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Original Article

The microRNA-1268a rs28599926 polymorphism modified diffusely infiltrating astrocytoma risk and prognosis

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Abstract: The rs28599926 polymorphism in the microRNA-1268a (miR-1268a) has been reported to correlate with tumor carcinogenesis; however, its role in diffusely infiltrating astrocytoma (DIA) has not yet been evaluated. Here we conducted a hospital-based case-control study, including 163 DIA cases and 189 age-, sex-, and race-matched healthy controls, to assess the association between rs28599926 polymorphism and DIA risk and prognosis. Genotypes were tested using TaqMan-PCR technique. We found a significant difference in the frequency of rs28599926 genotypes between cases and controls. Compared with the homozygote of rs28599926 C alleles (rs28599926-CC), the genotypes with rs28599926 T alleles (rs28599926-CT/-TT) increased DIA risk [odd ratios, 3.41; 95% confidence interval (CI), 2.19-5.31]. Furthermore, this polymorphism modified the overall survival and recurrence-free survival of DIA patients [hazard ratios (95% CI), 3.25 (2.14-3.79) and 2.52 (1.67-3.79), respectively]. Additionally, rs28599926 polymorphism was not only associated with tumor dedifferentiation of DIA, but also modified the effects of temozolomide treatment on DIA. These results suggest that miR-1268a rs28599926 polymorphism may be associated with DIA risk and prognosis.

Keywords: miR-1268a, polymorphism, DIA

Introduction

Diffusely infiltrating astrocytoma (DIA), originating from astrocytic glial cells and their precursors, is the most type of central nervous system tumors and accounts for approximately 60% of all primary brain tumors [1]. DIA varies considerably in its epidemiological features, clinic behavior, genetic profiles, morphologic attributes, and growth patterns. Histopathologically, this tumor can be assorted into grades II-IV according to the current World Health Organization (WHO) Classification: low-grade diffuse astrocytoma (Grade II), anaplastic astrocytoma (Grade III), and glioblastoma (Grade IV) [1]. Because of the progression from low to high grade and high-risk reoccurrence, DIA is characterized by the poor prognosis [2-4]. Therefore, a better understanding into its basic biology is urgently needed to identify its risk and prognostic markers. Several studies have shown that common genetic variants in biologically plausible pathways are potentially associated with DIA risk [5-16]. This implies that the genetic factors could play important roles in the pathogenesis of this tumor.

The microRNA-1268a (miR-1268a) is a recently reported small, endogenous, and noncoding RNA [17]. A study from Guangxi, a relatively high incidence area of DIA, has exhibited that a common polymorphism miR-1268a, namely rs28599926 C > T, has been identified to correlate with tumorigenesis [18]. However, it is unclear about whether this polymorphism correlates with DIA. Therefore, we specifically conducted a hospital-based case-control study to examine whether miR-1268a rs28599926 polymorphism modifies DIA risk and prognosis.

Material and methods

Study population

This is a hospital-based case-control study. All cases diagnosed with histopathologically confirmed DIA were recruited from hospitals affiliated with Youjiang Medical University for Nationalities and Guangxi Medical University during the period from January 2008 through December 2012. Healthy control subjects without a history of tumors were recruited from the general health check-up center at the same hospitals during the same period for comparison. To control the effects of confounders, the controls were individually matched (1:1 or 2:1) to cases based on age (±5 years), gender, and ethnicity (Han, Zhuang). All controls were surveyed to ascertain their willingness to participate in the study and to provide preliminary demographic data for matching. In this study, a total of 163 cases and 189 controls, representing 95% of eligible cases and 98% of eligible controls were interviewed and included the final analysis. The study protocol was been carried out in accordance with government policies and the Helsinki Declaration and approved by the ethics committees of the hospitals involved in this study.

Samples and data collection

After informed consent was obtained, 4 mL of peripheral blood samples were obtained from all cases and controls for DNA detraction and genotypic analysis of miR-1268a. Demographic information and clinical pathological data (including age, gender, smoking and drinking status, ethnicity, tumor grade, and treatment information) were collected in the hospitals using a standard interviewer administered questionnaire and/or medical records by a Guangxi Cancer Institution staff member. Tumor grade was evaluated according to the grading criteria of DIA from WHO and divided into low (Grade II) and high grade (Grade III and IV) for analysis. For survival analysis, all patients underwent serial monitoring every 3 months for the first 2 years and semiannually thereafter for detection of any recurrence. The last follow-up day was December 31, 2015, and survival status was confirmed by clinic records and either patient or family contact. The duration of overall survival (OS) was defined as from the date of surgical treatment to the date of death or last known date alive; whereas the duration of tumor recurrence-free survival (RFS) was defined as from the date of surgical treatment to the date of tumor recurrence or last known date alive.

