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Circulating microRNA-137 is a potential biomarker for human glioblastoma

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Abstract. – OBJECTIVE: In this study, we investigated whether circulating microRNA-137 (miR-137) could be a potential biomarker for patients with glioblastoma (GBM).

PATIENTS AND METHODS: Serum samples were collected from 64 GBM patients and 64 healthy controls. The expression level of circulating miR-137 was compared by quantitative RT-PCR. Among GBM patients, circulating miR-137 was compared between patients at early stages and those at advanced stages. Also, the correlations of serum miR-137 expression with clinicopathological features and overall survival of GBM patients were statistically examined. Furthermore, whether circulating miR-137 could serve as an independent predicting biomarker GBM patients' survival was assessed.

RESULTS: Serum miR-137 was downregulated in GBM patients than in healthy controls. It was further downregulated in GBM patients at advanced stages than in patients at early stages. Statistical analysis demonstrated that low serum miR-137 level was strongly correlated with patients' clinical grades (p = 0.003) and KFS (p = 0.002). Low serum miR-137 was also found to be significantly correlated with, and may predict poor survival in GBM patients.

CONCLUSIONS: Downregulated serum miR-137 may be a potential non-invasive prognostic biomarker for poor prognosis in GBM patients.

Key Words:
Glioblastoma, miR-137, Biomarker, Overall survival, Cox regression, Prognosis.

Introduction

Human glioblastoma (GBM), representing a heterogeneous multiform of rapidly growing glial-derived intracranial tumors, is one of the most malignant brain tumors in both adults and young patients[1-3]. Based on World Health Organization (WHO) classification[4], GBM patients can be categorized as those at early stages of pilocytic astrocytomas and diffuse astrocytomas (WHO grade I and II), or those at advanced stages of anaplasia astrocytomas and primary glioblastomas (WHO grade III and IV). Although great efforts had been made during the past decades to improve clinical outcomes of GBM patients by adopting advanced surgical procedures and innovative radiotherapy or immunotherapy[5,6], the prognosis of GBM patients remains very poor, with a 5-year survival rate to be less than 5%[1,2]. In addition, due to the heterogeneity of tumor development and metastasis, efficient non-invasive biomarkers are lacking for early diagnosis[7-9]. Thus, it is critical to understand the underlying mechanisms of GBM pathogenesis and develop novel biomarkers for early diagnosis to improve the chance of long-term survival among GBM patients.

MicroRNAs (miRNAs) represent families of single-stranded, noncoding short-length (~18 to 24 nucleotides long) RNAs that inhibit gene and protein productions by suppressing 3' un-translated region (3'UTR) of downstream targeted genes[10,11]. Studies have demonstrated that various groups of serum (or circulating) miRNAs were aberrantly dysregulated in cancer patients, therefore leading to the development of using circulating miRNAs as prognostic biomarkers for predicting human cancers[12-15]. In patients with GBM, several circulating miRNAs had been shown to be closely associated with patients' clinicopathological features. Specifically, in a recent microarray study, Dong and colleagues screened 752 miRNAs in serum samples from GBM patients, and found more than hundred circulating miRNAs were either downregulated or upregulated[16]. Also, they identified six dysregulated serum miRNAs, miR-576-5p, miR-340 and miR-626 (upregulated), as well as miR-320, let-7g-5p and miR-7-5P (downregulated) to be critical cancer modulators in GBM patients[16].

In many human cancers, microRNA-137 (miR-137) is normally lowly expressed in tumor tis-

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sues, thus acting as a tumor suppressive miRNA to modulate cancer proliferation, migration or chemosensitivity[17-20]. In human glioblastoma, miR-137 was found to be aberrantly downregulated in GBM tumors, inhibiting glioma proliferation and inducing tumor stem cell differentiation[21,22]. However, it is unknown whether miR-137 is dysregulated in sera of GBM patients, or circulating miR-137 is associated with clinical features or prognosis among GBM patients. Therefore, in this study, we attempted to fill the void by examining the serum expression level of miR-137 in 64 GBM patients through quantitative analysis of qRT-PCR. In addition, we statistically analyzed the correlation between serum miR-137 level and clinicopathological features, as well as survival in those GBM patients. The goal of this work is to explore whether serum miR-137 could be used as a future prognostic biomarker to assist early diagnosis in patients with glioblastoma.

