Am J Transl Res 2019;11(1):361-369
www.ajtr.org /ISSN:1943-8141/AJTR0082995

Original Article

MicroRNA-365 suppressed cell proliferation and migration via targeting PAX6 in glioblastoma

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Received June 10, 2018; Accepted October 28, 2018; Epub January 15, 2019; Published January 30, 2019

Abstract: MicroRNAs (miRNAs) act an important role in the progression of tumor. In this study, we showed that the serum expression of miR-365 was downregulated in the glioblastoma compared with in the healthy controls. We also demonstrated that miR-365 expression was downregulated in glioblastoma tissues compared with the adjacent normal tissues. Overexpression of miR-365 suppressed the glioblastoma cell proliferation and migration. Moreover, ectopic expression of miR-365 promoted the expression of Ecadherin while inhibited the expression of N-cadherin and Vimentin in U87 cell. Furthermore, we identified PAX6 as a direct target gene of miR-365 in U87 cell. Overexpression of miR-365 suppressed glioblastoma cell proliferation and migration and epithelial-to-mesenchymal transition through inhibiting PAX6 expression. These results suggested that miR-365 played a tumor suppressor in glioblastoma.

Keywords: Glioblastoma, microRNAs, miR-365, PAX6

Introduction

Gliomas are highly aggressive brain tumor with a high mortality and relapse rate [1-5]. Gliomas comprise of six subtypes including anaplastic astrocytoma, glioblastoma, anaplastic oligodendroglioma, astrocytoma, and malignant glioma [6-8]. Unfortunately, the prognosis of these tumors is not significantly improved during the last four decades because of no effective therapies [8-11]. Therefore, it is useful to search new early detection markers to improve diagnosis and treatment of gliomas.

MicroRNAs (miRNAs) are a class of non-coding, typically 22 nucleotides, and single-stranded and endogenous RNAs molecules [12-14]. MiRNAs play important role in the regulation of gene expression by binding to the 3'UTR (3'untranslated region) of mRNA and inhibiting mRNA expression or/and inducing mRNA degradation [15-17]. Increasing evidences have demonstrated that miRNAs are involved in many physiological processes including cell development, differentiation, migration and proliferation [18-20]. Recent research has indicated that deregulated miRNAs can be found in many tumors such as gastric, breast, ovarian, lung and bladder cancer and are involved in the development of these cancers [21-25]. In addition, cell-free, highly stable miRNAs have been identified in human serum [1, 16, 26]. Previous studies indicated that serum miRNAs might be a novel non-invasive diagnostic maker for cancers [26-28]. Previous studies suggested that miR-365 acted crucial roles in the development of tumors [29-32]. For instance, Nie et al. [33]. reported that miR-365 expression was downregulated in colon cancer samples compared to non-neoplastic mucosa tissues. Overexpression of miR-365 inhibited colon cell cycle progression, promoted cell apoptosis and suppressed tumorigenicity in the colon cancer cell via targeting Bcl-2 and Cyclin D1 expression. Sun et al. [34]. Showed that miR-365 expression was downregulated in non-small cell lung cancer (NSCLC) than that in normal tissue. Bai et al. [30]. also found that the expression of miR-365 was downregulated in malignant melanoma cell lines and tissues. Ectopic expression of miR-365 inhibited malignant melanoma cell prolif-

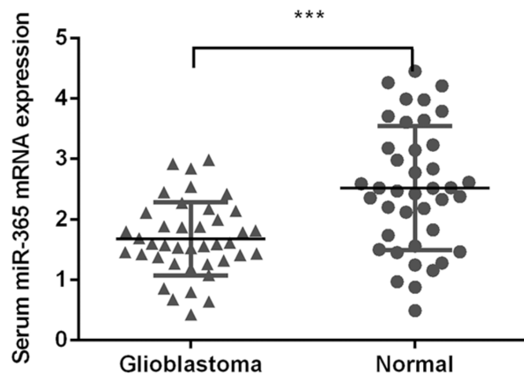

Figure 1. Serum expression level of miR-365 was downregulated in the glioblastoma. The serum expression of miR-365 in the glioblastoma and healthy controls was detected using qRT-PCR. ***P < 0.001. Statistical analysis was performed using Mann-Whitney U tests.

eration and metastasis via regulating NRP1 expression. Recently, Liu et al. [35]. showed that the serum expression of miR-365 was downregulated in the NSCLC patients compared to that in healthy serum volunteers. However, the expression and role of miR-365 in the glioblastoma were still unknown.

