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Original Article

Downregulation of microRNA-504 is associated with poor prognosis in high-grade glioma

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Abstract: Several previous reports indicated that microRNA-504 (miR-504) has an oncogenic function through negatively regulating p53. On the other hand, a recent study revealed that miR-504 inhibits cancer cell proliferation through targeting CDK6 in hypopharyngeal squamous cell carcinoma (HSCC), suggesting the tumor suppressive role of this miRNA. However, the role of miR-504 in human malignant glioma remains unclear. Therefore, the aim of this study was to investigate the clinical significance of miR-504 expression in high pathological grade glioma. Quantitative real-time reverse transcriptive-PCR (qRT-PCR) was performed to examine miR-504 expression levels in 63 glioma tissues including 13 anaplastic astrocytomas (AA, WHO grade III) and 50 glioblastomas (GBM, WHO grade IV), as well as 10 non-neoplastic brain tissues. Associations between miR-504 expression and clinicopathological factors and prognosis of glioma patients were statistically analyzed. MiR-504 showed significant decreased expression levels both in AAs and GBMs relative to non-neoplastic brains ($P \leq 0.001$, respectively). Additionally, low expression level of miR-504 was significantly associated with advanced WHO grade ($P = 0.01$). Moreover, Kaplan-Meier survival analysis showed that patients with low expression of miR-504 had significantly poor survival rate ($P = 0.002$). Cox regression analysis showed that miR-504 expression was independent prognosis-predicting factor for malignant glioma patients ($P = 0.038$; risk ration = 2.5). Our results suggest that miR-504 may be a prognostic predictor and be involved in tumorigencity as a tumor suppressor of malignant glioma.

Keywords: microRNA, miRNA-504, malignant glioma, prognosis, glioblastoma, anaplastic astrocytoma

Introduction

Glioma is one of the most frequent and malignant primary brain tumors in adults. Despite recent advances in surgery, radiotherapy, and chemotherapy, the prognosis for patients with this tumor remains poor [1]. According to the World Health Organization (WHO) classification, gliomas are divided into pilocytic astrocytoma (PA, WHO grade I), diffuse astrocytoma (DA, WHO grade II), anaplastic astrocytoma (AA, WHO grade III), and glioblastoma (GBM, WHO grade IV) in the order of increasing malignancy [1]. Clinically, patients with high pathological grade gliomas (WHO grade III and IV) have significantly poor prognosis relative to that with low grade gliomas (WHO grade I and II) [1]. Among these, GBMs account for the vast majority of human gliomas and are highly proliferative and invasive tumors characterized by remarkable biological heterogeneity and poor response to present treatments [2]. Despite recent introduction of concomitant temozolomide with radiotherapy, median overall survival of primary GBM patients remains around 14.6 months, and the 5-year survival rate is only 9.8% at present [3]. Further development of diagnosis and treatment based on novel molecular mechanisms is necessary for malignant glioma patients.

MicroRNAs (miRNA) are small noncoding RNA molecules that contribute to the regulation of crucial biological processes, such as cell proliferation, apoptosis, development, and differentiation [4, 5]. Recent studies have confirmed that miRNAs play crucial roles in tumorigenesis, angiogenesis, invasion, and apoptosis for various human cancers [6, 7]. Furthermore, the characterization of miRNA expression patterns in cancer cells is thought to have a substantial value for diagnoses and prognoses as well as

Table 1. Correlation of miR-504 relative expression level with clinicopathological factors of malignant glioma patients

Clinicopathological features	No. of cases	miR-504 expression		*P*
		High (n, %)	Low (n, %)	
WHO grade				0.01
III	13	7 (53.8%)	6 (46.2%)	
IV	50	10 (25.0%)	40 (75.0%)	
Age				0.19
> 50	48	11 (22.9%)	37 (77.1%)	
≤ 50	15	6 (40.0%)	9 (60.0%)	
Gender				0.72
Male	32	8 (25.0%)	24 (75.0%)	
Female	31	9 (29.0%)	22 (70.1%)	
KPS				0.52
< 90	41	10 (24.4%)	31 (75.6%)	
≥ 90	22	7 (31.8%)	15 (68.2%)	
Surgery				0.84
GTR	31	8 (25.8%)	23 (74.2%)	
PR	32	9 (28.1%)	23 (78.9%)	

Abbreviations: KPS, Karnofsky performance scale; GTR, gross total resection; PR, partial resection.

for subsequent therapeutic interventions [8-12].

