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RESEARCH

Decreased expression of microRNA-206 correlates with poor clinical outcome in patients with malignant astrocytomas

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Abstract MicroRNA-206 (miR-206) has been proved to function as a tumor suppressor in several types of human malignant cancers. More recently, it has been demonstrated that the ectopic expression of miR-206 significantly inhibited the proliferation and promoted apoptosis at the early stages in glioma cell U343. In order to investigate the clinical significance of miR-206 expression in human astrocytoma, quantitative real-time polymerase chain reaction (qRT-PCR) analysis was used to characterize the expression patterns of miR-206 in 108 astrocytoma and 20 normal brain tissues. As the results, the expression levels of miR-206 in astrocytoma tissues were significantly lower than those in normal brain tissues ($P<0.001$). Additionally, the decreased expression of miR-206 in astrocytoma was significantly associated with advanced pathological grade ($P=0.008$), low Karnofsky performance score (KPS, $P=0.02$), and large tumor size ($P=0.01$). Moreover, Kaplan-Meier survival and Cox regression analyses showed that low miR-206 expression ($P<0.001$) and advanced pathological grade ($P=0.02$) were independent factors predicting poor prognosis for astrocytomas. In conclusion, this is the first report of the differential expression of miR-206 in human astrocytoma tissues. MiR-206 could be a valuable marker of astrocytoma progression and low miR-206 expression is associated with poor overall survival in patients with malignant astrocytomas.

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Introduction

Human astrocytomas are the most common form of central nervous system neoplasms for both children and adults with an overall incidence of about 4–5 per 100,000 persons per year [1]. According to the World Health Organization (WHO) classification, astrocytomas 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 [2]. Among these, GBMs account for the vast majority of human astrocytomas, and are highly proliferative and invasive tumors characterized by remarkable biological heterogeneity and poor response to present treatments [3, 4]. Patients with low grade astrocytomas (grade I~II) have the best prognosis with a median survival time of 6 to 8 years after surgical intervention as compared to those with high grade tumors (grade III~IV) [5]. The median survival time for patients with AA is 2 to 3 years whereas patients with GBM have a median survival ranged from only 9 to 12 months [6]. As the mechanism of malignant potential of astrocytoma cells and prognostic factors of astrocytoma are poorly defined, and the histology-based classification is highly subjective, it is of great significance to identify novel risk factors and construct more robust histology-independent molecular classifiers.

Molecular analyses are performed to identify appropriate markers of astrocytomas for classification and prognosis.

Accumulating studies have detected genome-wide gene expression profiles to stratify astrocytomas and to define the intrinsic features of the astrocytoma subtypes. More recently, several studies have focused on other transcriptomic markers and were based on analyses of expression levels of microRNAs (miRNAs) in astrocytoma tissues. MiRNAs, 22 nucleotide endogenous non-coding single-stranded RNAs, negatively regulate the expression levels of their target genes either by degrading specific mRNA or inhibiting translation [7]. MiRNAs have been demonstrated to play important roles in many biological processes such as development, differentiation, cell proliferation and apoptosis [8]. As many of these processes often altered during the initiation and progression of human cancers, miRNAs have attracted the attention of many research groups to investigate their involvement in different malignancies. Recently, accumulating studies have been focus on the specific miRNA patterns for different types of astrocytomas. Jiang et al. [9] examined the levels of 450 miRNAs in human primary astrocytoma tissues of varying WHO grades by microarray analyses; Lages et al. [10] measured the levels of 282 miRNAs using membrane-array hybridisation and quantitative real-time polymerase chain reaction (qRT-PCR) in oligodendrogliomas, glioblastomas and control brain tissues, and found that the tissue levels of 7 miRNAs (miR-21, miR-128, miR-132, miR-134, miR-155, miR-210 and miR-409-5p) appropriately discriminated oligodendrogliomas from glioblastomas; Rao et al. [11] detected the expression of 756 miRNAs by microarray in 13 anaplastic astrocytoma and 29 glioblastoma tumor samples, and found a 23-miRNA expression signature that precisely differentiated glioblastoma from anaplastic astrocytoma with an accuracy rate of 95 %. Taken together, a body of evidence has suggested that miRNA expression could be used as effective markers for analyzing molecular pathogenesis and classifying subtypes of human astrocytomas.

