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Potential Diagnostic and Prognostic Value of Plasma Circulating MicroRNA-182 in Human Glioma

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Background: Previous studies showed the aberrant expression of microRNA-182 (miR-182) in glioma tissue. However, the exact role of circulating miR-182 in glioma remains unclear. Here, we confirmed the expression of plasma circulating miR-182 in glioma patients, and further explored its potential diagnostic and prognostic value.

Material/Methods: Real-time quantitative PCR (RT-PCR) was used to measure circulating cell-free miR-182 from 112 glioma patients and 54 healthy controls.

Results: Our findings showed that the level of circulating miR-182 in glioma patients was higher than that in healthy controls ($P<0.001$), which was significantly associated with KPS score ($P=0.025$) and WHO grade ($P<0.001$). The area under the receiver operating characteristic (ROC) curve (AUC) was 0.778. The optimal cut-off value was 1.56, and the sensitivity and specificity were 58.5% and 85.2%, respectively. Interestingly, a high predictive value of circulating miR-182 was observed in high-grade glioma (AUC=0.815). However, the AUC was lower in low-grade glioma (AUC=0.621). Kaplan-Meier analysis demonstrated that the cumulative 5-year overall survival rate in the high miR-182 group was significantly lower than that in the low miR-182 group in both overall survival (OS) ($P=0.003$) and disease-free survival (DFS) ($P=0.006$). Moreover, multivariate Cox analysis revealed that circulating miR-182 was an independent prognostic indicator for OS ($P=0.034$) and DFS ($P=0.013$).

Conclusions: These results suggest that circulating miR-182 may be a potential noninvasive biomarker for the diagnosis and prognosis of human glioma.

MeSH Keywords: Early Diagnosis • Glioma • MicroRNAs • Plasma • Prognosis

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Background

Glioma is the most common human primary malignant brain tumor, accounting for approximately 60% of all central nervous system tumors in both adults and children [1]. It is characterized by a rapid infiltrative growth pattern, making complete surgical resection impossible. Despite the recent advances in tumor diagnosis and treatment, including surgery, radiotherapy, and chemotherapy, glioma still has a high mortality rate and a poor 5-year survival rate. The poor prognosis is due to the early local invasiveness as well as the lack of effective early diagnosis. Currently, the criterion standard of glioma diagnosis is histological evaluation, but it is difficult to acquire tissue owing to the special anatomical position of glioma. Furthermore, imaging methods such as computed tomography (CT) and magnetic resonance imaging (MRI) are the most widely used tools to diagnose glioma before clinical diagnosis or treatment, but they are expensive and fail to improve the rate of early diagnosis, which results in glioma spreading [2]. Moreover, several clinicopathologic factors, such as WHO grade or Karnofsky performance status (KPS) score, are important for the prognosis of glioma [1]. Nevertheless, these factors may not accurately estimate prognosis because of heterogeneity in the patient population. Recently, some tumor-related molecules involved in the development and progression of glioma and have been used as diagnostic or prognostic biomarkers, such as FOXD3 [3] and BRAF [4]. However, the sensitivity and specificity of these biomarkers are inadequate for the evaluation of early diagnosis or prognosis. Therefore, there is a great need to explore novel and highly sensitive molecular biomarkers with reliable clinical significance.

MicroRNAs (miRNAs) are small non-coding RNAs (20–22 nucleotides) that negatively regulate the expression of genes by repressing the translation of target mRNAs. Accumulating evidence indicates that miRNAs are important in crucial biological processes such as cellular proliferation, differentiation, and tumorigenesis [5,6]. The aberrant expression of miRNAs has been identified in many diseases, including tumors, and its expression profiles are different in different types of tumor [6]. Moreover, circulating miRNAs have been reported to be detectable in clinical specimens such as plasma or serum with high stability, indicating great potential as convenient and non-invasive biomarkers [7,8]. Interesting, more and more researchers have found that circulating miRNAs are potential diagnostic or prognostic biomarkers for classification of cancers and other diseases, including prostate cancer [9], breast cancer [10], and gastric cancer [11]. Recently, several studies have explored the feasibility of using abnormal single miRNAs as diagnostic or prognostic biomarkers in glioma, such as miR-205 [12], miR-128 [13], and miR-210 [14]. However, to date, no circulating miRNAs in plasma/serum have been successfully used in glioma patients in clinical settings.

