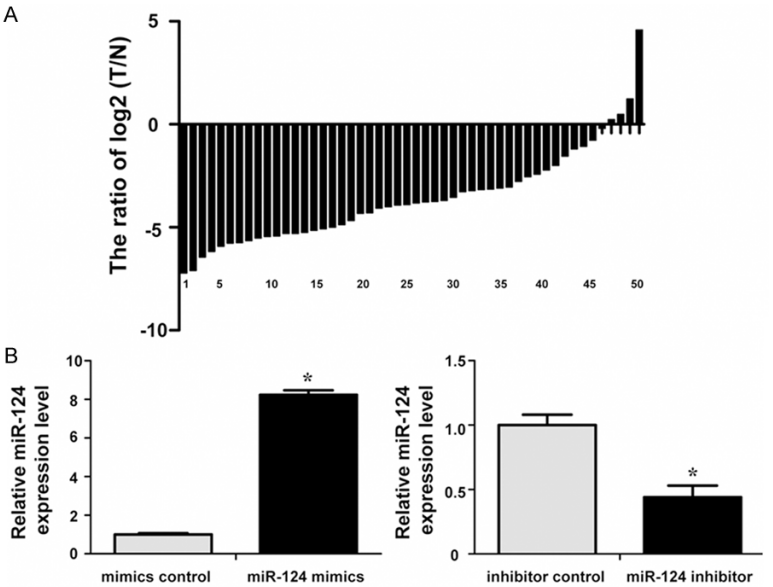

Figure 1. The miR-124 was lowly expressed in GCTB tissues. A. MiR-124 expression was determined by real-time PCR (RT-qPCR) in human GCTB tissues and paired adjacent normal bone tissues. T: GCTB tissues; N: normal bone tissues. $2^{-\Delta\Delta CT}$ method was used to analyze the data, and the data are shown in $\log_2$ (T/N). B. RT-qPCR analysis of the expression level of miR-124 in GCTSCs after transfected with miR-124 mimics, mimics control, miR-124 inhibitor and inhibitor control. Data were expressed as the mean ± SD (n = 3). *$P < 0.05$.

Statistical analysis

All experiments were repeated at least three times. Statistical analysis was performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) and data are expressed as mean ± standard deviation (SD). Differences between groups were analyzed using Student's *t* test or Analysis of Variance (ANOVA). A value of $P < 0.05$ was considered statistically significant.

Results

The miR-124 is lowly expressed in GCTB tissues

To investigate whether miR-124 plays an important role in human GCTB, we initially examined its expression level in 50 cases of GCTB tissues. The results revealed that the miR-124 was less expressed in GCTB tissues than in normal bone tissues (**Figure 1A**). Next, we explored the association of miR-124 expression level with GCTB clinicopathological features by chi-square test. In all 50 cases, we found that miR-124 is positively associated with tumor extension (P = 0.000) and grade (P = 0.000), suggesting that miR-124 might be involved in GCTB development.

To identify the roles of miR-124 in biological behavior of GCTB, miR-124 mimics and inhibitor were transfected into GCTSCs. The miR-124 levels in GCTSCs were significantly increased by transfecting the synthetic miR-124 mimics, whereas its endogenous expression was decreased after treatment with miR-124 inhibitor (**Figure 1B**).

Down-regulation of miR-124 inhibits GCTSCs proliferation

MiR-124 mimics or inhibitor was transfected into GCTSCs to evaluate its effects on GCTSCs growth. The MTT assays indicated that the rising expression of miR-124 could markedly inhibit GCTSCs proliferation compared to mimics control group. On the contrary, knock-down miR-124 expression by its specific inhibitor led to increasing growth of GCTSCs (**Figure 2**).

Down-regulation of miR-124 induces GCTSCs apoptosis

Further, the effect of miR-124 on GCTSCs apoptosis was calculated by flow cytometry. As shown in **Figure 3**, the results showed that the apoptosis rates of GCTSCs transfected with the miR-124 mimics and mimics control groups were 12.69 vs. 7.45% (P = 0.012), while the apoptosis rates of GCTSCs treated with miR-124 inhibitor and inhibitor control were 2.47% vs. 8.53% (P = 0.008), respectively.

RANKL is a direct target of miR-124 in GCTSCs

Gene expression regulated by miRNAs can be applied to cure many cancers. Thus far, miRNAs that directly targeted RANKL have been rarely reported. In this study, RANKL was predicted as a putative target gene of miR-124 by bioinformatics analysis (**Figure 4A**). To verify this speculation, we performed Dual-luciferase reporter assay *in vitro*. The results showed that miR-124 mimics remarkably decreased the relative luciferase activities of the WT recombinant reporter vector transfected GCTSCs; by contrast, miR-

