Identification of expression quantitative trait loci of *MTOR* associated with the progression of glioma

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Abstract. Mechanistic target of rapamycin (*MTOR*) encodes a key modulator of cell growth, proliferation, and apoptosis. Previous studies have demonstrated that the dysregulation of *MTOR* is involved in the development and progression of several types of cancer, including glioma. In the present study, a comprehensive analysis was conducted to examine whether the expression quantitative trait loci (eQTLs) of *MTOR* are associated with the progression of glioma. Candidate eQTLs of *MTOR* were obtained from the Genotype-Tissue Expression eQTL Browser. The Kaplan-Meier method and multivariate Cox model were used to analyze the progression-free survival time of glioma patients. Based on the analysis of 138 glioma patients, one eQTL of *MTOR*, rs4845964, was demonstrated to be significantly associated with the progression of glioma in a dominant manner. The adjusted hazard ratios (HRs) for patients with the AG or AA genotype at rs4845964 were 2.82 [95% confidence interval (CI), 1.27-6.27; P=0.0111] and 2.79 (95% CI, 1.10-7.07; P=0.0312), respectively, compared with those with the GG genotype. When the rs4845964 AG and AA genotypes were combined for analysis, the HR was 2.70 (95% CI, 1.25-5.82; P=0.0114) vs. the GG genotype. Stratified analyses revealed similar associations between the rs4845964 genotypes and the progression of glioma in all subgroups (following stratification by age, sex and tumor grade). These results demonstrate for the first time that the *MTOR* eQTL rs4845964 is associated with the progression of glioma.

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Introduction

Glioma is the most common primary malignant tumor of the central nervous system (1). In the past four decades, certain notable improvements have been made in conventional therapies for glioma. Nevertheless, the prognosis of the majority of glioma patients remains poor (1). At present, in addition to the histological tumor type and World Health Organization (WHO) malignancy grade (2), increasing numbers of molecular markers, such as *IDH* mutations and *MGMT* promoter methylation, have been identified as predictors of prognosis and indicators for the selection of therapeutic strategy for glioma patients (3). However, novel molecular markers are still required as a great deal of heterogeneity in glioma remains unexplained (4).

Mechanistic target of rapamycin (mTOR) is a serine/threonine protein kinase belonging to a family of phosphatidylinositol kinase-associated kinases, which has emerged as a central modulator of cell proliferation, apoptosis and metabolism (5,6). The dysregulation of multiple pathways signaled through mTOR has been reported in several types of cancer, including glioma (5,7). Additionally, it has been identified that persistent activation of the PI3K/AKT/mTOR pathway promotes malignant glioma progression in genetically modified mouse models (8). Consistently, increased PI3K/AKT/mTOR signaling is also associated with increased invasiveness of glioma (9).

Expression quantitative trait loci (eQTLs) are genomic variations that are associated with the expression levels of genes. Recently, several studies on genome-wide mapping of eQTLs have been conducted in multiple tissue types, including brain, liver and lymphoblastoid tissue (10-12). Furthermore, it has been demonstrated that eQTLs are likely to be associated with complex diseases (13,14). Therefore, we hypothesized that the eQTLs that regulate the expression level of *MTOR* may be associated with the progression of glioma.

In the present study, the associations between the eQTLs of *MTOR* and the progression-free survival (PFS) of glioma

patients were evaluated to test this hypothesis. The data demonstrate that one eQTL of *MTOR*, rs4845964, is associated with the progression of glioma in a dominant manner. Similar trends were identified in all subgroups by stratified analyses.

Materials and methods

Study subjects. The present study included 138 glioma patients whose sources and characteristics were described in our previous study (15). Briefly, all patients with histopathologically confirmed glioma were unrelated ethnic Han Chinese individuals recruited between January 2010 and July 2014 at the First Affiliated Hospital, Fujian Medical University (Fuzhou, China; n=122) and the First Affiliated Hospital, College of Medicine, Zhejiang University (Hangzhou, China; n=16) without sex, age or WHO grade restriction. The pathological diagnosis of each patient was confirmed by at least two local pathologists according to the WHO classification (2). Clinical and pathological information was obtained from patients' medical records. PFS time was defined as the period from the date of treatment to the date of progressive disease or mortality. The PFS times of patients without progression or who were lost to follow-up were censored at the time of the last adequate tumor evaluation. The last date of follow-up was 28th July, 2015. Informed consent was obtained from each patient at recruitment. The Institutional Review Board of the First Affiliated Hospital, Fujian Medical University, approved this study.