Recent investigations have identified dysregulation of specific miRNAs in malignant gliomas [13-18]. Chen et al. identified that miR-21 is overexpressed and acts as an antiapoptotic factor in glioblastoma cells [13]; Godlewski et al. reported that miR-128 inhibits glioma proliferation and self-renewal by targeting Bmi-1 oncogene [14]; In 2008, Kefas et al. demonstrated that miR-7 is down-regulated and inhibits epidermal growth factor receptor and the Akt pathway in glioblatoma [15]; Guan et al. showed evidences that up-regulation of miR-196 is associated with poor survival in glioblastoma patients [16]; Fareh et al. in 2012 revealed that miR-302-367 cluster drastically affects self-renewal and infiltration properties of glioma-initiating cells through CXCR4 repression and consequent disruption of the SHH-GLI-NANOG network [17]; A recent investigation by Wang et al. revealed that down-regulation of miR-326 is significantly correlated with poor outcomes in glioma patients [18]. These findings suggest that miRNA expression could be used as effective markers not only for analyzing the molecular pathogenesis but also for diagnosing and prognosis predicting in human malignant gliomas. Moreover, further study on miRNA functions in glioma stem cells may lead to novel treatment strategies for malignant glioma. In fact, several recent studies have facilitated the miRNA-based replacement for tumor suppressors miR-128, miR-7 and miR-34a over inhibitory approach in GBM [14, 15, 20]. Taken together, characterization of the miRNA expression signature for malignant gliomas is of great significance for revealing the molecular mechanisms of tumorigenesis and progression of these malignancies.

Several reports indicate that miR-504 has an oncogenic function by targeting TP53 [21, 22]. In contrast, a recent study confirmed the down-regulation of miR-504 in hypopharyngeal squamous cell carcinoma and showed its tumor suppressive function in this tumor [23]. Such contradictory results suggest that the function of miR-504 may be manifold and complicated in different types of human cancers. For glioma, a global miRNA expression screening study in 2010 by Guan et al. has shown that expression of miR-504 is significantly decreased in GBMs compared with that in AAs [16]. Subsequently, Ma et al. in 2012 reported that suppression of miR-504 increases the expression of mesenchymal markers in glioblastoma cells, suggesting miR-504 may negatively regulating mesenchymal signaling in this malignant brain tumor [24]. However, the role of miR-504 in human glioma has not been clearly understood. Therefore, in the present study, we sought to investigate the clinical significance of miR-504 expression in human malignant gliomas.

Materials and methods

Glioma specimens and patients

Glioma specimens were obtained from patients during surgery at Firt Affiliated Hospital of China Medical University. A portion of the tumor tissue was saved and made into paraffin sections for histopathologic diagnosis in strict accordance with World Health Organization (WHO) criteria by two established neuropathologists, with differences resolved by careful discussion.

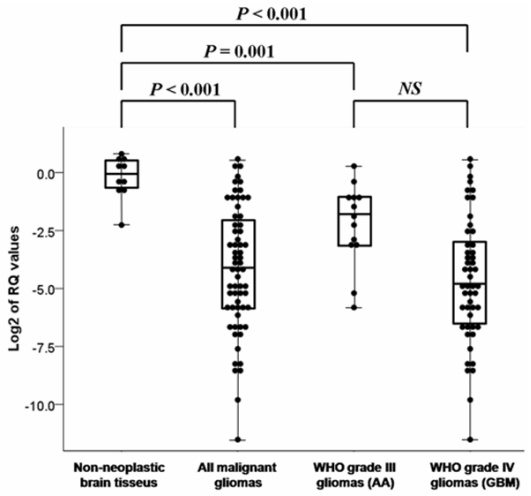

Figure 1. miR-504 expression in 63 malignant gliomas (50 GBMs and 13 AAs) and 10 non-neoplastic brain tissues detected by quantitative reverse transcriptive real-time polymerase chain reaction (qRT-PCR) analysis. The expression levels of miR-504 were found to be distinctly decreased in glioma tissues compared to normal brain tissues ($P < 0.001$). MiR-504 expression in GBMs and AAs were both significantly lower than that in non-neoplastic brains tissues ($P \leq 0.001$, respectively). There was no significant difference in miR-504 expression between GBMs and AAs ($P = 0.071$).

