

MiR-125b inhibits stromal cell proliferation in giant cell tumor of bone by targeting parathyroid hormone 1 receptor

Pan-Feng Wu¹, Jie-Yu Liang¹, Fang Yu¹, Zheng-Bing Zhou¹, Ju-Yu Tang¹, Kang-Hua Li^{1*}

¹ Department of Orthopaedics, Xiang Ya Hospital, Central South University, Changsha 410008, China

ARTICLE INFO

Article type:
Original article

Article history:
Received: Oct 24, 2014
Accepted: Apr 11, 2015

Keywords:
Cell proliferation
GCT
miR-125b
PTH1R
RANKL

ABSTRACT

Objective(s): miR-125b has been identified as a tumor suppressor in many tumors, but its role in giant cell tumor (GCT) of bone remains poorly understood. The current study aimed to investigate the potential role and mechanism of miR-125b in GCT.

Materials and Methods: Expression levels of miR-125b in GCT tissues were determined using RT-PCR. The cell proliferation was surveyed by direct cell counting, MTS and CCK-8, and the apoptotic cells were evaluated by Annexin V-FITC and propidium iodine staining assay. The target gene expression was determined using RT-PCR and western blot. Parathyroid hormone 1 receptor (PTH1R) 3'-UTR was cloned into luciferase reporter plasmid to confirm direct targeting.

Results: We found that miR-125b was significantly down-regulated in GCT tissues. Using both gain- and loss-of-function analyses, we further revealed that miR-125b suppressed GCT stromal cell proliferation and induced cell apoptosis. Furthermore, we revealed that PTH/PTHrP type 1 receptor is a direct and functional target of miR-125b.

Conclusion: Our results suggest that miR-125b acts as a tumor suppressor through suppression of the PTH1R/RANKL signaling pathway. These findings contribute to our understanding of the functions of miR-125b in GCT.

► Please cite this article as:

Wu PF, Liang JY, Yu F, Zhou ZB, Tang JY, Li KH. MiR-125b inhibits stromal cell proliferation in giant cell tumor of bone by targeting parathyroid hormone 1 receptor. Iran J Basic Med Sci 2015; 18:705-709.

Introduction

Giant cell tumor of bone is an aggressive and highly osteolytic bone tumor (1). Giant cell tumor of bone is composed of three major cell types including osteoclast-like multinucleated giant cells, spindle-like stromal cells, and monocytic round cells (2). Current preferred treatment of giant cell tumor (GCT) consists of limb sparing surgery by means of extended curettage in addition to local adjuvant therapies (3). Albeit anatomy and function are preserved with such an approach, local recurrence rates remain high (4, 5), thus the importance of developing an understanding of the biology of this tumor and subsequent creation of more effective therapeutic options is greatly emphasized.

MicroRNAs (miRNAs) are a group of endogenously expressed, non-coding small RNAs (20-25 nucleotides in length). MicroRNAs negatively regulate the expression of target mRNAs by suppressing translation or decreasing the stability of mRNAs (6). It has been found that miRNAs play crucial roles in many biological processes including cell differentiation, proliferation and apoptosis (7, 8). An increasing number of studies have demonstrated that miRNAs can function as

oncogenes or tumor suppressors (9-11). MiR-125b has been identified as a tumor suppressor in many tumors such as bladder cancer, breast cancer and oral squamous cell carcinoma (12-15). Currently, the role of miR-125b in bone tumor has been explored. miR-125b is significantly down-regulated in Ewing's sarcoma (ES), and overexpression of this miRNA inhibited ES cell proliferation, migration and invasion, blocked cell cycle progression, and induced cell apoptosis (16). However, the specific role of miR-125b in GCT remains unknown.

Materials and Methods

Cells and cell culture

Primary cell cultures were established from GCT patients after obtaining informed consent in accordance with a protocol approved by the Ethics Committee of Central South University (Changsha, China). GCT stromal cells were isolated in culture as previously described (17). Human Embryonic Kidney 293 (HEK293) cells were maintained in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA).

