RESEARCH ARTICLE

Increased Argonaute 2 Expression in Gliomas and its Association with Tumor Progression and Poor Prognosis

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Abstract

Background: Previous studies have showed that argonaute 2 is a potential factor related to genesis of several cancers, however, there have been no reports concerning gliomas. Methods: Paraffin specimens of 129 brain glioma cases were collected from a hospital affiliated to Binzhou Medical University from January 2008 to July 2013. We examined both argonaute 2 mRNA and protein expression by real-time quantitative PCR (qRT-PCR), Western blot analysis, and immunohistochemistry (IHC). The survival curves of the patients were determined using the Kaplan-Meier method and Cox regression, and the log-rank test was used for statistical evaluations. Results: Both argonaute 2 mRNA and protein were upregulated in high-grade when compared to low-grade tumor tissues. Multivariate analysis revealed that argonaute 2 protein expression was independently associated with the overall survival (HR=4.587, 95% CI: 3.001-6.993; *P*=0.002), and that argonaute 2 protein expression and WHO grading were independent prognostic factors for progression-free survival (HR=4.792, 95% CI: 3.993-5.672; *P*<0.001, and HR=2.109, 95% CI: 1.278-8.229; *P*=0.039, respectively). Kaplan-Meier analysis with the log-rank test indicated that high argonaute 2 protein expression had a significant impact on overall survival (*P*=0.0169) and progression-free survival (*P*=0.0324). Conclusions: The present study showed that argonaute 2 expression is up-regulated in gliomas. Argonaute 2 might also serve as a novel prognostic marker.

Keywords: Glioma - argonaute 2 - prognostic marker

Introduction

Glioma is the most common intracranial neoplasia in adults, which arises from the brain or spinal cord tissues. The highly invasive nature of this tumor prevents complete tumor resection and causes significant neurologic morbidity and mortality. Gliomas are categorized according to their grade, which is determined by pathologic evaluation of the tumor. Low-grade gliomas [WHO grade I-II] are well differentiated; these are benign and portend a better prognosis for the patient. High grade [WHO grade III-IV] gliomas are undifferentiated or anaplastic; these are malignant and carry a worse prognosis. Although the WHO classification can serve as a criterion to predict the patient clinical outcomes, several recent studies have indicated that this criteria alone may not be sufficient to estimate patient prognosis. Therefore, investigating molecular mechanisms of gliomas may produce better prognostic markers to anticipate patient survival. Previously, researchers have found some factors which were able to predict the prognosis of glioma and the detailed mechanisms (Arshad et al., 2010; Chen et al., 2012; Ye et al., 2012; Wang et al., 2014).

Argonaute protein is one of the most indispensible components in the RNA induced silencing complex (RISC), which tethers miRNA to the 3'UTR of the target mRNAs, leading to mRNA degradation or translational repression (Zhang et al., 2009). These proteins bind different classes of small noncoding RNAs, including microRNAs, small interfering RNAs and PIWI interacting RNAs, then small RNAs guide Argonaute proteins to their specific target through sequence complementarity, which typically leads to silencing of the target. There are eight Argonaute proteins which can be divided into the Argonaute subfamily and the PIWI subfamily. Argonaute subfamily is ubiquitously expressed in many organisms such as animals, plants, and fission yeast and can be divided into Argonaute 1, Argonaute 2, Argonaute 3 and Argonaute 4. However, researches identified that Argonaute 2 protein was the only one with the slicer activity and was highly specialized member of the Argonaute family with a crucial function within RISC in the regulation of miRNA homeostasis. Recent data from several studies have shown that Argonaute 2 was a potential factor related to tumorigenesis in liver cancer, gastric cancer, bladder cancer and renal cancer. However, there have been no reports about Argonaute 2 in glioma and the role of Argonaute 2 in glioma is still unknown.

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In the present study, we examined both Argonaute 2 mRNA and protein expression by Real-time quantitative PCR (qRT-PCR) and Western blot analysis and investigate the expression of Argonaute 2 proteins by Immunohistochemistry (IHC) and identify their potential roles in prognosis for patients with glioma.