Genotypic analysis of miR-1268a rs28599926 polymorphism

DNA was extracted from peripheral blood leukocytes from all tumor patients and control subjects according to standard procedures (Protocol #BS474, Bio Basic, Inc., Ontario, Canada). Genotypes of miR-1268a rs28599926 polymorphism were analyzed using previously published TaqMan-PCR technique [18].

Cell culture and transfection

Human glioma cell lines T98G was purchased from American Tissue Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's Modified Eagles Medium (DMEM, HyClone, Thermo Fisher Scientific (China) CO., Ltd, Shanghai) containing 10% fetal bovine serum (FBS, Gibco-Invitrogen Corp., Carlsbad, CA) in atmosphere of 5% CO_2 at 37°C using standard techniques. Cells were transfected with the wild or mutant type of miR-1268a mimics (miR-1268a-WT: 5'-CGGGC GUGGU GGUGG GGG-3' and miR-1268a-MT: 5'-CGGGC AUGGU GGUGG GGG-3') (GenePharma, China) using Invivofectamine® 12.0 Reagent (cat# 1377501, Life) according to the manufacturer's instructions. In this study, transfection efficacy was elucidated as the ratio of transfected cells detected by the LV200 system to total cells obtained from three different regions at random, and was about 85%.

Cell sensitivity assay

The sensitivity of T98G cells to temozolomide was elucidated by the half-maximal inhibitory concentration (IC50) using a cell counting kit (CCK-8) assay (cat# CK04, Dojindo Corp., Japan) according to the manufacturer's instructions. Briefly, a total of 5000 cells were seeded each well in a 96-well plate and transfected, followed by treatment with temozolomide at 15 different concentrations (0.1-300 μM) (48 hours after transfection). After 48 hours of treatment, 10 mL of CCK-8 solution was added into 100 μL of culture media and incubated for 2 hours at 37°C. Next, the absorbance of optical density (at 450 nm) was measured using UV spectrophotometer. IC50 values were calculated by nonlinear regression analysis using the

Table 1. Demographic characteristics of diffusely infiltrating astrocytoma (DIA) cases and controls

	Controls (n = 189)		Cases (n = 163)		χ^2	*P*
	n	%	n	%		
Age (yrs)					1.873	0.967
≤ 35	11	5.8	10	6.1		
36-40	15	7.9	12	7.4		
41-45	16	8.5	20	12.3		
46-50	43	22.8	33	20.2		
51-55	45	23.8	35	21.5		
56-60	31	16.4	29	17.8		
61-65	18	9.5	15	9.2		
≥ 66	10	5.3	9	5.5		
Sex					0.046	0.830
Male	122	64.6	107	65.6		
Female	67	35.4	56	34.4		
Race					0.234	0.629
Han	96	50.8	87	53.4		
Zhuang	93	49.2	76	46.6		
Smoking						
No	102	54.0	91	55.8	0.122	0.727
Yes	87	46.0	72	44.2		
Drinking					0.442	0.506
No	93	49.2	86	52.8		
Yes	96	50.8	77	47.2		
Tumor grade					-	-
II	-	-	71	43.6		
III	-	-	30	18.4		
IV	-	-	62	38.0		

GraphPad Prism software with Version 6.0 (GraphPad Software, Inc., San Diego, CA, USA).

TUNEL assay

Cells were seeded in six-well plates for 24 hours, and then transfected with miR-1268a mimics. Forty-eight hours after transfection, cells were treated with temozolomide (50 μM). After treatment for 36 and 48 hours, the cells were all harvested and analyzed by TUNEL staining using an in situ cell death detection kit (Roche, Mannheim, Germany) in combination with 4,6-diamino-2-phenyl indole staining. TUNEL-positive cells were counted in at least 300 cells in randomly chosen fields. The data were expressed as a percentage of TUNEL+ cells to total cells.

