Original Article
A three-miRNA signature as a potential biomarker for the diagnosis of glioma

Wu Xu, Weibang Liang, Yuxiang Dai

Department of Neurosurgery, Nanjing Drum Tower Hospital Clinical College of Nanjing Medical University, Nanjing 210008, Jiangsu, China

Received November 3, 2016; Accepted December 20, 2016; Epub March 1, 2017; Published March 15, 2017

Abstract: Aim: Biomarkers in blood have become increasingly appreciated in the diagnosis of gliomas. As the involvement of microRNAs (miRNAs) in carcinogenesis is well known, we conducted this study to identify differentially expressed miRNAs in blood samples of glioma patients to assess their diagnostic value. Method: Here, a total of 47 glioma patients and 45 healthy volunteers were enrolled from a hospital. Total RNA was isolated from plasma using TRIzol reagent and miRNA profiles of gliomas were obtained using miRNA microarrays. The performance of three selected miRNAs as cancer markers was analyzed by reverse transcription-PCR (RT-PCR). A new miRNA-based score was conducted by the logistic regression model. Receiver operating characteristic (ROC) curves and the area under the ROC curve (AUC) was generated to assess the diagnostic values of the miRNAs. Results: Microarray data showed 13 cases of aberrant expression of miRNAs. Among these, expression levels of miR-17, miR-130a, and miR-10b were markedly increased in the plasma of gliomas compared to in healthy individuals (all P<0.01). The AUC (95% CI) of miR-17, miR-130a, and miR-10b was 0.78 (0.69-0.86), 0.72 (0.62-0.81), 0.72 (0.62-0.80), respectively. We conducted a new score named the miR-Score, based on three selected miRNAs: miR-Score=-5.0+0.55*miR-17+0.40*miR-130a+0.20*miR-10b, which had the best diagnostic ability performance with AUC of 0.87 (0.78-0.93), sensitivity of 72.3%, and specificity of 85.1%. Conclusion: The three selected miRNAs are significantly increased in the plasma of glioma patients and thus have great potential to be novel, sensitive, and reliable biomarkers for the diagnosis of gliomas.

Keywords: miRNAs, glioma, diagnosis, receiver operating characteristic analysis

Introduction
Gliomas, which arise from glial cells, the neuro-epithelial support cells of the central nervous system, are the most common primary tumors of the CNS, comprising over 50% of primary brain tumors in adults [1]. These tumors are highly aggressive with a high relapse rate and mortality, without effective therapies to treat them [2]. Even with current neuroradiological imaging offering a high degree of detection, surgery remains an important diagnostic modality. The primary surgical goal is to provide adequate tissue for a pathologic diagnosis and grading [3]. Recently, circulating biomarkers have displayed great potential in clinical applications in oncology [4, 5]. For brain tumors, circulating biomarkers have the undeniable advantage of providing useful information via a minimally invasive procedure.

miRNAs can control the expression of their target mRNAs, predominantly by binding to the 3’ untranslated region (UTR) [6], playing a critical role in carcinogenesis and having a significant impact on cancer research [7, 8]. miRNAs, as promising biomarkers, can provide insights into the diagnosis of gliomas. To date, microarray studies of glioma tissue have implicated a number of miRNAs in glioma formation and propagation [9, 10]. For instance, Lai et al. reported that miR-210 is a promising diagnostic biomarker in glioma patients and is associated with tumor grade and poor outcome [11]. The level of serum miR-125b was found significantly lower in glioma patients when compared with that in normal population, and the accuracy of distinguishing glioma cancer patients was appropriately satisfied (AUC: 0.839; 95% CI: 0.743-0.935) [12]. In addition, Yang et al. identified four serum miRNAs, including miR-23a, miR-150*, miR-197, and miR-548b-5p. These were significantly decreased in the serum of malignant astrocytoma patients compared to in normal controls, and all of them showed excel-
Diagnostic value of miRNAs to glioma

The goal of the current study was to determine the feasibility of detecting and quantifying the level of expression of miRNAs in serum from glioma patients and to find diagnostic biomarkers with practical application in clinical settings. Here, the microarray assay was used to determine differentially expressed miRNAs in the plasma of glioma patients. These miRNAs were validated using quantitative PCR (qPCR) and investigated as candidate circulating biomarkers for the diagnosis of gliomas using receiver operating characteristic (ROC) curves and multivariate logistic regression analysis.

