MicroRNA-125b reduces glucose uptake in papillary thyroid carcinoma cells

GUOWU ZHANG[1], SIHAI ZHOU[1], QUAN YANG[2] and FANG LIU[2]

Departments of [1]General Surgery and [2]Radiology,
Yongchuan Hospital of Chongqing Medical University, Chongqing 402160, P.R. China

Abstract. Previous studies have shown that microRNA (miR)-125b plays important roles in several human cancer types. The aim of the present study was to analyze the potential roles of miR-125b in papillary thyroid carcinoma (PTC). It was found that miR-125b was downregulated in PTC and its expression was affected by clinical stages. Glucose transporter 1 (GLUT1) was upregulated in PTC and was negatively correlated with miR-125b. In PTC cells, overexpression of miR-125b suppressed glucose uptake and downregulated GLUT1. Furthermore, GLUT1 overexpression reduced the effects of miR-125b overexpression on glucose uptake. Moreover, miR-125b overexpression suppressed PTC cell proliferation. GLUT1 overexpression promoted the proliferation of PTC cells and reduced the effects of miR-125b overexpression on cancer cell proliferation. Overall, miR-125b decreased glucose uptake in PTC cells by downregulating GLUT1.

Introduction

The most common type of endocrine tumors is thyroid cancer (TC) (1). In 2012, TC caused more than 40,000 deaths and affected about 300,000 new cases worldwide (2). Moreover, the incidence rate of TC is continuously increasing in recent years in several countries, such as China (3). Therefore, it is important to develop effective approaches to prevent the occurrence of TC and improve treatment outcomes. Papillary TC (PTC) is the major histological subtype of TC and accounts for about 80% of all cases (4). With appropriate treatment, such as thyroid hormone suppression, surgical resection and radioactive iodine therapy, most PTC cases can be cured.

Correspondence to: Dr Fang Liu, Department of Radiology, Yongchuan Hospital of Chongqing Medical University, 439 Xuanhua Road, Yongchuan Area, Chongqing 402160, P.R. China
E-mail: ewrjppv89333796@126.com

However, tumor metastasis may occur in a small portion of PTC patients and the prognosis is generally poor (5).

Compared with normal cells, glucose metabolism is accelerated in cancer cells, and the increased glucose metabolism rate in cancer cell supports the continuous growth of tumors (6). In cancer treatment, the inhibition of glucose uptake has been considered as a promising target (7). Glucose transporter 1 (GLUT1) is one of the major players in glucose uptake, which mediates glucose across plasma membranes (8). GLUT1 is usually upregulated in different types of cancer types, including in PTC (9). Some microRNAs (miRNAs/miRs), such as miR-1291 may regulate the expression of GLUT1 to inhibit cancer progression (10). In a recent study, Huang *et al* (11) showed that miR-125b is involved in glucose-induced reactive oxygen species generation, indicating the involvement of miR-125b in glucose metabolism. The present study was conducted to investigate the role of miR-125b in PTC and explore its possible involvement in glucose metabolism.

Materials and methods

Patients and specimens. A total of 109 patients with PTC were admitted to Yongchuan Hospital of Chongqing Medical University between December 2016 and November 2018. From these 109 patients, 56 PTC patients (30 males and 26 females; age range, 34 to 62 years; mean age, 48.1±6.2 years) were selected to serve as the research subjects of the present study. The inclusion criteria were as follows: i) First-time diagnosed cases; and ii) patients who received no therapies within 100 days before admission. The exclusion criteria were as follows: i) Recurrence cases (n=2); ii) patients with other clinical disorders (n=30); iii) and patients who were treated for any other clinical disorders within 100 days before admission (n=21). Based on clinical findings, there were 16, 20, 10 and 10 patients at clinical stages (AJCC) I-IV (12), respectively. All 56 patients signed informed consent. This study was approved by the Ethics Committee of Yongchuan Hospital of Chongqing Medical University.

Adjacent normal tissues (within 2 cm around tumors) and PTC tissues were obtained from each patient during the diagnosis, through histopathological biopsy. Weights of tissue samples ranged from 0.015 to 0.022 g. At least three pathologists checked the tissues and confirmed that all tissue samples were correctly classified (nomal tissues contained no cancer cells and PTC tissues contained >80% cancer cells).