Patients and Methods

Ethic Statements

All clinical and laboratory protocols were reviewed and approved by the Clinic Ethic Committees at Zhengzhou University People's Hospital and He'nan Provincial People's Hospital in Zhengzhou, He'nan Province, China. The experiments were performed in accordance with the Declaration of Helsinki, and in cooperation with local, State as well as Federal Health Agencies. All the patients signed the consent form to be included in this study.

Patients and Serum Samples

Between 2012 and 2015, a total of sixty-four patients with GBM were eligible and thus enrolled in the study at the Departments of Neurosurgery at Zhengzhou University People's Hospital and He'nan Provincial People's Hospital. Eligibility criteria are that patients did not receive any major brain or chest surgeries, radiotherapy, chemotherapy or immunotherapy within 180 days before the study. All patients received surgery. Surgically retrieved tumors were prepared in sectioning slides, and evaluated by independent histologists in accordance with WHO classification of brain tumors[4]. Of all 64 patients, 35 patients had tumors which were categorized as pilocytic astrocytomas and diffuse astrocytomas (WHO grade I/II). The remaining 29 patients had the tumors categorized as anaplasia astrocytomas or primary glioblastomas (WHO grade III/IV). Patients' serum samples were drawn 24 hours before the surgery, and immediately snap-frozen in liquid nitrogen, then stored at –80°C until further processed.

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

Total RNA was isolated from frozen sera using a TRIZOL kit (Thermo Fisher Scientific, San Jose, CA, USA) according to the manufacturer's instruction. RNA quality was verified by a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Complimentary DNA was then obtained by reverse transcription with a TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, San Jose, CA, USA) according to the manufacturer's instruction. Gene level of miR-137 in patients' sera was quantified by quantitative real-time reverse transcription-PCR (qRT-PCR) using a TaqMan MicroRNA assay kit (Thermo Fisher Scientific, San Jose, CA, USA) with an ABI Prism 7900HT Sequence Detection System (Thermo Fisher Scientific, San Jose, CA, USA). 18s gene was used as house keeping gene. Relative miR-137 expression was quantified by the $2^{-\Delta\Delta Ct}$ method, where Ct represented reaction cycles, and normalized to 18s expression level.

Statistical Analysis

All calculated data were averaged from triplicates and represented as mean ± standard deviations. All statistical analyses were carried out on a Windows-based SPSS software (SPSS, Chicago, IL, USA). While evaluating the correlation between circulating miR-137 and GBM patients' clinicopathological features, hazard risk ratios (HRR) and 95% confidence intervals (CI) were estimated using forward stepwise univariate and multivariate Cox regression model, along with the Chi-squared test. GBM Patients' survival was estimated by Kaplan-Meier model, along with the log-rank test. Statistical differences were determined if p was less than 0.05.

Results

Serum miR-137 is Downregulated in GBM Patients

Studies had demonstrated that miR-137 is aberrantly downregulated in tumors of GBM pa-