MiR-182 is an oncogene that is dysregulated in many human cancers, and its overexpression contributes to the growth, invasion, and/or chemotherapeutic sensitivity of these tumors [15–17]. A previous study reveals that miR-182 is significantly up-regulated in tissues, which is related to the poor prognosis or the therapeutic outcome of glioma patients [18]. This suggests that miR-182 may be a promising biomarker for early diagnosis and prognosis. Although circulating miR-182 has been found in the plasma or serum of some tumors [19,20], its expression and correlation with clinical features in glioma have not yet been determined. Hence, we detected the expression of plasma circulating miR-182 in glioma patients and healthy controls to evaluate its feasibility in diagnosis and prognostic prediction. Furthermore, we analyzed the relationships among clinical data, clinicopathological variables, and diagnostic or prognostic value. Our results provide new evidence that miR-182 can be a novel diagnostic and prognostic biomarker with a satisfactory sensitivity and specificity in patients with glioma.

Material and Methods

Clinical samples

We enrolled 166 subjects in this study from December 2008 to March 2010 in Liaocheng People's hospital (Shandong, China), including 54 healthy volunteers and 112 newly diagnosed glioma patients with various stages. Glioma patients were diagnosed by histological examination based on the WHO categories, and all patients were classified according to WHO classification system [21], including 18 cases of pilocytic astrocytoma (grade I), 23 cases of diffuse astrocytoma (grade II), 32 cases of anaplastic glioma (grade III), and 39 cases of glioblastoma (grade IV). Patient characteristics, including age, sex, KPS score, and WHO grade, are described in detail in Table 1. Surgical resection was done in all patients with primary glioma, and none of these had undergone chemotherapy or radiotherapy before surgery. All patients were grouped into low-grade (WHO grade I–II, 41/112) or high-grade (grade III–IV, 71/112). All glioma patients were followed up at intervals of 1 month in the initial 1–2 years and every 3 months thereafter. Clinical follow-up of 112 patients was finished by April 2015. Overall survival time was defined as the period between the initial operation and death, and disease-free survival was the period between the initial operation and tumor recurrence or death. This study was approved by the Ethics Committee of Liaocheng People's Hospital. Written informed consent was obtained from all subjects.

Samples collection

Venous blood of all subjects was collected into tubes containing EDTA K_3, and hemolyzed blood samples were excluded.

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Table 1. Correlations between circulating miR-182 and clinicopathological variables (median and interquartile range).

Parameters	No. of patients	Circulating miR-182 levels	P-value of circulating miR-182
Gender			
Male	72	2.16 (1.02–3.31)	0.462
Female	40	2.21 (0.67–3.76)	
Age			
>50 years	41	2.17 (1.22–3.12)	0.623
≤50 years	71	2.19 (0.90–3.49)	
Tumor size			
>5 cm	46	2.24 (1.14–3.34)	0.899
≤5 cm	66	2.14 (0.80–3.49)	
KPS score			
>80	70	2.69 (1.31–4.07)	0.025
≥80	42	1.37 (0.60–2.15)	
WHO grade			
I	18	0.98 (0.14–1.83)	<0.001
II	23	1.56 (0.90–2.23)	
III	32	2.27 (1.21–3.34)	
IV	39	4.12 (2.28–5.97)	

Immediately after collection, 10 ml of blood was centrifuged at 1200×g for 10 min at 4°C. The supernatant was collected and then centrifuged at 12 000×g for 10 min at 4°C to completely remove all cell components. The supernatant was transferred into a clean tube and stored as separate aliquots at –80°C for future use.

RNA extraction

Total RNA containing small RNA was extracted from 400 µl of plasma by TRIzol LS reagent according to the manufacturer's instructions. In brief, 750 µl of TRIzol reagent was added to plasma and mixed. After standing for 10 min, 200 µl of chloroform was added, then the mixture was incubated 10 min at room temperature, followed by centrifugation for 15 min at 12 000 ×g, after which we transferred the aqueous phase containing RNA to a fresh tube. RNA was precipitated by mixing with 500 µl of isopropyl alcohol, and then centrifuged for 15 min at 12 000 ×g. After washing with 1000 µl of 75% ethanol, the pellet was dissolved in 20 µl of RNase-free water.