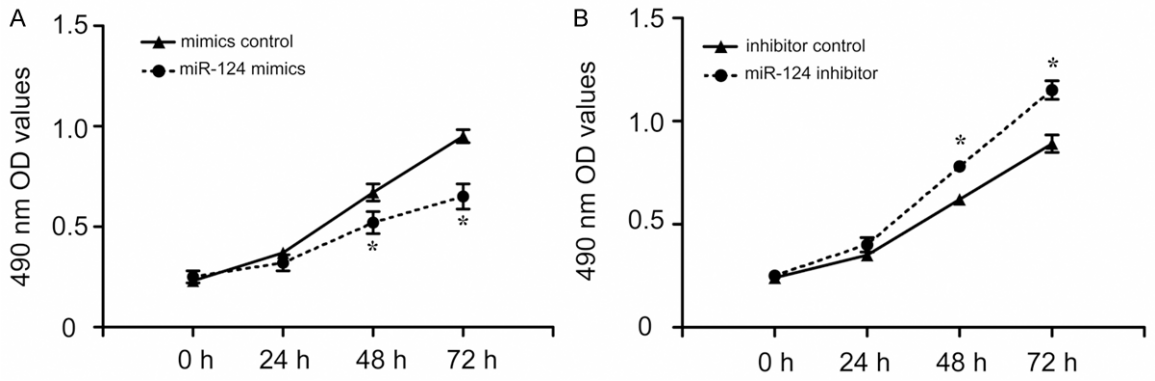

Figure 2. Down-regulation of miR-124 inhibited GCTSCs proliferation. MTT assays were employed to evaluate the growth ability of GCTSCs at 0, 24, 48 and 72 h after transfection. Values were expressed as the mean ± SD (n = 3). *P < 0.05.

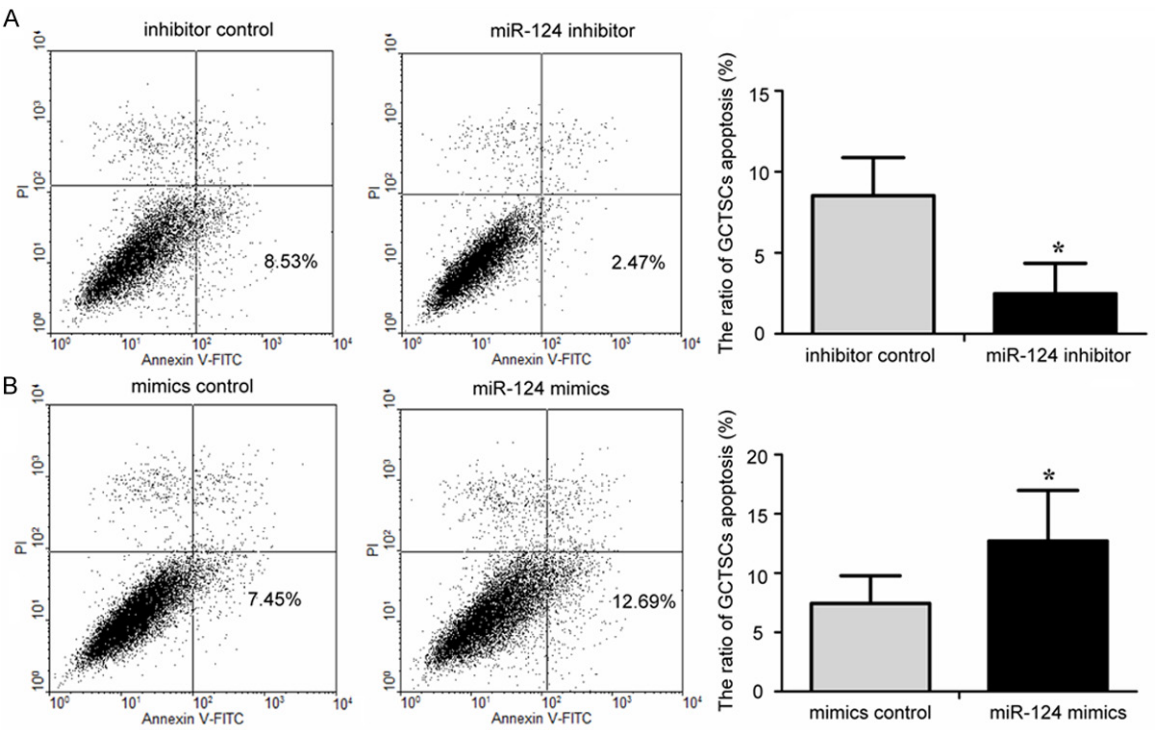

Figure 3. Down-regulation of miR-124 induced GCTSCs apoptosis. Flow cytometry was employed to evaluate GCTSCs apoptosis ability after transfected with miR-124 mimics or inhibitor, the relative ratio of apoptosis cells per field was shown on the lower right. Values were expressed as the mean ± SD (n = 3). *P < 0.05.

124 mimics did not evidently influence the MUT reporter vector treated cells (**Figure 4B**). We further illustrated whether miR-124 regulated RANKL expression in GCTSCs. Interesting, the mRNA and protein expression levels of RANKL was significantly decreased by miR-124 mimics (**Figure 4C**).