Statistical analysis

All statistical analyses were done using the statistical package for social science (SPSS) version 18 (SPSS Institute, Chicago, IL). To assess differences between groups were compared using one-way ANOVA or the x^2 test. The conditional logistic regression (with multivariate factors) was used to calculate odds ratios (ORs) and their 95% confidence intervals (CIs) for risk of DIA. Non-conditional logistic regression was used to elucidate the effects of miR-1268a genotypes on the pathological features of DIA (including age, gender, race, smoking and drinking status, and tumor grade). Kaplan-Meier survival analysis (with the log-rank test) was used to analyze the association between miR-1268a genotypes and DIA prognosis. Hazard ratios (HRs) and 95% CIs for miR-1268a genotypes were calculated from multivariate Cox regression model. In this study, a *P*-value of less than 0.05 was considered statistically significant.

Results

The characteristics of subjects

Demographic characteristics of the 163 DIA cases and the 189 controls were shown in **Table 1**. No significant differences were observed between the cases and control groups for sex, age, ethnicity, and smoking and drinking status. Among patients with DIA, about 50% of them received the surgical resection with temozolomide treatment; others only received surgical resection but without temozolomide treatment. During the follow-up period of these patients, 141 faced tumor recurrence with 29.0% of the 3-year RFS rate, and 155 died with 26.9% of the five-year OS rate. These cases receiving temozolomide treatment had a better prognosis [median overall survival time (MST) and median tumor reoccurrence-free survival time (MRT) were 24 and 32 months] than those without temozolomide treatment (MST = 12 months and MRT = 13 months) (**Figure 1A** and **1B**).

MiR-1268a rs28599926 polymorphism increased DIA risk

Table 2 summarized the genotypic and allelic distribution of MiR-1268a rs28599926 poly-

