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Original Research Article

Up-regulation of miR-30b suppresses glioblastoma by negatively regulating MEF2D through Wnt/β-catenin signaling pathway

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Abstract

Purpose: *To study miR-30b' significance on glioblastoma, and its underlying mechanism of action.*
Methods: *Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) while 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di- phenytetrazoliumromide (MTT), Transwell, and xenograft tumor formation assays were carried out to study miR-30b's effect on glioblastoma while luciferase reporter assay was employed to study the interaction between MEF2D and miR-30b. Glioblastoma cells treatment with miR-30 mimic or inhibitor were subjected to Western blot assay to study the effect of Wnt/β-catenin signaling on miR-30b/MEF2D axis-mediated cell progression.*
Results: *MiR-30b was lowly expressed in glioblastoma tissues (p = 0.007), and this was associated with poor prognosis of patients (p = 0.022). The direct target of miR-30b was identified as MEF2D (p = 0.036). Increasing miR-30b blocked MEF2D expression in glioblastoma cells (p = 0.029). Moreover, MEF2D overturned miR-30b' inhibitory effect on glioblastoma cell progression (p = 0.041; p = 0.006; p = 0.037). In vivo, restoration of miR-30b inhibited tumor growth (p = 0.01) and MEF2D. Interestingly, restoration of miR-30b inhibited epithelial-to-mesenchymal transition (EMT) and Wnt/β-catenin signaling pathways.*
Conclusion: *These results indicate the critical role of miR-30b/MEF2D axis in glioblastoma via EMT and Wnt/β-catenin pathways. Thus, the miR-30b/MEF2D axis might be a beneficial therapeutic target for the management of glioblastoma patients.*

Keywords: *MiR-30b, MEF2D, Glioblastoma, Wnt/β-catenin, EMT*

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INTRODUCTION

Glioblastoma is a common type of brain tumor with a high incidence [1]. Although significant progress has been made in the treatment of glioblastoma, the disease is still a big problem in clinical practice due to its poor prognosis. Therefore, it is necessary to seek innovative and specific therapeutic methods for the disease.

Accumulating evidence indicate that miRNAs are abnormally expressed in many malignancies, including glioblastoma. For example, miR-29a was reported to be lowly expressed in glioblastoma, resulting in suppression of the

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disease via TRAF4/AKT signaling pathway [2]. Increasing miR-785 repressed glioblastoma cell proliferation and metastasis by targeting ZBTB20 [3]. In glioblastoma, miR-30b was reported to be decreased, while restoration of miR-30b suppressed cell proliferation and apoptosis [4,5]. However, miR-30b' mechanism in glioblastoma is still unclear.

Myocyte enhancer factor 2D (MEF2D), a transcription factor, belongs to the MEF2 family [6] which has been showed to be involved not only in muscle development but also in tumor suppression, initiation and progression [7]. Increasing evidence show that MEF2D is up-regulated in multiple cancers, and is linked to the etiology and prognosis of tumors [8,9]. However, its regulatory effect on glioblastoma has not been reported. This study was carried out to study miR-30b' functional role on glioblastoma, and the molecular mechanism involved.

EXPERIMENTAL

Tissue samples

Forty-four glioblastoma tissues were obtained from glioblastoma patients undergoing a surgical procedure at Weihai Central Hospital, Weihai, Shandong, China. All participants included in this study received neither radiotherapy nor chemotherapy before surgery. The tissues were taken from the pathology department and stored at –80 °C in a refrigerator until RNA isolation. Before specimen collection, the written informed consent forms were signed by all participates participated in the study. The procedures used in this research were approved by the ethic committee of Weihai Central Hospital (approval no. 2017-014). All procedures are carried out according to the "Declaration of Helsinki" [10].

Cell lines and transfection

The glioblastoma cell lines (U251, T98G, A172 and LN18) and primary normal human astrocytes NHA cells were cultured following the instructions of manufacturer. Cells were incubated at 37 °C under 5 % CO_2 atmosphere.