Selection of candidate eQTLs. Candidate eQTLs of *MTOR* were selected from the Genotype-Tissue Expression (GTEx) eQTL Browser (http://www.ncbi.nlm.nih.gov/projects/gap/eqtl/index.cgi) (16). The gene symbol '*MTOR*' was used as search term, and a P-value cutoff of 1.00×10^{-2} was applied to identify specific eQTLs overlooked by previous eQTL studies. As numerous eQTLs are shared between different tissues (17), an analysis of all studies included in the database was conducted. Subsequently, all eQTLs of *MTOR* from the database were assessed for linkage disequilibrium (LD) status by computing their pairwise correlation coefficients (r) relative to each other using HapMap CHB data. Tag-eQTLs were only genotyped when the r^2 value was >0.8. Haploview v4.2 software (Broad Institute, Cambridge, MA, USA) was used to assess the LD status and generated LD structure.

Genotype analysis. Genomic DNA was isolated from the peripheral blood lymphocytes using a commercial Tiangen TIANamp Genomic DNA kit (Tiangen Biotech Co., Ltd., Beijing, China). A Sequenom MassARRAY iPLEX platform (Sequenom Inc., San Diego, CA, USA) was used to genotype the candidate eQTLs of *MTOR*. Primers for genotyping are shown in Table I. Several measures were implemented for genotyping quality control: i) Positive and negative (no DNA) samples were included in every assay plate; and ii) a 5% random sample was tested twice, and the reproducibility was 100%.

Statistical analysis. The associations between the candidate tag-eQTLs of *MTOR* and the PFS of glioma patients were estimated using the Kaplan-Meier method, and log-rank test was used to determine the P-values. Dominant, recessive, and additive models were used to calculate the P-values for each candidate tag-eQTL of *MTOR*. The lowest P-values from the three models were used to evaluate the statistical significance of the associations. A multivariate Cox model with adjustment for sex, age, and WHO grade was used to analyze the tag-eQTLs of *MTOR* that were significantly associated with glioma PFS, and hazard ratios (HRs) and their 95% CIs were calculated for each genotype at this eQTL. Statistical analyses were implemented in SPSS (version 13.0; SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference, and all statistical tests were two-sided.

Results

Characteristics of the study subjects. The clinical characteristics of the 138 glioma patients are presented in Table II. The total cohort consisted of 90 (65.2%) males and 48 (34.8%) females. In total, 86 (62.3%) patients were aged >40 years and 52 (37.7%) were aged ≤40 years. Among the 124 (89.9%) patients for whom detailed tumor WHO classification data were available, 7 (5.1%) were WHO grade I, 45 (32.6%) were WHO grade II, 29 (21.0%) were WHO grade III, and 43 (31.2%) were WHO grade IV. All patients underwent maximal safe resection or subtotal resection, while 95 (68.8%) and 106 (76.8%) patients also received radiotherapy and alkylate-based chemotherapy, respectively.

Candidate eQTLs. As shown in Table III, 21 candidate eQTLs of *MTOR* were displayed in the GTEx eQTL Browser based on the aforementioned search terms. Initially, 7 overlapping eQTLs were excluded, and r-values were then computed for the remaining 14 candidate eQTLs to assess the LD status. The LD structure between the remaining 14 candidate eQTLs of *MTOR* is shown in Fig. 1. After excluding rs652625, whose minor allele frequency is <0.05 in the Chinese population, 6 tag-eQTLs (rs4845964, rs6668659, rs1061622, rs527676, rs1801131 and rs198388) remained and were selected for genotyping.