In this study, we demonstrated that miR-365 expression was downregulated in the serum of glioblastoma compared to the healthy controls. Moreover, miR-365 expression was also lower in the in the glioblastoma tissue compared with the adjacent normal tissues. Overexpression of miR-365 suppressed the glioblastoma cell proliferation, migration and epithelial-to-mesenchymal transition (EMT).

Materials and methods

Sample and tissue collection

This research was approved by the ethics committee of The 2nd Affiliated Hospital, Harbin Medical University and all samples were contained from glioblastoma's patients or control with written informed consents. Serum samples or tissues were collected from glioblastoma's patients and healthy volunteers at our department between 2012 and 2014. None of the glioblastoma patients were received radiotherapy or chemotherapy before surgery.

qRT-PCR

Total RNA was isolated from serums, tissuse and cells using Trizol (Invitrogen, USA) following to instruction's information. The expression of miR-365 was measured using qRT-PCR and qRT-PCR was performed on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, USA). The expression level of miR-365 was normalized to the endogenous snRNA U6. GAPDH was used to as the control for mRNA expression. The primers sequences were shown following: GAPDH forward, 5'-ACAACT TTGGTATCGTGGAAGG-3', and reverse, 5'-GCCATCACGCCA CAGTTTC-3'; PAX6 forward, 5'-AACGATAACATACCAAGC GTGT-3', and reverse, 5'-GGTCTGCCCGTTCAACATC-3'; Vimentin Forword: 5'-GAAGAGGTTAGTGGAGTGA-3', Reverse: 5'-TGCTGTTCCTGAATCTGA-3'.

Cell lines cultured and tranfection

Four glioblastoma cell lines (U373, U87, A172 and U251) and one normal astrocyte line (NHAs) were collected from the American Tissue Culture Colection (ATCC) and was cultured in DMEM medium. miR-365 mimics and its scramble oligonucleotides were obtained from RiboBio (Guangzhou, China). Cells were transfected with the miR-365 mimic and scramble mimic, pcDNA-PAX6 and control using Lipofectamine 2000 (Invitrogen, USA) following to the manufacturer's information.

Cell growth and migration assay

Cells were cultured in the 96-well plate and the cells proliferation ratio was measured with CCK-8 (Cell Counting Kit-8, Dojindo, Japan) following to the manufacturer's information. Wound healing assay was performed to detect the cell migration. The wound was made using the Petri dishbottom. Cells were continued to culture for 48 hours and the migration rate was detected through measuring the wound distance.

Western blot

Total protein was extracted from cells in lysis buffer. Proteins were separated using electrophoresis on SDS-PAGE gels and transferred to nitrocellulose membranes (Pall Corp, NY, USA). The following antibodies were used as follows: PAX6, GAPDH, E-cadherin, N-cadherin and Vimentin (dilutions 1:1000, Abcam). Protein was visualized by an enhanced chemiluminescence (Amersham, UK).