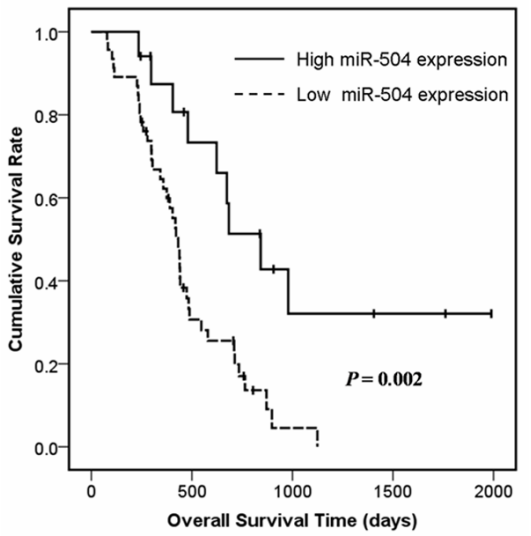

Figure 2. Kaplan-Meier survival curves for glioma patients with high and low expression levels of miR-504. Among 63 malignant glioma patients (50 GBMs and 13 AAs), those with low miR-504 expression (left, dotted line, $n = 46$) had significantly shorter survival periods than did patients with high miR-504 expression (right, solid line, $n = 17$; $P = 0.002$).

And the remaining tissue was snap-frozen in liquid nitrogen then stored at -80°C for RNA extraction and other biological molecular experiments. A total of 63 high-grade glioma specimens were collected, including 50 glioblastomas (GBM, WHO grade IV) and 13 anaplastic astrocytomas (AA, WHO grade III). The clinicopathological features and the treatment strategies of all patients were indicated in **Table 1**. Ten non-neoplastic brain tissue samples used as controls were obtained by collecting donations with consents from individuals who died in traffic accidents and were confirmed to be free of any prior pathological lesions. None of the patients had received chemotherapy or radiotherapy prior to surgery, and all patients were well followed up. Overall survival time was calculated from the date of the initial surgical operation to death. Patients, who died of diseases not directly related to their gliomas or due to unexpected events, were excluded from this study. The present study was approved by the Ethics Committee of China Medical University.

RNA extraction, reverse transcription and real-time PCR quantification for miRNA detection

Total RNA was extracted from glioma frozen tissues using a mirVana miRNA Isolation Kit (Life Technologies, USA). To examine the expression levels of miR-504 in glioma tissues, cDNA synthesis and subsequent quantitative real-time PCR were performed using a TaqMan MiRNA Reverse Transcription Kit (Life Technologies) and individual TaqMan MiRNA assay (Life Technologies), and Applied Biosystems 7500HT Fast Real-Time PCR System (Life Technologies), as previously described [25]. RNU48 were used as endogenous controls, and non-neoplastic brain tissues were used for calibration. Relative quantification of miR-504 expression was calculated with the $2^{-\Delta\Delta}$Ct method.

Statistical analysis

All computations were carried out using the software of SPSS version19.0 for Windows (SPSS Inc, IL, USA). Data were expressed as mean ± standard deviation (SD). The differential expression of miR-504 between glioma tissues and normal brain tissues was evaluated by independent sample t test. The X^2 test was used to analyze the relationship between miR-504 expression and the clinicopathological characteristics. A life table was calculated according to the Kaplan-Meier method. Risk ratios for the time-to-event endpoint were esti-

Table 2. Univarite and multivariate Cox regression analysis for overall survival in malignant glioma patients

Univariate analysis					Multivariate analysis			
Variate	No. of case (%)	Mean OS	95% CI	*P* (log-rank)	Variate	RR	95% CI	*P*
WHO grade				< 0.001	WHO grade			0.001
III	13 (20.6%)	1330	940-1724		IV vs. III	14.9	3.2-68.1	
IV	60 (79.4%)	444	378-509					
Age				0.066	Age			0.141
> 50	48 (76.2%)	801	509-1094		> 5 0 vs. ≤ 50	0.5	1.2-1.3	
≤ 50	15 (23.8%)	547	409-685					
Gender				0.631	Gender			0.128
Male	32 (50.8%)	629	425-832		Male vs. Female	1.6	0.9-3.0	
Female	31 (49.2%)	559	467-651					
KPS				0.021	KPS			0.548
< 90	41 (65.1%)	487	389-585		< 90 vs. ≥ 90	1.3	0.6-2.6	
≥ 90	22 (34.9%)	841	557-1125					
Surgical resection				0.165	Surgery			0.097
GTR	31 (49.2%)	679	498-860		PR vs. GTR	1.7	0.9-3.2	
PR	32 (50.8%)	569	387-750					
miR-504 expression				0.002	miR-504 expression			0.038
Low	46 (73.0%)	470	389-551		Low vs. High	2.5	1.1-5.7	
High	17 (27.0%)	1056	688-1424					

Abbreviations: KPS, Karnofsky performance scale; GTR, gross total resection; PR, partial resection; OS, overall survival; RR, risk ratio.

mated using the multivariate Cox regression analysis in a forward stepwise method to evaluate the effect of multiple independent prognostic factors on overall survival outcome. Differences were considered statistically significant when *P* was less than 0.05.

