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Serum microRNA-376 family as diagnostic and prognostic markers in human gliomas

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Abstract.
BACKGROUND: MicroRNA (miR)-376 family play crucial roles in cancer formation and progression.
OBJECTIVE: To investigate expression patterns of circulating miR-376 members in glioma patients, and to explore their diagnostic and prognostic values.
METHODS: Expression of miR-376 members in serum samples from 100 glioma patients and 50 healthy controls were detected by quantitative real-time PCR.
RESULTS: Serum miR-376a, miR-376b and miR-376c in glioma patients were significantly lower than those in healthy controls (all $P < 0.05$). Their expression could efficiently distinguish the glioma patients from healthy controls according to the receiver operating characteristic (ROC) analysis [for miR-376a, the area under ROC curve (AUC) = 0.872, the optimal cut-off value = 1.95, the sensitivity = 81.0% and the specificity = 82.0%; for miR-376b, AUC = 0.890, the optimal cut-off value = 2.07, the sensitivity = 82.0% and the specificity = 78.0%; for miR-376c, AUC = 0.837, the optimal cut-off value = 2.12, the sensitivity = 90.0% and the specificity = 70.0%; all $P < 0.001$]. Decreased expression of miR-376a, miR-376b and miR-376c in patients' sera were significantly associated with advanced WHO grade (all $P < 0.01$) and low KPS (all $P < 0.05$). Kaplan-Meier and Cox regression analyses showed that low miR-376a, miR-376b and miR-376c expression, and high grade were all independent factors predicting poor outcome of glioma patients. Notably, subgroup analyses showed that serum miR-376a, miR-376b and miR-376c levels had more significant prognostic values in patients with high grade gliomas than those with low grade gliomas.
CONCLUSIONS: Aberrant expression of the miR-376 family may be involved into tumorigenesis and tumor progression of human gliomas. Circulating miR-376a, miR-376b and miR-376c may be promising non-invasive biomarkers for diagnosis and prognosis in glioma patients.

Keywords: Glioma, microRNA-376 family, diagnosis, prognosis, biomarker

1. Introduction

As the most common and highly aggressive human primary brain tumors, gliomas represent approximately 30% of all malignancies in central nervous system [1]. On the basis of the World Health Organization (WHO) classification in 2007, human gliomas are categorized into four grades: I, pilocytic astrocytoma; II, diffuse astrocytoma; III, anaplastic astrocytoma; IV, glioblastoma [2]. Among them, tumors in grades I and II have a well differentiated variant, while those in grades III and IV have poorly differentiated variant [2]. In particularly, glioblastoma is one of the most malignant forms of human gliomas, and is characterized by highly aggressive invasion and proliferative nature, as well as its extremely poor clinical outcome [3]. Despite recent advances in multimodal and aggressive treatments including surgical resection, radiotherapy and chemotherapy, the therapeutic efficacy and clinical outcome of glioma patients with the exception of pilocytic astrocytomas remain unsatisfactory [4]. Especially, the five-year survival rate of glioblastoma patients after diagnosis is less than 10% and the median survival time is only 12 to 15 months [5]. Although patients' age at diagnosis, the score on the preoperative Karnofsky Performance scale and the WHO garde have been used as prognostic factors, survival of glioma patients with the same clinicopathological features often vary significantly, implying the molecular

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differences existing in these patients [6,7]. Therefore, it is of great clinical significance to clarify the molecular mechanisms underlying tumorigenesis and tumor progression of human gliomas and to identify novel molecular biomarkers of diagnosis and prognosis for this malignancy.

MicroRNAs (miRNAs), a group of endogenous, small (18–26 nucleotides in length) and non-protein-coding RNAs, regulate gene expression at a post-transcriptional level via binding with the 3'-untranslational region (UTR) of specific mRNA targets [8]. MiRNAs can either induce the degradation of mRNA targets or impair their translation [9]. A single miRNA may potentially bind to hundreds of mRNA targets according to bioinformatics analysis. Functionally, miRNAs are implicated in the regulation of various biological processes, such as cell development, differentiation, proliferation, apoptosis and motility by interacting with the corresponding targets [10]. Growing evidence show that aberrant expression of miRNAs may play a role in tumorigenesis and tumor progression of various malignancies. According to the functions of the corresponding target genes, miRNAs act as either oncogenes or tumor suppressors, and regulate various cancer-related signal pathways [11]. More interestingly, it has been indicated that these abnormal miRNAs have the potentials to be used as diagnostic or prognostic biomarkers of human malignancies.