Association between the candidate eQTLs and glioma PFS. The associations between the 6 tag-eQTLs of *MTOR* and the PFS of glioma patients are presented in Table IV. Dominant, recessive and additive models were used to investigate these associations. Only rs4845964 was found to be significantly associated, based on the dominant model, with the PFS of glioma patients (P=0.0376). The survival curve for rs4845964 based on the dominant model is shown in Fig. 2. No significant association was identified between the other 5 tag-eQTLs of *MTOR* and the PFS of glioma patients. As shown in Table V, the median PFS time for the patients with the rs4845964 GG genotype was not reached, while the median PFS times were 21 months for patients with the AG genotype and 34 months for those with the AA genotype. Multivariate Cox model analysis demonstrated that the adjusted HRs for the patients with the rs4845964 AG genotype or AA genotype were 2.82 (95% CI, 1.27-6.27; P=0.0111) and 2.79 (95% CI, 1.10-7.07; P=0.0312), respectively, vs. those with the GG genotype. Due to the nature of action of rs4845964, the HR and 95% CI for rs4845964

Table I. Information on primers used for Sequenom MassARRAY iPLEX assays.

eQTL	Primer	Sequence (5'-3')
rs4845964	2nd-PCR Primer	ACGTTGGATGATGCATACAAGGTGAGGTGG
	1st-PCR Primer	ACGTTGGATGAAGTCAGGACAACACCTCCC
	Extension Primer	GCGAGATTATTCTCTTTATTCT
rs6668659	2nd-PCR Primer	ACGTTGGATGGTGTCTCTCCCATAGTAAGC
	1st-PCR Primer	ACGTTGGATGCACTTAGAGTTGGATGGTGG
	Extension Primer	GTAAGCCCTCAGCAAA
rs1061622	2nd-PCR Primer	ACGTTGGATGGGTAAGTGTACTGCCCCTG
	1st-PCR Primer	ACGTTGGATGTGTAACGTGGTGGCCATCC
	Extension Primer	TACGTGCAGACTGCATCC
rs527676	2nd-PCR Primer	ACGTTGGATGAGAGGCTCCTGAGGAGTAGA
	1st-PCR Primer	ACGTTGGATGCAGGTGAGAGTGCCCATATC
	Extension Primer	GCCGGAGGAGTAGACTCAGGGAGC
rs1801131	2nd-PCR Primer	ACGTTGGATGCCGAGAGGTAAAGAACGAAG
	1st-PCR Primer	ACGTTGGATGAGAGCAAGTCCCCCAAGGAG
	Extension Primer	GGACCGAAGACTTCAAAGACACTT
rs198388	2nd-PCR Primer	ACGTTGGATGTTTCTCCCAAGTGCCTCAAG
	1st-PCR Primer	ACGTTGGATGAGGTAGCAGGCTTTCTTTTC
	Extension Primer	CCCTCAAGTGCTTGAGATATT

eQTL, expression quantitative trait loci; PCR, polymerase chain reaction.

Table II. Clinical characteristics of glioma patients (n=138).

	Patients	
Variable	No.	(%)
Sex		
Male	90	(65.2)
Female	48	(34.8)
Age (years)		
≤40	52	(37.7)
>40	86	(62.3)
WHO grade		
I	7	(5.1)
II	45	(32.6)
III	29	(21.0)
IV	43	(31.2)
Unknown	14	(10.1)
Surgery	138	(100.0)
Radiotherapy	95	(68.8)
Chemotherapy	106	(76.8)

WHO, World Health Organization.

were also calculated using a dominant model. Patients with the rs4845964 AG or AA genotype had an increased risk of progression (HR, 2.70; 95% CI, 1.25-5.82; P=0.0114) compared with those with the GG genotype.

Table III. eQTLs of *MTOR* displayed in GTEx eQTL Browser.

MTOR eQTL	eQTL position	P-value
rs198389	Chr 1: 11919270	1.3407×10^{-5}
rs235249	Chr 1: 12258230	2.2680×10^{-5}
rs603151	Chr 1: 12248290	2.3412×10^{-5}
rs6668659	Chr 1: 11922297	2.8550×10^{-5}
rs652625	Chr 1: 12225350	3.9440×10^{-5}
rs1061622	Chr 1: 12252954	4.1319×10^{-5}
rs525891	Chr 1: 12249642	4.1319×10^{-5}
rs6668659	Chr 1: 11922297	5.0206×10^{-5}
rs603151	Chr 1: 12248290	6.3759×10^{-5}
rs527676	Chr 1: 10891776	6.4493×10^{-5}
rs6668659	Chr 1: 11922297	6.5262×10^{-5}
rs5746053	Chr 1: 12262297	7.2931×10^{-5}
rs5746053	Chr 1: 12262297	7.6596×10^{-5}
rs6697733	Chr 1: 12242457	9.0416×10^{-5}
rs4845964	Chr 1: 10980543	9.5734×10^{-5}
rs5746051	Chr 1: 12261971	0.0001
rs5746051	Chr 1: 12261971	0.0001
rs6697733	Chr 1: 12242457	0.0001
rs1801131	Chr 1: 11854475	0.0001
rs198388	Chr 1: 11917339	0.0001
rs198389	Chr 1: 11919270	0.0001

eQTL, expression quantitative trait locus; *MTOR*, mechanistic target of rapamycin; GTEx, Genotype-Tissue Expression; Chr 1, chromosome 1.