Statistical analysis

Data was shown as mean ± SD (standard deviation). The differences between two groups were measured using Student's t test and one-way ANOVA was performed to measure the differ-

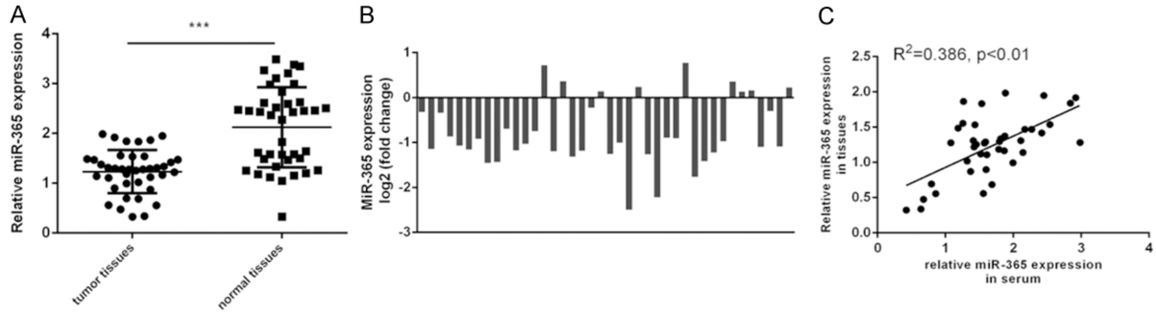

Figure 2. Expressionl level of miR-365 was decreased in the glioblastoma tissue. A. The expression of miR-365 was downregulated in the glioblastoma tissue compared to the adjacent normal tissues. Statistically significant difference was determined using Student's t test. B. The miR-365 expression in 31 cases (31/40; 77%) compared to adjacent tissues. C. The expression of miR-365 in glioblastoma tissue was associated with those in glioblastoma patients' serum. ***P < 0.001. Association between miR-365 expression in serum and matched glioma tissues was detected by Spearman correlation test.