Materials and Methods

Patients and specimens

The study protocol was approved by the Ethics Committee of Binzhou medical University. The paraffin specimens of 129 brain glioma cases were collected from Hospital Affiliated to Binzhou medical University from January 2008 to July 2013. These cases were used for testing immunohistochemical protein levels and for the analysis of prognosis. They were totally 70 men and 59 women, whose age range from 37 to 71 years. Clinico-pathological characteristics in our study are presented in Table1.

Real-time quantitative PCR

Total RNA was isolated from tissue using TRIZOL reagent according to the manufacturer's protocol (Invitrogen). RNA was reverse transcribed using SuperScript First Strand cDNA System (Invitrogen) according to the manufacturer's instructions. The Argonaute 2 sense primer was 5'-AAGGCTGCTCTAACCCTCTTG-3', and the antisense primer was 5'-ACGCTGTTGCTGACACATC-3'. For the GAPDH gene, the sense primer was 5'-TGCACCACCAACTGCTTAGC-3', and the antisense primer was 5'-GGCATGGACTGTGGTCATGAG-3'. The PCR amplification was performed for 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, on a Applied Biosystems 7900HT (Applied Biosystems) with 1.0 μl of cDNA and SYBR Green Real-time PCR Master Mix (Takara). Data was collected and analyzed by SDS2.3 Software (Applied Biosystems). The expression level of each candidate gene was internally normalized against that of the GAPDH. The relative quantitative value was expressed by the $2^{-\Delta\Delta Ct}$ method. Each experiment was performed in triplicates and repeated three times.

Western blot assay

Total proteins from tissues were lysed in lysis buffer containing protease inhibitor. Protein concentration was determined using a Bio-Rad protein assay system (Bio-Rad). Equivalent amounts of proteins were separated by SDSPAGE, and then transferred to polyvinylidene difluoride membranes (Bio-Rad). After being blocked in Tris buffered saline (TBS) containing 5% non-fat milk, the membranes were incubated with specific primary antibodies (Abcam) at 4 °C for 12 hours and then with horseradish peroxidase conjugated anti-Rabbit antibody for 2 hours at room temperature. ECL detection reagent (Amersham LifeScience, Piscataway, NJ) was used to demonstrate the results.

Immunohistochemistry staining

All samples were fixed in 10% formaldehyde solution, embedded in paraffin blocks, cut in 4μm thick sections,

and mounted on glass slides. Each slide was dewaxed in xylene and rehydrated in grade alcohol, followed by boiling in 10 mmol/L of citrate buffer (PH 6.0) for antigen retrieval. After inhibition of endogenous peroxidase activities for 30 minutes with methanol containing 0.3% H2O2, the sections were blocked with 2% bovine serum albumin for 30 minutes and incubated overnight at 4°C with primary Rabbit monoclonal anti-Ago2 antibody (Abcam). After washing thrice with PBS, the slides were incubated with horseradish peroxidase-conjugated mouse-anti-rabbit IgG for 30 minutes, followed by reaction with diaminobenzidine and counterstaining with Mayer/hematoxylin. Negative control was done by omission of the primary antibody and substituting it with nonspecific rabbit IgG.

The results of immunostaining were evaluated and scored semiquantitatively by two pathologists who were blinded to the patients' clinical data. The evaluation of the immunostaining results was based on a double scoring system (staining intensity multiplied by staining area). Staining intensity was scored as 0 for no staining, 1 for definite but weak staining, 2 for moderate staining and 3 for strong staining. The staining area was scored as 1 for staining of <35%, 2 for 35–75%, and 3 for>75% of the tumor cells. High expression of Argonaute 2 was defined as an immunostaining score of ≥4, whereas low expression of proteins was defined as a score of<4.