The miR-376 family, containing miR-376a, miR-376b and miR-376c, is located on the human chromosome 14q32 and at the distal end of mouse chromosome 12, in loci called Dlk-Dio3 in humans and Dlk1-Gtl2 in mice, respectively [12]. MiR-376 family members are evolutionarily conserved in placental mammals. Under the physiological conditions, miR-376a is highly expressed in brain, retina and uterus, while miR-376b is most abundantly expressed in spleen and adrenal glands, and miR-376c in the ovaries [13]. Pathologically, expression alterations of miR-376 family members have been observed in a spectrum of human malignancies, including acute myeloid leukemia, melanoma, osteosarcoma, chondrosarcoma, oral squamous cell carcinoma, esophageal cancer, breast cancer, lung cancer, hepatocellular carcinoma, gastric cancer, bile duct carcinoma, pancreatic ductal adenocarcinoma, colorectal cancer, prostate cancer, ovarian cancer, endometrial serous adenocarcinoma, uterine leiomyomas [14]. In glioblastoma, Choudhury et al. [15] reported that the attenuated adenosine-to-inosine editing of miR-376a* could promote invasiveness of tumor cells. However, the clinical significance of miR-376 family members in human gliomas remains unclear. Therefore, the aim of this study was to investigate the expression patterns of circulating miR-376 members in glioma patients, and to explore their diagnostic and prognostic implications in this malignancy.

2. Materials and methods

2.1. Ethics, consent and permissions

This study was approved by the Research Ethics Committee of Beijing Luhe Hospital, Capital Medical University. Written informed consent was obtained from all of the patients. All specimens were handled and made anonymous based on the ethical and legal standards.

2.2. Patients and tissue samples

A total of 100 patients with the primary gliomas were enrolled in the current study from Jan 1^{st}, 2008 to Dec 31^{st}, 2010 in Department of Neurosurgery, Beijing Luhe Hospital, Capital Medical University (Beijing, China). According to the WHO classification [2], all glioma patients were divided into four groups, including 10 cases of pilocytic astrocytoma (grade I), 20 cases of diffuse astrocytoma (grade II), 30 cases of anaplastic glioma (grade III), and 40 cases of glioblastoma (grade IV). The patients underwent surgical resection without chemotherapy or radiotherapy before surgery. The clinicopathological characteristics of all the patients were summarized in Table 1. In addition, 50 age and gender-matched healthy volunteers were collected for healthy controls. The serum samples collected from 100 glioma patients and 50 healthy controls were stored at −80°C for future RNA detection.

All glioma patients received follow-up (median, 26 months; range, 1 ∼ 78 months) which was completed by Mar 31^{st}, 2015. By the end of follow-up, 20 cases were alive and 80 cases died. None patients died from other diseases or unexpected causes. Overall survival was defined as the period from the initial surgical operation to death.

2.3. RNA extraction and quantitative real-time PCR

To detect the levels of miR-376a, miR-376b and miR-376c in serum samples collected from 100 glioma patients and 50 healthy controls, 10 μL of sera were