Materials and methods

Subjects

Blood samples of 47 human primary gliomas and 45 healthy volunteers were obtained from the First Affiliated Hospital of Nanjing Medical University between May 2014 and July 2015. Each 5-mL venous blood sample was drawn into a gold-top serum-separating tube, processed for serum extraction within 2 h, and stored at -80°C for further processing. Clinical data were collected, including gender, age, Karnofsky Performance Status (KPS), Extent of resection, and WHO grade and adjuvant treatment. Diagnosis after biopsy or tumor resection was made according to the revised WHO classification [14]. This research was conducted in strict accordance with the protocol approved by the Ethics Committee of Nanjing Drum Tower Hospital Clinical College of Nanjing Medical University, and all participants provided written informed consent. Patients who had severe infection, a history of any other malignancy, or were unwilling to participate in the study were excluded.

RNA isolation

Total RNA, including miRNAs, was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Briefly, 750 µL lysis buffer was added to 200 µL of serum and incubated for 5 min, followed by centrifugation at 12,000×g for 10 min at 4°C. 0.2 mL isopropanol was added to the lysate and then transferred to an RNA Spin Column. The sample was centrifuged at 12,000×g for 1 min and 35 (74.5%). Extent of resection: Gross total resection 35 (74.5%) Subtotal resection 12 (25.5%) Adjunct treatment: Radiotherapy 10 (21.3%) Chemotherapy 6 (12.7%) R&C combination 8 (17.0) WHO: I+II 16 (34.0%) III+IV 31 (66.0%) Note: KPS score: Karnofsky Performance Status score, WHO: World Health Organization, R&C combination: Radiotherapy and chemotherapy combination.

miRNA microarray

Samples were randomly selected from glioma patients and healthy controls. The quality and quantity of the RNA samples were assessed by standard electrophoresis and spectrophotometry methods, and samples with RNA integrity number (RIN)>8 were processed for hybridization. After isolation, the miRCURY™ Hy3™/Hy5™ Power labeling kit (Exiqon, Vedbaek, Denmark) was used according to the manufac-
Diagnostic value of miRNAs to glioma
turer’s guidelines for miRNA labeling, followed by hybridization on the miRCURY™ LNA Array (v.18.0) (Exiqon). Scanned images were then imported into a confocal LuxScan scanner (CapitalBio Corp) and SpotData Pro software (CapitalBio Corp) was used for data analysis.

Reverse Transcription and Quantitative Real-time PCR (qRT-PCR)

First strand cDNA was synthesized according to the manufacturer’s instructions using a TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, USA). The reverse transcription conditions were 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Then qRT-PCR was performed to quantify the expression level of miRNA with SYBR Green PCR Master Mix (Applied Biosystems) following the manufacturer’s instructions. The amplification reaction contained the 10 μL master mix, 0.4 μL forward primer, 0.4 μL reverse primer, and 7.2 μL nuclease-free water. Sequences of primers used were listed as follows: miR-17 forward, 5’-CAAAGTGCTTACAGTGC-3’ and reverse, 5’-GTGCAGAGTCCGAGGT-3’; miR-130a forward, 5’-GGGCAGTGCAATGTTAA-3’ and reverse, 5’-GTGCAGAGTCCGAGGT-3’; miR-10b forward, 5’-TACCCTGTAGAACCGAA-3’ and reverse, 5’-GTGCAGAGTCCGAGGT-3’; U6 forward, 5’-CCCTGTAACCCGAA-3’ and reverse, 5’-GTGCAGAGTCCGAGGT-3’; U6 forward, 5’-TACCCTGTAGAACCGAA-3’ and reverse, 5’-GTGCAGAGTCCGAGGT-3’. The RT-PCR reaction was performed at 95°C for 2 min and in 40 cycles at 95°C for 15 s and 60°C for 1 min on a Roche Lightcycler 480 Real Time PCR System (Applied Biosystems, CA, USA). All PCR reactions were carried out in triplicate. Relative quantification of miRNA expression levels was calculated using the 2^-(ΔΔCt) method.

Statistical analysis

Continuous variables were presented as the mean ± standard deviation and categorical variables were expressed as absolute relative frequencies. A Student’s t-test or two sided χ² test was used to compare the differences in serum miRNA concentrations between two groups. One-way analysis of variance (ANOVA) was used to compare the difference between more than two groups, and the differences between groups were subsequently determined by the LSD-t test when appropriate. For each miRNA, ROC curves were constructed and the area under the ROC curve (AUC) was generated to assess its diagnostic values and to find the appropriate cut-off point. A logistic regression model was performed for the multivariable analysis for markers, and a new miRNA score was then generated based on the three selected miRNAs. Results were statistically significant for P<0.05. All statistical analysis was performed by IBM SPSS software version 20.0, and GraphPad Prism 5.0 (GraphPad Software Inc., CA) was used for graphing.