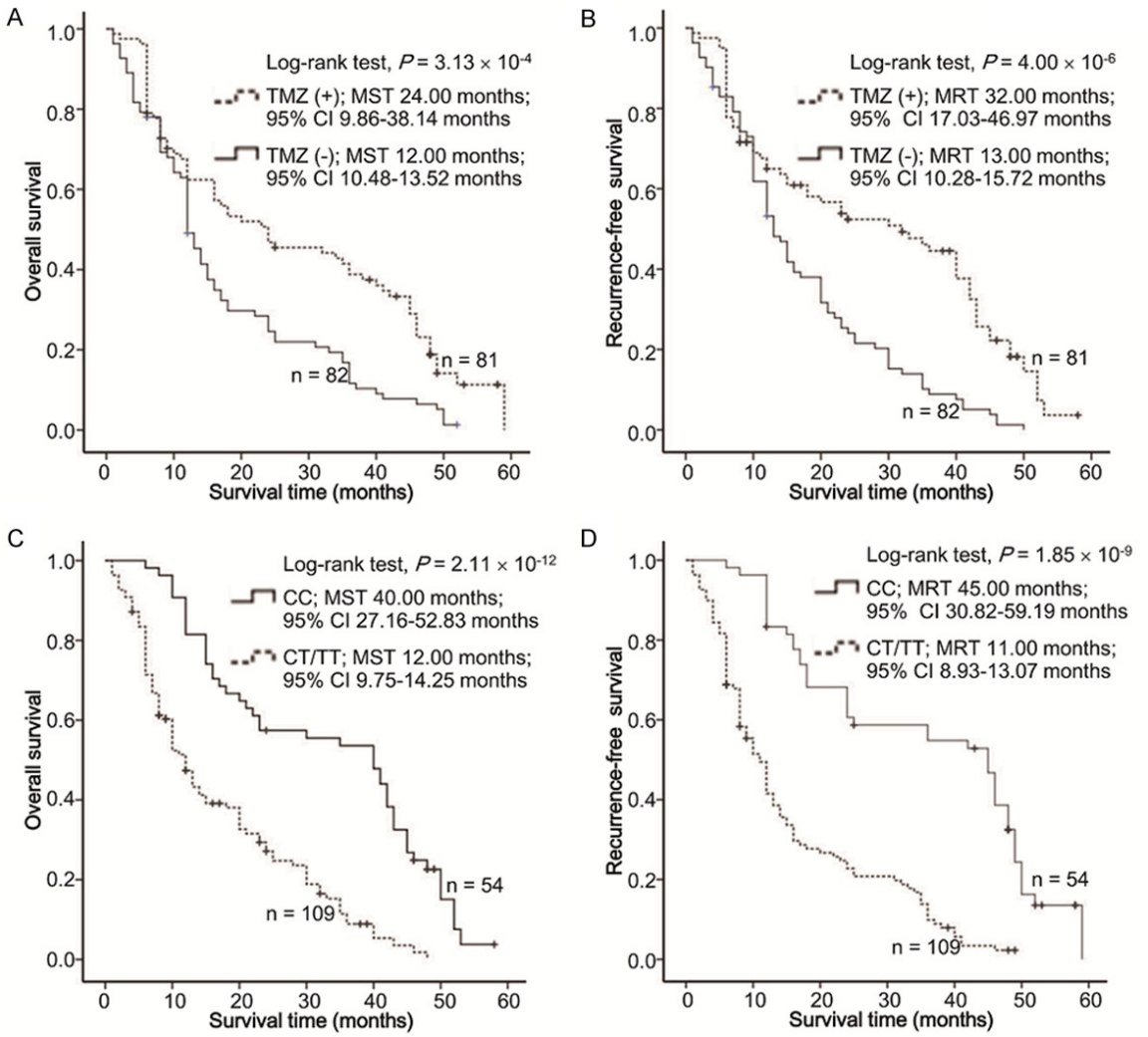

Figure 1. The effects of miR-1268a rs28599926 polymorphism and temozolomide (TMZ) treatment on diffusely infiltrating astrocytoma (DIA) prognosis in 163 cases. Temozolomide treatment was found to correlate with the overall survival (OS) (A) or tumor recurrence-free survival (RFS) (B) of DIA cases. The rs28599926 genotypes were also observed to relate to DIAs' OS (C) and RFS (D). Cumulative hazard function was plotted by Kaplan-Meier's methodology, and *P* value was calculated with two-sided log-rank tests. *Abbreviations*: MST, the median overall survival time; MRT, the median tumor recurrence-free survival time; CC, the genotype with the homozygotes of rs28599926 C alleles; CT/TT, genotypes with rs28599926 T alleles.

morphism for both patients with DIA and controls. Genotype frequent distribution in controls fitted the Hardy-Weinberg equilibrium well. The heterozygous genotype with rs28599926 C and T allele (rs28599926-CT) and the variant homozygous genotype with rs28599926 T allele (rs28599926-TT) were more frequent among DIA cases than the controls ($P < 0.01$). Logistic regression analysis showed that the OR for DIA for these individuals carrying rs28599926-CT compared with those exhibiting the homozygote for C alleles (rs28599926-CC) was 2.79 (95% CI, 1.76-4.44), and the corresponding OR for those featuring rs28599926-TT was 10.16 (95% CI, 3.94-26.21). These results exhibited that DIA risk was associated with the number of rs28599926 T alleles.

MiR-1268a rs28599926 polymorphism modulated the clinic pathological features of DIA

To explore possible effects of miR-1268a rs28599926 polymorphism on DIA, we analyzed the distribution difference of this polymorphism among different clinic-pathological characteristics of cases, including sex, age, race, smoking and drinking status, and tumor grade. Results showed these DIA cases with

Table 2. The miR-1268a rs28599926 polymorphism and diffusely infiltrating astrocytoma risk

Rs28599926		Controls (n = 189)		Cases (n = 163)		OR (95% CI)	*P*
		n	%	n	%		
Genotype	CC[a]	118	62.4	54	28.6	Reference	
	CT[a]	65	34.4	82	43.4	2.79 (1.76-4.44)[b]	1.30×10^{-5}
	TT[a]	6	3.2	27	14.3	10.16 (3.94-26.21)[b]	2.00×10^{-6}
	CT/TT[a]	71	37.6	109	57.7	3.41 (2.19-5.31)[b]	6.12×10^{-8}
Allele	C[c]	301	79.6	190	58.3	Reference	
	T[d]	77	20.4	136	41.7	2.80 (2.01-3.91)	1.45×10^{-9}

[a]CC, CT, TT, CT/TT represent the homozygotes of rs28599926 C alleles, the heterozygotes of rs28599926 C and T allele, and the homozygotes of rs28599926 T alleles, and the combination genotypes CT and TT of rs28599926, respectively. [b]Odds ratio (OR) conditional on matched set. [c]C represents both heterozygous C and homozygous C of rs28599926. [d]T represents both heterozygous T and homozygous T of rs28599926.