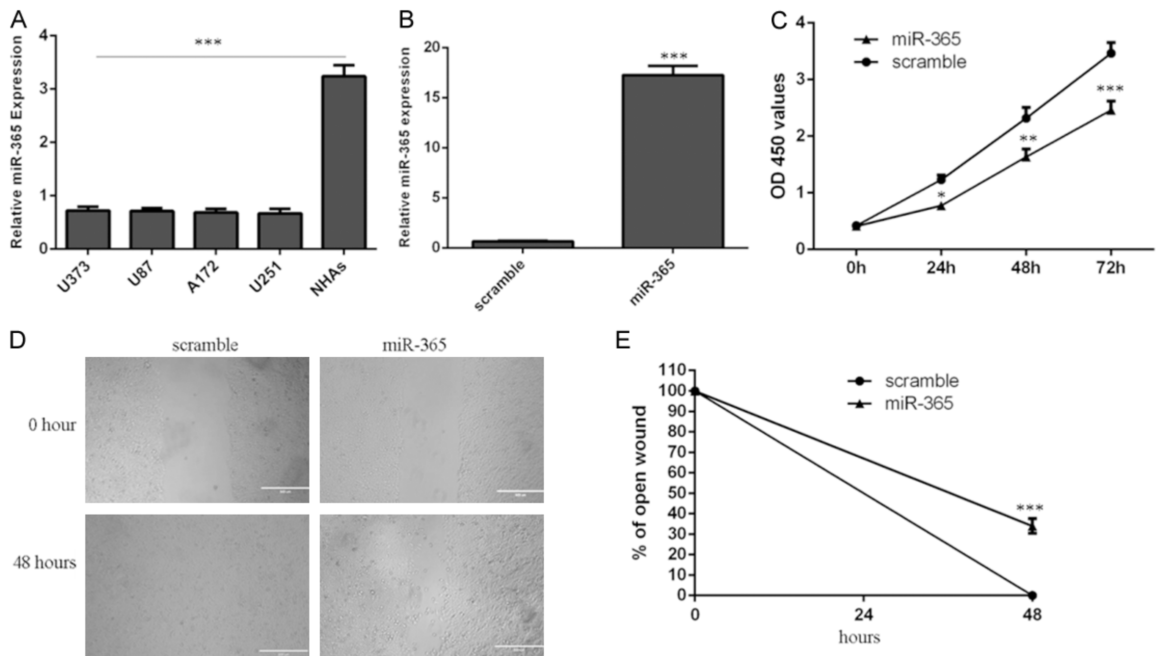

Figure 3. miR-365 suppressed the glioblastoma cell proliferation and migration. A. The expression of miR-365 in the glioblastoma cell lines (U373, U87, A172 and U251) and one normal astrocyte line (NHAs) was detecte using qRT-PCR. B. The expression of miR-365 was measured by qRT-PCR in the U87 cell. C. Overexpression of miR-365 suppressed the U87 cell proliferation. D. Overexpression of miR-365 suppressed the U87 cell migration. E. The relative open wound was shown. *P < 0.05, **P < 0.01 and ***P < 0.001. Statistically significant difference was determined using Student's t test.

ences between more than two groups. P < 0.05 was defined to be statistically significant.

Result

Serum expression level of miR-365 was downregulated in the glioblastoma

We firstly measure the serum expression level of miR-365 in glioblastoma and healthy controls. The serum expression of miR-365 was downregulated in glioblastoma compared with the healthy controls (**Figure 1**).

Expression level of miR-365 was decreased in glioblastoma tissue

The expression of miR-365 was downregulated in glioblastoma tissue compared to the adjacent normal tissues (**Figure 2A**). Among these

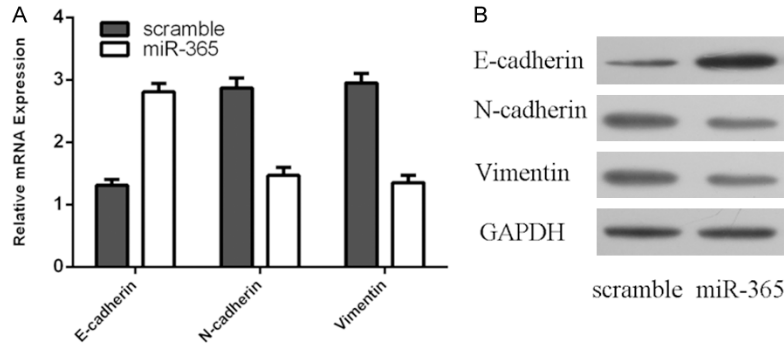

Figure 4. miR-365 inhibited the glioblastoma cell epithelial-to-mesenchymal transition. A. The mRNA expression of Ecadherin, N-cadherin and Vimentin was measured by qRT-PCR. B. The protein expression of Ecadherin, N-cadherin and Vimentin was detected by western blot.