RT-qPCR for circulating miR-182

Quantitative reverse-transcription polymerase chain reaction (RT-qPCR) was used to detect the level of miR-182 in plasma of all subjects. The reverse transcription reaction was carried out using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). RT reactions (15 µl) contained 5 µl of RNA extract, 1.5 µl of 10× reverse transcription buffer, 0.15 µl of 100 mM dNTPs, 1 µl of MultiScribe reverse transcriptase, 0.19 µl of RNase inhibitor, 1 µl of gene-specific primer, and 4.16 µl of nuclease-free water. For synthesis of cDNA, the reaction mixtures were incubated at 16°C for 30 min, at 42°C for 30 min, and at 85°C for 15 min, and then held at 4°C. We amplified 1.33 µl of cDNA solution by using 10 µl of TaqMan 2×Universal PCR Master Mix with No AmpErase UNG (Applied Biosystems), 1 µl of gene-specific primer, and 7.67 µl of nuclease-free water in a final volume of 20 µl. Circulating miR-182 was detected by use of RT-qPCR in the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA). The mixtures were incubated at 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min (45 cycles). The cycle threshold (C_T) values were calculated with SDS 2.4 software (Applied Biosystems). RNU6B was used as the endogenous plasma control. Relative expression quantification of circulating miR-182 in plasma was performed by the comparative C_T method ($2^{-\Delta\Delta CT}$) [22–24]. In this study, the expression of circulating miR-182 was calibrated relative to pooled plasma from 15 healthy controls [25].

Statistical analysis

SPSS software (version 15.0) was used to analyze the data. The Kolmogorov-Smirnov test was used to evaluate the distribution of data. The nonparametric Mann-Whitney U test or

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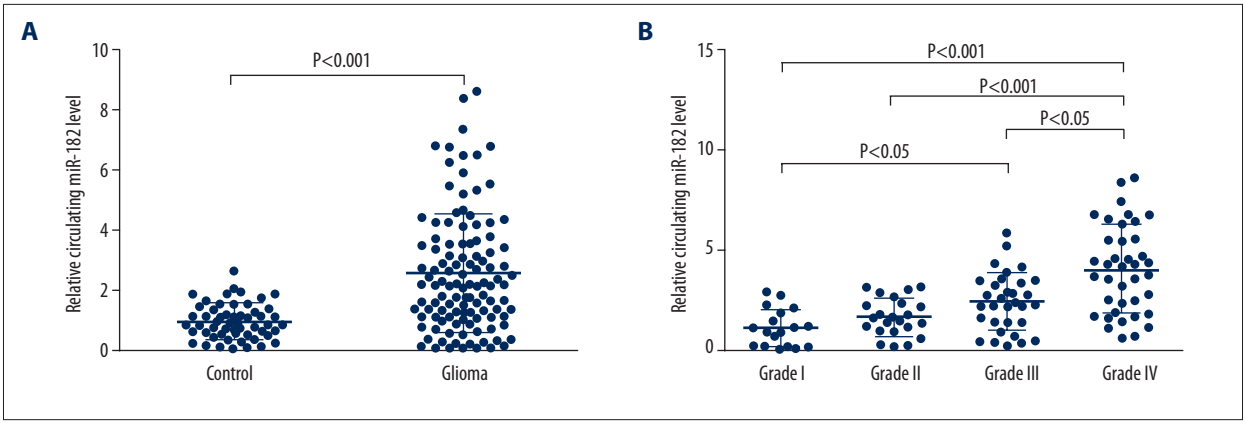

Figure 1. The level of circulating miR-182 (**A**) in patients with glioma and healthy controls. (**B**) Circulating miR-182 levels among patients with different WHO grades. Expression levels of miR-182 are normalized to RNU6B, and vertical bars represent median values.

Kruskal-Wallis test was used to determine the statistical differences in circulating miR-182 among the groups, as appropriate. The Spearman coefficient test was used to analyze circulating miR-182 with respect to WHO grade. ROC was used to distinguish glioma patients from healthy controls. Youden index (sensitivity+specificity-1) was used to determine the optimal cut-off value for circulating miR-182. The survival curves of glioma patients were estimated by Kaplan-Meier method and log-rank test. The Cox proportional hazards regression model was used to assess the independent prognostic factor. Differences were considered statistically significant when *P* was less than 0.05.

