RETRACTED ARTICLE: Flot2 targeted by miR-449 acts as a prognostic biomarker in glioma

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ABSTRACT
Flotillin-2 (FLOT2) was reported as oncogene and involves in the pathogenic process of several cancers, yet the precise mechanism of FLOT2 in glioma is still limited. In this study, we demonstrated that FLOT2 expression levels were greatly upregulated in glioma tissues and cell lines, and the FLOT2 expression in glioma tissue was markedly associated with tumour stage and size. Overexpression of FLOT2 was correlated with poor prognosis of glioma patients. The functional assay revealed that silenced FLOT2 repressed the viability, migration, and invasion of glioma cells. And then, we detected the relationship between miR-449 and FLOT2. Luciferase reporter assay and Western blot results showed that miR-449 directly binding the 3’UTR sequence of FLOT2 and regulated FLOT2 expression in glioma cells. Finally, we detected the expression levels of miR-449 in glioma tissue and cell lines and found that miR-449 was significantly downregulated in glioma tissues and cell lines. In conclusion, we demonstrated that overexpression FLOT2 was associated with poor prognosis of glioma patients and involved in the progression of glioma, identifying a novel prognostic biomarker and therapeutic target for glioma progression.

Introduction
Glioma is the most common malignant and aggressive primary brain malignancy, comprising ∼80% of the nervous malignant tumours [1]. Although the great improvement in surgery, radiotherapy, and chemotherapy were contributed to the therapy for glioma, the overall survival of gliomas patients is still about 15 months [2–4]. So, revealing the molecular mechanism and identifying novel therapeutic targets for glioma is urgently needed. Flotillin-2 (FLOT2), a member of lipid raft marker flotillin family, function as physical platforms of other molecules, playing critical role in crucial intracellular signalling pathways. Studies showed that FLOT2 involves in various biological processes, including neuronal differentiation, adhesion, endocytosis, embryo survival, phagocytosis, and several signalling pathways [5–7]. Recently, FLOT2 expression was reported to be upregulated and was suggested to be a prognostic marker for various tumours [8]. For example, Xie et al. reported that FLOT2 expression was increased in breast cancer tissue and induced proliferation of breast cancer [9], and Berger et al. showed that FLOT2 involved the lung metastases in breast cancer [8]. Cao et al. revealed that the overexpression of FLOT2 was significantly associated with poor prognosis in gastric carcinomas and reduced survival of gastric carcinomas patients [10]. Wang et al. revealed the overexpression of Flot2 in hepatocellular carcinoma, which was significantly correlated with poor prognosis of hepatocellular carcinoma patients. Elevated Flot2 was an independent prognostic marker of nasopharyngeal carcinoma, and showed shorter overall survival time of nasopharyngeal carcinoma patients [11]. These findings suggested that FLOT2 function as oncogene in the development and progression of various cancers. Recent studies showed the potential relationship between Flot2 and glioma [12,13]. For example, Chetty et al. showed that the disruption of the lipid rafts mediates the apoptotic action of glioblastoma xenograft cells [14]. Flot2 was also reported to involve in regulating αf production in an αf-dependent cerebral amyloidosis mouse model [15]. In addition, miR-138 promotes lipid raft formation and involves in esophageal squamous cell carcinoma progression [16]; microRNA (miRNA) was also reported as the prognostic biomarker of glioma. Therefore, we speculated that Flot2 may play an important role in glioma progression.

miRNAs are a class of cytoplasmic non-coding RNAs with 19–24 nucleotides length. Emerging evidence showed that miRNAs involved tumorigenesis and cancer progression via negatively regulating oncogenes or tumour suppressors expression by binding to the 3’-UTR of miRNAs of the target gene. For example, miR-200a, an anti-Galphai1 miRNA, down-regulation in human glioma and involved cell proliferation of glioma [17]. miR-519a, as a tumour suppressor, promotes autophagy in glioma [18]. miR-181a was reported to reduce
cell proliferation and invasion in glioma [19]. However, expression and potential role of miR-449 in glioma remains unknown.