All transfection procedures were conducted with the aid of Lipofectamine 2000™ reagent (Invitrogen). Glioblastoma cells were transfected with miR-30b mimic/inhibitor to force or silence miR-30b expression, respectively.

qRT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen). The RNA was reverse-transcribed to cDNA with All-in-OneTM miRNA First-Strand cDNA Synthesis Kit. The quantification of all gene transcripts was conducted using TaqMan PCR kit with the method of 2™ΔΔCt.

Western blot

The western blot was performed as described previously [11]. Briefly, proteins were separated and then transferred. Subsequently, they were blocked with 5 % skim milk, followed by primary antibodies and secondary antibodies. The loading control used was GADPH.

MTT assay

Glioblastoma cells were plated in 96-well plate. 20 uL MTT medium was added when cultured for 1, 2, 3, and 4 days. 100 uL dimethyl sulfoxide (DMSO) was then added and incubation for an additional 10 min. The absorbance of each well was detected at 490 nm wavelength.

Transwell assay

Transwell chamber without Matrigel precoating was applied for measuring cell migratory ability, while Transwell chamber precoated with Matrigel was used to determine cell invasive ability. Glioblastoma cells migrated or invaded from the upper to the bottom chamber were stained, and then photographed by a microscope.

Xenograft assays in athymic nude mice

Xenograft tumor formation assay was applied for measuring tumor growth *in vivo*. The U251 cells treatment with miR-30b mimic were added to culture medium (100 µL) and then 5×10^6 cells were injected into the nude mice' right flank (n = 8). A vernier caliper or electronic scale was used to measure the xenograft tumor size or tumor weight, respectively, every 4 days. The mice were sacrificed after 28 days after inoculation.

Luciferase reporter assay

Wild or mutant type of MEF2D-3'UTR fragment was amplified and then cloned into pGL3-reporter vectors. The MiR-30b mimic and pGL3-reporter vectors were co-transfected into U251 cells for 48 h. Luciferase activity was measured by Dual-Luciferase Reporter Assay System.

Statistical analysis

Data are presented as mean ± standard deviation (SD) in three experiments. Student's *t*-test or Tukey's post hoc test was employed to compare differences between groups. All

statistical analysis were done using SPSS 17.0 statistical software and GraphPad Prism 5. Values of $p < 0.05$ were regarded as indicated of statistically significant differences.

RESULTS

MiR-30b expression was decreased in glioblastoma

RT-qPCR was applied to measure miR-30b level in glioblastoma. Results displayed that its mRNA level in glioblastoma tissues was obviously decreased, relative to normal tissues (Figure 1 A). Moreover, miR-30b was decreased in all glioblastoma cells versus to normal cells (NHA) (Figure 1B). Furthermore, results of Kaplan-Meier survival curve revealed that miR-30b high expression was linked to high percentage survival of glioblastoma patients, whereas miR-30b low expression was associated with low survival rate of glioblastoma patients (Figure 1 C). Thus, miR-30b might be an indicator of the prognosis of glioblastoma patients.

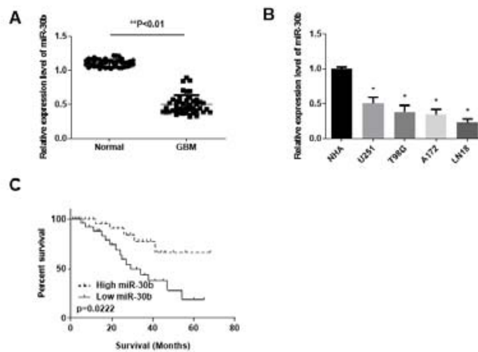

Figure 1: miR-30b expression in glioblastoma. (A) miR-30b was under-expressed in glioblastoma tumor tissues. (B) miR-30b expression was down-regulated in glioblastoma cells (U251, T98G, A172, and LN18), when compared to normal cells (NHA). (C) Percentage survival of patients with high or low miR-30b expression. $^{*}P < 0.05$; $^{**}p < 0.01$