Table 1
Clinicopathological features of 100 patients with glioma patients

Clinicopathological Features	Case	Percent (%)	miR-376a-low (n, %)	P	miR-376b-low (n, %)	P	miR-376c-low (n, %)	P
Age (years)								
⩽ 50	55	55.0	42 (76.36)	NS	40 (72.73)	NS	42 (76.36)	NS
> 50	45	45.0	37 (82.22)		33 (73.33)		34 (75.56)	
Gender								
Male	70	70.0	55 (78.57)	NS	52 (74.29)	NS	51 (72.86)	NS
Female	30	30.0	24 (80.00)		21 (70.00)		25 (83.33)	
Tumor size (cm)								
⩽ 5	68	68.0	53 (77.94)	NS	50 (73.53)	NS	52 (76.47)	NS
> 5	32	32.0	26 (81.25)		23 (71.88)		24 (75.00)	
WHO grade								
I	10	10.0	4 (40.00)	0.01	2 (20.00)	0.01	3 (30.00)	0.01
II	20	20.0	10 (50.00)		7 (35.00)		8 (40.00)	
III	30	30.0	25 (83.33)		24 (80.00)		25 (83.33)	
IV	40	40.0	40 (100.00)		40 (100.00)		40 (100.00)	
KPS								
⩽ 90	40	40.0	24 (60.00)	0.02	21 (52.50)	0.02	22 (55.00)	0.02
> 90	60	60.0	55 (91.67)		52 (86.67)		54 (90.00)	

Note: 'NS' refers to the differences without statistical significance.

mixed with 10 μL of 2 × preparation buffer containing 2.5% Tween 20 (EMD Chemicals, Gibbstown, NJ), 50 mmol/L Tris (Sigma-Aldrich, St. Louis, MO) and 1 mmol/L EDTA (Sigma-Aldrich, St. Louis, MO). RNAs were extracted by Trizol reagent (Invitrogen, Carlsbad, CA, USA), and 2 μg of RNA was reversely transcripted. Quantitative real-time PCR was performed using a high-specificity miRNA Detection Kit (Stratagene Corp., La Jolla, CA) in conjunction with an ABI 7500 thermal cycler, according to the manufacturer's instruction. U6 was used as an internal control for the expression of miR-376 family members. The primers for reverse transcription and PCR were synthesized by Sangon Biotech, Shanghai, China. For miR-376a, the primer sequences were: 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TCG CAC TGG ATA CGA CAC GTG G-3' (reverse transcription), 5'-AUC AUA GAG GAA AAU-3' (forward) and 5'-GTG CAG GGT CCG AGG T-3' (reverse); For miR-376b, the primer sequences were: 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA ACA TG-3' (reverse transcription), 5'-GGG GGT GGA TAT TCC TTC T-3' (forward) and 5'-CGC TTC ACG AAT TTG CGT GTC AT-3' (reverse); For miR-376c, the primer sequences were: 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT GGA GA-3' (reverse transcription), 5'-AUC AUA GAG GAA AAU-3' (forward) and 5'-GTG CAG GGT CCG AGG T-3' (reverse); For U6, the primer sequences were: 5'-CAC TGG ATA CGA CAC GTG GAC GTG G-3' (reverse transcription), 5'-CTC CGA TAG ATC TGC CCT CTT GAA-3' (forward) and 5'-CGC TTC ACG AAT TTG CGT GTC AT-3' (reverse). Relative miR-376a/miR-376b/miR-376c expression levels were calculated using the comparative threshold cycle (Ct) method [16].

2.4. Statistical analysis

All data analyses were statistically performed using the software of SPSS version 11.0 for Windows (SPSS Inc, IL, USA). All experiments were done for three times and continuous variables were expressed as Mean ± S.D. Student's t test and one-way analysis of variance (ANOVA) were used to determine the statistical significance of differences among groups. Receiver operating characteristic (ROC) analysis was used to evaluate the efficiency of miR-376a/miR-376b/miR-376c expression levels in distinguishing glioma patients from healthy controls. Survival curves were plotted using the Kaplan-Meier method, and differences between the survival curves were tested using the log-rank test. Cox's proportional hazards model was used to identify the factors with an independent influence on survival. Differences were considered to be statistically significant when p was less than 0.05.

3. Results

3.1. Diagnostic value of serum miR-376a, miR-376b and miR-376c levels in human gliomas

As shown in Fig. 1A, the serum levels of miR-376a (tumor vs. normal: 1.20 ± 0.78 vs. 2.98 ±1.22, $P <$