In this study, we detected the expression and function of FLOT2 in glioma, and found that miR-449 regulated FLOT2 expression by target its 3’ UTR directly in glioma. Our study revealed the FLOT2 overexpression was associated with poor prognosis of glioma patients, indicating that FLOT2 could be a promising biomarker and therapeutic target for glioma.

Methods

Patients and surgical specimens

Fifty-six pairs of glioma and adjacent non-tumour brain tissues were collected from Fujian Provincial Hospital of Fujian Medical University from Jan 2015 to Jan 2017. All tissues were directly preserved in liquid nitrogen and stored at −80°C. In this study, all investigation and experiments have obtained patients’ consent and been approved by the Ethic Committee for Fujian Provincial Hospital of Fujian Medical University. The clinical characteristics of the patients are summarized in Table 1.

| Variable | Number | Low | High | p value |
|----------|--------|-----|------|---------|
| Age (years) | <60 | 23 | 10 | 13 | NS |
| | ≥60 | 33 | 18 | 15 | |
| Gender | Male | 27 | 14 | 13 | NS |
| | Female | 29 | 14 | 15 | |
| Tumour size (cm) | <3 | 24 | 16 | 8 | .005 |
| | ≥3 | 32 | 10 | 22 | |
| Tumour stage | I–II | 28 | 22 | 6 | <.001 |
| | III–IV | 28 | 8 | 20 | |

Cell line culture

The glioma cell lines (U87, H4, U251, U118, and A172), human astroglia cell line (HA), and human embryonic kidney cell line (HEK-293) were cultured in RPMI-1640 (Gibco BRL, Carlsbad, CA, USA). The U87 and U251 cells were seeded in 6-well plates with 3 × 10^5 cells/well, and MTT was used to detect the cell proliferation ability at 24, 48, 72, and 96 h.

MTT assay

The cell migration ability and cell invasion ability were detected using Transwell assay as previously described [20].

Western blot analysis

The total proteins were extracted from cells and tissue using RIPA buffer (Beyotime, Shanghai, China). Fifty micrograms of total protein was used for Western blotting as previously described [11]. The antibodies, anti-Flot2 (Santa Cruz Biotechnology, USA), and anti-GAPDH (Cell Signaling Technology, USA), were used in this study. The anti-rabbit or anti-mouse secondary antibodies (Sigma – Aldrich, Australia) were incubated for 1 h at room temperature. The visualization was enhanced by ECL Plus (Life Technologies, Gaithersburg, MD).

Luciferase reporter assay

The 3’UTR of FLOT2 and the mutated target site of FLOT2 were inserted into pGL3 (Promega, Fitchburg, WI). Cells were co-transfected with the reporter plasmid and miR-449 mimic or inhibitors to glioma cell using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and then the luciferase activities were measured with the dual-luciferase assay system (Promega) according to the manufacturer’s instructions.

Statistical analysis

All the data were analyzed using SPSS version 18.0. Student’s t-test and Pearson’s chi-squared test were used to analyze the molecular expression and the relationship between FLOT2 and clinicopathologic characteristics. Kaplan–Meier method and a log-rank test were used for survival analysis. Data were presented as the mean ± SD. p < .05 was considered statistically significant.

Quantitative real-time PCR

Total RNA was isolated from the tissues or cells using TRIZOL reagent and then reverse to cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Kusatsu, Japan) for mRNA and or by using miRNA cDNA First Strand Synthesis (Tiangen, Beijing, China) for miRNA. The qPCR was performed using SYBR Green real-time PCR Master Mix (Takara) or miRcute miRNA Fluorescence Quantitation Kit (Tiangen) on the Applied Biosystems 7900HT (Applied Biosystems). The primers were as follows: FLOT2 5’-CCCCAGATTGCTGCCAAA-3’, and 5’-TCCACTGAG GACCACAATCTCA-3’. The relative mRNA expression was normalized to GAPDH. miR-449 5’-GGATCCCTCTGTTC ATGTACAATTAGC-3’, and 5’-AATGAATTCTAAAGGAAAA AATTGAATGGT-3’. The relative miRNA expression was normalized to RUNU6. The relative quantitative value was expressed by the 2-DDCt method. Each experiment was performed in triplicates and repeated three times.