Statistical Analysis

Statistical analysis was conducted using the Statistical Package for the Social Sciences 13.0 for Windows
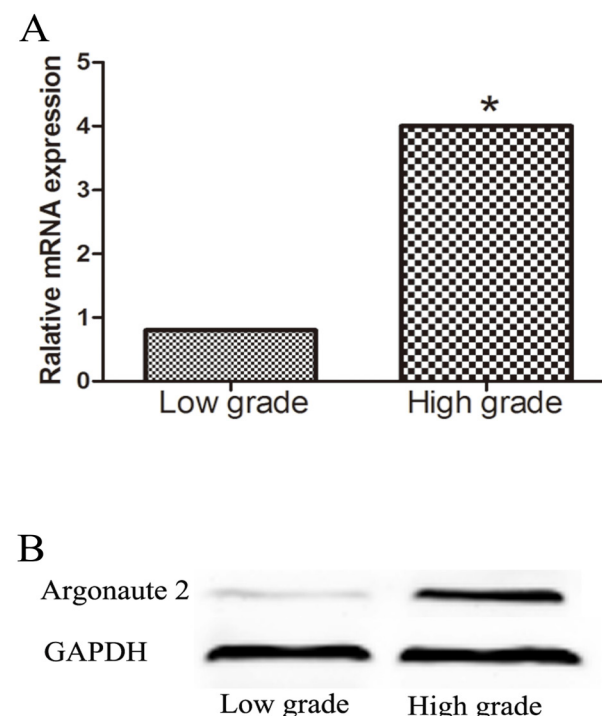

Figure 1. The Expression of Argonaute 2 mRNA and Protein in the Human Gliomas Cancer Tissues. A: The relative mRNA expression of Argonaute 2 was higher in high-grade gliomas tissues when compared to low-grade tumor tissues (P<0.05); B: The Argonaute 2 protein expression was higher in high-grade gliomas tissues when compared to low-grade tumor tissues

Table 1. Correlation Between the Expression of Argonaute 2 Protein and Clinicopathological Parameters in Glioma Patients

Variable	n	Argonaute 2 expression		P value
		Low (n=82)	High (n=47)	
Age (years)				
≥45	78	50	28	0.19
<45	51	32	19	
Gender				
Male	70	43	27	0.32
Female	59	39	20	
Extent of resection				
Subtotal	49	28	21	0.11
Total	80	54	26	
WHO grading				
I-II (n=47)	67	35	32	0.013
III-IV (n=41)	62	47	15	
KPS score				
≥80	69	34	35	0.007
<80	60	48	12	

WHO, World Health Organization; KPS, Karnofsky performance score

(SPSS Inc., Chicago, IL, USA). The chi-square test was used to assess Argonaute 2 expression with respect to clinicopathological parameters. The survival curves of the patients were determined using the Kaplan-Meier method and Cox regression, and the log-rank test was used for statistical evaluations. Data were expressed as the mean and standard deviation and analyzed using one-way analysis of variance. *P*<0.05 was considered to indicate a significant difference.

Results

Expression of Argonaute 2 mRNA by qRT-PCR and Argonaute 2 protein expression by Western blot qRT-PCR analysis of Argonaute 2 mRNA expression in high-grade (III-IV) and low-grade (I-II) tumor tissues showed that Argonaute 2 mRNA was upregulated in high-grade gliomas tissues when compared to low-grade tumor tissues (*p*<0.05, Figure 1A). To investigate whether Argonaute 2 was also elevated at the protein level, Western blot analysis was performed. We found that the protein level of Argonaute 2 in high-grade gliomas tissues was significantly higher than that in low-grade tumor tissues (Figure 1B).

Association of Argonaute 2 protein expression with clinicopathological features

Immunohistochemistry staining showed that the Argonaute 2 protein was mainly accumulated in the cytoplasm of malignant cells. And the expression of Argonaute 2 in high-grade gliomas tissues was significantly higher than that in low-grade tumor tissues (Figure 2). Argonaute 2 was highly expressed in 47 of the 129 (36.4%) glioma patients. High expression of Argonaute 2 was found to significantly correlate with WHO grade (*P*=0.013) and KPS score (*P*=0.007). No significant difference in Argonaute 2 expression was observed with gender, age, and extent of resection (*P*>0.05) (Table 1).
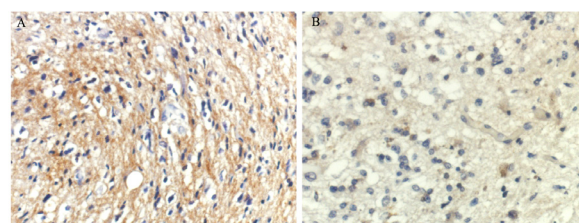