Table IV. Six tag-eQTLs of *MTOR* and their associations with the progression of glioma.

		Genotype[a] (138 patients)				
MTOR eQTL	eQTL location	Common	Heterozygous	Rare	Model[b]	P-value[c]
rs4845964	Downstream	28	68	36	Recessive	0.8931
					Dominant	0.0376[d]
					Additive	0.0656
rs6668659	Upstream	90	40	3	Recessive	0.3899
					Dominant	0.2215
					Additive	0.4217
rs1061622	Upstream	94	28	10	Recessive	0.4452
					Dominant	0.8637
					Additive	0.7391
rs527676	Downstream	111	23	0	Recessive	NC
					Dominant	0.1511
					Additive	0.1511
rs1801131	Upstream	76	51	5	Recessive	0.9649
					Dominant	0.9519
					Additive	0.9978
rs198388	Upstream	90	42	2	Recessive	0.1912
					Dominant	0.2540
					Additive	0.2926

[a]Some samples failed to genotype. [b]Model used for association analysis. [c]Log-rank test. [d]Statistically significant (P<0.05). eQTL, expression quantitative trait locus; *MTOR*, mechanistic target of rapamycin; NC, not calculated.

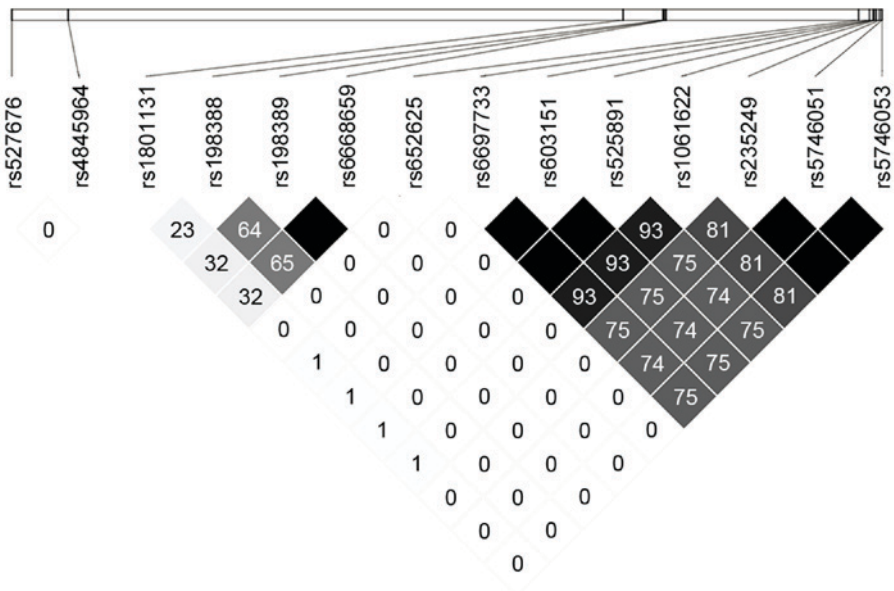

Figure 1. LD structure among 14-candidate eQTLs of mechanistic target of rapamycin. The values of r^2 x100 are shown in the boxes and the shade of grey represents the LD status between each pair of eQTLs (r^2=1 black; 0<r^2<1 shades of grey; r^2=0 white). LD, linkage disequilibrium; eQTLs, expression quantitative trait loci.

Stratified analyses. A dominant model was then used to perform stratified analyses for rs4845964 by sex, age and WHO grade (Fig. 3). A significantly increased risk of progression was identified in patients aged >40 years (HR, 8.84; 95% CI, 1.20-64.93; P=0.0322). There was no significant association between rs4845964 and the PFS of glioma patients in the other subgroups. However it was notably identified that, in all subgroups, patients with an rs4845964 AG or AA genotype were more likely to have a shorter PFS time. Particularly in female patients and those

Table V. HRs of rs4845964 genotypes for the progression of glioma.