Results

Down-regulation of miR-504 in high pathological grade gliomas

To evaluate the dysregulation of miR-504 in high pathological grade gliomas, we examined miR-504 expression levels in a panel of 63 glioma tissues including 13 AAs (WHO grade III) and 50 GBMs (WHO grade IV) as well as 10 non-neoplastic brain tissues for calibration, by qRT-PCR. As shown in **Figure 1**, miR-504 expression levels were distinctly decreased in glioma tissues compared with non-neoplastic brain tissues (Student's *t*-test, $P < 0.001$), corresponding to WHO grade of the tumor. In addition, miR-504 expression levels in GBMs and AAs were both significantly lower than that in brain tissues ($P \leq 0.001$, respectively). Furthermore, our data showed that its expression levels were 0.485-fold lower in GBMs than that in AAs, though with no statistical significance ($P = 0.071$).

MiR-504 down-regulation correlates with malignant degree of gliomas

Subsequently, correlations between miR-504 expression level and several clinicopathological factors including malignant degree of tumor, patients' age at diagnosis, gender, pre-operative Karnofsky performance scale (KPS) and extent of tumor resection in glioma patients were evaluated by X^2 test. We assigned gliomas to miR-504 low-expression group and high-expression group that were tumors with miRNA expression under and above the median value of expression in all 63 gliomas, respectively (median expression value = 0.209; n = 46 and 17 for low-expression group and high-expression group, respectively). As shown in **Table 1**, miR-504 low-expression was significantly more frequent in GBMs than AAs (75.0% vs. 46.2%, $P = 0.01$, X^2 test). However, no statistically significant correlation was observed between miR-504 expression and other clinicopathological factors (**Table 1**).

Low expression level of miR-504 predicts poor survival in glioma patients

Furthermore, prognostic value of miR-504 expression in malignant glioma patients was evaluated. We performed analyzed log lank test and Kaplan-Meier analysis to evaluate the association between miR-504 expression level and clinical information in 63 malignant glioma patients mentioned above. We observed that miR-504 expression level displayed a significant correlation with glioma patients' overall survival. As shown in **Figure 2**, patients with low-expression level had significantly poor overall survival compared to that with high-expression level of miR-504 (mean overall survivals 470 and 1056 days, respectively; $P = 0.002$, log rank test). In addition, univariate and multivariate analysis using the Cox proportional hazard regression model was performed to determine whether miR-504 expression level and other clinical parameters are independent factors for prognostic prediction in glioma patients. Our result showed that both miR-504 low expression ($P = 0.038$; risk ratio 2.5, multivariate Cox regression analysis) and high pathological grade ($P = 0.001$; risk ratio 14.9, multivariate Cox regression analysis) were independent predictors of poor prognosis in glioma patients (**Table 2**).

Discussion

Recent study has implicated miRNAs in a variety of human cancers, expression signature of these small non-coding RNA molecules can provide insight into the diagnosis and prognosis of human cancers including glioma, the most frequent and malignant primary brain tumors in human adults [13-18]. In the present study, we evaluated the correlations between clinicopathological information and expression profiles of miR-504 in human high pathological grade gliomas from clinical tissue samples. As the results of our data analyzing, we found that: first of all, miR-504 was distinctly down-regulated in both WHO grade III and IV huamn glioma tissues compared to non-neoplastic brains; in addition, the decreased expression of miR-504 was significantly associated with the advanced tumor malignant grade; furthermore, glioma patients with low expression levels of miR-504 had significantly shorter overall survivals compared to that with high miR-504 expression; finally, according to the result of multivariate analysis, low expression of miR-504 was an independently significant risk factor for poor overall survival in malignant glioma patients, suggesting that miR-504 expression might be clinically a valuable marker for prognosis prediction in brain tumor patients. To our knowledge, this is the first ivestigation evaluating the expression pattern and clinical significance in large panel of high pathological gliomas.