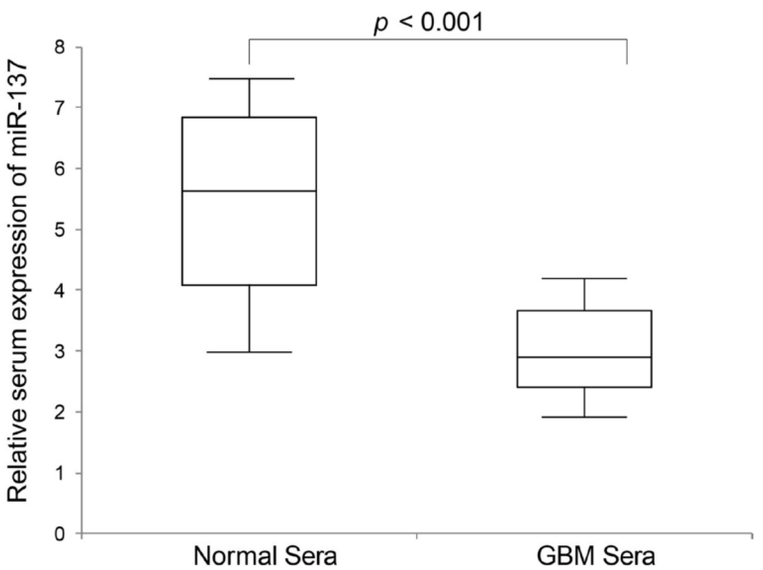

Figure 1. *MiR-137 is lowly expressed in sera of GBM patients*. Between 64 GBM patients and 64 healthy control patients, qRT-PCR was conducted to compare serum expression levels of miR-137 ($^{*}p < 0.001$).

tients, and generally serving as a tumor suppressor in inhibiting GBM proliferation and migration[21,22]. In this study, we looked into the sera of GBM patients to explore whether serum miR-137 level would also be aberrantly expressed. A total of 64 GBM patients, as well as 64 health control patients were included in the study. Through the analysis of qRT-PCR, we found that miR-137 was significantly downregulated in sera of GBM patients, than in sera of healthy control patients. (Figure 1A, *: $p < 0.001$).

Serum miR-137 is Further Downregulated in Patients with Advanced Stages of GBM

In this study, based on WHO standard, 35 of 64 GBM tumors were categorized as Grade I or II GBM, and 29 of them were categorized as Grade III or IV GBM. We then compared the serum miR-137 expression between patients with early-stages of GBM (WHO grade I and II), and patients with advanced stages of GBM (WHO grade III and IV). Through the analysis of qRT-PCR, we discovered that miR-137 was further downregulated in patients with advanced stages of GBM (Figure 2, *: $p < 0.001$).

Downregulation of Serum miR-137 is Correlated with Advanced Clinicopathological Features of GBM Patients

Based on the mean serum expression level of miR-137, GBM patients were divided into two groups. Patients in one group (n = 33) had lower serum expression level of miR-137 than the mean value. Patients in the other group (n = 31) had higher serum expression level of miR-137 than the mean value. We, then, analyzed the correlation between serum miR-137 expression (low serum miR-137 vs. high serum miR-137), and the clinicopathological features of GBM patients (Table I). The statistic results showed that GBM patients' age ($p = 0.632$), gender ($p = 0.433$), or tumor size ($p = 0.237$) were not correlated with serum miR-137 level (Table I). However, regarding patients' WHO grade and Karnofsky Performance Scale (KPS), low serum miR-137 was discovered to be strongly correlated with patients with advanced WHO grades (Grade III and IV, $p = 0.003$) or KPS < 90 ($p = 0.002$) (Table I, $^{*}p < 0.05$).

Downregulation of Serum miR-137 is Correlated with Poor Overall Survival of GBM Patients

Between 33 GBM patients with low serum miR-137 and 31 GBM patients with high serum miR-137, their cumulative overall survival (OS) were plotted with Kaplan-Meier method and compared by log-rank test. It showed that low expression level of serum miR-137 was strongly correlated with poor OS among BGM patients (Figure 3, $p < 0.001$). Based on this information, we further investigated which of clinicopathological features would be correlated with patients'