Figure 2. The Expressions of Argonaute 2 in Gliomas Tissues (×400). A: Immunohistochemistry staining of Argonaute 2 in high-grade gliomas tissues. B: Immunohistochemistry staining of Argonaute 2 in low-grade gliomas tissues
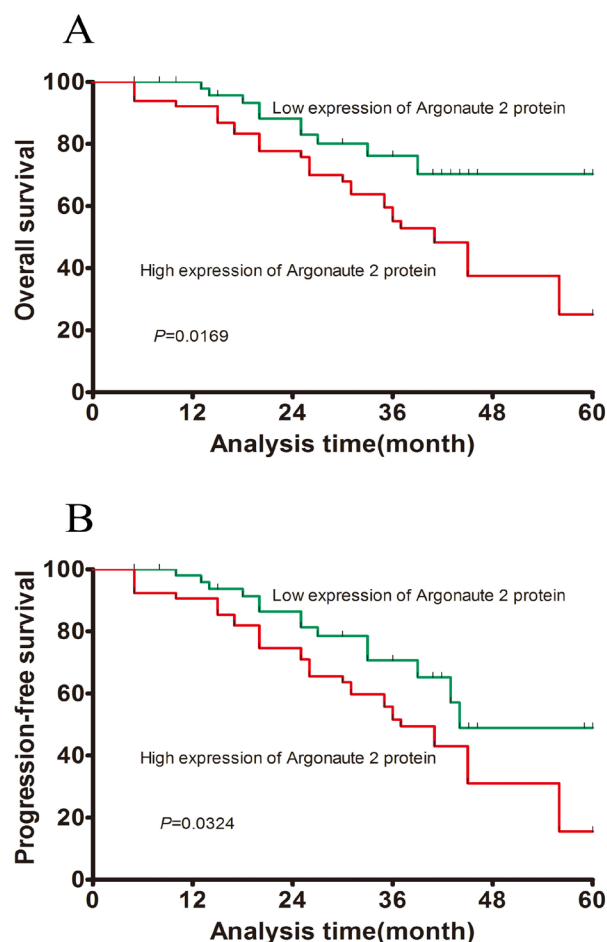

Figure 3. Kaplan-Meier Survival Analyses of Glioma Patients. Patients with high Argonaute 2 levels in tumor tissue had significantly lower overall (A, *P*=0.0169) and progression-free survival rates (B, *P*=0.0324) than patients with low Argonaute 2 levels

Correlation of Argonaute 2 protein expression with clinical outcomes

In the present study, 11 patients were lost to follow-up and were excluded from the survival analyses. The remaining 116 patients with adequate follow-up data were followed for 5-63 months. Multivariate analysis revealed that Argonaute 2 protein expression was independently associated with the overall survival (HR=4.587, 95% CI: 3.001-6.993; *P*=0.002, Table 2), and that Argonaute 2 protein expression and WHO grading were independent prognostic factors for progression-free survival (HR=4.792, 95% CI: 3.993-5.672; *P*<0.001, and HR=2.109, 95% CI: 1.278-8.229; *P*=0.039, respectively,

Table 2. Cox Regression Model for Multivariate Analysis of Prognostic Factors for Gliomas

Variable	Overall survival			Progression-free survival		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (≥45 vs.>45)	2.097	0.825-6.009	0.310	1.978	0.786-2.997	0.090
Gender (male vs. female)	1.867	0.762-3.997	0.430	1.276	0.667-4.924	0.510
Extent of resection (subtotal vs. total)	1.789	0.785-5.997	0.090	2.992	0.991-5.221	0.061
WHO grading (III-IV vs. I-II)	2.863	0.992-3.976	0.055	2.109	1.278-8.229	0.039
KPS score (<80 vs. ≥80)	3.991	0.671-5.889	0.090	3.672	0.921-4.932	0.060
Argonaute 2 expression (high vs.low)	4.587	3.001-6.993	0.002	4.792	3.993-5.672	<0.001

HR, Hazard ratio; CI, confidence interval

Table 2). Kaplan-Meier analysis with the log-rank test indicated that high Argonaute 2 protein expression had a significant impact on overall survival (*P*=0.0169; Figure 3A) and progression-free survival (*P*=0.0324; Figure 3B).