*Corresponding author: Kang-Hua Li. Department of Orthopaedics, Xiang Ya Hospital, Central South University, Changsha 410008, China; Fax: 86-731-89753005; email: adeleyd@163.com

Patient sample collection

Twelve pairs of GCT tissues and adjacent normal bone tissues were collected by routine therapeutic surgery after obtaining informed consent in accordance with a protocol approved by the Ethics Committee of Central South University (Changsha, China).

Quantitative RT-PCR analysis

The total RNAs were extracted from cells with TRIZOL reagent (Invitrogen). For the detection of miR-125b, RT and PCR reactions were performed by using qSYBR-green-containing PCR kit (Genecopie), and U6 snRNA as an endogenous control for miRNA detection. For PTH1R, RANKL or IL-8 mRNA, cDNA was synthesized from 1 µg of total RNA by means of Reverse Reaction kit according to the manufacturer's instructions (Promega, Madison, WI, USA). Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The relative gene expression was calculated using the 2- $\Delta\Delta C_t$ method relative to U6 snRNA or GAPDH.

Cell proliferation assays

Transfected cells were plated on 12-well or 96-well plates at the desired cell concentrations and the cell proliferation was measured using direct cell counting, MTS-formazan reduction (Promega, USA) and Cell Counting Kit-8 fluids (CCK-8, Dojindo, Mashikimachi, Japan) by absorbance at 450 nm.

Apoptosis analysis

The apoptotic cells were evaluated by Annexin V-FITC and propidium iodine staining (BD, USA) and analyzed with a FACS Calibur instrument (BD, USA). The collected data were analyzed using FlowJo software.

Luciferase reporter assay

PTH1R 3'-UTR was amplified from human blood genomic DNA, and then was cloned into pMir-Report (Ambion). Yielding mutant constructs, mutations were introduced in potential miR-125b binding sites using the QuikChange site-directed mutagenesis kit (Stratagene). The plasmids of the wild-type or mutant UTR of PTH1R were cotransfected either with miR-125b mimics or negative control (NC) into HEK293 cells using Lipofectamine™ 2000 (Invitrogen). After 48 hr, cells were harvested and assayed using the Dual Luciferase Reporter Assay System (Promega).

Western blot analysis

Proteins were separated by 10% SDS-PAGE and then transferred to PVDF membranes (Amersham, Buckinghamshire, UK). The membranes were incubated overnight at 4 °C with anti-PTH1R antibody (Sigma) and anti-GAPDH (Sigma) antibody followed by HRP-linked secondary antibodies. incubated overnight at 4 °C with anti-PTH1R antibody (Sigma) and anti-

GAPDH (Sigma) antibody followed by HRP-linked secondary antibodies.

Statistical analysis

Statistical analyses were performed using SPSS 15.0. The difference between groups was analyzed using Student t test when comparing only two groups or one-way analysis of variance when comparing more than two groups. Statistically significant difference was set at $P < 0.05$.

Results

miR-125b is down-regulated in giant cell tumor of bone

First, the expression pattern of miR-125b in GCT was investigated. Expression levels of miR-125b are examined in 12 pairs of GCT tissues and adjacent normal bone tissues by quantitative RT-PCR analysis (qRT-PCR). As shown in Figure 1, miR-125b was significantly down-regulated in GCT tissues compared to the paired non-tumor tissues ($P = 0.006$, paired t test).