In the human genome, miR-1-1/miR-133a-2, miR-1-2/miR-133a-1, and miR-206/miR-133b form clusters in three different chromosomal regions: 20q13.33, 18q11.2, and 6p12.2, respectively [12]. Among these, miR-206/133b is in an intergenic region [12]. As a homologue of miR-1, miR-206 is similar to miR-1 in terms of expression and function, but its sequence differs from the miR-1 sequence by four nucleotides [13]. Previous studies identified miR-206 as a skeletal muscle-specific miRNA which plays a crucial role in muscle growth and development [14]. In addition, miR-206 has been proved to function as a tumor suppressor in several types of human malignant cancers, including lung cancer [15], rhabdomyosarcoma [16], endometrial carcinoma [17] and breast cancer [18]. More recently, it has been demonstrated that the ectopic expression of miR-206 significantly inhibited the proliferation and promoted apoptosis at the early stages in glioma cell U343 [19], suggesting the involvement of miR-206 in tumorigenesis and progression of glioma. However, the role of miR-206 in human astrocytoma has not been clearly understood. Therefore, the aim of the present study was to investigate the clinical significance of miR-206 expression in this disease.

Materials and methods

Patients and Tissue Samples

This study was approved by the Research Ethics Committee of Second Hospital of Hebei Medical University, P. R. China. Written informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

One hundred and eight human astrocytoma tissue samples for qRT-PCR were obtained from Department of Neurosurgery, Second Hospital of Hebei Medical University. Samples were quickly removed at surgery and immediately divided into two parts: one part was fixed in 4 % paraformaldehyde for 24 h, paraffin embedded and used for histopathological diagnosis, and the remaining part was snap frozen in liquid nitrogen and maintained at −80 °C until used for RNA isolation. All the slides were re-evaluated according to WHO classifications [2] by two pathologists, with differences resolved by careful discussion. A total of 64 males and 44 females (1.45:1) were enrolled in this study, and the median age was 43 years (range, 13–72). Thirty of the 108 astrocytomas were classified as low-grade [18 pilocytic astrocytomas (WHO I) and 12 diffuse astrocytomas (WHO II)], and 78 were classified as high-grade astrocytomas [32 anaplasia astrocytomas (WHO III), and 46 primary glioblastomas (WHO IV)]. None of the patients had received chemotherapy or radiotherapy prior to surgery. The clinicopathological features of all patients were indicated in Table 1. Twenty normal brain tissue samples used as controls were obtained by collecting donations with consents from individuals [the median age was 39 years (range, 13–76)] who died in traffic accidents and were confirmed to be free of any prior pathological lesions. The information on the age and the localization of these normal brain samples are listed in Table 2.

All patients had complete 5-year follow-up until death. 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 astrocytomas or due to unexpected events, were excluded from this study.

RNA extraction and qRT-PCR for miRNA detection

Total RNA from fresh astrocytoma and normal brain tissues was isolated with TRIzol reagent according to the manufacture's instruction. Synthesis of cDNA with reverse transcriptase was

Table 1 Association of miR-206 expression in human astrocytoma tissues with different clinicopathological features

Clinicopathological features	No. of cases	miR-206 expression		P
		High (n, %)	Low (n, %)	
WHO grade				
I	18	15 (83.3)	3 (16.7)	**0.008**
II	12	6 (50.0)	6 (50.0)	
III	32	12 (37.5)	20 (62.5)	
IV	46	9 (19.6)	37 (80.4)	
Age				
<50	42	18 (42.9)	24 (57.1)	NS
≥50	66	24 (36.4)	42 (63.6)	
Gender				
Male	64	25 (39.1)	39 (60.9)	NS
Female	44	17 (38.6)	27 (61.4)	
Tumor size				
≥6 cm	75	25 (33.3)	50 (66.7)	**0.01**
<6 cm	33	17 (51.5)	16 (48.5)	
KPS				
<90	78	27 (34.6)	51 (65.4)	**0.03**
≥90	30	15 (50.0)	15 (50.0)	

Bold numbers refer to the differences with statistical significance.

performed by NCode™ miRNA quantitative RT-PCR Kits (Invitrogen™, Life Technologies Corporation, California, USA). qRT-PCR was performed using TaqMan MicroRNA Assay primer with the TaqMan Universal PCR Master Mix and analyzed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems; Foster City, CA, USA) according to the manufacturer's instructions. Analysis was performed by the comparative threshold cycle (Ct) method according to User Bulletin no.2 (Applied Biosystems; Foster City, CA, USA). Each sample was examined in triplicate and the amounts of the PCR products produced were normalized to U6B which served as internal control.