Results

Clinical characteristics of study population

There were 29 male and 18 female patients in the glioma group, and the median age was 49.2 years (range: 27-74 years). Among these, tobacco and alcohol use was reported in 14 (29.8%) and 15 (31.9%) glioma patients, respectively. In all glioma patients, the histopathologic diagnosis was established by brain biopsy or resection. According to WHO grade criteria, 16 of the 47 glioma tissues were classified as low grade (5 WHO grade I and 11 WHO grade II), and 31 were classified as high-grade (16 WHO grade III and 15 WHO grade IV). 35 patients received gross total resection while 12 only received subtotal resection due to the diffuse lesions or inappropriate region. Plasma samples were all obtained before operation. Detailed clinical characteristics of the 47 glioma patients and 45 healthy people are shown in Table 1. 5 of the plasma samples derived from glioma patients were analyzed by microarray.

Differential Expression of miRNAs in Gliomas

Interestingly, Figure 1 shows that 13 miRNAs exhibited significantly different expression levels (P<0.05 and fold change≥1.5), including 6 upregulated and 7 suppressed miRNAs. Among these, miR-17, miR-130a, and miR-10b showed the largest upregulation while miR-34a, miR-182, and miR-195 showed the most prominent downregulation. Given that traditional serum diagnostic indicators are always found at a higher level and this is easier for the public to
understand, we chose the former three miRNAs for further investigation. The expression level of each of these three selected miRNAs was then detected by qRT-PCR in 47 plasma samples from glioma patients and 45 healthy volunteers. As shown in Figure 2, the relative expression of miR-17, miR-130a, and miR-10b was significantly higher in glioma patients compared with that in healthy ones (all P<0.01), consistent with the microarray findings. To determine whether this alteration was due to gliomas in general or a specific grade of glioma, the expression data were subsequently segregated based on WHO grade criteria. Increased expression of miR-17 remained statistically significant relative to controls in all grades (P<0.01; Figure 3A). In contrast, increased expression of plasma miR-130a and miR-10b only remained significant in high-grade glioma patients (III-IV) compared to in controls in this analysis. Only a trend for increasing expression was shown in low-grade glioma (grade I-II).
Diagnostic value of miRNAs to glioma

Diagnostic performance of miRNAs in glioma patients

ROC curves were constructed and the AUC was generated to assess the diagnostic values of the three selected miRNAs. As shown in Figure 4, ROC analyses revealed that the AUC of miR-17, miR-130a, and miR-10b was 0.787 (0.690-0.865), 0.720 (0.617-0.807), and 0.721 (0.619-0.808), respectively. The detailed information about the ability of the three miRNAs to diagnose the gliomas in patients is revealed in Table 2. In addition, multivariate logistic regression analysis was performed. The results indicated that miR-17, miR-130a, and miR-10b in plasma were all potential diagnostic biomarkers for the identification of glioma patients after adjustment for age and gender (Table 3).

Table 2. Receiver operating characteristic (ROC) analysis of miRNA in glioma patients

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>AUC</th>
<th>95% CI</th>
<th>P value</th>
<th>Youden</th>
<th>Cut-off</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-17</td>
<td>0.787</td>
<td>0.690-0.865</td>
<td>&lt;0.001</td>
<td>0.447</td>
<td>3.03</td>
<td>89.3%</td>
<td>55.3%</td>
</tr>
<tr>
<td>miR-130a</td>
<td>0.720</td>
<td>0.617-0.807</td>
<td>&lt;0.001</td>
<td>0.362</td>
<td>2.68</td>
<td>70.2%</td>
<td>65.2%</td>
</tr>
<tr>
<td>miR-10b</td>
<td>0.721</td>
<td>0.619-0.808</td>
<td>&lt;0.001</td>
<td>0.383</td>
<td>10.1</td>
<td>44.6%</td>
<td>93.6%</td>
</tr>
<tr>
<td>miR-Score</td>
<td>0.872</td>
<td>0.787-0.932</td>
<td>&lt;0.001</td>
<td>0.575</td>
<td>5.43</td>
<td>72.3%</td>
<td>85.1%</td>
</tr>
</tbody>
</table>

Note. AUC, area under the receiver operating characteristic curve; CI, confidence interval; P-value, compared with AUC of 0.5. MicroRNA-Score=-5+0.55*miR-17+0.40*miR-130a+0.20*miR-10b.