PTC cells and cell transfections. HTH83 and IHH-4 human PTC cell lines (ATCC) were used. Cells were cultivated under conditions of 37°C and 5% CO_2. Cell culture medium was DMEM (10% FBS, Invitrogen; Thermo Fisher Scientific, Inc.).

Negative non-targeting control miRNA (5'-CCGGUG UACGUAGUGGGCAGUG-3') and miR-125b mimic (5'-UCC CUGAGACCCUAACUUGUGA-3') were obtained from Sigma-Aldrich (Merck KGaA). GLUT1 expression pcDNA3 vector and empty pcDNA3 vector were purchased from Sangon Biotech Co., Ltd. HTH83 and IHH-4 cells were harvested at the confluence of 70-80%, and 40 nM negative control (NC) miRNA, 40 nM miR-125b mimic, 15 nM empty pcDNA3 vector (NC), or 10 nM GLUT1 expression pcDNA3 vector was transfected into the cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Cells without transfections were control (C) cells. The interval between transfection and the subsequent experiments was 24 h.

Reverse transcription-quantitative PCR (RT-qPCR). HTH83 and IHH-4 cells ($1x10^5$ cells) or 0.01 g tissue of each sample (ground in liquid nitrogen) was mixed with 1 ml Ribozol reagent (VWR Life Science) to extract total RNAs. Following digestion with DNase I, reverse transcriptions (55°C for 15 min and 80°C for 10 min) were performed using Quantitect Reverse Transcription kit (Qiagen China Co., Ltd.) and qPCR reaction mixtures were prepared using the Universal One-Step RT-qPCR kit (SYBR, New England BioLabs, Inc.). The expression of GLUT1 mRNA was detected using GAPDH as endogenous control. The PCR thermocycling conditions were: 95°C for 1 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 40 sec.

miRNAs were extracted from the aforementioned cells and tissues using mirVana miRNA Isolation kit (Thermo Fisher Scientific, Inc.), following miRNA reverse transcriptions (55°C for 10 min and 80°C for 10 min) performed using Taqman® MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.; cat. no. 4366596). The expression of miR-125b was detected using the TaqMan™ microRNA assay (Applied Biosystems™; Thermo Fisher Scientific, Inc.; cat. no. 4427975) with U6 as the endogenous control. The PCR thermal cycling conditions were: 95°C for 1 min, followed by 40 cycles of 95°C for 10 sec and 58°C for 20 sec.

The primer sequences were: GLUT-1 forward, 5'-CTGCTCATCAACCGCAA-3'; GLUT-1 reverse, 5'-CTTCTTCTCCCGCATCATC-3'; GAPDH forward, 5'-ACCCAGAAGACTGTGGATG-3'; and GAPDH reverse, 5'-GTAGAGGCAGGGATGATGTT-3'. The forward primer of miR-125b was 5'-UCCCUGAGACCCUAACUUG-3'. The reverse primer of miR-125b and U6 primers were from the kit.

All data were processed using the $2^{-\Delta\Delta Cq}$ method (13). Each experiment was replicated three times.

Glucose uptake analysis. The Krebs-Ringer-HEPES (KRH) buffer was prepared before glucose uptake assay, using the following reagents: 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 120 mM NaCl, 5 mM KCl, 1.3 mM KH_2PO_4 and 25 mM HEPES (pH 7.4). HTH83 and IHH-4 cells were harvested at 24 h post-transfections. Following washing with KRH buffer, $4x10^5$ cells were dissolved in fresh KRH buffer. Subsequently, [^{3}H]-2-deoxyglucose (1 μCi; Perkin Elmer Life Sciences) was

added and cells were cultivated at 37°C for 2 min. Glucose uptake was terminated by adding ice-cold KRH (3 volumes). Cells were separated from the buffer by centrifugation at 1,200 x g for 10 min at room temperature. A scintillation spectrometry was used to measure radioactivity and disintegrations per minute, which represents the amount of glucose in cells. DMP was normalized to cellular protein mass.

Cell proliferation analysis. HTH83 and IHH-4 cells were resuspended in Eagle's Minimum Essential Medium (10% FBS, Invitrogen; Thermo Fisher Scientific, Inc.) with a ratio of $4x10^4$ cells per 1 ml medium to make single-cell suspensions. The cells were cultivated under the aforementioned conditions, and Cell Counting Kit-8 solution (10 μl; Sigma-Aldrich; Merck KGaA) was added to each well at 4 h before the end of cell culture. Cell culture was terminated every 24 h until 96 h, followed by the addition of 10 μl DMSO. Subsequently, the OD values (450 nm) were measured.