As a less known miRNA, miR-504 has been reported to be involved in psychiatric disorders and psychotropic drug abuse through regulating the expression of dopamine D1 receptor gene (*DRD1*) and dopamine D2 receptor gene (*DRD2*) [26, 27]. Huang et al. in 2009 originally reported that miR-504 modulates differential allelic specific expression of *DRD1*, which is associated with nicotine dependence, by directly binding to the 3'-untranslated region (3'UTR) [26]. A recent study by Zhang et al. showed evidences that maternal deprivation enhances behavioral vulnerability to stressors during adulthood through the up-regulation of miR-504 expression in the nucleus accumbens and miR-504 may mediate the down-regulation of *DRD2*, which is involved in stress-induced depression, in animal models [27].

On the other hand, several recent investigations have indicated the involvement of miR-504 in human cancers. Functioning as an oncomiRNA, this miRNA was originally shown to target the tumor suppressor gene, TP53, as reported by Hu et al [21]. They observed that the overexpression of miR-504 decreased the p53 protein levels through directly binding to 3'UTR of p53 and functions in cells, including the p53 transcriptional activity, p53-mediated apoptosis, and cell-cycle arrest in response to stress, and also promoted the tumorigenecity of cells *in vivo*, suggesting the direct regulation of p53 by miR-504 as a important mechanism of p53 regulation in cells [21]. Similarly, a recent study by Soutto et al clearly demonstrated that TFF1 activates p53 through down-regulating miR-504 in gastric cancer [28]. In addition, Yang et al. demonstrated that the overexpression of miR-504 induces cellular invasion and lymph nude metastasis by inhibiting the expression of its target gene, FOXP1, in oral squamous cell carcinomas [22]. Furthermore, Jiang et al identified miR-504 as a novel predictive marker for the prognosis of pancreatic ductal adenocarcinoma (PDAC). They showed that

expression level of miR-504 was significantly higher in PDAC than that in normal pancreatic tissues and high miR-504 level significantly correlated with poor survival, high clinical and pathological stages in PDAC patients [29].

However, little is known about the involvement of miR-504 in tumorigenesis and clinical implication in malignant glioma, the most frequent huamn brain tumor. A global miRNA expression screening study in 2010 by Guan et al. has shown that expression of miR-504 is significantly decreased in GBMs compared with that in AAs [16]. Kim et al. in 2011 subclassified GBMs into 5 distinct subclasses based on 261 microRNA expression profiles and categorized miR-504 into neural precursor cluster [30]. On the other hand, Ma et al. in 2012 analyzed the association between miRNA and mRNA expression in human GBMs and observed that miR-504 was down-regulated in primary GBMs compared with DAs and secondary GBMs. In addition, they showed that expression of miR-504 was negatively correlated with that of mesenchymal markers including VIM and YKL-40 in GBMs and transfection of miR-504 inhibitor enhanced expression of VIM and YKL-40 in U87 cells [24]. In accordance with the findings mentioned above, at the present study, we found that miR-504 expression levels in high-pathological grade gliomas were distinctly lower than that in non-neoplastic brain tissues and the decreased miRNA expression significantly correlated with higher pathological tumor grade. Beside these observations, we futhermore showed that miR-504 expression level was significantly correlated with overall survival and lower-expression of miR-504 was an independent risk factor of poor survival in malignant glioma patients. Taken together, these findings indicated that miR-504 functions as a tumor suppressor in glioma. Similarly, a recent study also found the downregulation of miR-504 in hypopharyngeal squamous cell carcinoma, and overexpression of this miRNA inhibited cancer cell proliferation by targeting CDK6 [23]. Interestingly, miR-504 was originally reported to have an oncogenic function through negatively regulating p53 in cancer cells [21]. Consequently, miR-504 could act as both oncogene and tumor suppressor in various cancers, and which role it takes depends on the origin organism of tumor.

In conclusion, our results suggest that down-regulation of miR-504 might have potential value for predicting clinical outcomes in high-pathological grade glioma patients, suggesting that miR-504 is an important candidate tumor suppressor, and its down-regulation might be involved in tumorigenesis and malignant pregression of huamn glioma. However, the detailed biological mechanism by which down-regulation of miR-504 contributes to tumorigenesis of malignant glioma still remains unclear. Hence, further functional analyses are deserved to uncover the mechanism of biological regulation by miR-504 in glioma tumorigenesis.

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Disclosure of conflict of interest

None.

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