MEF2D was a target of miR-30b in glioblastoma

TargetScanHuman was applied to predict the possible target of miR-30b in glioblastoma cells. As shown in Figure 2A, there have binding sites of miR-30b with MEF2D. To verify whether MEF2D was indeed regulated by miR-30b, luciferase reporter assay was applied in U251 cells. The luciferase activity of MEF2D in wild type was remarkably inhibited by miR-30b mimic in U251 cells, whereas there was no significant difference in mutant type (Figure 2B). Moreover, miR-30b was over-expressed or under-expressed by miR-30b mimic or inhibitor, respectively, in U251 cells (Figure 2C). Western blotting and RT-PCR analysis demonstrated that over-expression of miR-30b inhibited MEF2D expression, while MEF2D expression was raised by miR-30b inhibitor (Figure 2D). Next, results discovered that MEF2D was increased in glioblastoma tissues, relative to normal tissues (Figure 2E). Results in Figure 2F shown that the correlation between miR-30b mRNA and MEF2D was negatively (Figure 2 F). The findings indicate that miR-30b directly targeted MEF2D.

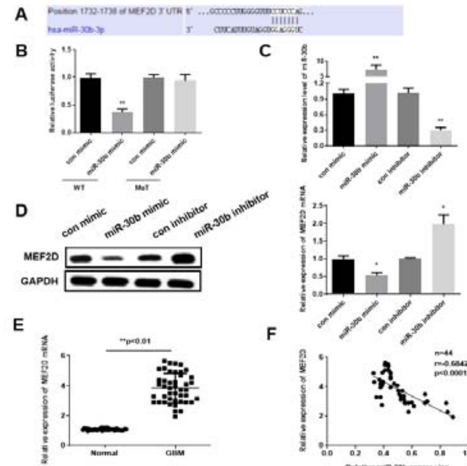

Figure 2: Identification of miR-30b' target in glioblastoma. (A) Binding sites of miR-30b and MEF2D, as predicted using TargetScan. (B) Luciferase activity of MEF2D was detected in U251 cells. (C) MiR-30b mRNA expression was measured in U251 cells. (D) The levels of MEF2D were detected in U251 cells. (E) MEF2D mRNA expression was tested in glioblastoma tissues. (F) Inverse relationship between miR-30b and MEF2D. $^{*}P < 0.05$; $^{**}p < 0.01$

MiR-30b suppressed glioblastoma cell growth by targeting MEF2D

MEF2D expression was measured in U251 and T98G cells treatment with miR-30b mimic or combined with MEF2D plasmid. As shown in Figure 3A, MEF2D expression level was decreased by miR-30b mimic, but the increases were reversed by MEF2D plasmid. Results from MTT displayed that miR-30b over-expression suppressed cell viability, while MEF2D plasmid reversed this inhibitory effect (Figure 3B). Transwell assay showed that increases in miR-30b concentration blocked glioblastoma cell invasion and migration, whereas MEF2D reversed this inhibitory effect (Figure 3 C and D). These results suggest that miR-30b suppressed glioblastoma cell progression by regulating MEF2D.