Table 3. The miR-1268a rs28599926 polymorphism and diffusely infiltrating astrocytoma risk stratified by race (Han and Minority), ender (female and male), and age (≤ 50 yrs and > 50 yrs)

	Rs28599926-CC (n = 54)		Rs28599926-CT/TT (n = 109)		OR (95% CI)[a]	*P*
	n	%	n	%		
Race						
Han	25		62		Reference	
Zhuang	29		47		0.65 (0.34-1.26)[a]	0.21
Gender						
Male	35		72		Reference	
Female	19		37		0.95 (0.47-1.91)[b]	0.87
Age						
≤ 50	26		54		Reference	
50	28		55		0.92 (0.48-1.78)[c]	0.80
Smoking						
No	28		63		Reference	
Yes	26		46		0.42 (0.15-1.21)[d]	0.11
Drinking						
No	30		56		Reference	
Yes	24		53		1.20 (0.62-2.33)[e]	0.59
Tumor grade[f]						
Low	33		38		Reference	
High	21		71		2.94 (1.50-5.76)[g]	1.75×10^{-2}

[a]Adjusted by gender, age, smoking and drinking status, and tumor grade. [b]Adjusted by race, age, smoking and drinking status, and tumor grade. [c]Adjusted by gender, race, smoking and drinking status, and tumor grade. [d]Adjusted by gender, age, race, drinking status, and tumor grade. [e]Adjusted by gender, age, race, smoking status, and tumor grade. [f]Low and high grade were defined as tumor grade II and III-IV, respectively. [g]Adjusted by gender, age, smoking and drinking status, and race.

genotypes of rs28599926-CT and -TT (rs28599926-CT/TT), compared to those with rs28599926-CC, faced higher risk of tumor dedifferentiation (OR = 2.94, **Table 3**). However, the expression of this polymorphism did not affect other features.

MiR-1268a rs28599926 polymorphism related to poor prognosis of DIA

Kaplan-Meier survival analysis showed that patients with rs28599926-CT/TT featured a significantly poorer prognosis than those with rs28599926-CC ($P = 2.11 \times 10^{-12}$ for OS and $P = 1.85 \times 10^{-9}$ for RFS, respectively) (**Figure 1C** and **1D**). Multivariate cox regression analysis (with stepwise forward selection based on likelihood ratio test) was next performed to determine whether miR-1268a rs28599926 polymorphism was an independent predictor of DIA cases. The results exhibited that the rs28599926-CT/TT increased the dying risk of patients with DIA compared with rs28599926-CC (HR = 3.25, 95% CI = 2.14-3.79). Risk role was also found in the RFS analysis; the corresponding HR (95% CI) was 2.52 (1.67-3.79) (**Table 4**). Taken together, these results implied that this polymorphism could be used as an independent prognostic marker for DIA.

MiR-1268a rs28599926 polymorphism affected the effects of temozolomide on DIA

To investigate possible effect of miR-1268a rs28599926 genotypes on temozolomide treatment ameliorating DIA prognosis, we stratified the analysis of the correlation between temozolomide treatment and DIA out-

Table 4. Cox's proportional hazard model analysis for multivariate analysis of potential predictor factors for diffusely infiltrating astrocytoma cases

Variable	OS		RFS	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Rs28599926 (CT/TT vs. CC)	3.25 (2.14-3.79)	3.10×10^{-8}	2.52 (1.67-3.79)	1.00×10^{-6}
Tumor grade (high vs. low)	2.54 (1.74-3.70)	1.00×10^{-6}	3.32 (2.25-4.91)	1.58×10^{-9}
TMZ (Yes vs. no)	0.57 (0.41-0.80)	1.28×10^{-3}	0.46 (0.32-0.66)	2.60×10^{-5}

Abbreviations: OS, overall survival; RFS, tumor reoccurrence-free survival; TMZ, temozolomide treatment.