	Patients (n=132)				
Genotype	No.	(%)	Median PFS time (months)	HR[a] (95% CI)	P-value
GG	28	(21.2)	Not reached	1.00 (Reference)	-
AG	68	(51.5)	21	2.82 (1.27-6.27)	0.0111
AA	36	(27.3)	34	2.79 (1.10-7.07)	0.0312
AG+AA	104	(78.8)	22	2.70 (1.25-5.82)	0.0114

[a]Data were calculated by multivariate Cox model, adjusted for sex, age, and World Health Organization grade. HR, hazard ratio; PFS, progression-free survival; CI, confidence interval.

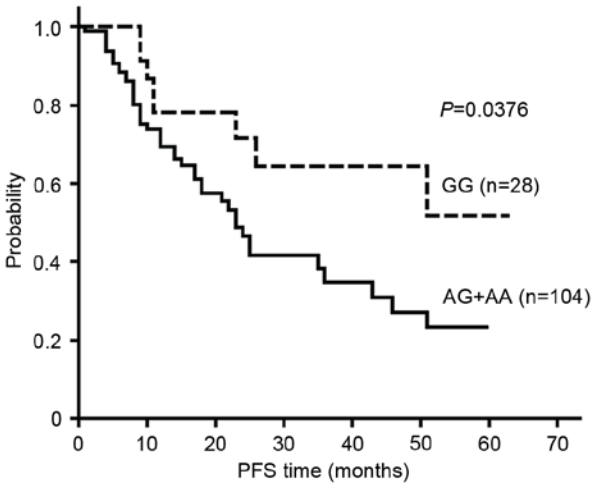

Figure 2. Kaplan-Meier estimates of the PFS of glioma patients according to rs4845964 genotypes in a dominant model (P=0.0376, log-rank test). PFS, progression-free survival.

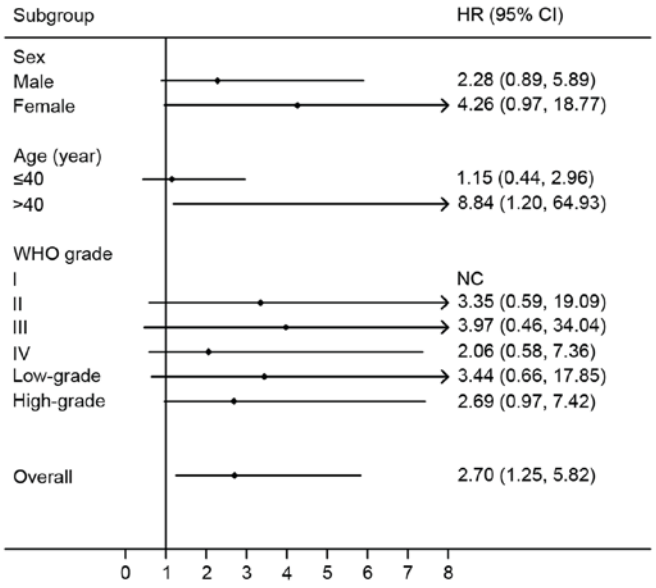

Figure 3. Forest plot for the associations between rs4845964 and the progression of glioma in the dominant model stratified by sex, age and WHO grade. Horizontal lines represent 95% CIs, and central black dots represent HRs. HR, hazard ratio; CI, confidence interval; WHO, World Health Organization; NC, not calculated.

with high-grade glioma, there were non-significant trends toward worse PFS for patients with the rs4845964 AG or AA genotype. In the stratified analysis by sex, the HR for the AG/AA vs. GG genotype in male patients was 2.28 (95% CI, 0.89-5.89; P=0.0875), while it was 4.26 (95% CI, 0.97-18.77; P=0.0552) for females. The HR for the AG/AA vs. GG genotype was 1.15 (95% CI, 0.44-2.96; P=0.7765) for patients aged ≤40 years. Furthermore, in the stratified analysis by WHO grade, the HRs for the AG/AA vs. GG genotype were 3.35 (95% CI, 0.59-19.09; P=0.1728) for WHO grade II glioma, 3.97 (95% CI, 0.46-34.04; P=0.2086) for WHO grade III glioma, and 2.06 (95% CI, 0.58-7.36; P=0.2643) for WHO grade IV glioma. Similar results were revealed when glioma was categorized as low-grade glioma (WHO grade I and II) and high-grade glioma (WHO grade III and IV); deteriorative PFS times for patients with the rs4845964 AG or AA genotypes was found in the low-grade glioma (HR, 3.44; 95% CI, 0.66-17.85; P=0.1423) and high-grade glioma (HR, 2.69; 95% CI, 0.97-7.42; P=0.0564) subgroups.