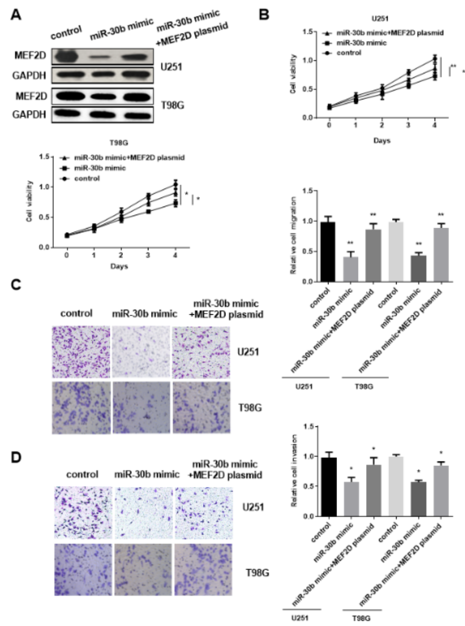

Figure 3: MiR-30b suppressed glioblastoma cells by targeting MEF2D. (A) MEF2D protein levels in glioblastoma cells after increasing miR-30b, or combination with MEF2D plasmid. (B) Comparison of cell viability in glioblastoma cells after increasing miR-30b, or combination with MEF2D plasmid. (C) Comparison of the migration of glioblastoma cells after increasing miR-30b, or combination with MEF2D plasmid. (D) Comparison of the invasion of U251 and T98G cells after increasing miR-30b, or combination with MEF2D plasmid. $^{*}P < 0.05$; $^{**}p < 0.01$

MiR-30b inhibited glioblastoma tumor growth *in vivo*

It has been found that miR-30b suppressed glioblastoma cell development *in vitro*, further investigations were done to see whether miR-30b had the same effect *in vivo*. Results revealed that miR-30b significantly reduced tumor volume, when compared to the control (Figure 4 A). Moreover, tumors with miR-30b plasmid grew more slowly than those with miR-NC (Figure 4 B). When the tumors were weighed using an electronic scale, it was found that the tumor was lighter in miR-30b mimic group (Figure 4C). In addition, western blot analysis of MEF2D displayed that miR-30b over-expression inhibited MEF2D expression *in vivo*. These findings indicate that miR-30b suppressed glioblastoma tumor growth *in vivo*.

MiR-30b overexpression blocked Wnt/β-catenin pathway in glioblastoma

Mounting evidence showed that EMT and Wnt/β-catenin pathway participated in glioblastoma development. In this study, the precise molecular mechanism involved in miR-30b' effect in glioblastoma was investigated. U251 and T98G cells were treated with miR-30b mimic or inhibitor. The results revealed that miR-30b over-expression inhibited the expressions of N-cadherin and vimentin, while it increased E-cadherin expression (Figure 5 A). Conversely, miR-30b inhibitor exhibited the opposite effect on these levels. Thus, miR-30b inhibited cell invasion and migration through EMT. Besides, protein levels of p-β-catenin, c-Myc and Cyclin D1 were detected in U251 and T98G cells so as to determine miR-30b' underlying mechanism on cell proliferation. Results displayed that these above proteins were markedly repressed by miR-30b mimic, but they were enhanced by miR-30b inhibition (Figure 5D). These observations indicate that miR-30b overexpression blocked Wnt/β-catenin pathway in glioblastoma.

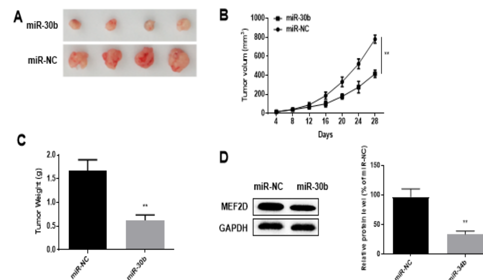

Figure 4: Effect of MiR-30b on glioblastoma growth. (A) Mass of tumor taken from nude mice every four days. (B) Tumor volumes were detected every four days. (C) Tumor weights were measured at 28 days. (D) MEF2D protein levels in tumor tissues of mice after treatment with miR-30b mimic. $^{**}P < 0.01$