Table 1. Correlation of FLOT2 expression with clinicopathologic features in glioma patients.
Figure 1. FLOT2 expression in glioma and correlation with glioma progression and poor prognosis. (a) Real-time PCR analysis of FLOT2 expression levels in glioma tissue. (b) Real-time PCR analysis of FLOT2 expression levels in glioma cell lines (U87, H4, U251, U118, and A172) and human astroglia cell line (HA). Each bar represents the mean ± SD of three independent experiments. *p < .01 vs. HA cells. (c) Kaplan–Meier analysis for overall survival curves of patients with high or low FLOT2 expression.

Figure 2. siFLOT2 represses cell viability, migration, and invasion of glioma. (a) siFLOT2 evidently reduced FLOT2 mRNA and protein expression levels in U251 and U87 cells. (b) siFLOT2 significantly repressed cell viability in both U87 and U251 cells. (c) siFLOT2 significantly repressed cell invasion of U251 cells. (d) siFLOT2 significantly repressed cell migration of U251 cells. *p < .05.
Results

**Up-regulation of FLOT2 mRNA in glioma tissues and cell lines**

To reveal the FLOT2 expression in glioma, we carried out qPCR analysis in 56 pairs of glioma tissues and adjacent normal tissues. The data showed that FLOT2 expression in glioma tissues was significantly upregulated compared with the adjacent normal tissues (Figure 1(A)). Next, we explored the FLOT2 expression in glioma cell lines (U87, H4, U251, U118, and A172) and human astroglia cell line (HA). As shown in Figure 1(B), FLOT2 expression in glioma cell lines was also much higher than that in normal astroglia cell lines. To further evaluate the potential roles of FLOT2 in glioma, we explored the relationship between FLOT2 expression and the clinicopathological features of glioma patients. We found that the FLOT2 expression level was significantly associated with tumour stage and size (Table 1). However, the FLOT2 expression showed no significant relationship with other features including patient age and gender in glioma patients ($p > .05$). We next analyzed the overall survival of glioma patients. As shown in Figure 1(C), the glioma patients with high FLOT2 expression observed shorter survival than those with low FLOT2 expression. These results demonstrated FLOT2 expression as a prognostic factor for glioma.

**Silenced FLOT2 repressed the viability, migration and invasion of glioma cells**

We used the siRNA of FLOT2 (siFLOT2) to downregulate FLOT2 expression in glioma cells. As shown in Figure 2(A), siFLOT2 remarkably reduces FLOT2 expression levels in U87 and U251 glioma cells. We next detected the cell viability using MTT, and detected the migratory and invasive activities of U87 and U251 cells using transwell assay. As shown in Figure 2(B), the cell viability was significantly reduced in siFLOT2 group compared with NC group. Meanwhile, the cell migratory and invasive activities are also obviously suppressed by siFLOT2 in U87 and U251 cells (Figure 2(C, D)). It can be concluded that FLOT2 knockdown is effective in restraining glioma cell growth and invasiveness.

![Figure 3](image)

**Figure 3.** MiR-449 target FLOT2 directly at the 3’UTR of FLOT2 in glioma cells. (a) The predicted wild-type (WT) or mutant (Mut) binding site of miR-449a on the 3’-UTR of FLOT2. (b) luciferase activities was detected by Luciferase reporter assay. qPCR analysis revealed the effects of miR-449 on FLOT2 expression in both U87(c) and U251 (d) cells. Error bars represent ± SE and *p < .01.
MiR-449 target FLOT2 directly at the 3' UTR of FLOT2

According to previous researches, FLOT2 was involved in the tumorigenesis as a target gene of miR-449 and other miRNAs in various cancer [21–23]. Therefore, we assumed the potential relationship between miR-449 and FLOT2 in glioma cells. Luciferase activities of FLOT2 were detected in glioma cells transfected with pGL3- FLOT2-3'-UTR and miR-449 mimics, miR-449 inhibitor or negative controls (Figure 3(A)). We found that miR-449 mimics significantly repressed the luciferase activities in FLOT2 group but did not affect the luciferase activities in Mut-FLOT2 group (Figure 3(B)). Meanwhile, miR-449 inhibitors significantly improved the luciferase activities in FLOT2 group but did not affect the luciferase activities in Mut-FLOT2 group (Figure 3(B)). Next, we detected the effects of miR-449 on FLOT2 expression. The results showed that miR-449 mimics significantly repressed the expression levels of FLOT2 mRNA and protein in U87 and U251 glioma cell (Figure 3(C, D)). These data indicated that miR-449 directly binding the 3'UTR sequence of FLOT2 and regulated FLOT2 expression in glioma cells.