Western blotting. HTH83 and IHH-4 cells ($1x10^5$ cells) were mixed with 1 ml pre-cold (4°C) RIPA buffer (Invitrogen; Thermo Fisher Scientific, Inc.) to extract total protein. BCA assay was performed to quantify all protein samples. Protein samples were boiled for 5 min, followed by SDS-PAGE using gel (10%) electrophoresis with 30 μg per lane. Proteins were then transferred onto PVDF membranes. Subsequently, the membranes were blocked in 5% non-fat milk for 2 h at 22°C, and treated with primary antibodies of rabbit polyclonal GAPDH (1:800; cat. no. ab8245; Abcam) and GLUT1 (1:800; cat. no. ab15309; Abcam) overnight at 4°C. IgG-HRP goat anti-rabbit (1:800; cat. no. MBS435036; MyBioSource, Inc.) was used as the secondary antibody and incubation was performed for 2 h at room temperature. Signals were developed using ECL (Sigma-Aldrich; Merck KGaA) and analyzed by Image J version 1.46 (National Institutes of Health).

Statistical analysis. Each experiment was repeated three times and the data from three biological replicates were used to calculate the mean ± standard deviation values. The differences between normal and PTC tissues were analyzed using the paired t-test. The differences among different cell and patient groups were analyzed using ANOVA (one-way) in combination with Tukey's test. Correlations were analyzed using linear regression. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-125b is downregulated in PTC. The levels of miR-125b expression were measured in the two types of tissues from 56 patients with PTC, by RT-qPCR. Compared with normal tissues, the expression levels of miR-125b were significantly lower in PTC tissues (Fig. 1A; $P<0.05$). The 56 patients with PTC included 16, 20, 10 and 10 patients at clinical stages (AJCC) I-IV, respectively. It was observed that the levels of miR-125b expression decreased with increasing clinical stage (Fig. 1B; $P<0.05$).

GLUT1 is negatively correlated with miR-125b in PTC. GLUT1 mRNA level was detected by RT-qPCR. Paired t-test analysis
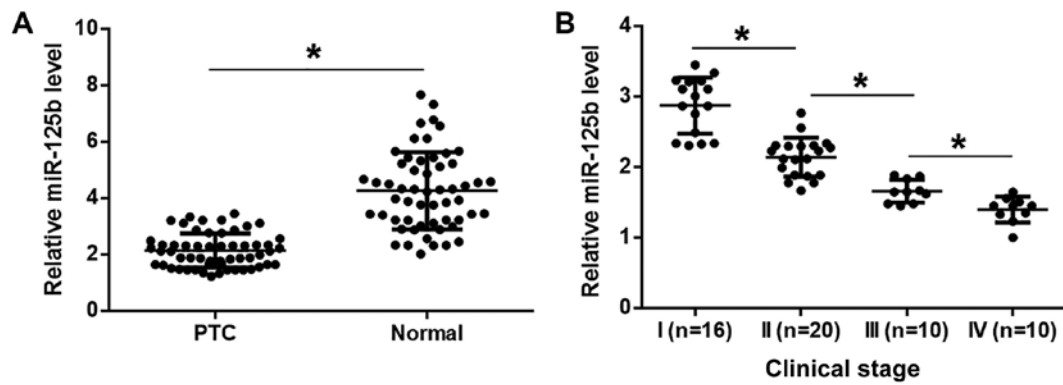
Figure 1. miR-125b is downregulated in PTC. miR-125b was detected by reverse transcription-quantitative PCR and data were compared by paired t-test. (A) Compared with normal tissues, the expression levels of miR-125b were significantly lower in PTC tissues. (B) ANOVA (one-way) and Tukey's test analysis showed decreased levels of miR-125b expression with increasing clinical stages. *$P<0.05$. miR, microRNA; PTC, papillary thyroid carcinoma.
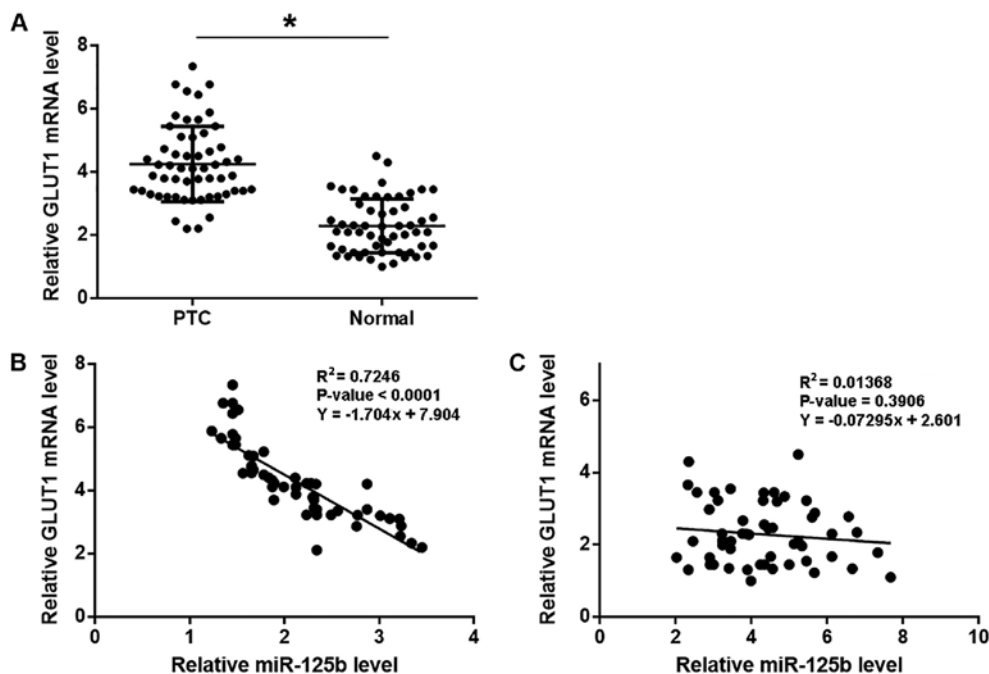

