Two gene polymorphisms (rs4977756 and rs11515) in CDKN2A/B and glioma risk in South Indian population

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ABSTRACT

Gliomas are most common neoplasms in the CNS with unknown aetiology. Gene polymorphisms have been studied in glioma to check its risk in different population. CDKN2A, commonly altered tumor suppressor gene polymorphisms were recently shown to be associated with glioma in Caucasians. Present study evaluated potential association between two SNPs in CDKN2A/B gene with glioma risk in South Indian population with a total of 128 cases and 140 control subjects. Allelic discrimination assay was used for the genotyping and the association of each SNP with glioma risk were calculated using odds ratio and 95% CI. There was no association between rs4977756 polymorphism and glioma risk in south Indian population. GG genotype had a non-significant low risk in glioma (OR = 0.69). rs11515 polymorphism was not in Hardy Weinberg Equilibrium in our sample, so it was not considered for association studies. There was difference in genotype in tissue samples paired with blood samples for rs4977756 polymorphism, suggesting the importance of tissue SNP status in association studies. These results show that these two polymorphisms may not contribute to risk for glioma in South Indian population.

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1. Introduction

Gliomas are the most common primary tumors of the brain originating from glial cells (Ostrom et al., 2014). Advanced age and male predominance is associated with gliomas (Ohgaki and Kleihues, 2005). Radiation exposure and cytomegalo virus infection are also linked to the risk factors for gliomas (Braganza et al., 2012; Cobbs et al., 2002). Genetic and environmental factors play a major role in gliomagenesis (Wrensch et al., 2002; Goodenberger and Jenkins, 2012). Identification of genetic risk factors will help in early detection and prevention of this disease.

CDKN2A (p16) belongs to an important family of proteins, which negatively regulate the G1 phase of cell cycle (Serrano et al., 1993). P16 gene products bind to CDK4 and CDK6 and thereby inhibiting their interaction with cyclin D1. The inhibition of cyclin D1–CDK4/6 complex activity prevents RB protein phosphorylation and release of E2F, leading to the inhibition of cell cycle in G1/S transition (Cobrinik et al., 1992). P16 gene alterations are common in glioma which is negatively associated with patient survival (Kamiryo et al., 2002; Sibin et al., 2015). p16 gene polymorphisms have shown to be associated with the development of various cancers including gastric, melanoma, head and neck and cervical cancer (Geddert et al., 2005; Aitken et al., 1999; Zheng et al., 2002; Thakur et al., 2012).

Few studies on Caucasians have shown that, p16 polymorphisms are associated with glioma risk (Royds et al., 2011; Shete et al., 2009). We have studied these two common SNPs in p16 gene (rs4977756 and rs11515) and their role in glioma susceptibility in South Indian population.

2. Materials and method

2.1. Study population

A total of 128 patients were enrolled in this study. All of the glioma cases were newly diagnosed from December 2010 to June 2014. Glioma diagnosis was confirmed for all cases by MRI and histopathology. Histopathology and grading was documented according to WHO 2007 criteria (Louis et al., 2007). The control subjects ($n = 140$) were healthy adults without a history of cancer or neurological disorders, who were randomly selected from general population. This study was approved by Institute ethics committee for human studies, NIMHANS, Bangalore, India. 5 ml of blood samples were collected from patients and control subjects for SNP analysis. Fifty glioma tissue samples were collected post surgically from patients who underwent surgery in Neurosurgery OT. Tissues were stored in RNA later and kept under $-80\,°C$ till isolation of DNA. Blood samples from these patients were collected before surgery.

2.2. Genotyping

Genomic DNA from blood and tissue was isolated using commercially available kits with standard protocol. Purity and quantity was

checked with Nanodrop ND2000c spectrophotometer. DNA with A260/280 1.75–1.85 was used for the study. Genotyping of the rs11515 and rs4977756 was done using Taqman® allelic discrimination assay (Applied Biosystems, Foster City, CA) with a commercially available primer probe set (assay ID C_12096259_10, c_11841829_10). Genotyping was performed in duplicates in Applied biosystem 7500 Fast machine.