miR-125b suppresses GCT stromal cell proliferation and induces cell apoptosis

To evaluate the biological significance of miR-125b in the development of GCT, GCT stromal cells were isolated from GCT samples, then transfected with miR-125b, anti-miR-125b or NC lentivirus into these cells. The efficiency was confirmed by real-time PCR (Figure 2A). Then, cell proliferation was measured by using direct cell counting, MTS and CCK-8 assays. Overexpression of miR-125b inhibited cell growth and proliferation, whereas inhibition of miR-125b promoted cell proliferation ($P < 0.05$; Figure 2B, C, D). Furthermore, miR-125b overexpression increased the percentage of apoptotic cells, but knockdown of this miRNA did not significantly change GCT stromal cell apoptosis ration (Figure 2E).

miR-125b inhibits GCT stromal cell proliferation by targeting PTH1R

Using in silicon prediction programs, PTH1R was identified as a potential target for miR-125b. PTH1R was selected out for its role in osteoclastogenesis

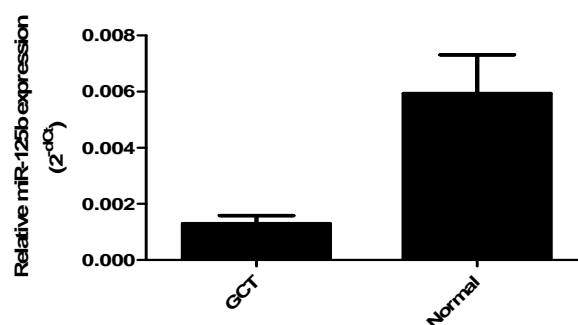


Figure 1. The expression of miR-125b in human GCT tissues. miR-125b was detected in 12 GCT patients by qRT-PCR

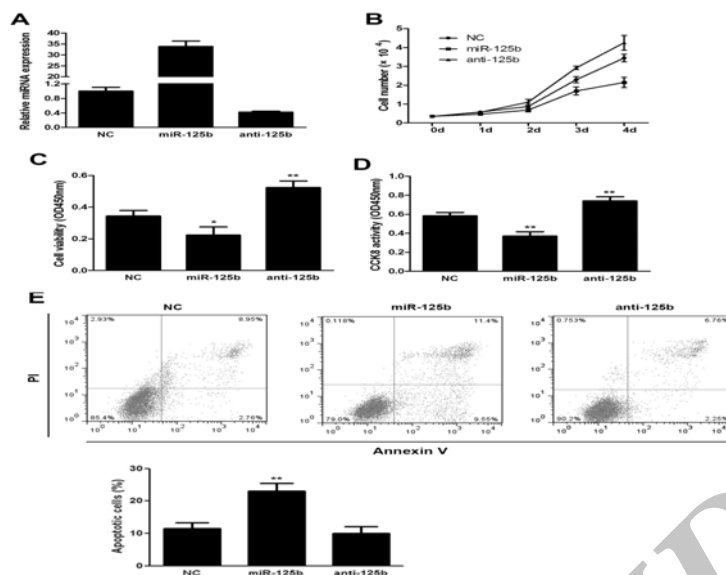


Figure 2. MiR-125b inhibits GCT stromal cell proliferation and induces cell apoptosis. (A) miR-125b expression was examined by qRT-PCR in GCT stromal cells transfected with negative control (NC), miR-125b or anti-miR-125b lentivirus. (B) Growth assays were performed by direct cell counting. GCT stromal cells were transfected with NC, miR-125b or anti-miR-125b lentivirus. (C, D) The cell proliferative potential was determined in GCT stromal cells by MTS assay (C) and CCK-8 (D) assay. The absorbance at 450 nm was assayed and data were presented as mean \pm s.d from at least three independent experiments. (E) The apoptotic cells were evaluated by Annexin V-FITC and propidium iodide (PI) staining and analyzed with FACS. Data are presented as mean \pm SD * P <0.05; ** P <0.01

and pathogenesis of bone tumor (17, 18). To verify whether PTH1R is a direct target of miR-125b, we cloned the wild-type 3'UTR or the mutant (lacking the 7-bp seed sequence) into a luciferase reporter vector. When we cotransfected HEK293 cells with the cloned