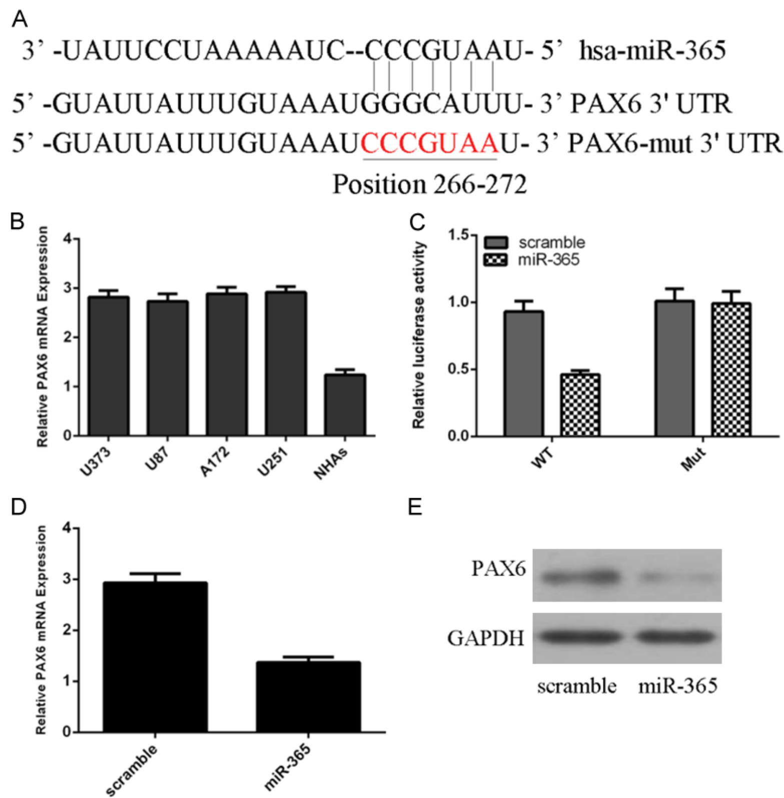

Figure 5. PAX6 was a direct a target gene of miR-365 in the glioblastoma cell. A. One putative binding sites of the miR-365 in the 3'UTR of PAX6 are shown. B. The expression of PAX6 in the glioblastoma cell lines (U373, U87, A172 and U251) and one normal astrocyte line (NHAs) was measured using qRT-PCR. C. The luciferase activity was decreased in the U87 cell co-transfected with miR-365 mimic and PAX6-3'UTR-WT luciferase reporter. D. Ecoptic expression of miR-365 suppressed the PAX6 mRNA expression. E. Overexpression of miR-365 inhibited the protein pression of PAX6.

patients, miR-365 expression was downregulated in 31 cases (31/40; 77%) compared with adjacent tissues (**Figure 2B**). In addition, the expression of miR-365 in glioblastoma tissue was positively associated with the expression of miR-365 in the serum of glioblastoma patients (**Figure 2C**).

miR-365 suppressed the glioblastoma cell proliferation and migration

miR-365 expression was downregulated in the glioblastoma cell lines (U373, U87, A172 and U251) compared with one normal astrocyte line (NHAs) (**Figure 3A**). The U87 cell was transfected with miR-365 mimic, qRT-PCR data showed that miR-365 mimic promoted the expression of miR-365 in the U87 cell (**Figure 3B**). Overexpression of miR-365 inhibited the U87 cell proliferation (**Figure 3C**). Moreover, miR-365 overexpression suppressed U87 cell migration (**Figure 3D** and **3E**).

miR-365 inhibited the glioblastoma cell epithelial-to-mesenchymal transition

Ectopic expression of miR-365 promoted the mRNA expression of Ecadherin and suppressed the mRNA expression of N-cadherin and Vimentin mRNA in the U87 cell (**Figure 4A**). In line with this, overexpression of miR-365 increased the Ecadherin protein expression and suppressed the N-cadherin and Vimentin protein expression in the U87 cell (**Figures 4B** and S1).

PAX6 was a direct a target gene of miR-365 in the glioblastoma cell

Targetscan was used to found the potential target gene of miR-365. One putative binding sites of the miR-365 in the 3'UTR of PAX6 are shown