Figure 2. GLUT1 is negatively correlated with miR-125b in PTC. GLUT1 mRNA levels were measured by reverse transcription-quantitative PCR. Paired t-test was used for data comparison. (A) GLUT1 mRNA levels were significantly higher in PTC tissues compared with normal tissues. *$P<0.05$. Linear regression analysis showed that GLUT1 mRNA and miR-125b were significantly and inversely correlated across PTC tissues (B), however not across normal tissues (C). GLUT1, glucose transporter 1; miR, microRNA; PTC, papillary thyroid carcinoma.

showed that GLUT1 mRNA levels were significantly higher in PTC tissues compared with normal tissues (Fig. 2A; $P<0.05$). Linear regression analysis showed that GLUT1 mRNA and miR-125b were significantly and inversely correlated in PTC tissues, (Fig. 2B). In normal tissues, GLUT1 mRNA and miR-125b were not significantly correlated (Fig. 2C).

miR-125b suppresses glucose uptake in PTC cell by downregulating GLUT1. HTH83 and IHH-4 cells were transfected with miR-125b mimic and GLUT1 expression vectors. Compared with NC and C groups, the expression levels of miR-125b (Fig. 3A-a) as well as GLUT1 protein (Fig. 3A-b) and GLUT1 mRNA (Fig. 3A-c) were significantly increased at 24 h post-transfection (Fig. 3A; $P<0.05$). Moreover, compared with the two controls, miR-125b overexpression resulted in down-regulated GLUT1 (Fig. 3B-a) and decreased glucose uptake

(Fig. 3B-b). GLUT1 overexpression decreased the effects of miR-125b overexpression on glucose uptake (Fig. 3B; $P<0.05$).

miR-125b suppresses PTC cell proliferation by downregulating GLUT1. Compared with NC and C groups, miR-125b overexpression resulted in decreased, while GLUT1 overexpression resulted in increased proliferation rate of PTC cells. GLUT1 overexpression reduced the effects of miR-125b overexpression on cancer cell proliferation (Fig. 4; $P<0.05$).