Table 2 Information on the age and the localization of 20 normal brain samples

Case No.	Age	Localization
1	38	Temporal cortex
2	52	Occipital cortex
3	36	Parietal cortex
4	13	Frontal cortex
5	40	Occipital cortex
6	71	Occipital cortex
7	62	Temporal cortex
8	21	Parietal cortex
9	38	Occipital cortex
10	25	Occipital cortex
11	76	Parietal cortex
12	66	Temporal cortex
13	73	Occipital cortex
14	48	Frontal cortex
15	42	Temporal cortex
16	26	Frontal cortex
17	19	Occipital cortex
18	20	Parietal cortex
19	37	Occipital cortex
20	51	Occipital cortex

Statistical analysis

All computations were carried out using the software of SPSS version13.0 for Windows (SPSS Inc, IL, USA). Data were expressed as means±standard deviation (SD). The differential expression of miR-206 between astrocytoma tissues and normal brain tissues was evaluated by independent sample t test. The X^2 test was used to analyze the relationship between miR-206 expression and the clinicopathological characteristics. A life table was calculated according to the Kaplan-Meier method. Hazard ratios for the time-to-event endpoint were estimated using the multivariate Cox regression analysis in a forward stepwise method to evaluate the effect of multiple independent prognostic factors on survival outcome. Differences were considered statistically significant when p was less than 0.05.

Results

miR-206 down-regulation in human astrocytoma tissues

The expression levels of miR-206 were detected in 108 astrocytoma and 20 normal brain tissues normalized to U6B. As shown in Fig. 1, the expression levels of miR-206 were found to be distinctly decreased in astrocytoma tissues compared to normal brain tissues (mean±SD: 2.4±1.4 vs. 4.4±2.0, $P<0.0001$), corresponding to the astrocytoma WHO grades. The statistic results showed that its expression in high-grade (III-IV; mean±SD: 1.8±1.0) and low-grade (I-II; mean±SD: 3.6±0.4) astrocytomas were both significantly lower than that in normal brains tissues ($P<0.0001$ and 0.04, respectively). Additionally, there was also a significant difference in miR-206 expression between high-grade (III-IV) and low -grade (I-II) astrocytoma tissue specimens ($P<0.0001$).

miR-206 down-regulation associates with aggressive clinicopatholigcal features of human astrocytomas

Then, we analyzed the associations of miR-206 expression with various clinicopathological parameters of astrocytoma tissues. Astrocytoma tissue samples expressing miR-206 at

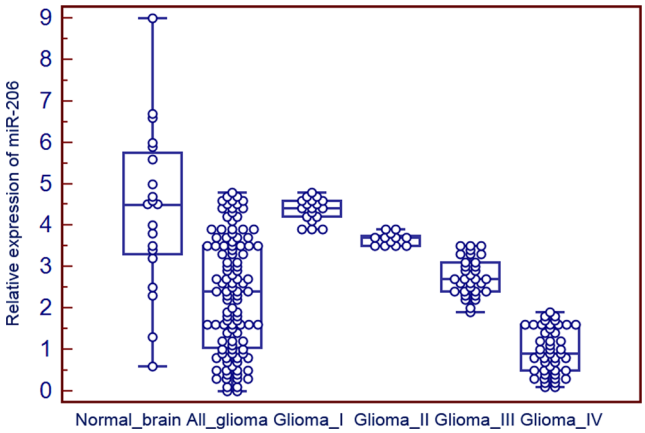

Fig. 1 miR-206 expression in 108 astrocytoma and 20 normal brain tissues detected by quantitative real-time polymerase chain reaction (qRT-PCR) analysis. The expression levels of miR-206 were found to be distinctly decreased in astrocytoma tissues compared to normal brain tissues (mean±SD: 2.4±1.4 vs. 4.4±2.0, $P<0.0001$), corresponding to the astrocytoma WHO grades. miR-206 expression in high-grade (III-IV; mean±SD: 1.8±1.0) and low-grade (I-II; mean±SD: 3.6±0.4) astrocytomas were both significantly lower than that in normal brains tissues ($P<0.0001$ and 0.04, respectively). Additionally, there was also a significant difference in miR-206 expression between high-grade (III-IV) and low -grade (I-II) astrocytoma tissue specimens ($P<0.0001$). 'I~IV' refers to astrocytoma tissues with grade I~IV