Table 3. Multivariate logistic analyses for plasma levels of miRNAs and various clinical parameters in glioma patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Glioma patients vs healthy individuals</th>
<th>B</th>
<th>Wald</th>
<th>OR (95% CI)</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, female vs male</td>
<td>-1.653</td>
<td>1.932</td>
<td>0.75</td>
<td>(0.61-1.38)</td>
<td>0.241</td>
</tr>
<tr>
<td>Age, &lt;50 vs ≥50 years</td>
<td>-0.385</td>
<td>0.224</td>
<td>1.13</td>
<td>(0.42-2.77)</td>
<td>0.673</td>
</tr>
<tr>
<td>MiR-17 &lt;3.03 vs ≥3.03</td>
<td>3.187</td>
<td>6.541</td>
<td>29.22</td>
<td>(5.18-115.85)</td>
<td>0.004</td>
</tr>
<tr>
<td>MiR-130a &lt;2.68 vs ≥2.68</td>
<td>1.765</td>
<td>7.417</td>
<td>5.61</td>
<td>(1.89-14.55)</td>
<td>0.003</td>
</tr>
<tr>
<td>MiR-10b &lt;10.1 vs ≥10.1</td>
<td>1.960</td>
<td>8.051</td>
<td>6.78</td>
<td>(1.64-21.21)</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Note: B=partial regression coefficient; CI=confidence interval; OR=odds ratio. The cutoff value of miRNAs in glioma patients vs control subjects was derived from receiver operating characteristic curves with Youden’s index.

Figure 5. Diagnostic performance of miR-Score in plasma samples from glioma patients. A: Order distribution of miR-Score values between two groups. B: Pairwise comparison of ROC curves of four subjects: miR-17 vs miR-Score (P=0.008), miR-130a vs miR-Score (P=0.002), miR-10b vs miR-Score (P=0.0005). There was no statistical significance between miR-17 vs miR-130a (P=0.2564), miR-17 vs miR-10b (P=0.3273), or miR-10b vs miR-130a (P=0.9871).

Patients relative to in healthy individuals (P=0.23 and 0.07, respectively; Figure 3C, 3D). Details are illustrated in Figure 3.

Diagnostic performance of miRNAs in glioma patients

ROC curves were constructed and the AUC was generated to assess the diagnostic values of the three selected miRNAs. As shown in Figure 4, ROC analyses revealed that the AUC of miR-
We used the coefficient before the three selected miRNAs in the logistic regression model to calculate a new score: miR-Score = -5.0 + 0.55*miR-17 + 0.40*miR-130a + 0.20*miR-10b. Interestingly, compared with these three miRNAs separately, the miR-Score showed the highest AUC value of 0.872 (0.787-0.932), with relatively higher sensitivity of 72.3% and specificity of 85.1% (Figure 5B). The value of the miR-Score among 45 healthy and 47 glioma patients was orderly distributed (Figure 5A).

Discussion

A biomarker is described as any measurable diagnostic indicator that is used to assess the risk or presence of disease [15]. Currently, blood-derived biomarkers, including circulating tumor DNA (ctDNA) [16], miRNAs [17], extracellular vesicles (EVs) [17, 18], and circulating tumor cells [19, 20] have shown great potential in distinguishing glioma from other types of cancer. Because they would help improve the management of patients through multimodal diagnostic procedures, it would be a tremendous advance in neuro-oncology if clinically useful circulating biomarkers from blood were validated.

miRNAs are small (approximately 21-24 nucleotides) noncoding RNA molecules. Because they are regulatory molecules with both oncogenic and suppressor gene functions, and are a major component of intercellular communication [21], miRNAs are gaining increasing attention as biomarkers in cancer. As some are detected in extracellular spaces and body fluids, this creates a novel opportunity for noninvasive assay development [22].