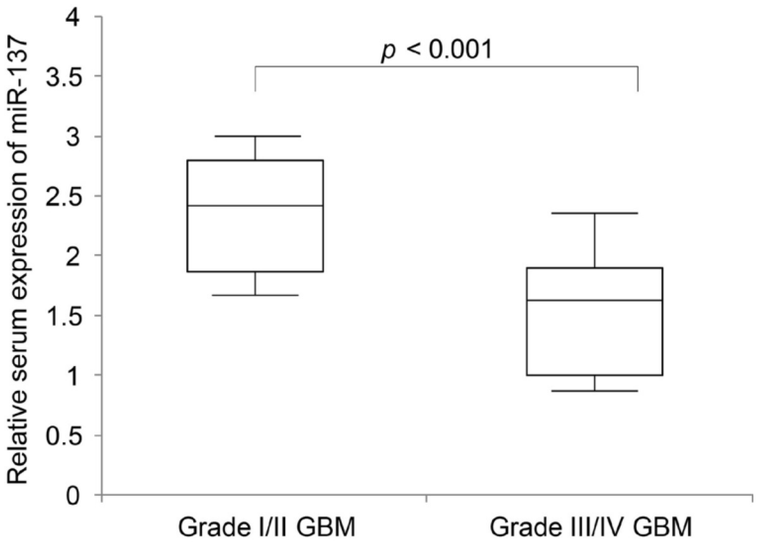

Figure 2. *MiR-137 is lowly expressed in sera of GBM patients at advanced stages*. Between 35 early-stage GBM patients (WHO Grade I and II), and 29 advanced-stage GBM patients (WHO Grade III and IV), qRT-PCR was conducted to compare serum expression levels of miR-137 ($^*p < 0.001$).

OS. The analysis of univariate Cox regression showed that, WHO grade ($p = 0.012$, HRR = 2.361, 95% CI = 1.902-5.722), KPS $p = 0.025$, HRR = 2.189, 95% CI = 1.578-4.509) and serum miR-137 expression ($p < 0.001$, HRR = 5.074, 95% CI = 3.891-8.931), were strongly associated with OS (Table II, univariate analysis $^*p < 0.05$). In addition, the analysis of multivariate cox regression confirmed that WHO grade ($p = 0.024$, HRR = 2.199, 95% CI = 1.567-4.853) and serum miR-137 expression ($p < 0.001$, HRR = 4.781, 95% CI = 2.911-7.572) were independent predictor of poor OS among GBM patients (Table II, multivariate analysis $^*p < 0.05$).

Discussion

It has been demonstrated that miR-137 was downregulated in the tumorous tissues in GBM patients, and acting as a tumor suppressor to inhibit cancer proliferation and induce differentiation of tumor stem cells[21,22]. However, it is still unknown, what the expression pattern of circulating miR-137 would be, or whether circulating miR-137 could be a valuable biomarker for predicting prognosis in GBM patients. Thus, in this study, we attempted to answer this question by comparing the serum miR-137 levels between 64 GBM patients and equal number healthy control patients. Through the analysis of qRT-PCR, we demonstrated that serum miR-137 was aberrantly downregulated in GBM patients, in-line with previous studies showing miR-137 downregulation in tumorous tissues was associated with poor prognosis of GBM patients[21].

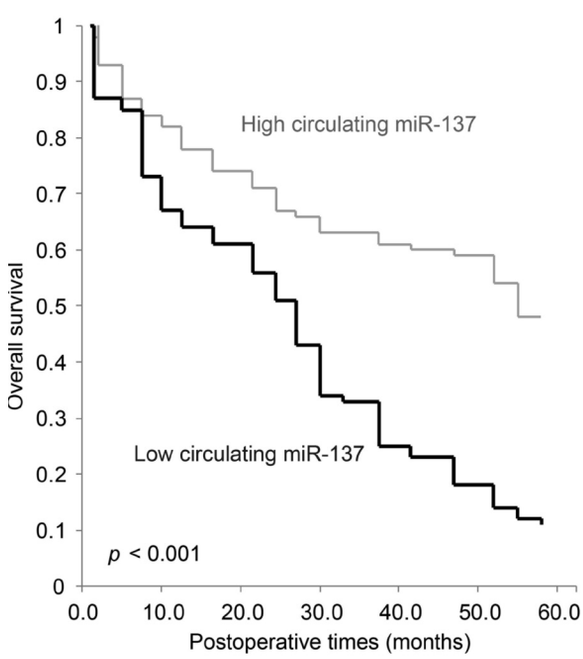

Figure 3. *Serum miR-137 is associated with overall survival of GBM patents*. Overall survival (OS) curves of GBM patients were plotted using Kaplan-Meier method, and compared between patients with low circulating miR-137 and those with high circulating miR-137 by log-rank test ($p < 0.001$).