Discussion

In the present study, the role of miR-125b was investigated in PTC. It was found that miR-125b was downregulated in PTC and may inhibit the glucose uptake and proliferation of PTC cells, by downregulating GLUT1.
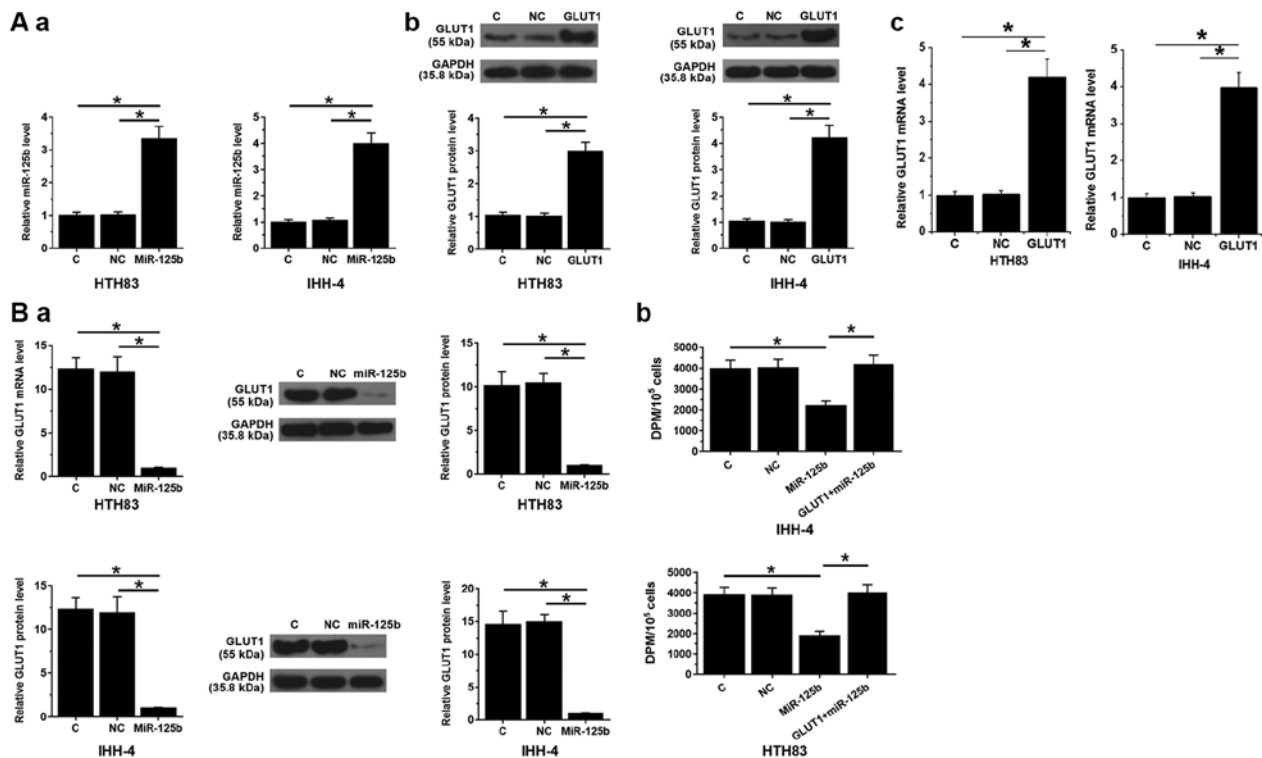
Figure 3. miR-125b suppresses glucose uptake in PTC cell by downregulating GLUT1. (A) Compared with NC and C groups, the expression levels of miR-125b (A-a) and GLUT1 (A-b, protein levels and A-c, mRNA levels) were significantly increased at 24 h post-transfection, following the transfections with miR-125b mimic and GLUT1 expression vectors. Moreover, compared with the two controls, miR-125b overexpression resulted in downregulated GLUT1 (B-a) and decreased glucose uptake (B-b). (B-b) GLUT1 overexpression reduced the effects of miR-125b overexpression on glucose uptake. *P<0.05. GLUT1, glucose transporter 1; miR, microRNA; PTC, papillary thyroid carcinoma; NC, negative control miRNA transfection (in cases of miR-125b overexpression) or empty pcDNA3 vector transfection (in cases of GLUT1 overexpression); C, control (cells without transfections); DPM, disintegrations per minute.
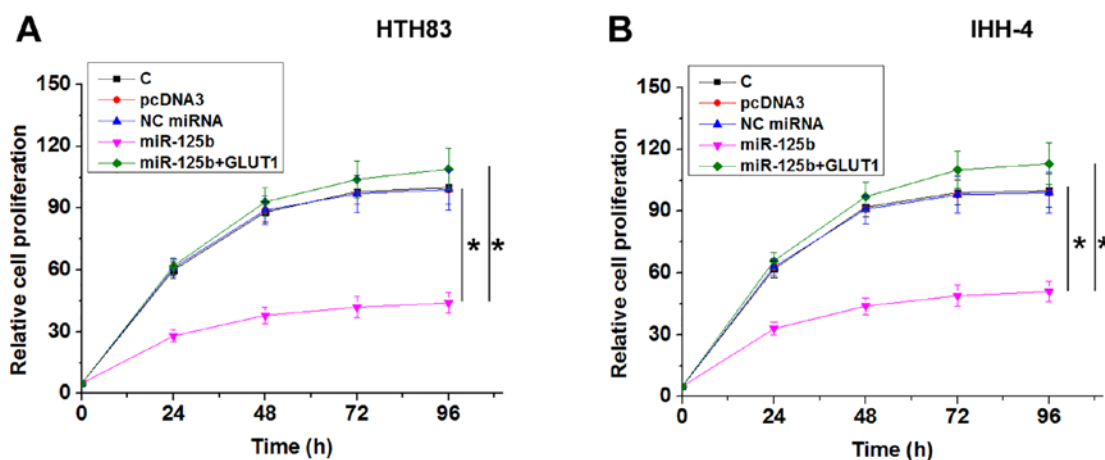