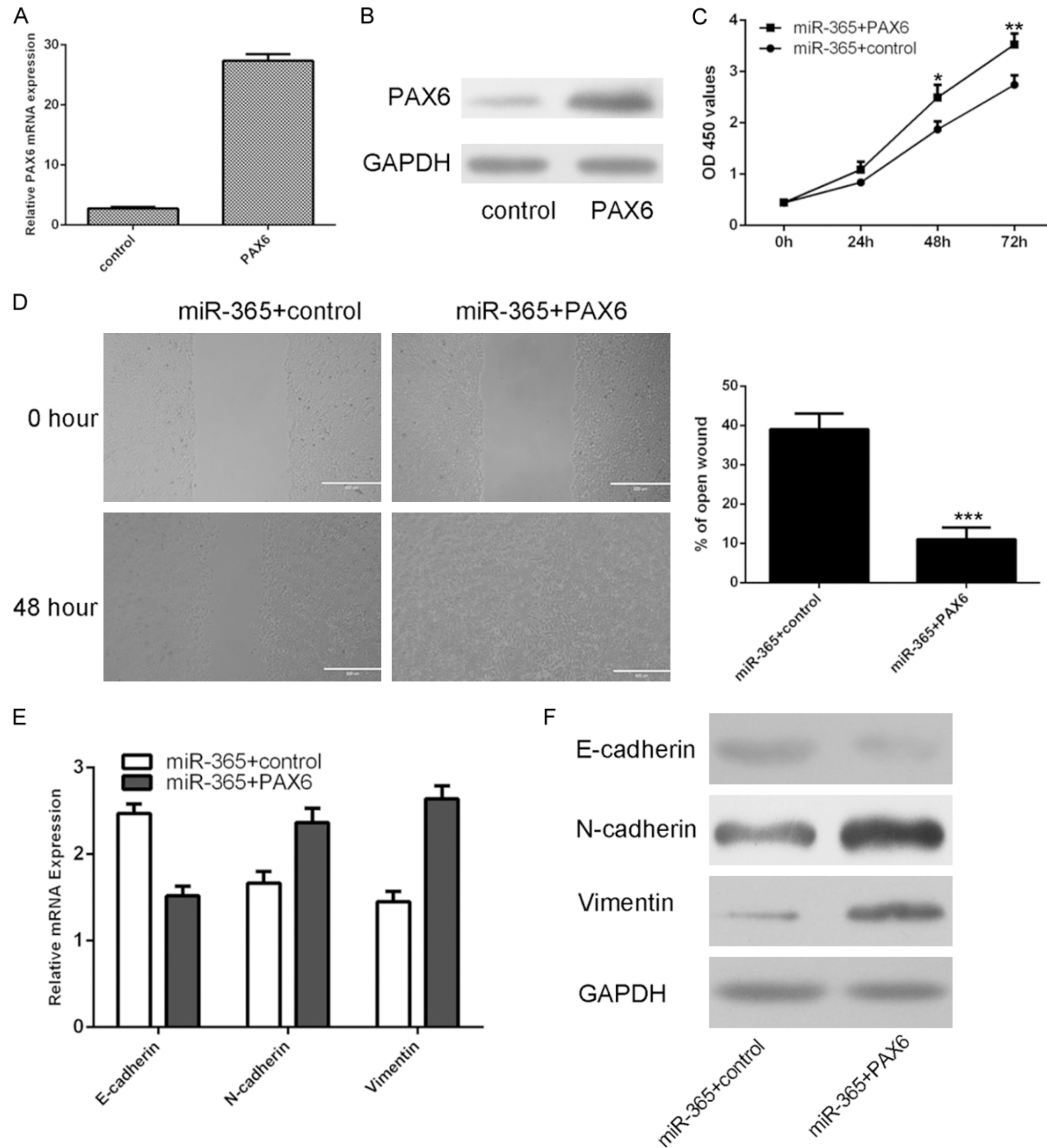

Figure 6. miR-365 suppressed glioblastoma cell proliferation and migration by inhibiting PAX6. A. The mRNA expression of PAX6 was determined by using qRT-PCR. B. The protein expression of PAX6 was detected by using western blot. C. PAX6 overexpression promoted the miR-365-overexpressing U87 cell proliferation. D. PAX6 Overexpression abrogated the reduction of migration ability caused by ectopic expression of miR-365 in U87 cells. The relative migrative wound was shown. E. The mRNA expression of E-cadherin, N-cadherin and vimentin was determined by qRT-PCR. F. The protein expression of E-cadherin, N-cadherin and vimentin was determined by western blot. *P < 0.05, **P < 0.01 and ***P < 0.001. Statistically significant difference was determined using Student's t test.

in **Figure 5A**. PAX6 expression was upregulated in glioblastoma cell lines (U373, U87, A172 and U251) compared to one normal astrocyte line (NHAs) (**Figure 5B**). A luciferase reporter assay was conducted to confirm this prediction in U87 cell. The luciferase activity was decreased in the U87 cell co-transfected with miR-365 mimic and PAX6-3'UTR-WT luciferase reporter; however, this effect was abolished in the U87 cell co-transfected with PAX6-3'UTR-MUT lucif-

erase reporter and miR-365 mimic (**Figure 5C**). Overexpression of miR-365 suppressed the PAX6 expression (**Figures 5D**, **5E** and S2).