This study focuses on the observation of deregulated miRNA expression in plasma samples from glioma patients. Our results demonstrate that miRNAs circulating in peripheral blood may serve as novel biomarkers for the detection of glioma. We performed microarray analysis to acquire miRNA signatures in the plasma from glioma patients and matched negative controls. 13 aberrantly expressed miRNAs were found, and the three most significantly elevated miRNAs were then further validated, namely miR-17, miR-130a, and miR-10b. Interestingly, when comparison was performed in different grades of glioma and negative controls, these miRNAs showed statistically significant differences in high grade (III-IV) but not in low-grade gliomas (grade I-II). Then, we performed ROC curves and AUC to assess the diagnostic values of the three miRNAs. We could observe good diagnostic ability of the miR-17, miR-130a, and miR-10b, with AUC of 0.78 (0.69-0.86), 0.72 (0.62-0.81), and 0.72 (0.62-0.80), respectively. In addition, multivariate logistic regression analysis revealed that these were all risk factors in glioma patients. Furthermore, we constructed a new score based on these three selected miRNAs, named the miR-Score, which shows excellent diagnostic ability as compared to the 3 selected miRNAs alone. Collectively, our study provides evidence that miR-17, miR-130a, and miR-10b, derived from the plasma of glioma patients, have great clinical value as promising circulating biomarkers.

Overexpression of miRNAs is implicated in the metastasis of human tumors [23]. In our study, miR-17 was the most abundant miRNA in plasma samples from glioma patients. These data are consistent with those from Lu et al., who demonstrated high miR-17 expression levels in glioma tissue [24, 25]. miR-17, a member of the miR-17-92 cluster, has been reported to promote cell motility, invasion, and tube-like structure formation, resulting in drug-resistance by repressing murine double minute 2 (MDM2) levels [26]. Of interest, Comincini et al. suggested that miR-17 could negatively regulate autophagy-related protein 7 expression, resulting in a modulation of the autophagic status in glioblastoma cells. This could significantly impact cancer development, progression, and treatment [27]. More importantly, the increased expression of miR-17 in gliomas was found significantly associated with advanced pathological grades and low Karnofsky performance scores. Additionally, miR-17 expression was significantly associated with poor overall survival in glioma patients with high pathological grades [24]. miR-10b, previously reported potentially involved in the invasion of colorectal cancer [28], showed high expression in highly metastatic human breast adenocarcinoma cells and colon adenocarcinoma cells compared to less metastatic ones [29]. However, heterogeneous distribution of miR-10b expression has also been found among circulating tumor cells from the same patient [29]. Recently, miR-10b was reported to be upregulated in glioma tissues compared to in non-tumoral brain tissues. Upregulation of miR-10b has also been associ-
Diagnostic value of miRNAs to glioma

Dong et al. has also suggested that inhibition of miR-10b could induce cell cycle arrest and apoptosis and inhibit cell invasiveness in a human glioma cell line [31]. The miR-130 family (miR-130b, miR-301a, and miR-301b) is included in the miRNA superfamily, and is upregulated in several types of cancers such as bladder, breast, lung, and head and neck cancers. Egawa et al. has revealed that the miR-130 family has a crucial role in the malignant progression of bladder cancer, and its expression level has been found closely correlated with PTEN [32]. Duan et al. have found miR-130 expression level dramatically increased in gastric cancer tissues and overexpressed miR-130 could promote proliferation and migration of gastric cancer cells. Importantly, high levels of miR-130a have been detected in glioblastoma multiforme (GBM) tissues and were found to be significantly associated with overall survival of GBM patients [33]. Similarly, Chen et al. have found miR-130a correlated with overall survival in Temozolomide-treated patients with GBM and have suggested that miR-130a could serve as a better predictive marker than 06-methylguanine-DNA methyltransferase (MGMT) methylation in clinical resistance to chemotherapy [34]. In our study, no exact association of expression levels of miR-10b and miR-130a with the glioma grading was found. This negative finding may be attributed to the small sample size of this study and should be reevaluated in a larger patient cohort.

Conclusion

In conclusion, our results extend the findings of previous studies about miR-17, miR-130a, and miR-10b in glioma patients. Our data show that the three selected miRNAs were expressed at significantly higher levels in glioma patients, and demonstrate the ideal diagnostic value to distinguish glioma patients from healthy individuals. Considering the high diagnostic value of combined miR-17, miR-130a, and miR-10b analyses in this study, we anticipate miRNA analyses to have great potential to characterize circulating markers for gliomas.

Acknowledgements

This study was supported by grants from National Basic Research Program of China (2014CB957940) to D.M. and the grant from the National Natural Science Foundation of China (No. H1618) to L.W.

Disclosure of conflict of interest

None.

Address correspondence to: Weibang Liang, Department of Neurosurgery, Nanjing Drum Tower Hospital Clinical College of Nanjing Medical University, No. 321, Zhongshan Road, Gulou District, Nanjing 210008, Jiangsu, China. E-mail: lweibang@sina.com

References

Diagnostic value of miRNAs to glioma