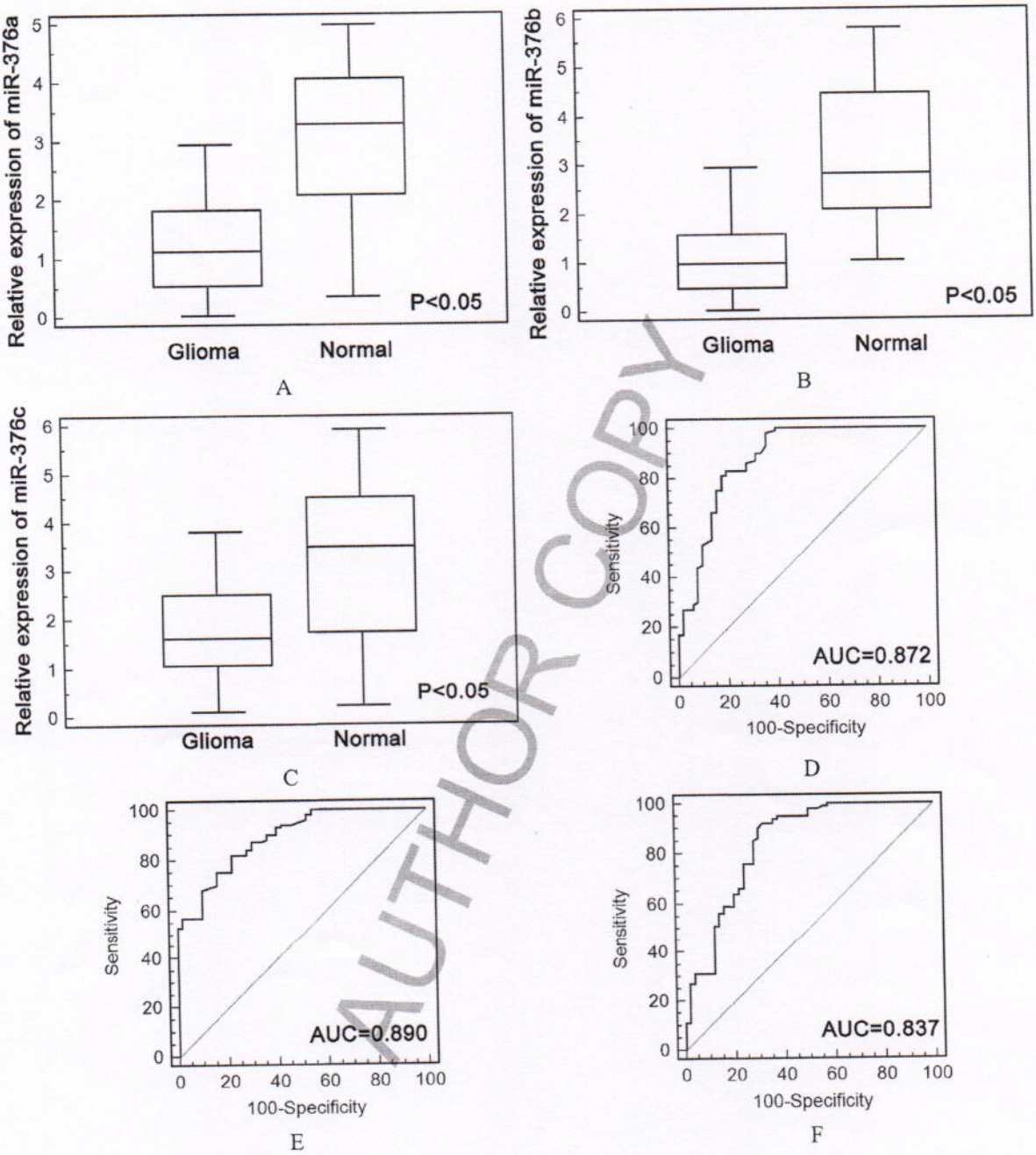

Fig. 1. Diagnostic value of serum miR-376a, miR-376b and miR-376c levels in human gliomas. (A), Serum levels of miR-376a (tumor vs. normal: 1.20 ± 0.78 vs. 2.98 ± 1.22, $P < 0.05$), miR-376b (tumor vs. normal: 1.13 ± 0.85 vs. 3.12 ± 1.40, $P < 0.05$) and miR-376c (tumor vs. normal: 1.37 ± 1.37 vs. 3.18 ± 1.66, $P < 0.05$) in glioma patients were all significantly lower than those in healthy controls. (B $\sim$ D), Serum levels of miR-376a, miR-376b and miR-376c efficiently distinguished the glioma patients from healthy controls [for miR-376a, AUC = 0.872, the optimal cut-off value = 1.95, the sensitivity = 81.0% and the specificity = 82.0%; for miR-376b, AUC = 0.890, the optimal cut-off value = 2.07, the sensitivity = 82.0% and the specificity = 78.0%; for miR-376c, AUC = 0.837, the optimal cut-off value = 2.12, the sensitivity = 90.0% and the specificity = 70.0%; all $P < 0.001$].