miR-365 suppressed glioblastoma cell proliferation and migration by inhibiting PAX6

The mRNA expression of PAX6 was upregulated in the U87 cell after treated with pcDNA-PAX6 vector (**Figure 6A**). The protein expression of PAX6 was also increased in the U87 cell after treated with pcDNA-PAX6 vector (**Figure 6B**). We restored PAX6 expression through into the miR-365-overexpressing U87 cells by transfecting the pcDNA-PAX6 vector. We demonstrated that PAX6 overexpression promoted the miR-365-overexpressing U87 cell proliferation (**Figure 6C**). PAX6 Overexpression abrogated the reduction of migration ability caused by ectopic expression of miR-365 in U87 cells (**Figure 6D**). qRT-PCR and western blot assay demonstrated that epithelial marker (E-cadherin) was decreased, whereas mesenchymal markers (N-cadherin and vimentin) was increased accompanied with restoration of PAX6 (**Figures 6E** and **6F**, S3).

Discussion

In this study, we showed that the serum expression of miR-365 was downregulated in the glioblastoma compared with healthy controls. We further measured the expression of miR-365 in glioblastoma tissues. We demonstrated that miR-365 expression was downregulated in the glioblastoma tissue compared with the adjacent normal tissues. Interestingly, we also found that miR-365 expression in glioblastoma tissue was associated with those in glioblastoma patients' serum. Overexpression of miR-365 inhibited the U87 cell proliferation and migration. Moreover, ectopic expression of miR-365 promoted the expression of Ecadherin and suppressed the expression of N-cadherin and Vimentin in the U87 cell. These results suggested that overexpression of miR-365 inhibited glioblastoma cell epithelial-to-mesenchymal transition. Furthermore, we identified PAX6 as direct target gene of miR-365 in the U87 cell. Overexpression of miR-365 suppressed glioblastoma cell proliferation and migration and epithelial-to-mesenchymal transition by inhibiting PAX6 expression. These results suggested that miR-365 played a tumor suppressor role through inhibiting PAX6 expression in glioblastoma.

Previous studies demonstrated that miR-365 played crucial roles in the initiation and development of cancers [29-32]. For example, Nie et al. [33] Showed that miR-365 expression was downregulated in colon cancer tissues compared with non-neoplastic mucosa tissues. Restored expression of miR-365 suppressed colon cell cycle progression, increased cell apoptosis and inhibited tumorigenicity in the colon cancer cell through targeting Bcl-2 and Cyclin D1 expression. Sun et al. [34]. demonstrated that miR-365 expression was lower in non-small cell lung cancer (NSCLC) than that in normal tissue. Bai et al. [30] also found that miR-365 expression was decreased in malignant melanoma cell lines and tissues. Ectopic expression of miR-365 suppressed malignant melanoma cell proliferation and metastasis through regulating NRP1 expression. However, Zhou et al. [36] demonstrated that miR-365 expression was upregulated in cutaneous squamous cell carcinoma (CSCC) tissues and miR-365 promoted CSCC development through regulating the expression of Nuclear Factor I/B (NFIB). Recently, Liu et al. [35] showed that the serum expression of miR-365 was downregulated in the NSCLC patients compared with that in healthy serum volunteers. However, the expression and role of miR-365 in the glioblastoma were still unknown. In our study, we showed that the serum expression of miR-365 was downregulated in the glioblastoma compared with healthy controls. We further measured the expression of miR-365 in the glioblastoma tissues. We demonstrated that miR-365 expression was downregulated in glioblastoma tissue compared with the adjacent normal tissues. Among these patients, the miR-365 expression was downregulated in 31 cases (31/40; 77%) compared with adjacent tissues. Moreover, the expression of miR-365 in glioblastoma tissue was associated with the expression of miR-365 in glioblastoma patients' serum. Overexpression of miR-365 inhibited the U87 cell proliferation and migration. Moreover, ectopic expression of miR-365 promoted the expression of Ecadherin and suppressed the expression of N-cadherin and Vimentin in the U87 cell. These results suggested that miR-365 acted as a potential tumor suppressor gene in the development of glioblastoma.