UTR and miR-125b mimics, we observed that miR-125b overexpression caused a consistent reduction in luciferase activity for wild type 3'UTR-reporter. Conversely, cotransfection of miR-125b mimics with

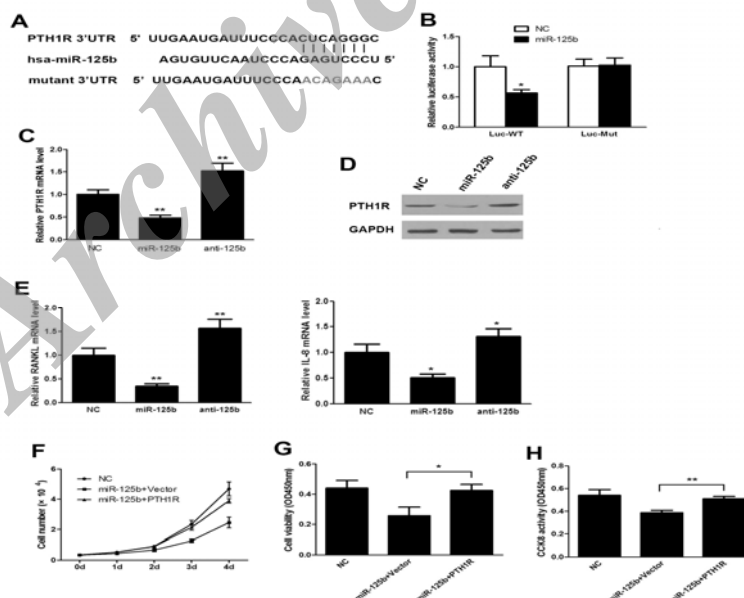


Figure 3. PTH1R is a direct target of miR-125b. (A) PTH1R 3'UTR contains one predicted miR-125b binding site. The mutagenic nucleotides are indicated in grey. (B) Dual luciferase reporter assay. HEK293 cells were transfected with wild type 3'UTR-reporter or mutant (Mut) constructs together with miR-125b mimics or negative controls (NC). Relative firefly luciferase expression was normalized to Renilla luciferase. (C, D) The expression levels of PTH1R in GCTSCs transfected with NC, miR-125b or anti-miR-125b lentivirus by qRT-PCR and western blot. (E) qRT-PCR to measure RANKL and IL-8 expression in GCTSCs transfected with NC, miR-125b or anti-miR-125b lentivirus. (F, G and H) PTH1R rescues the suppressive roles of miR-125b in GCTSC proliferation. GCTSCs miR-125b or NC were transfected with or without PTH1R plasmids. Cell proliferation analysis was performed by cell counting (F), MTS assay (G) and CCK-8 assay (H). Data are presented as mean \pm sd. * P <0.05; ** P <0.01

the mutated form of 3'UTR-reporter resulted in no significant change in luciferase activity (Figure 3A, B), suggesting that miR-125b directly targeted the PTH1R 3'-UTR. In agreement, miR-125b overexpression significantly reduced both mRNA and protein expression for PTH1R in GCTSC cells. Conversely, miR-125b inhibitors transfection increased its mRNA and protein levels (Figure 3C, D). Overexpression of miR-125b also inhibited PTH1R downstream targets such as RANKL and IL-8 expression, whereas knockdown of this miRNA increased RANKL and IL-8 expression (Figure 3E), further indicating that PTH1R is a target of miR-125b in GCT cells.

To verify the functional connection between miR-125b and PTH1R, PTH1R were transfected with plamids containing PTH1R gene or empty vector after transfection of miR-125b mimics. As shown in Figure 3F-H, in miR-125b-expressing cells, overexpression of PTH1R rescued growth defects of miR-125b, underlining the specific importance of the PTH1R for miR-125b action. Therefore, our results suggest that the role of miR-125b in the development of GCT, at least in part, depends on its down-regulation of PTH1R pathway.