MiR-449 was downregulated in glioma tissue and cell lines

To further confirm the role of miR-449 in glioma, we detected the expression levels of miR-449 in glioma tissue and cell lines. We found that the expression level of miR-449 in glioma tissues was significantly upregulated compared with the adjacent normal tissues (Figure 4(A)). Next, we explored the expression levels of miR-449 in glioma cell lines (U87, H4, U251, U118, and A172) and human astroglia cell line (HA) (Figure 4(B)).

Discussion

Recently, it is becoming clear that dysregulation of FLOT2 involved to diverse human diseases especially in tumorigenesis [8]. In this study, we discovered the expression and role FLOT2 in glioma, indicating siFLOT2 as a biomarker and a therapeutic target for glioma.

FLOT2 was reported to be deregulated and associated with progression and poor survival in numerous types of cancer [24,25], making it a critical regulator of tumour initiation and prognosis. For example, the upregulation of FLOT2 was observed in renal cell carcinoma, and was association with poorer prognosis and overall survival of patients [26]. High expression of FLOT2 was also reported in cervical carcinoma and is associated with poor clinical survival of patients with cervical carcinoma [27]. FLOT2 also reported as a predicted biomarker in the progression of head and neck cancer [28]. Recent studies showed that lipid rafts mediates the apoptosis of glioblastoma cells [14]. In addition, miR-138 a prognostic biomarker of glioma, could upregulating multiple components of lipid rafts, including FLOT2 in esophageal squamous cell carcinoma [16]. Collectively, these results indicated that FLOT2 may play an important role in glioma progression. In this study, we found that FLOT2 expression levels were greatly upregulated in glioma tissues and cell lines, and the FLOT2 expression in glioma tissue was markedly associated with tumour stage and size. The overall survival analysis showed that the high FLOT2 expression was associated with poorer survival. Together, these results suggested the important role of FLOT2 protein in the prognosis of patients with glioma. We next analyzed the role of FLOT2 on cell viability, migration, and invasion of glioma. We found that silenced FLOT2 repressed the viability, migration and invasion of both U87 and U251 cells. These results indicated that FLOT2 function as a biomarker and a therapeutic target for glioma.

Accumulating studies showed that miRNAs involved tumorigenesis and cancer progression via negatively regulating oncogenes or tumour suppressors’ expression by binding to the 3'-UTR of miRNAs of the target gene. MiR-449 was reported as a tumour suppressor and it regulated cancer cell proliferation, invasion and migration by directly repressing its target gene expression [29,30]. For example, Bou et al.
showed that miR-449 and is downregulated in gastric cancer, and it inhibits cell proliferation by directly targeting GMMN, MET, CCNE2, and SIRT1 [31]. Zhang et al. revealed that miRNA-449 suppresses proliferation of hepatoma cell by repressing SIRT1 expression [32]. Luo et al. revealed the downregulation of Mir-449 in non-small cell lung cancer and miR-449 repressed cell migration and invasion by targeting c-Met in non-small cell lung cancer [33]. Zhang et al. showed that miR-449 was significantly downregulated in breast cancer, and it suppressed the migration and invasion of breast cancer cells by targeting TPDS2 [34]. Consistent with these studies, we found that miR-449a may function as a tumour suppressor and its expression level was evidently downregulated in glioma. Furthermore, we found that miR-449 could repress the FLOT2 expression levels by directly targeting the 3′UTR of FLOT2 mRNA.

In conclusion, overexpression of FLOT2 was observed in glioma and the FLOT2 expression in glioma tissue was markedly associated with poor prognosis of glioma patients. Flot2 promoted tumour growth and metastasis of glioma and was regulated by miR-449. These results indicated that Flot2 may be regarded as a potential poor prognostic marker for glioma.

Disclosure statement

The authors declare that they have no conflict of interests

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