Figure 4. miR-125b suppresses the proliferation of (A) HTH83 and (B) IHH-4 PTC cells by downregulating GLUT1. Compared with NC and C groups, miR-125b overexpression resulted in decreased, while GLUT1 overexpression resulted in increased proliferation rate of PTC cells. GLUT1 overexpression reduced the effects of miR-125b overexpression on cancer cell proliferation. *P<0.05. GLUT1, glucose transporter 1; miR, microRNA; PTC, papillary thyroid carcinoma; NC, negative control; C, control.

miR-125b has been characterized as a tumor-suppressive miRNA or oncogenic miRNA in different types of cancer. In ovarian cancer, miR-125b is downregulated and the overexpression of miR-125b suppresses the proliferation of cancer cells by directly targeting BCL3 (14). In invasive breast cancer, miR-125b is methylated, while the transcription of miR-125b gene downregulates the expression of oncogenic ETS1, thereby inhibiting cancer progression (15). In contrast, Shi et al (16)

established a xenograft prostate tumor model to study the roles of miR-125b in prostate cancer, and found that miR-125b inhibited the expression of pro-apoptotic genes in prostate cancer cell and promoted the growth of tumor, indicating that miR-125b played an oncogenic role in prostate cancer. To the best of our knowledge, the involvement of miR-125b in PTC and other types of TC is still unknown. The present study found that miR-125b was downregulated in PTC and that the

upregulation of miR-125b led to decreased glucose uptake in PTC cells and inhibited PTC cell proliferation. Therefore, miR-125b has tumor-suppressive roles in PTC.

The inhibition of glucose metabolism is an effective approach to inhibiting cancer progression. It is known that certain tumor-associated miRNAs can regulate glucose metabolism to participate in cancer biology (17-19). In the present study, it was shown that miR-125b can downregulate GLUT1 to suppress glucose uptake in PTC cells and inhibit cell proliferation. Therefore, overexpression of miR-125b may serve as a potential therapeutic target to inhibit the growth of PTC tumors; however, clinical trials are needed to test this conclusion. In addition, the mechanism that mediates the downregulation of GLUT1 by miR-125b is unclear. In the present study, a significant correlation between GLUT-1 and miR-125b was observed across PTC tissues, however not across normal tissues. Therefore, the interaction between GLUT-1 and miR-125b is indirect and may be mediated by certain PTC-associated pathological factors; however, the factors involved in this interaction have not been identified. Thus, further studies are still required. The present study also showed that the altered expression of miR-125b can be used to assist the diagnosis of HCC. However, future studies with larger sample sizes are needed to further confirm these conclusions.