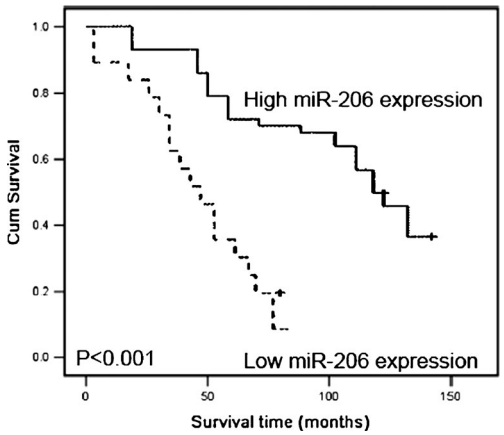

Fig. 2 Kaplan-Meier survival curves for astrocytoma patients with high and low expression of miR-206. The 5-year overall survival rate of astrocytoma patients with low miR-206 expression was significantly lower than those with high miR-206 expression ($P<0.001$)

levels less than the median expression level (2.4) were assigned to the low expression group (mean expression value 1.5, $n=66$), and those samples with expression above the median value were assigned to the high expression group (mean expression value 3.8, $n=42$). Data in Table 1 showed that the low level of miR-206 expression was significantly more common in astrocytoma tissues with advanced pathological grade and large tumor size than those with low pathological grade and small tumor size, respectively ($P=0.008$ and 0.01, respectively). A significant relationship was also found between miR-206 expression and Karnofsky performance score (KPS). The decreased expression of miR-206 more frequently occurred in tumors with low KPS than those with high KPS ($P=0.02$, Table 1). However, there was no significant association between miR-206 expression and other clinicopathological parameters, including gender and age at diagnosis ($P>0.05$, Table 1).

miR-206 down-regulation predicts poor overall survival in patients with astrocytomas

Furthermore, we evaluated the prognostic value of miR-206 expression in overall survival of patients with astrocytomas. The detail clinical information of all 108 astrocytoma patients was reviewed. The median survival times of patients with grade I~IV astrocytomas were respectively 62.4 months (range: 49.5~70.3 months, mean: 62.6 months), 46.8 months (range: 36.9~52.7 months, mean: 46.5 months), 32.1 months (range: 19.3~40.8 months, mean: 32.0 months), 14.8 months (range: 5.8~20.7 months, mean: 14.6 months). According to the log-rank test and Kaplan-Meier analysis, the expression level of miR-206 in the astrocytomas significantly displayed a correlation with the patients' survival time. Specially, the overall survival of patients whose tumors expressed low levels of miR-206 was significantly shorter than those expressed high levels of miR-206 ($P<0.001$, Fig. 2).

Univariate and multivariate analyses were performed and determined whether the miR-206 expression level and various clinical parameters were independent prognostic factors of patient outcomes. As the results in Table 3, the low expression of miR-206 ($P<0.001$) and advanced pathological grade ($P=0.02$) were independent factors predicting poor prognosis for astrocytomas. Interestingly, the prognostic value of miR-206 expression might be higher than that of pathological grade (P value: <0.001 vs. 0.02, Table 3).

Table 3 Univariate and multivariate analyses of different prognostic parameters in patients with astrocytomas by Cox-regression analysis

Parameter	Univariate analysis			Multivariate Analysis		
	Risk ratio	95 % confidence interval	P	Risk ratio	95 % confidence interval	P
Age	1.8	0.5–3.8	0.6	1.2	0.2–3.3	0.8
Gender	2.0	0.7–4.3	0.2	1.6	0.3–3.9	0.3
WHO grade	4.2	1.3–12.0	**0.006**	3.7	0.9–10.1	**0.02**
Tumor size	2.3	0.9–4.7	0.1	1.3	0.9–3.7	0.2
KPS	2.4	1.0–4.8	0.1	1.3	1.0–3.8	0.2
miR-206 expression	6.9	1.1–18.3	**<0.001**	6.2	1.0–15.6	**<0.001**

Bold numbers refer to the differences with statistical significance.