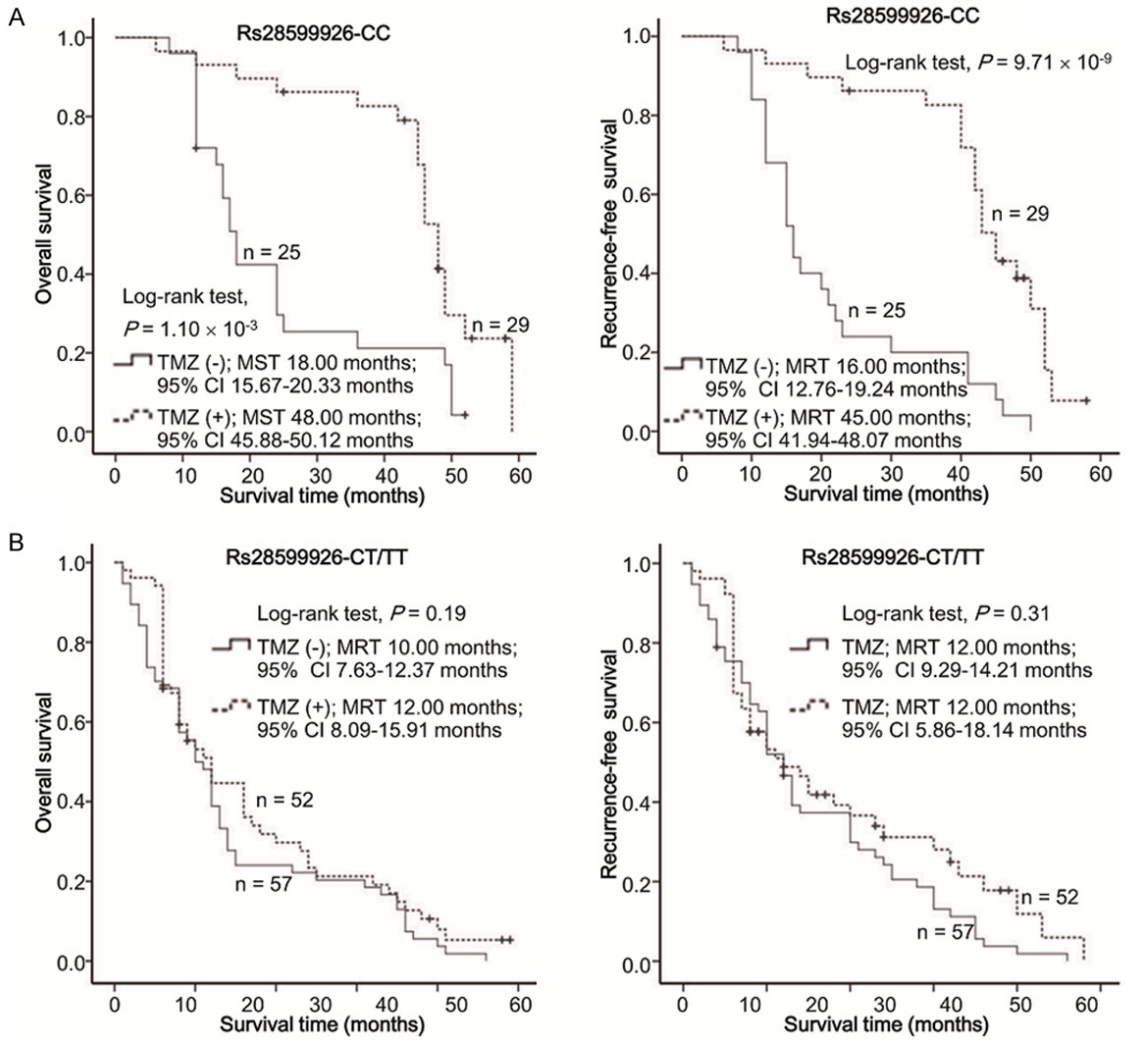

Figure 2. The effects of miR-1268a rs28599926 polymorphism on temozolomide (TMZ) treatment modifying diffusely infiltrating astrocytoma (DIA) prognosis. TMZ significantly affected the overall survival (OS) (left) or tumor recurrence-free survival (RFS) (right) of DIA cases among cases with rs28599926-CC (A), but not among patients with rs28599926-CT/TT (B). Cumulative hazard function was plotted by Kaplan-Meier's methodology, and *P* value was calculated with two-sided log-rank tests. *Abbreviations*: MST, the median overall survival time; MRT, the median tumor recurrence-free survival time; CC, the genotype with the homozygotes of rs28599926 C alleles; CT/TT, genotypes with rs28599926 T alleles.