Results

The expression of plasma circulating miR-182

Results from our research group and other groups show that RNU6B might be useful as an internal control [24, 23, 26]. We then detected circulating miR-182 in all glioma patients. After normalization relative to the level of RNU6B, the Kruskal–Wallis test showed that the level of circulating miR-182 in patients with glioma (mean ±SD, 2.57±1.95) was much higher than that of healthy controls (0.97±0.38) ($P<0.001$, Figure 1A).

The correlation between circulating miR-182 and clinicopathological features

We evaluated the correlation of circulating miR-182 with clinicopathological parameters, including sex, age, tumor size, KPS score, and WHO grade (Table 1). Plasma circulating miR-182 was statistically correlated with KPS score ($P=0.025$) and WHO grade ($P<0.001$), but we found no significant correlation between miR-182 and sex, age, or tumor size (all at $P>0.05$). A significant correlation was observed between circulating miR-182 and WHO grade (r=0.786, $P=0.006$), indicating that the up-regulation of miR-182 might be correlated with clinical glioma progression. Figure 1B shows that circulating miR-182 in grade IV (glioblastoma) was much higher than that of patients with pilocytic astrocytoma (grade I, $P<0.001$), diffuse astrocytoma (grade II, $P<0.001$), or anaplastic glioma (grade III, $P<0.05$). The level in grade III was higher than that in grade I ($P<0.05$). The results suggest that circulating miR-182 may be a useful marker for disease status.

Predictive value of circulating miR-182 for glioma

As shown in Figure 2A, the level of circulating miR-182 in patients with high-grade (3.25±2.05) was higher than that of patients with low-grade glioma (1.38±0.94) or healthy controls (0.97±0.38) (both at $P<0.001$), indicating a good ability to discriminate between high-grade glioma patients and low-grade glioma patients or healthy controls. Interesting, no significant difference was detected between low-grade glioma patients and controls ($P>0.05$), suggesting that circulating miR-182 might not be an effective marker for low-grade glioma detection.

ROC curve and area under the ROC curve (AUC) were used to further estimate the value of circulating miR-182 in predicting glioma. Figure 2B shows the predictive performance of miR-182 in the different stages of glioma. The AUC of all stage was 0.778 (95% CI, 0.679–0.878). The cut-off value of circulating miR-182 in glioma patients was 1.56. The corresponding sensitivity and specificity were 58.5% and 85.2%, respectively. In evaluating the predictive performance of circulating miR-182 in distinguishing patients with high-grade glioma from healthy controls, the AUC was 0.815 (95% CI, 0.718–0.913). We then evaluated the diagnostic performance for low-grade glioma. The AUC was 0.621 (95% CI, 0.500–0.741), and was significantly lower than all stages or high-grade (both $P<0.05$), suggesting that miR-182 might be an unreliable biomarker for

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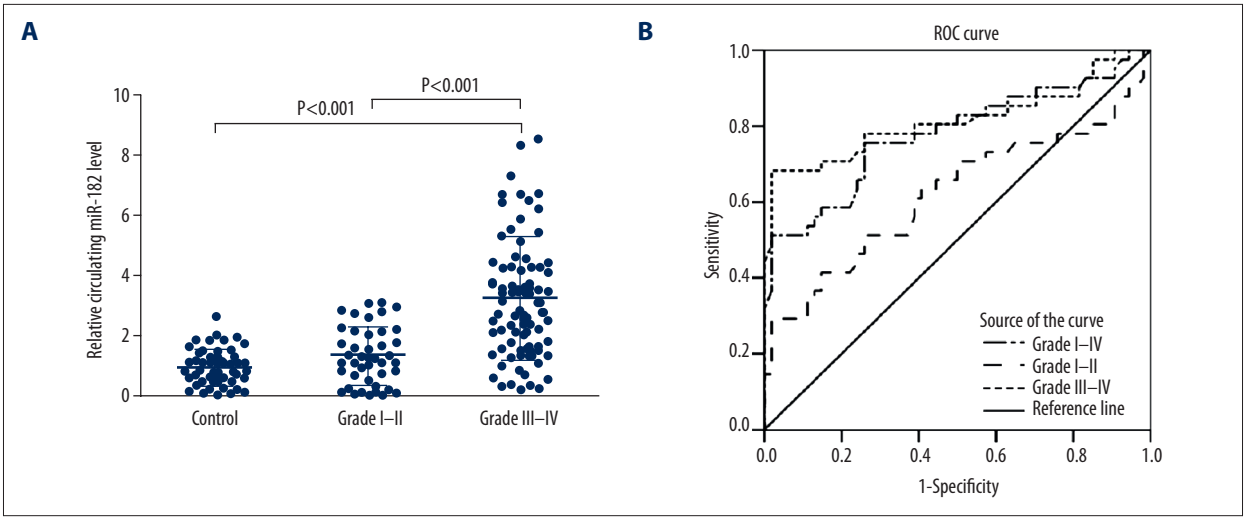