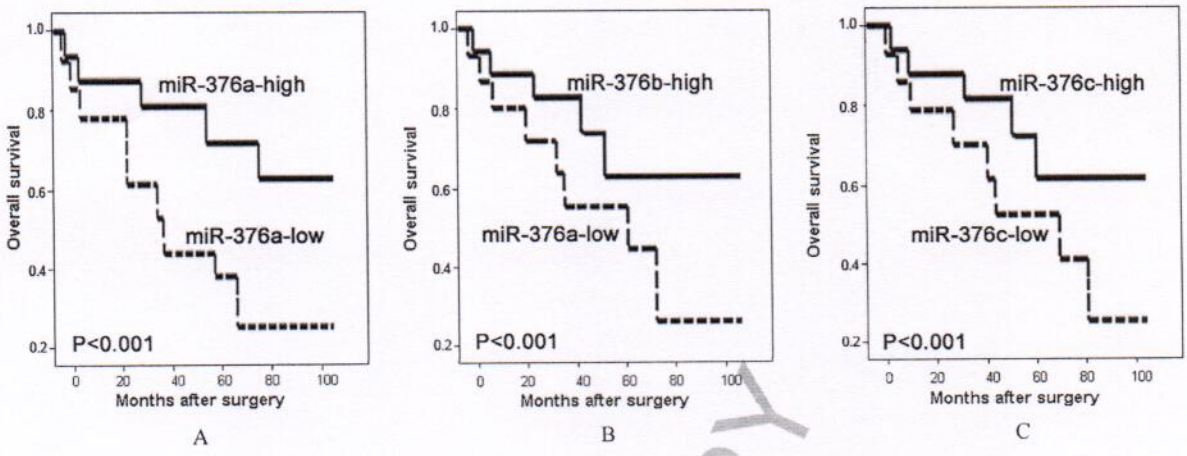

Fig. 2. Kaplan-Meier curves for overall survival according to serum miR-376a (A), miR-376b (B) and miR-376c (C). The optimal cutoff values (1.95, 2.07 and 2.12, respectively) of serum miR-376a, miR-376b and miR-376c were used to divide glioma patients into high-level and low-level groups.

0.05), miR-376b (tumor vs. normal: 1.13 ± 0.85 vs. 3.12 ± 1.40, $P < 0.05$) and miR-376c (tumor vs. normal: 1.37 ± 1.37 vs. 3.18 ± 1.66, $P < 0.05$) in glioma patients were all significantly lower than those in healthy controls. ROC curve and area under the ROC curve (AUC) were calculated to evaluate the diagnostic values of serum miR-376 members in glioma patients. As shown in Figs 1B $\sim$ D, the serum levels of miR-376a, miR-376b and miR-376c efficiently distinguished the glioma patients from healthy controls [for miR-376a, the area under ROC curve (AUC) = 0.872, the optimal cut-off value = 1.95, the sensitivity = 81.0% and the specificity = 82.0%; for miR-376b, AUC = 0.890, the optimal cut-off value = 2.07, the sensitivity = 82.0% and the specificity = 78.0%; for miR-376c, AUC = 0.837, the optimal cut-off value = 2.12, the sensitivity = 90.0% and the specificity = 70.0%; all $P < 0.001$].