come by different genotypes of miR-1268a rs28599926 polymorphism (**Figure 2**). Among these cases with rs28599926-CC (**Figure 2A**), longer MST and MRT were found in these

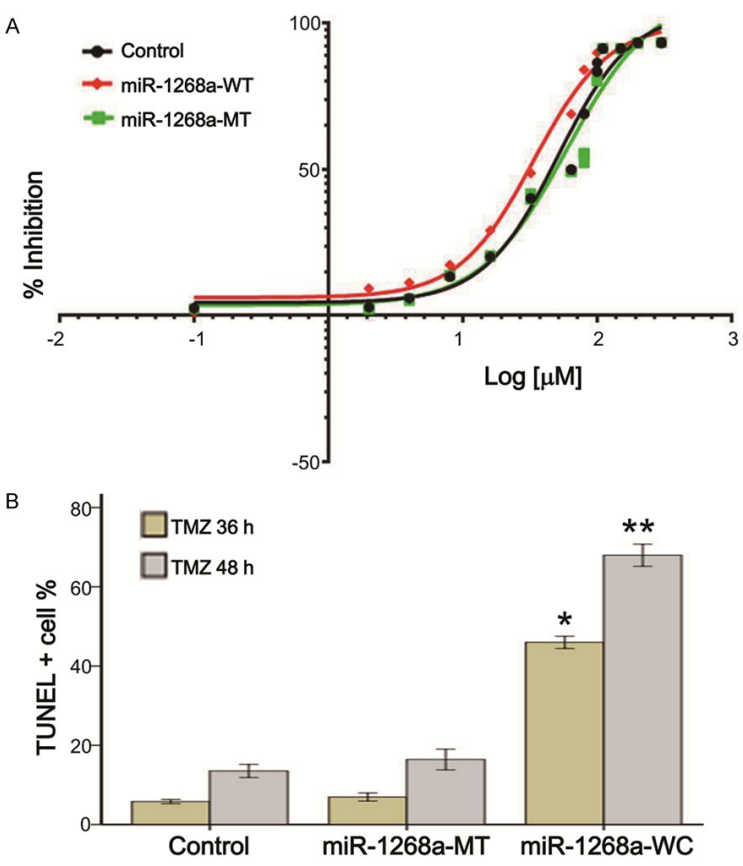

Figure 3. The miR-1268a rs28599926 polymorphism modified effects of temozolomide (TMZ) treatment on human glioma cells T98G in vitro. T98G cells were transfected with normal saline (Control), the wild type of miR-1268a mimics (miR-1268a-WT), or the mutant type of miR-1268a mimics (miR-1268a-MT). A: The sensitivity of cells to TMZ was evaluated by the half-maximal inhibitory concentration. B: TUNEL staining was used to analyze the TMZ-induced cell deaths. Data were analyzed using one-way ANOVA with Bonferroni corrections. "*" and "**" refer to $P < 0.05$ and < 0.01 for miR-1268a-WT vs. miR-1268-MT, respectively.

receiving temozolomide treatment than without accepting the same treatment (48 vs. 18 months for MST and 45 vs. 16 months for MRT, respectively). However, similar results were not observed among DIA patients carrying rs28599926-CT/TT (**Figure 2B**).

Based on the above-mentioned findings that temozolomide treatment was beneficial for these cases with rs28599926-CC but not those with rs28599926-CT/TT, we hypothesize that miR-1268a rs28599926 polymorphism can affect the sensitivity of DIA cells to temozolomide, an important chemotherapeutic drug for DIA [19-21]. To address this, human T98G glioma cells were transfected with the mimics with different genotypes of miR-1268a rs28599926 polymorphism, followed by the treatment with increasing concentrations (from 0.1 to 300 μM) of temozolomide. Thirty-six hours later, CCK-8 assay showed that miR-1268a-WT significantly increased sensitivity of T98G cells to temozolomide. The IC50 values of temozolomide were 33.66 vs. 59.52 μM for miR-1268a-WT vs. miR-1268a-MT (**Figure 3A**). Furthermore, the wild type of this polymorphism significantly increased temozolomide-induced cell death in the T98G cell lines, as assessed by TUNEL assay (**Figure 3B**).