Figure 2. (**A**) The expression of circulating miR-182 in healthy controls and patients with low-grade (I–II) and high-grade (III–IV) glioma. (**B**) ROC analysis for the diagnosis of glioma using circulating miR-182 in all stages, low-grade, and high-grade patients.

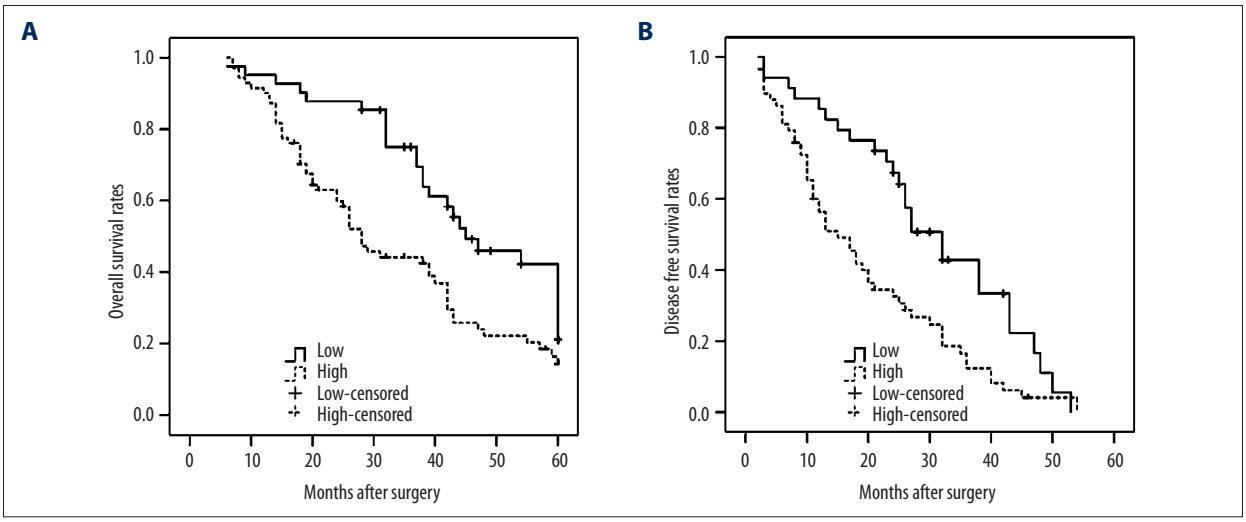

Figure 3. Kaplan-Meier curves for OS (**A**) and DFS (**B**) according to plasma circulating miR-182. The optimal cutoff value (1.56) of circulating miR-182 was used to classify glioma patients into a high-level group and a low-level group.

low-grade glioma. Taken together, these results show that circulating miR-182 might provide a new complementary tumor marker for the diagnosis of glioma.

Correlation between circulating miR-182 level and prognosis in glioma patients

To determine whether increasing circulating miR-182 level can predict the outcome after resection of primary glioma, we explored the association between circulating miR-182 and the prognosis of patients. The patients were categorized into low and high circulating miR-182 groups, based on the optimal cut-off value (1.56). The prognostic performance of serum miR-182 was evaluated using Kaplan-Meier analysis. Figure 3A and 3B show that the cumulative 5-year overall survival rate of disease-free survival (DFS) and/or overall survival (OS) with higher level of circulating miR-182 were shorter than that of patients with lower levels (higher (32.786, 95%CI: 22.941–33.059) versus lower (44.923, 95%CI: 31.487–58.513)) for DFS (P=0.006), and higher (19.325, 95%CI: 9.620–20.380) versus lower (30.638, 95%CI: 24.668–39.332) for OS (P=0.003). Moreover, univariate Cox proportional hazard regression model analysis revealed a significant relationship between DFS and KPS score (P<0.001), as well as WHO grade (P<0.001) and circulating miR-182 (P=0.009). OS was related to KPS score (P<0.001), WHO grade (P<0.001), and circulating miR-182 (P=0.004). Subsequently, to determine whether circulation miR-182 was an independent prognostic factor of glioma patients, univariate and multivariate Cox regression analyses were performed. The results show that circulating miR-182,