Discussion

The aim of the present study was to elucidate novel molecular markers to improve the classification and prognosis evaluation of glioma patients using an eQTL-based strategy. Based on the analysis of 138 glioma patients, one eQTL of *MTOR* (rs4845964) was found to be significantly associated with progression of glioma. Stratified analyses revealed that the associations between rs4845964 genotypes and the PFS of glioma patients were similar in all subgroups. To the best of our knowledge, this is the first report evaluating the association between eQTLs of *MTOR* and progression of glioma.

Currently, the WHO malignancy grade of glioma remains a cornerstone for the selection of therapeutic strategy, and the treatments applied to the patients with the same WHO grade are uniform (18). However, patients with the same WHO grade often have different clinical courses, which may be partly explained by diverse genetic backgrounds and genomic alterations (19-21). In the past two decades, the advancements in novel molecular markers have markedly improved personalized therapies for glioma (3). Nevertheless, there remains a great deal of heterogeneity not yet explained in glioma. In the present study, an eQTL of *MTOR* was identified as a novel

prognostic predictor of glioma, which further demonstrated the value of genetic variation in glioma classification.

As a pivotal component of multiple pathways, mTOR contributes important functions in several cellular processes, including, cell proliferation and apoptosis (5,6). Accumulating evidence demonstrates that mTOR activity is associated with the progression and survival of glioma cells *in vitro* and *in vivo* (8,9,22). On the basis of an analysis of glioma tissues, it has been demonstrated that higher expression of phosphorylated mTOR is associated with a less favorable clinical outcome in patients with glioblastoma (23). Additionally, multiple studies have revealed that mTOR inhibition can reverse chemoresistance to temozolomide and enhance the radiosensitivity of glioma (24-27). Thus, it is biologically plausible that genetic variations affecting the expression level of *MTOR* may be associated with the clinical outcomes of glioma. Additionally, similar trends of association between rs4845964 and the progression of glioma were found in all subgroups by stratified analyses. These results suggest that the eQTL of *MTOR* identified in the current study may be a valuable indicator of the progression of glioma, regardless of sex, age and WHO grade.

In the present study, one eQTL of *MTOR* that is significantly associated with the progression of glioma was identified from six tag-eQTLs. The candidate eQTLs of *MTOR* evaluated in this study were all extracted from an eQTL study performed on lymphoblastoid cell lines from a Caucasian population (12). Increasingly, evidence has demonstrated that eQTLs have significant tissue specificity (28-30). A study comparing eQTLs derived from blood and brain also identified that, while many eQTLs are shared, a number of tissue-specific eQTLs exist (17). These results may partly explain why the other 5 tag-eQTLs of *MTOR* were not revealed to be associated with progression of glioma. In contrast, it has been revealed that eQTLs identified from blood can also help to explain brain related phenotypes (17). This result is comparable with our finding that a proportion of *MTOR* eQTLs identified from lymphoblastoid cell lines are associated with the progression of glioma. In order to identify true eQTLs of *MTOR* with moderate P-values, which have been previously overlooked by eQTL studies due to the use of a stringent P-value threshold, a higher P-value cutoff for candidate eQTLs selection was used in the current study. Therefore, false-positive eQTLs of *MTOR* were unavoidable in candidate eQTL selection, which is another possible reason for the failure of the other 5 tag-eQTLs of *MTOR*. In addition, the interethnic differences in genetic background between Chinese and Caucasian individuals may constitute another potential reason.

To conclude, to the best of our knowledge, this study has demonstrated for the first time a significant association between an eQTL of *MTOR* and the progression of glioma in a Chinese population. Similar trends of association were found in all subgroups by stratified analyses. These findings may advance our understanding of glioma progression and improve the prognostic evaluation of glioma.

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