Discussion

To the best of our knowledge, no studies have investigated the role of miR-1268a rs28599926 polymorphism in the risk of DIA. In this study, we analyzed the association between aforementioned polymorphism and the risk of DIA among Guangxi population and found miR-1268a rs28599926 T alleles increased DIA risk (adjusted OR = 3.41). These results imply that this polymorphism may have functional significance in DIA carcinogenesis.

DIA is one of major cancer types in the Guangxi Zhuang Autonomous Region; the possible risk factors of which include occupations, environmental carcinogens, diet, and ionizing radiation, and so on [1, 5]. Recently, increasing epidemiological evidence has shown that an individual susceptibility related to genetic factors might also be associated with DIA carcinogenesis [5-7, 9, 11-15, 18].

The miR-1268a is an important abundant microRNA encoded by the corresponding gene MIR1268A that maps to human chromosome 15q11.2 regions. In 2008, Morinal, *et al* [17] identified miR-1268a in human embryonic stem cells using the Illumina sequencing technology. Higher expression of this microRNA was observed in the human embryonic stem cells

than differentiated cells from embryoid bodies, suggesting miR-1268a might play an important role in embryogenesis and cell differentiation [17]. With the Human Genome Project developing, more than fifty polymorphisms have been identified in the MIR1268A gene (dbSNP in NCBI Database). In this study, we only analyzed miR-1268a rs28599926 polymorphism, primarily because this polymorphism is relatively common in Guangxi population and may involve in tumorigenesis [18]; whereas other polymorphisms are rare. In this study, we collected 163 DIA and 189 control samples from Guangxi area, a relatively high incident area of DIA. We observed about 20 percent of control individuals had miR-1268a rs28599926 T alleles. However, higher frequency was observed in the individuals with DIA, and following regression analysis proved this polymorphism increased DIA risk. Similar to our findings, Long, *et al* [18] showed that individuals with rs28599926-CT/TT face higher risk (OR = 2.10, 95% CI = 1.82-2.43) of liver cancer than those with rs28599926-TT. Additionally, they also found the rs28599926 polymorphism can interfere in the interaction of miR-1268a binding to the 3'-UTR of ADAMTS4, involving in carcinogenesis [22-24]. These results suggested miR-1268a rs28599926 polymorphism might modify the risk of tumors such as DIA and liver cancer.

Additionally, we also investigated the association between miR-1268a rs28599926 polymorphism and DIA prognosis. We found that DIA patients with risk genotypes of miR-1268a rs28599926 (OR > 1) had a significant poor RFS and OS compared to those without risk genotypes. Multivariate cox regression analysis further showed this polymorphism increased 1.52-times tumor reoccurrence risk and 2.25-times death risk. This is possibly because it correlates with the fact that this polymorphism modifies tumor grade and differentiation.

Another important finding in this study was that among different genotypes of miR-1268a rs28599926 polymorphism, temozolomide treatment exhibited different effects on DIA patients, suggesting this polymorphism might modify the effects of temozolomide treatment on DIA patients. This is likely due to miR-1268a rs28599926 polymorphism increasing sensitivity of DIA cells to temozolomide treatment.

However, there were several limitations to our present study. Because of the hospital-based the selection of control subjects, potential selection bias might have occurred. Despite the analysis of miR-1268a rs28599926 polymorphism, we did not analyze other polymorphisms in microRNA genes possibly able to modify the risk of DIA [7, 25-28]. Although this study investigated the effects of this polymorphism on the tumor sensitivity to chemotherapeutic drug temozolomide, it is deficient in functional and mechanical analysis. In addition, our findings were based on relatively small numbers and limited by small number subjects in part of the genotype strata. Therefore, more functional analyses deserve further elucidation based on a large sample and the combination of genes.

To conclude, this study is, to the best of our knowledge, the first report investigating an association between miR-1268a rs28599926 polymorphism and DIA risk and prognosis in Guangxi patients. We have found evidence that the genotypes of miR-1268a rs28599926 T alleles may be related to increased risk and poor prognosis for DIA, and that this polymorphism may modify the sensitivity of tumor cells to chemotherapeutic drugs such as temozolomide. Given that DIA is a highly fatal tumor, the finding of a genetic susceptibility (if confirmed) may have implications for screening and prevention.

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Disclosure of conflict of interest

None.

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