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Table 2. Univariate and multivariate analyses of prognostic variables of DFS and OS in glioma patients.

Parameters	Categories	Univariate analysis		Multivariate analysis	
		HR (95% CI)	P-value	HR (95% CI)	P-value
Disease free survival					
Gender	Female *vs.* Male	1.25 (0.80–2.00)	0.329	0.64 (0.40–1.02)	0.063
Age	>50 *vs.* ≤50	0.88 (0.55–1.41)	0.601	1.13 (0.69–1.85)	0.615
Tumor size	>5 cm *vs.* ≤5 cm	1.18 (0.76–1.83)	0.460	0.69 (0.44–1.09)	0.111
KPS Score	>80 *vs.* ≤80	2.60 (1.60–4.22)	<0.001	2.43 (1.47–4.03)	0.001
WHO Grade	I + II *vs.* III + IV	3.04 (1.86–4.98)	<0.001	3.15 (1.85–5.35)	<0.001
Circulating miR-182	Low *vs.* high	1.88 (1.17–3.00)	0.009	1.30 (0.79–2.13)	0.034
Overall survival					
Gender	Female *vs.* Male	0.77 (0.48–1.24)	0.280	0.59 (0.31–0.87)	0.131
Age	>50 *vs.* ≤50	1.11 (0.69–1.78)	0.668	1.59 (0.95–2.65)	0.081
Tumor size	>5 cm *vs.* ≤5 cm	0.96 (0.61–1.50)	0.845	1.04 (0.66–1.65)	0.858
KPS Score	>80 *vs.* ≤80	2.65 (1.59–4.41)	<0.001	3.15 (1.82–5.48)	<0.001
WHO Grade	I + II *vs.* III + IV	3.20 (1.89–5.41)	<0.001	3.06 (1.73–5.41)	<0.001
Circulating miR-182	Low *vs.* high	0.50 (0.31–0.80)	0.004	1.25 (0.89–2.53)	0.013

KPS score, and WHO grade were independent prognostic indicators for DFS (P=0.034) or OS (P=0.013) (Table 2). These data suggest that miR-182 is a novel tumor marker for the prognosis of patients with glioma.

Discussion

Glioma has a high mortality because of late clinical presentation and lack of effective early detection measures. It is urgent to identify new effective biomarkers for early diagnosis of glioma. The crucial finding of this study is that circulating cell-free miR-182 is significantly upregulated in glioma patients. Further analysis shows that miR-182 has a higher sensitivity and specificity in high-grade glioma than that in all stages or low-grade in discriminating glioma patients from healthy controls, suggesting that the upregulation of miR-182 was related to advanced clinical stage of glioma. Moreover, circulating cell-free miR-182 has been demonstrated to be an independent prognostic factor for glioma patients. Taken together, these findings reveal that it may be a more reliable circulating tumor marker for diagnosis and prognosis of glioma.

Many genetic and epigenetic alterations have been demonstrated during tumorigenesis. Thus, molecules that can specifically reflect these alterations may be prospective tumor markers. MiRNAs are small non-coding RNAs that widely exist in several types of clinical samples, including serum, urine, and stool [7,27]. The dysregulation of plasma/serum miRNAs have been found in several cancers, such as lung cancer and colorectal cancer. This indicates that miRNAs expression might be dependent on cancer type [28,29]. MiRNAs in blood may originate from the damaged cells or circulating cells, indicating that circulating miRNAs may be used as early diagnostic markers of tumor status [30]. Several plasma tumor-related miRNAs are involved in glioma development and progression and have been identified as potential tumor markers [12–14]. It is encouraging that circulating miRNAs in plasma/serum can serve as potential tumor biomarkers, and this could overcome the problem of collecting tissue specimens through biopsy or surgery. In the current study, the level of miR-182 in plasma was significantly upregulated in patients with glioma and could discriminate glioma patients from healthy controls, suggesting that miR-182 may be a valuable marker for glioma in a less invasive manner and at an early stage.