Discussion

MiRNAs comprise approximately 1% of the genome of different species, each of which has hundreds of different conserved or non-conserved targets, making them key players in various cellular processes (19). Thus, it is extremely important to understand physiological and disease-associated mechanisms of these small, single-stranded RNAs. Recent studies have revealed a critical role for miRNAs in tumor development and progression, particularly in GCT (20, 21). In the present study, we determined that the level of miR-125b expression was significantly lower in GCT than that adjacent non-tumor tissue in our patient cohort. Using in vitro and in vivo assays, we found that miR-125b could inhibit GCT stromal cell proliferation and induce GCTSC apoptosis. Our results are in accordance with other studies, suggesting the tumor suppressor role of miR-125b in cancers, and provide a solid foundation for the utilization of miR125b in anticancer therapy in future.

We next explored the possible targets of miR-125b in GCT through different computational algorithms. Silicon analysis revealed PTH1R as a candidate target of miR-125b. PTH1R was selected out for its role in osteoclastogenesis and pathogenesis of bone tumor (17, 18, 22, 23). PTHR1 belongs to a group of transmembrane receptors that binds with G proteins. PTH/PTHrP binding to PTHR1 results in the activation of pathways in osteoblasts that promote osteoclastogenesis through increasing RANKL (23). Previously, a study has shown that PTHrP/PTH1R is constitutively expressed in GCT (18). Furthermore,

PTHrP/PTH1R pathway can inhibit GCT stromal cell proliferation and induce GCT apoptosis (19). Here, PTH1R as a direct target of miR-125b was further confirmed in luciferase activity assays and miR-125b-mediated PTHR1 expression analysis. MiR-125b also inhibited RANKL and IL-8 expression. Importantly, overexpression of PTH1R rescued growth defects of miR-125b in GCT stromal cells. Our results suggest that miR-125b inhibits cell growth in giant cell tumor by targeting PTH1R.

Conclusion

Our findings demonstrate that miR-125b is significantly down-regulated in GCT clinical specimens and acts as a tumor suppressor through suppression of the PTH1R/RANKL signaling pathway by targeting the PTH1R gene. These results may help us understand the molecular mechanism of GCT carcinogenesis, and provide us with a strong rationale to further investigate miR-125b as a potential biomarker and therapeutic target for GCT.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (81472625).

References

1. Amanatullah DF, Clark TR, Lopez MJ, Borys D, Tamurian RM. Giant cell tumor of bone. *Orthopedics* 2014; 37:112-120.
2. Cowan RW, Singh G. Giant cell tumor of bone: a basic science perspective. *Bone* 2013; 52:238-246.
3. Raskin KA, Schwab JH, Mankin HJ, Springfield DS, Hornicek FJ. Giant cell tumor of bone. *J Am Acad Orthop Surg* 2013; 21:118-126.
4. Karpik M. Giant Cell Tumor (tumor gigantocellularis, osteoclastoma)-epidemiology, diagnosis, treatment. *Ortop Traumatol Rehabil* 2010; 12: 207-215.
5. van der Heijden L, Dijkstra PD, van de Sande MA, Kroep JR, Nout RA, van Rijswijk CS, *et al.* The clinical approach toward giant cell tumor of bone. *Oncologist* 2014; 19:550-561.
6. Slezak-Prochazka I, Durmus S, Kroesen BJ, van den Berg A. MicroRNAs, macrocontrol: regulation of miRNA processing. *RNA* 2010;16: 1087-1095.
7. Kunej T, Godnic I, Ferdin J, Horvat S, Dovc P, Calin GA. Epigenetic regulation of microRNAs in cancer: an integrated review of literature. *Mutat Res* 2011; 717: 77-84.
8. Nicolas FE, Lopez-Martinez AF. MicroRNAs in human diseases. *Recent Pat DNA Gene Seq* 2010;4: 142-154.
9. Yang W, Lee DY, Ben-David Y. The roles of microRNAs in tumorigenesis and angiogenesis. *Int J Physiol Pathophysiol Pharmacol* 2011; 3:140-155.
10. Deng M, Tang H, Zhou Y, Zhou M, Xiong W, Zheng Y, *et al.* miR-216b suppresses tumor growth and invasion by targeting KRAS in nasopharyngeal carcinoma. *J Cell Sci* 2011; 124:2997-3005.
11. Tang H, Deng M, Tang Y, Xie X, Guo J, Kong Y, *et al.* miR-200b and miR-200c as prognostic factors and