3.2. *Decreased serum miR-376a, miR-376b and miR-376c levels associate with aggressive tumor progression of human gliomas*

In order to statistically evaluate the associations between serum levels of miR-376 family members and various clinicopathological characteristics of gliomas, all 100 patients were divided into miR-376a-low ($n = 79$) /high ($n = 21$), miR-376b-low ($n = 73$) /high ($n = 27$) and miR-376c-low ($n = 76$) /high ($n = 24$) groups using the corresponding optimal cut-off values mentioned above. As shown in Table 1, the glioma patients with low miR-376a, miR-376b and miR-376c expressions more frequently had high WHO grade than those with high expressions (all $P < 0.05$). Moreover, serum levels of miR-376 family members were positively correlated with KPS (all $P < 0.05$). There were no statistically significant associations between miR-376 family member expression and patients' age, gender or tumor size.

3.3. *Decreased serum miR-376a, miR-376b and miR-376c levels predict unfavorable prognosis in human gliomas*

The Kaplan-Meier curves in Fig. 2 indicated that the overall survivals of glioma patients with low miR-376a, miR-376b and miR-376c expression levels were all shorter than those with high expression levels (all $P < 0.001$). In addition, we stratified the samples into two groups: a low-grade group (WHO grade I–II) and a high-grade group (WHO grade III–IV). Notably, subgroup analyses showed that serum miR-376a, miR-376b and miR-376c levels had more significant prognostic values in patients with high grade gliomas than those with low grade gliomas (all $P = 0.02$, Fig. 3). Moreover, Cox regression analysis was performed to assess the correlations between miR-376 family members' levels, various clinicopathological characteristics and overall survival among the glioma patients. As shown in Table 2, the univariate and multivariate analyses revealed that serum miR-376a, miR-376b and miR-376c, and WHO grade were independent prognostic factors for glioma patients (all $P < 0.05$).

Table 2
Univariate and multivariate analyses of prognostic factors in glioma patients

Variable	Univariate log-rank test P value	Cox multivariable analysis P value	Relative risk
Age ($\leqslant$ 50 vs $>$ 50 years)	0.328	–	–
Gender (Male vs Female)	0.166	–	–
Tumor size ($\leqslant$ 5 vs $>$ 5 cm)	0.082	–	–
WHO grade (I $\sim$ II vs III $\sim$ IV)	$<$ 0.001	$<$ 0.001	6.986
KPS ($\leqslant$ 90 vs $>$ 90)	0.010	0.030	2.216
Serum miR-376a (Low vs high)	$<$ 0.001	0.010	3.823
Serum miR-376b (Low vs high)	$<$ 0.001	0.010	3.626
Serum miR-376c (Low vs high)	$<$ 0.001	0.010	3.686

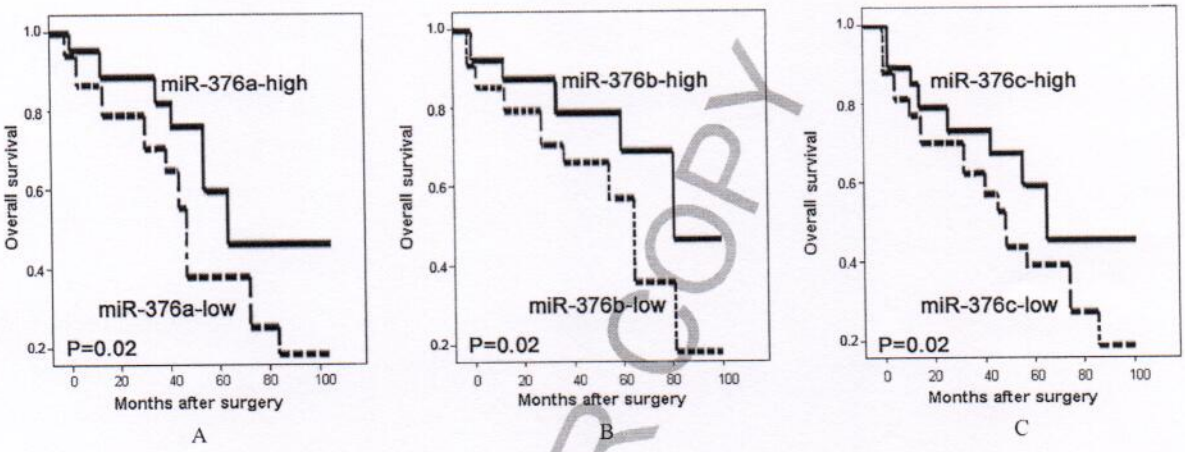

Fig. 3. Kaplan-Meier curves for overall survival in patients with high-grade gliomas according to serum miR-376a (A), miR-376b (B) and miR-376c (C). The optimal cutoff values (1.95, 2.07 and 2.12, respectively) of serum miR-376a, miR-376b and miR-376c were used to divide glioma patients into high-level and low-level groups.