MiR-182 is dysregulated in tissues of several cancers, including gastric cancer and ovarian cancer [31,32]. Further studies show that miR-182 is an oncomiR and is involved in several crucial steps of tumorigenesis, such as epithelial-mesenchymal transition, proliferation, invasion, and metastasis, through directly targeting FOXO3, BRCA1, MTSS1, and MITF [15,17,33,34]. These results suggest that miR-182 is involved in the mechanism by which various cancers develop and progress, and it could lead to the development of therapeutic targets and, even more importantly, it may be a useful tumor marker. Indeed, aberrant miR-182 in some tumors is correlated to tumor size, lymph node metastasis, and advanced TNM stage [35]. Moreover, the

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combination of miR-182 and other miRNAs can distinguish people with tumors from healthy people, with high sensitivity and specificity. Our results revealed that circulating miR-182 can differentiate people with glioma from healthy controls with a sensitivity of 58.5% and a specificity of 85.2%. Further exploring the role of miR-82 in glioma development and progression, such as cellular proliferation, invasion, and apoptosis, would be helpful for better understanding the effect of miR-182 on the biological behavior of gliomas.

The predictive performance of circulating miR-182 was best in distinguishing people with high-grade gliomas from healthy controls. Thus, it is more meaningful and accurate to estimate the diagnostic value of miR-182. A previous study showed that miR-182 is markedly up-regulated in glioma tissues [18]. Some brain tumors, such as glioma, have blood vessels of increased diameter and thickened basement membranes; the blood–brain barrier (BBB) is broken down, and blood vessel structure and function also become markedly abnormal [36]. MiR-182 enters into the blood stream through the BBB, which might be one of causes of increased circulating miR-182. Notably, our results showed that the level of circulating miR-182 in high-grade glioma, especially glioblastoma, was higher than that in other grades. Glioblastoma is characterized by abnormal proliferation and death of endothelial cells, which help break down the BBB. This provides a good explanation of the above phenomenon. We then evaluated the diagnostic performance for low-grade glioma. The AUC was 0.621 (95% CI, 0.500–0.741), which is lower than all stages or high-grade. This suggests that miR-182 might be an unreliable biomarker for low-grade glioma. We speculate that although brain tumor vessels appear leaky in early glioma, some elements of the BBB remain intact, resulting in low levels of miR-182 in low-grade glioma.

The outcome of glioma remains unfavorable, and it is difficult to find effective therapeutic strategies. Thus, identifying a powerful prognostic marker for glioma is of great importance. Overexpression of miR-182 has been reported to be associated with poor prognosis of several cancers [37,38]; therefore, we examined the expression of circulating miR-182 in glioma. The data show that increasing level of miR-182 was closely correlated with KPS score and WHO grade, and might contribute to poor prognosis. Further analysis demonstrated that higher plasma miR-182 expression is related with worse patient survival, indicating that miR-182 may be an independent prognostic factor for survival. Previous studies have reported that several miRNAs, such as miR-205, are associated with the outcomes of glioma [12,14]. Our results have revealed miR-182 as a novel independent prognostic factor, which could have high clinical and pathogenetic significance in glioma biology. Since miR-182 may be involved in the early stages of glioma, the blockage of miR-182 may effectively disturb the tumorigenesis and be a potential therapeutic target; therefore, the inhibition of miR-182 may improve glioma outcome. Huynh et al. [39] demonstrated that the inhibition of miR-182 *in vivo* can significantly suppress tumor invasion and metastasis. If these findings are further confirmed in glioma, miR-182 might be used to improve the therapy of glioma and decrease the mortality.

In summary, the findings of our study prove that the increasing expression of circulating miR-182 may be a useful non-invasive biomarker for early diagnosis and predicting clinical outcome of glioma. Further multi-center prospective studies on how miR-182 contributes to the diagnosis or prognosis of glioma are warranted.

Conclusions

The level of circulating miR-182 was significantly higher in glioma patients than in healthy controls. It is a potential diagnostic or prognostic factor for glioma.

Conflicting interests

The authors declare that they have no conflict of interest.

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