mediators of gastric cancer cell progression. Clin Cancer Res 2013; 19:5602-5612.

12. Han Y, Liu Y, Zhang H, Wang T, Diao R, Jiang Z, et al. Hsa-miR-125b suppresses bladder cancer development by down-regulating oncogene SIRT7 and oncogenic long noncoding RNA MALAT1. FEBS Lett; 2013; 587:3875-3882.

13. Feliciano A, Castellvi J, Artero-Castro A, Leal JA, Romagosa C, Hernandez-Losa J, et al. miR-125b acts as a tumor suppressor in breast tumorigenesis via its novel direct targets ENPEP, CK2-alpha, CCNJ, and MEGF9. PLoS One 2013; 8:e76247.

14. Shiiba M, Shinozuka K, Saito K, Fushimi K, Kasamatsu A, Ogawara K, et al. MicroRNA-125b regulates proliferation and radioresistance of oral squamous cell carcinoma. Br J Cancer 2013; 108:1817-1821.

15. Ferracin M, Bassi C, Pedriali M, Pagotto S, D'Abundo L, Zagatti B, et al. miR-125b targets erythropoietin and its receptor and their expression correlates with metastatic potential and ERBB2/HER2 expression. Mol Cancer 2013; 12:130.

16. Li J, You T, Jing J. MiR-125b inhibits cell biological progression of Ewing's sarcoma by suppressing the PI3K/Akt signalling pathway. Cell Prolif 2014; 47: 152-160.

17. Zhou W, Yin H, Wang T, Liu T, Li Z, Yan W, et al. MiR-126-5p regulates osteolysis formation and stromal cell proliferation in giant cell tumor through inhibition of PTHrP. Bone 2014; 66:267-276.

18. Mak IW, Cowan RW, Turcotte RE, Singh G, Ghert M. PTHrP induces autocrine/paracrine proliferation of bone tumor cells through inhibition of apoptosis. PLoS One 2011; 6:e19975.

19. Zhang B, Farwell MA. microRNAs: a new emerging class of players for disease diagnostics and gene therapy. J Cell Mol Med 2008; 12:3-21.

20. Wu Z, Yin H, Liu T, Yan W, Li Z, Chen J, et al. MiR-126-5p regulates osteoclast differentiation and bone resorption in giant cell tumor through inhibition of MMP-13. Biochem Biophys Res Commun 2014; 443:944-949.

21. Lehner B, Kunz P, Saehr H, Fellenberg J. Epigenetic silencing of genes and microRNAs within the imprinted Dlk1-Dio3 region at human chromosome 14.32 in giant cell tumor of bone. BMC Cancer 2014; 14:495.

22. Houpis CH, Tosios KI, Papavasileiou D, Christopoulos PG, Koutlas IG, Sklavounou A, et al. Parathyroid hormone-related peptide (PTHrP), parathyroid hormone/parathyroid hormone-related peptide receptor 1 (PTHR1), and MSX1 protein are expressed in central and peripheral giant cell granulomas of the jaws. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010; 109:415-424.

23. Romero G, Sneddon WB, Yang Y, Wheeler D, Blair HC, Friedman PA. Parathyroid hormone receptor directly interacts with dishevelled to regulate beta-Catenin signaling and osteoclastogenesis. J Biol Chem 2010; 285:14756-14763.