Discussion

MicroRNAs (miRNAs), which are single-stranded RNA molecules of 20-23 nucleotide length, can regulate gene expression at a post-transcriptional level and play a pivotal role in the regulation of cell development, metabolism, immunity, proliferation, differentiation, and apoptosis. Researchers have found that their dysregulation can contribute to the initiation and progression of a variety of cancers as either a tumor suppressor or a tumor promoter. Many cancer-related microRNAs have been identified functionally. The mature miRNA directs a RNA-induced silencing complex (RISC) to the complementary target genes and causes inhibition of translation and degradation of the messenger RNA. Argonaute proteins are the essential components of the miRNA-induced silencing complex (miRISC) that directly recruit miRNAs and function as the interface between miRNAs and their mRNA targets. Mammals have four Argonauts (Argonaute 1, Argonaute 2, Argonaute 3 and Argonaute 4) that are involved in the miRNA pathway. However, researches identified that Argonaute 2 protein was the only one with the slicer activity and was highly specialized member of the Argonaute family with a crucial function within RISC in the regulation of miRNA homeostasis. Recent data from several studies have shown that Argonaute2 was a potential factor related to tumorigenesis in liver cancer, gastric cancer, bladder cancer and renal cancer. However, there have been no reports about Argonaute 2 in glioma and the role of Argonaute 2 in glioma is still unknown.

Development and progression of glioma to metastasis and lethal state are believed to be driven by multiple genetic alterations, the nature of which has remained poorly understood. To directly address the potential roles for Argonaute 2 protein in the occurrence and development of glioma, an elaborate experiment was conducted and a rigorous analysis was performed of human Argonaute 2 protein on a cohort of 129 human glioma specimens. In the present study, we found the expression level of Argonaute 2 protein in cytoplasm was significantly associated with WHO grade (*P*=0.013) and KPS score (*P*=0.007). It is suggested that Argonaute 2 protein were associated with tumor development and progression and may promote tumor invasion. However, previous researches reported

that the expression of Argonaute 2 protein in prostate cancer and esophageal cancer was not associated with pathologic characteristics, including age, sex, Gleason score and tumour stage. Whether this inconsistence was ascribed to the tissue specificity should be further studied, and more research is needed to explore the exact role of Argonaute 2 protein in different cancers. In the present study, a total of 116 patients histologically proven glioma with follow-up information were conducted a systematically analysis to confirm the relationship of the Argonaute 2 protein and outcome of patient initially. Our finding demonstrated that patients with higher expression of Argonaute 2 in tumor tissue had a worse overall survival and progression-free survival than patients with lower expression, providing the evidence that elevated expression of Argonaute 2 in glioma might facilitate an increased malignant and worse prognostic phenotype.

The present study has not investigated the detailed mechanisms underlined the oncogene functions of Argonaute 2 in glioma. Identification of target mRNA species and interacting partners of Argonaute 2 might lead us to the further insights into the complex roles of Argonaute 2 in glioma carcinogenesis. Previous studies has identified that let-7, c-myc and FAK mRNAs were affected by increased Argonaute 2 expression and have been implicated in tumorigenesis and tumor metastasis. Therefore, more studies are needed in the future to better understand the mechanisms underlined the oncogene functions of Argonaute 2 in glioma.

In conclusion, we have demonstrated that positive expression of Argonaute 2 in glioma was correlated with a more malignant phenotype and with a poor prognosis in a large number of clinical samples. Our data suggested that Argonaute 2 may function as a valuable prognostic biomarker for glioma.

Acknowledgements

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