Discussion

Recent studies have demonstrated the aberrant expression of various miRNAs in astrocytomas. For example, the alterations of 245 miRNAs in GBM have been detected [20]; the expression of miR-21 was markedly up-regulated in primary GBMs and glioma cell lines compared with normal brain tissues and nontumorous glial cells [21]; the up-regulation of miR-182 has been demonstrated to be an independent prognostic indicator for the poor overall survival of astrocytoma patients [9]. These findings suggest that miRNAs are involved in astrocytoma development and progression. Therefore, the aims of the present study were 1) to explore the expression patterns of miR-206 in human astrocytomas of various histological subtypes, and 2) to search for correlations between miR-206 expression and patients' survival. As results of our analysis, there are four points of findings. Firstly, miR-206 was down-regulated in human astrocytoma tissues compared with normal brain tissues; Secondly, the decreased expression of miR-206 in astrocytoma tissues was significantly correlated with advanced tumor progression and aggressive clinicopathological features; Thirdly, the results of Kaplan-Meier analyses shown that astrocytoma tissues with low miR-206 expression tend to have poorer overall survival. Finally, the multivariate analysis clearly demonstrated that the low expression of miR-206 was a statistically significant risk factor affecting overall survival in astrocytoma patients, suggesting that miR-206 expression could be a valuable marker of astrocytoma progression and prognosis. To our knowledge, this is the first study to analyze the expression patterns and clinical significance of miR-206 in a large number of astrocytoma patients.

As a skeletal muscle specific miRNA, miR-206 was originally recognized to be involved in muscle development [22, 23]. It has been demonstrated that miR-206 induced the differentiation of myoblast and modulated the proliferation and differentiation of skeletal muscle satellite cells by down-regulation of pax7 [24]. Because the differential expression patterns of serum miRNAs are intrinsic to a specific disease, they could be used as noninvasive diagnostic tools. In this context, miR-206 expression level in serum has been proposed to be used to distinguish rhabdomyosarcoma from non-rhabdomyosarcoma tumors with both high sensitivity (1.0) and specificity (0.9) [25]. In carcinogenesis, miR-206 has been found to be down-regulated in various human cancers [15–18]. In line with these previous studies, our data here also detected the decreased expression of miR-206 in human astrocytoma tissues compared with normal brain tissues. Gain-of-function experiments, which are a feasible way to evaluate the functional significance of miRNAs in various cancers, have been demonstrated that the ectopic miR-206 expression may inhibit cell growth in rhabdomyosarcoma [16], lung cancer [15], breast cancer [18] and endometrioid endometrial cancer [17]. It also can induce apoptosis in rhabdomyosarcoma [16], lung cancer [15] and endometrioid endometrial cancer cells [17], and can induce G0/G1 arrest in rhabdomyosarcoma [16], breast [18] and lung cancer [15] cells. Moreover, cell migration and invasion activities can be inhibited by miR-206 in rhabdomyosarcoma [16], lung cancer [15] and endometrioid endometrial cancer cells [17]. Consistent with these findings on other malignancies, Wang et al. [19] recently indicated that overexpression of miR-206 might promote cell apoptosis of glioma cells in vitro. In this investigation, our statistical analysis found that miR-206 down-regulation was associated with advanced pathological grade, large tumor size and low KPS. On the basis of the previous reports, we hypothesize that the contribution of low miR-206 expression to the aggressive progression of human astrocytomas might be caused by the loss of its inhibitory efficiency on tumor cell apoptosis, migration and invasion.

Given above intriguing observation correlating miR-206 down-regulation with advanced tumor progression in human astrocytomas, we further investigated its potential prognostic value of miR-206 for this malignancy. As the results, overall survival after surgical resection among astrocytoma patients in low miR-206 expression group was poorer than those in high miR-206 expression group.

In conclusion, this is the first report of the differential expression of miR-206 in human astrocytoma tissues. MiR-206 could be a valuable marker of astrocytoma progression and low miR-206 expression is associated with poor overall survival in patients with malignant astrocytomas. However, the mechanism by which miR-206 was down-regulated in astrocytomas is still unclear. One study showed that miR-206 negatively regulated the endogeneous Otx2 expression in human glioma cell line U343 [19]. Further studies were needed to investigate the detailed mechanism of miR-206 down-regulation in human astrocytomas.

Conflict of interest None

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