2.2.1. Blood–tissue pair comparison of genotypes

Genotype of both the SNPs were analysed in 50 blood-tissue pair samples from same patients and checked for any difference in genotype in both rs4977756 and rs11515.

2.3. Statistical analysis

Data was analysed with statistical software R.3.0.1 and G*Power 3.1.9.2 software. Continuous variables were expressed as mean ± SD and categorical variables were expressed as per cent values or absolute numbers, as indicated. Differences in demographic characteristics between patients and controls were compared by using χ2 test for all categorical variables. To estimate the deviation of frequency of gene alleles in tested population, we performed the Hardy-Weinberg equilibrium using χ2 tests. Genotypes and allele frequencies were compared by χ2 analysis. Association between risk and variables were expressed as odds ratio with 95% Confidence Interval (CI). The results were considered statistically significant at $P < 0.05$.

3. Results

3.1. Population characteristics

General characteristics of glioma patients and the control subjects who were included in this study were presented in Table 1. Clinical and genotyping data were available for all the cases and controls. Mean age in the study group was 40.80 ± 13.42 years for patients and 37.461 ± 11.04 years for controls. There was no significant difference between distribution of age and sex among cases and controls. All of them had a south Indian ethnicity by birth. Out of 128 glioma subjects, most of the patients were grade 3 (51.6%) followed by grade 4 (29.7%), grade 2 (15.6%) and grade 1 (3.1%). Majority of the glioma patients were harbouring the tumor in frontal lobe (49.2%), followed by insular (18.8%), temporal (12.5%), two lobes (11.7%) and other lobes (7.8%).

3.2. Association of CDKN2A polymorphisms with glioma

The distribution of genotypes of rs4977756 in control was in HWE ($p = 0.44$). The allelic frequency for A allele was 74.3 and G was 25.7 in control subjects and 75.8 for A and 24.2 for G in patients. rs11515 was not in HWE ($p = 0$). Because of this reason rs11515 was not considered for further analysis. For rs11515, C allele was 3.2% and G was allele

Table 1
Selected characteristics of subjects.

Characteristic	Cases, N (%)	Control, N (%)	P value
Total	128	140	
Gender			0.82
Male	84	90	
Female	44	50	
Age at diagnosis			0.06
<40	65	88	
≥40	63	52	
Tumor grade			
Low	24	–	
High	104	–	
Tumor type			
GBM	34	–	
Non GBM	94	–	

Table 2
Case control distribution of genotypes of polymorphisms analysed.

Genotype	Controls, n (%)	Cases, n (%)	OR (95% CI)	P value
AA	79 (56.4)	73 (57.0)	Reference	
AG	50 (35.7)	48 (37.5)	1.04 (0.63–1.73)	0.88
GG	11 (7.9)	7 (5.5)	0.69 (0.25–1.87)	0.46
AG + GG	61 (43.6)	55 (43.0)	0.975 (0.6–1.58)	0.92

is 96.8% in controls and A is 2.7% and G is 97.3% for patients. Genotypes were distributed as CC (0.1%), CG (6.2%) and GG (93.7%) in controls and CC (0.07%), CG (5.3%) and GG (94.6%) in patients. The association study results of rs4977756 were shown in Table 2. Sample size calculation for allele comparison based on previous meta-analysis study (Lu et al., 2015) was performed using G*Power 3.1.9.2 software. A two tailed z test was used with parameters: sampling ratio = 1; power to detect the difference (1-β) = 0.9 and type I error rate (α) = 0.05. We obtained the cumulative frequency for 'A' allele as 0.56 in cases and 0.61 in control. The minimum sample required to detect the difference in allele was 2039 cases and 2039 control.