Discussion

Since the first miRNA (lin-4) was been reported, miRNAs have been demonstrated to act important roles in various of tumors, and large number of miRNAs have been found to be aberrantly presented in certain types of cancer [24, 25]. Here, we found that miR-124 was markedly decreased in patients with GCTB compared with normal individuals. In addition, we reported that down-regulation of miR-124 is closely correlated with the grade and extension of the GCTB. These results suggesting the miR-124 may be a novel biomarker for GCTB development.

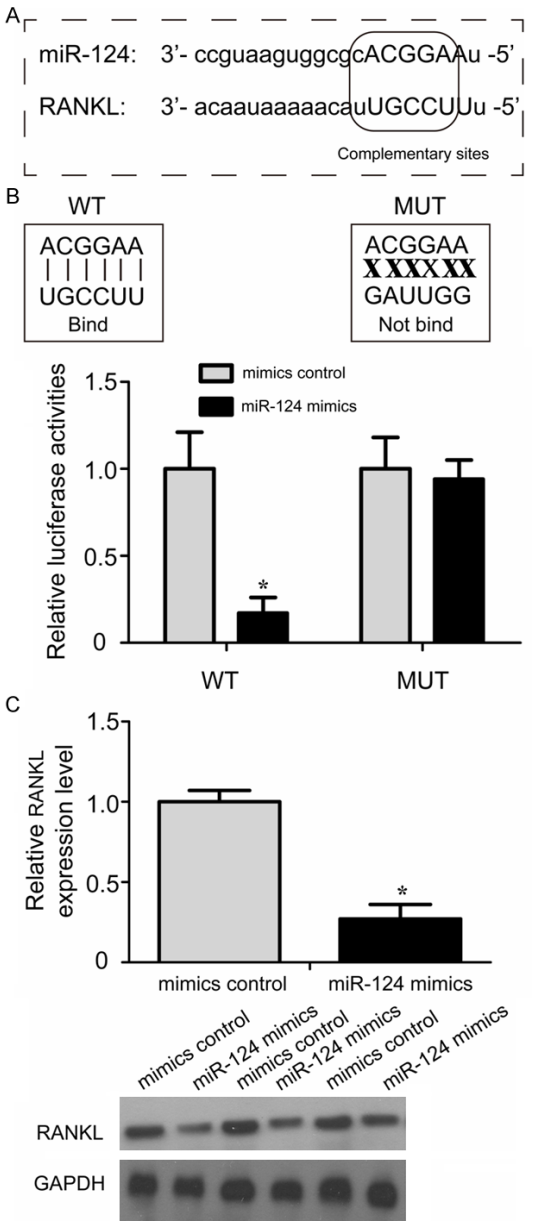

Figure 4. RANKL was a direct target of miR-124 in GCTSCs. A. Complementary sites for the seed region of miR-124 and RANKL predicted by miRanda. B. Dual-luciferase reporter assay for GCTSCs following co-transfection with the WT and MUT recombinant reporter plasmids and miR-124 mimics or mimics control. WT: wild type; MUT; mutant type. C. Over-expression of miR-124 reduced the mRNA and protein levels of RANKL in GCTSCs by using RT-qPCR and Western blot. *$P < 0.05$.

Unlike genetic stability, the expression of miRNAs can easily be altered by external influence, so recognition of its potential tumor-initiating functions may undoubtedly open a new door to tumor therapies [26, 27]. More and more evidences have demonstrated that miR-124 could inhibit cells growth and promote apoptosis in many tumors. Such as, miR-124 suppresses cells proliferation in breast cancer through down-regulation of CDK4 [28]. The tumor suppressor of miR-124 inhibits cells proliferation by directly targeting STAT3 and functions as a prognostic biomarker for postoperative NSCLC patients [29]. MicroRNA-124 functions as a tumor suppressor role in osteosarcoma [30]. MicroRNA-124 arrests cells proliferation and induces apoptosis by repressing EZH2 in gastric cancer [31]. In this study, we performed functional experiments to assess the effects of miR-124 on the biological behavior of GCTSCs. Consistent with these mechanisms, we found that knock-down of miR-124 dramatically promoted cells proliferation and inhibited apoptosis of GCTSCs, exogenous over-expression of miR-124 promoted GCTSCs proliferation and inhibited apoptosis.

RANKL, a member of the tumor necrosis factor (TNF) family, which was produced by GCTSCs or bone marrow micro-environment in response to tumor cells played crucial roles in GCTB [32]. Recent findings have suggested numbers of transcription factors that regulate RANKL expression, like vitamin D3 and parathyroid hormone (PTH) [33, 34]. However, the role of miRNAs in GCTSCs has rarely been illustrated. Our study firstly indentified RANKL as a new target of miR-124. In addition, we revealed the binding sites of miR-124 in the 3'-UTR of RANKL mRNA, and further verified the decrease of RANKL expression induced by restoration of miR-124 in GCTSCs.

In conclusion, this present study demonstrated that miR-124 was down-regulated in GCTB tissues. Restoration of miR-124 could significantly inhibit growth and induce apoptosis of GCTSCs. To our knowledge, this research is the first study to demonstrate that RANKL is a direct target of miR-124 in GCTSCs. Our findings suggested that miR-124 may be a potential new target for gene treatment of GCTB.

Disclosure of conflict of interest

None.

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