PAX6 is one member of the paired box (PAX) families, which is located on the chromosome

11p13 and plays a crucial role in the development of neuroectodermal epithelial tissues [37-39]. Recent studies showed that PAX6 existed in tumor tissues and acted an important role in the development of cancer [40, 41]. For example, Xia et al. [40] demonstrated that PAX6 expression was upregualted in invasive ductal breast cancer and higher expression of PAX6 was associated with poor prognosis in breast cancer patients. Meng et al. [42] showed that miR-335 suppressed cell proliferation, colony formation, cell-cycle progression, and invasion through inhibiting PAX6 expression in breast cancer cells. Luo et al. [43] demonstrated that miR-7 suppressed the cell proliferation and invasion in non-small cell lung cancer cell through targeting PAX6 expression. In line with these data, we showed that PAX6 was a direct a target gene of miR-365 in glioblastoma cell.

In conclusion, we demonstrated that miR-365 expression was downregulated in the glioblastoma compared with healthy controls. The expression of miR-365 was also lower in the in the glioblastoma tissue compared with the adjacent normal tissues. Overexpression of miR-365 suppressed the glioblastoma cell proliferation, migration and EMT partly through targeting PAX6 expression. These results suggested that miR-365 played a tumor suppressor role in glioblastoma.

Acknowledgements

First prize of Heilongjiang Postdoctoral Science Foundation (BS142785). Second prize of China Postdoctoral Science Foundation (2014M561373). Foundation for Returness of Ministry of Education of China. Doctoral Fund of The second affiliated to Harbin Medical University (BS2012-18). The Since Returning Foundation of Heilongjiang Province (LC2013C40).

Disclosure of conflict of interest

None.

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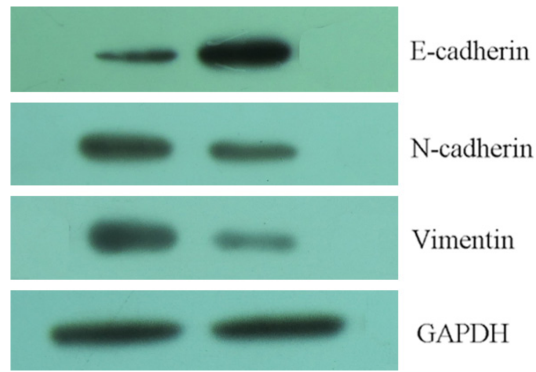

Figure S1. The western images of **Figure 4**.

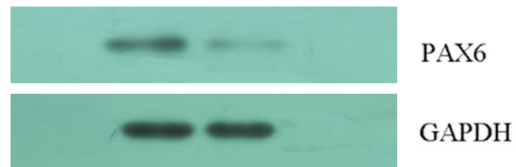

Figure S2. The western images of **Figure 5**.

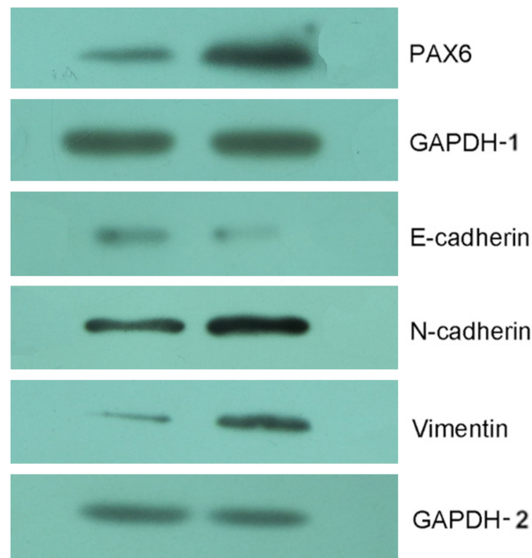

Figure S3. The western images of **Figure 6**.