3.3. Rs4977756 and glioma subtypes

The genotype was correlated with different grades and tumor type to check the confounding effect. But there was no significant association with grade of the tumor or type of the tumor in the study population. The data was presented in the Table 3. Genotype was compared between tumor type and tumor grade within the patient group and it was found that there was no association between tumor grade ($p = 0.13$) and tumor type ($p = 0.89$) with the genotype.

3.4. Change in alleles of blood tissue paired samples

In order to see the role of SNPs in tissue, we checked a number of tissue blood pairs. It was found that there was change in sequence for 12% of the samples. 3 samples changed from AA to AG and one sample AG to GG. It suggested, G allele may be contributing risk in the formation of glioma. Interestingly, two samples changed in genotype from AG to AA. All samples which had this change were high grade gliomas. There was no change in genotype of rs11515. The frequency of these mutations was presented in Fig. 1.

4. Discussion

Gliomas are very well known for its aggressiveness and poor survival of the patients. Even after the discovery of various next generation technologies, the reason for gliomagenesis is still unclear. Genome-wide association study (GWAS) uses SNPs as markers to identify the genetic cause of many disorders including cancer. A GWAS study identified five genetic loci associated with glioma risk and CDKN2A was one among them (Shete et al., 2009). CDKN2A is a tumor suppressor gene which produces a protein with 16 kDa known as p16/p14ARF which is involved in the cell cycle regulation (Serrano et al., 1993; Cobrinik et al., 1992). Researches showed that germline mutation of p16 gene causes the familial melanoma and glioblastoma (Hussussian et al., 1994; Tachibana et al., 2000). P16 regulation is associated with radiation sensitivity as proved by previous studies, so any genetic alteration in this gene might be associated with gliomagenesis (Miyakoshi et al., 1997). Many studies including previous studies from our lab indicated that alterations in p16 gene and its expression are common in glioma.

The rs4977756 polymorphism is mapped 59 kb telomeric to CDKN2B at 9p21.3. This region encompasses the CDKN2A-CDKN2B tumor suppressor genes. Previous genetic association studies indicated that the rs4977756 polymorphism may contribute to the increased

Table 3
Odds ratios and 95% CIs for glioma in relation to rs4977756 polymorphisms by histopathology.

Genotype	Controls, n (%)	Glioma patients		P value
		n (%)	OR (95% CI)	
Tumor grade vs genotype				
Low grade			0.53 (0.2–1.37)	0.19
AA	79 (56.4)	17 (70.8)		
AG + GG	61 (43.6)	7 (29.2)		
High grade			1.1 (0.67–1.85)	0.69
AA	79 (56.4)	56 (53.8)		
AG + GG	61 (43.6)	48 (46.2)		
Tumor type vs genotype				
GBM			1.02 (0.48–2.18)	0.95
AA	79 (56.4)	19 (55.9)		
AG + GG	61 (43.6)	15 (44.1)		
Non GBM			0.96 (0.57–1.63)	0.88
AA	79 (56.4)	54 (57.4)		
AG + GG	61 (43.6)	40 (42.6)		

risk of glioma (Shete et al., 2009). Two meta-analysis papers published recently had shown significant association between this SNP among western population (Lu et al., 2015; Qi et al., 2015). One of the meta-analysis included 12 studies (6 articles) and found that rs4977756 polymorphism was associated with increased risk of glioma in all genetic models especially in homozygote models (Lu et al., 2015). Another meta-analysis included 13 studies and subgroup analyses of ethnicities and similar results were observed in Caucasians. However, the association was not found between rs4977756 polymorphism and the risk of glioma in all models for the Asian studies (Qi et al., 2015). The CDKN2A/B rs4977756 polymorphism shows obvious increase the risk of glioma in Caucasians. Both the meta-analysis suggested the need of Asian studies to confirm the results in other ethnic populations, as the ethnicity influences the susceptibility to the disease. There was only one Asian study (Li et al., 2012) on the association with glioma and there was no study on Indian population. As the previous Asian study showed significant correlation with glioma, an Indian study would help to understand the association of this SNP with ethnical variation. In contrast to the other Asian report, our study showed no association of rs4977756 with glioma risk, even after stratifying tumor grade and type.