In conclusion, miR-125b is downregulated in PTC and miR-125b overexpression may inhibit the growth of PTC by suppressing glucose uptake, by downregulating GLUT1.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GZ: experimental work, data analysis, and manuscript writing. SHZ and QY: experiment work, literature research, and data analysis. FL: study design, research concept and manuscript editing. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval was obtained from the Ethics Committee of Yongchuan Hospital of Chongqing Medical University (approval no. YCH201611063565CQMU). All the patients signed informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2015. CA Cancer J Clin 65: 5-29, 2015.
2. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F: Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136: E359-E386, 2015.
3. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ and He J: Cancer statistics in China, 2015. CA Cancer J Clin 66: 115-132, 2016.
4. Rosenbaum MA and McHenry CR: Contemporary management of papillary carcinoma of the thyroid gland. Expert Rev Anticancer Ther 9: 317-329, 2009.
5. Brito JP, Hay ID and Morris JC: Low risk papillary thyroid cancer. BMJ 348: g3045, 2014.
6. Annibaldi A and Widmann C: Glucose metabolism in cancer cells. Curr Opin Clin Nutr Metab Care 13: 466-470, 2010.
7. Cairns RA, Harris IS and Mak TW: Regulation of cancer cell metabolism. Nat Rev Cancer 11: 85-95, 2011.
8. Kraegen EW, Sowden JA, Halstead MB, Clark PW, Rodnick KJ, Chisholm DJ and James DE: Glucose transporters and in vivo glucose uptake in skeletal and cardiac muscle: Fasting, insulin stimulation and immunoisolation studies of GLUT1 and GLUT4. Biochem J 295: 287-293, 1993.
9. Ciavardelli D, Bellomo M, Consalvo A, Crescimanno C and Vella V: Metabolic alterations of thyroid cancer as potential therapeutic targets. Biomed Res Int 2017: 2545031, 2017.
10. Yamasaki T, Seki N, Yoshino H, Itesako T, Yamada Y, Tatarano S, Hidaka H, Yonezawa T, Nakagawa M and Enokida H: Tumor-suppressive microRNA-1291 directly regulates glucose transporter 1 in renal cell carcinoma. Cancer Sci 104: 1411-1419, 2013.
11. Huang YF, Zhang Y, Liu CX, Huang J and Ding GH: microRNA-125b contributes to high glucose-induced reactive oxygen species generation and apoptosis in HK-2 renal tubular epithelial cells by targeting angiotensin-converting enzyme 2. Eur Rev Med Pharmacol Sci 20: 4055-4062, 2016.
12. Lang BHH, Lo CY, Chan WF, Lam KY and Wan KY: Staging systems for papillary thyroid carcinoma: A review and comparison. Ann Surg 245: 366-378, 2007.
13. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
14. Guan Y, Yao H, Zheng Z, Qiu G and Sun K: miR-125b targets BCL3 and suppresses ovarian cancer proliferation. Int J Cancer 128: 2274-2283, 2011.
15. Zhang Y, Yan LX, Wu QN, Du ZM, Chen J, Liao DZ, Huang MY, Hou JH, Wu QL, Zeng MS, et al: miR-125b is methylated and functions as a tumor suppressor by regulating the ETS1 proto-oncogene in human invasive breast cancer. Cancer Res 71: 3552-3562, 2011.
16. Shi XB, Xue L, Ma AH, Tepper CG, Kung HJ and White RW: miR-125b promotes growth of prostate cancer xenograft tumor through targeting pro-apoptotic genes. Prostate 71: 538-549, 2011.
17. Fang R, Xiao T, Fang Z, Sun Y, Li F, Gao Y, Feng Y, Li L, Wang Y, Liu X, et al: MicroRNA-143 (miR-143) regulates cancer glycolysis via targeting hexokinase 2 gene. J Biol Chem 287: 23227-23235, 2012.
18. Xiao X, Huang X, Ye F, Chen B, Song C, Wen J, Zhang Z, Zheng G, Tang H and Xie X: The miR-34a-LDHA axis regulates glucose metabolism and tumor growth in breast cancer. Sci Rep 6: 21735, 2016.
19. Chen B, Liu Y, Jin X, Lu W, Liu J, Xia Z, Yuan Q, Zhao X, Xu N and Liang S: MicroRNA-26a regulates glucose metabolism by direct targeting PDHX in colorectal cancer cells. BMC Cancer 14: 443, 2014.