4. Discussion

Accumulating studies have indicated that serum miRNAs have great potentials to be convenient and non-invasive biomarkers for human cancers, because this group of non-coding RNAs can be detectable in clinical specimens with high stability [17]. In the current study, we firstly detected the expression levels of three miR-376 family members in serum samples obtained from 100 glioma patients and 50 healthy controls. Our data showed the significantly decreased serum levels of miR-376a, miR-376b and miR-376c in glioma patients compared with healthy controls. The crucial finding of this study was that the three miR-376 family members all had high sensitivities and specificities in distinguishing glioma patients from healthy controls. In addition, the downregulation of miR-376a, miR-376b and miR-376c were related to advanced tumor progression of gliomas. Moreover, serum levels of these miRNAs were demonstrated to be independent prognostic factors for glioma patients. These findings suggest the underlying clinical significance of the miR-376 family as biomarkers for screening gliomas and predicting patients' prognosis.

Growing evidence shows the cancer type-specific changes occurring in miRNA expression patterns. As a large miRNA cluster, miR-376 family members have been found to be subject to changes in various human cancer types. For example, Liu et al. [18] performed a miRNA microarray expression profiling and identified miR-376a as one of overexpressed miRNAs in murine lung cancers, while Son et al. [19] revealed that miR-376b was downregulated in a set of non-small cell lung carcinomas; miR-376a was frequently down-regulated in hepatocellular carcinoma cell lines and clinical sample tissues [20]; miR-376c was reported to be down-regulated in an intrahepatic cholangiocarcinoma cell line [21]. Here, our results from quantitative real-time PCR analysis confirmed that the levels of miR-376a, miR-376b and miR-376c in glioma patients' sera were distinctly lower than those in healthy controls, implying the involvement of these miRNAs in tumorigenesis of human gliomas. Functionally, miR-376a acts as a tumor suppressor to inhibit proliferation and to induce

apoptosis in hepatocellular carcinoma [22]; Downregulation of miR-376a and miR-376c may contribute to the overexpression of insulin growth factor 1 receptor and to aberrant negative regulation of insulin signaling pathway in melanoma, leading to the promotion of tumorigenesis and metastasis [23]; MiR-376c also functions as a tumor suppressor to the cell growth and invasion of non-small-cell lung cancer cells by targeting LRH-1-mediated Wnt signaling pathway; MiR-376c suppresses cell proliferation and invasion in osteosarcoma by targeting transforming growth factor-alpha [24]. These findings imply that miR-376 family members may play different roles in various cancer types based on the functions of their regulatory downstream target genes. However, their clinical implications remain unclear. To address this problem, we here not only found the decreased serum levels of miR-376a, miR-376b and miR-376c in patients with gliomas, and also revealed that their downregulation could discriminate glioma patients from healthy controls, suggesting that miR-376a, miR-376b and miR-376c may be promising diagnostic markers for glioma at an early stage with a non-invasive manner. Then, we also found the significant associations between miR-376 family members' downregulation and aggressive clinicopathological characteristics and patients' prognosis. Kaplan-Meier analyses show that glioma tissues with low miR-376a/b/c expression levels had shorter overall survival; multivariate analysis clearly identified serum miR-376a/b/c levels as independent risk factors affecting overall survival in glioma patients, particularly in those with high WHO grade gliomas.

In conclusion, aberrant expression of the miR-376 family may be involved into tumorigenesis and tumor progression of human gliomas. Circulating miR-376a, miR-376b and miR-376c may be promising non-invasive biomarkers for diagnosis and prognosis in glioma patients. Further investigations are required to explore the exact molecular mechanisms underlying the effect of miR-376 family members on gliomas.

Conflict of interest

None declared.

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