The study performed by Li et al. (2012) included 226 cases and 251 controls. The A allele frequency was 77% in both cases and control. But in our study the allelic frequency for A allele was 74.3% in control subjects and 75.8% in patients. In both the studies A allele was major allele and it had frequency above 70%. In European population the A allele frequency was <70%. There was similarity in distribution pattern of A allele in the study by Li et al. and our study. This suggests that in Asian population this SNP is not playing role in glioma risk.

We had calculated the minimum sample size required for allelic difference and it was 2039 samples in each group. Our study was limited to only south Indian population. Inclusion of 2039 patients was not feasible in a short period of time. So, this study can be taken as preliminary data and further studies should uncover the role of this polymorphism with higher number of samples.

Rs11515 is single nucleotide polymorphism located in the 3′-UTR of exon 3 of p16 gene. It was reported that rs11515 was significantly associated melanoma prognosis (Sauroja et al., 2000) and risk of familial melanoma (Aitken et al., 1999). There was no association found in gastric and oesophageal adenocarcinoma (Geddert et al., 2005), epithelial ovarian cancer (Yan et al., 2008) and squamous cell carcinoma of the head and neck (Zheng et al., 2002). Among Caucasian population there was only one study in glioma (Royds et al., 2011). In an Indian study, there was an association of rs11515 with development of cervical cancer (Thakur et al., 2012). The minor allele frequency for this SNP was found to be 0.11 for G allele in North Indian population. But in our study, G allele was the major allele and MAF for C was 0.001. South Indian population is different in ethnicity and this may be a reason for the varying results.

Role of gene polymorphisms in the blood sample may or may not correlate with the tissue sample which is affected. Owing to various genetic changes during tumorigenesis the SNP sequence may change in the affected organ. There are various studies which has analysed and correlated SNP in blood with expression of genes. We observed SNP in tissue which may change its sequence and affect the gene expression pattern during pathogenesis. The results from tissue blood pair study revealed that there was a change in SNP sequence in blood and tissue. Our study indicated that, the rs4977756 polymorphism A allele to G in tumor tissue may be one of the cause of tumorigenesis. Interestingly, there was no change in sequence in tissue blood paired samples for rs11515. This may be explained as only polymorphisms that follow Hardy Weinberg equilibrium will show somatic mutation in the disease tissue. In our cohort only high grade glioma showed these mutations. This may be because of mutations happening during low grade to high grade transition. There are no reports in this concern that gene polymorphisms can change from blood to tissue. Majority (67%) of the change was towards G allele which was shown as risk allele. As this SNP is found to be involved with glioma risk this change explains the role of rs4977756 polymorphism in gliomagenesis. Future studies aimed at correlation of SNP and gene expression should consider SNP status in tissue. Our study showed that, SNPs which are polymorphic and contributing disease pathogenesis may be mutated at tissue level during tumorigenesis.

The present study reveals that there is no association of these two gene polymorphisms and glioma risk in South Indian population.

Conflict of interest

The authors have declared that there is no conflict of interest financially or findings specified in this paper.

Acknowledgements

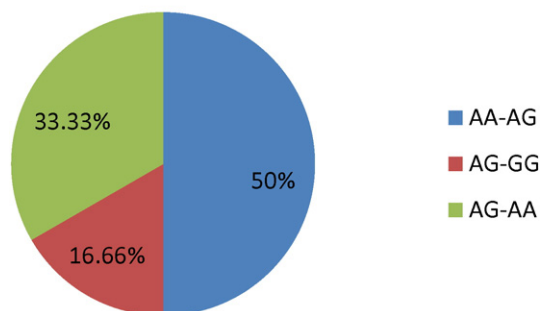## Genotype change from blood to tissue

Fig. 1. Graph showing frequency of de novo mutations in the tumor tissue when compared with blood.

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