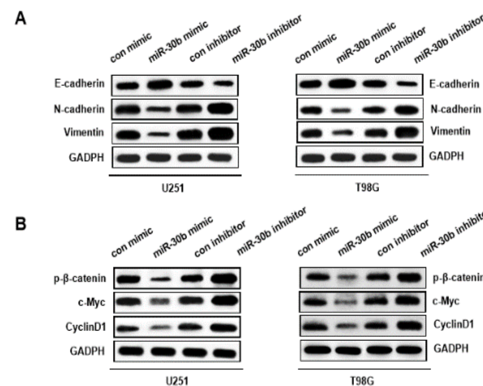

Figure 5: MiR-30b regulated EMT and Wnt/β-catenin signaling pathway in glioblastoma cells. (A) Measurement of E-cadherin, N-cadherin, vimentin levels in U251 and T98G cells after increasing or decreasing miR-30b. (B) Measurement of p-β-catenin, c-Myc and CyclinD1 levels in U251 and T98G cells after increasing or decreasing miR-30b

DISCUSSION

Glioblastoma is the most common and most malignant astrocytoma of the central nervous system [12]. Even after various treatments including radical surgery, radiation, and chemotherapy, the average survival time of glioblastoma is about one year after diagnosis. Therefore, efforts directed toward better understanding of glioblastoma are essential for the development of efficient therapies. In previous studies, various miRNAs were found to influence glioblastoma tumorigenesis, including miR-422a, miR-1268, and miR-142 [13,14].

These observations have attracted intense investigation into miRNA-based therapies for glioblastoma. In particular, MiR-30b is a well-known cancer-related miRNA which has become the hotspot of current studies. For instance, miR-30b was associated with the development of breast tumor [15]. Another study discovered a decrease expression of miR-30b in breast cancer, and that it negatively regulated cell invasion [16]. Moreover, a study discovered that miR-30b downregulation in lung cancer inhibited cell growth by targeting EGFR [17]. It is important to note that a single miRNA could regulate many target genes. For example, miR-30b attenuated gallbladder carcinoma via targeting NT5E [18]. Besides, miR-30b hampered cell proliferation and invasion in gastric cancer through regulating IGF-1R [19]. This present study found that miR-30b was lowly expressed in glioblastoma, which consistent with the prior researches reported that miR-30b was under expressed in glioblastoma [5].

Accumulating evidence suggest that MEF2D is involved in the growth of many tumors. A study reported that high expression of MEF2D in pancreatic cancer cells controlled cell growth [20]. Moreover, several studies have identified MEF2D as the target of miR-30a in osteosarcoma and lung cancer [21,22]. The present study verified that MEF2D was the target of miR-30b in regulating glioblastoma. In another study, it was reported that the upregulation of MEF2D in glioblastoma was associated with the prognosis of patients [23]. This is in line with the results that MEF2D was upregulated in glioblastoma. Moreover, it was demonstrated that MEF2D acted as miR-30b' target in inhibiting glioblastoma progression. Furthermore, the inhibitory effect of miR-30b mimic was attenuated by inhibiting the expression of MEF2D.

Wnt/β-catenin pathway shows a carcinogenic role in glioblastoma. For example, miR-505 suppressed glioblastoma through Wnt/β-catenin pathway [24]. Moreover, this signaling which is involved in glioblastoma, is regulated by miR-125b [25]. This study suggests that the Wnt/β-catenin pathway participates in the regulation of glioblastoma by miR-30b.

Limitations of the study

There were limitations in this study. First, the effect of MEF2D over-expression or knockdown on Wnt/β-catenin signaling pathway was not investigated. This would have provided a deeper insight into the mechanism at play. Secondly, the interaction of miR-30b with clinical characteristics was not considered. These areas should be investigated in future studies.

CONCLUSION

The findings of this study demonstrate that miR-33b/MEF2D is a potential alternative and effective approach for controlling abnormal proliferation and metastasis of glioblastoma cells. Thus, these findings underscore the importance of miRNA as a promising molecular tool in glioblastoma research.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jianfeng Bai, as the first author, contributed significantly to data analyses and manuscript preparation. Qingqing Yu performed data analyses and writing of the manuscript. Tongbo Ning, the corresponding author, contributed to the conceptualization of the study. All authors read and approved the final manuscript.

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