Table I. Correlation of serum miR-137 with clinicopathological features of 64 patients with glioblastoma. Abbreviations: WHO, World Health Organization; KPS, Karnofsky Performance Scale. *p < 0.05

	Serum miR-137 expression		
Features	**Low (n = 33) No. of cases (%)**	**High (n = 31) No. of cases (%)**	***p***
Age (years)			
< 50	13 (39.4)	10 (32.2)	0.632
≥ 50			
Gender	20 (60.6)	21 (67.8)	
Male	18 (54.5)	17 (54.8)	0.433
Female	15 (45.5)	14 (45.2)	
WHO grade			
I/II	12 (36.4)	23 (74.2)	0.003*
III/IV	21 (63.6)	8 (25.8)	
KPS			
< 90	23 (69.7)	9 (29.0)	0.002*
≥ 90	10 (30.3)	22 (71.0)	
Tumor size			
< 5 cm	14 (42.4)	15 (48.4)	0.237
≥ 5 cm	19 (57.6)	16 (51.6)	

Not only did we show serum miR-137 was downregulated in GBM patients than in healthy control patients, but also we showed that serum miR-137 was further downregulated in GBM patients at advanced stages than in GBM patients at early stages. This information is very important, suggesting that circulating miR-137 may be closely associated with tumor biology in GBM patients. We then, in our work, analyzed this possibility by comparing serum miR-137 expression (low expression vs. high expression) among the key clinicopathological features of GBM patients. We discovered that patients with low serum miR-137 level were significantly associated with advanced WHO Grade and KPS. In addition, we analyzed the correlation between serum miR-137 and overall survival of GBM patients. We found that patients with low serum miR-137 level had significantly worse overall survival than patients with high serum miR-137 level. Moreover, we used Cox regression model to show that serum miR-137 was a reliable independent prognostic predictor for poor prognosis of GBM patients. Therefore, all our data suggest that serum miR-137 may be a novel inexpensive biomarker for cancer patients with GBM. Interestingly, low expression of serum miR-137 was also found in patients with Alzheimer's disease (AD) and closely associated with AD risk models[23], suggesting that downregulated, or lowly expressed serum miR-137 may be an even more valuable tool to not only predict poor prognosis or advanced clinicopathological features in GBM patients, but also be a non-invasive biomarker for patients with neurodegenerative diseases.

Table II. Univariate and multivariate cox regression analysis on OS and prognostic features in 64 patients with glioblastoma. Abbreviations: OS, overall survival; HRR, hazard risk ratio; CI, confidence interval. *$p < 0.05$.

		Univariate analysis		Multivariate analysis	
Prognostic features		***p***	**HRR (95% CI)**	***p***	**HRR (95% CI)**
Age	<50 years and ≥ 50 years	0.371	1.554 (0.892-2.345)		
Gender	Male and female	0.311	0.875 (0.451-2.621)		
WHO grade	I/II and III/IV	0.012*	2.361 (1.902-5.722)	0.024*	2.199 (1.567-4.853)
KPS	<90 and ≥ 90	0.025*	2.189 (1.578-4.509)	0.058	1.788 (1.056-3.567)
Tumor size	<5 cm and ≥ 5 cm	0.061	3.290 (1.634-7.691)		
Serum miR-137 level	Low vs. high	<0.001*	5.074 (3.891-8.931)	0.001*	4.781 (2.911-7.572)

Conclusions

Our data discovered an aberrant expression pattern of circulating miR-137 in patients with GBM. The results of our study strongly suggest that low serum miR-137 may be used as a prognostic biomarker for human GBM patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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