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Genetic polymorphisms of *EGF* 5'-UTR and *NAT2* 857G/A associated with glioma in a case control study of Malaysian patients

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ABSTRACT. Studies of genetic mutations that have been used in predicting glioma prognosis have revealed a complex relationship between clinical and genetic factors. Epidermal growth factor (EGF) and the *NAT2* gene play a central role in carcinogenesis. An adenine (A) to guanine (G) single nucleotide polymorphism at position 61 in the 5'-untranslated region (5'-UTR) of the *EGF* gene has been found to be associated with levels of EGF production, and the mutations in the *NAT2* gene have been postulated as a risk factor for cancer. We investigated EGF and the *NAT2* gene in 13 glioma tissue samples and 12 normal controls. In the *EGF* 5'-UTR 61G polymorphism, the heterozygote GA was the most common genotype in the glioma patients. In the *NAT2* polymorphism at nucleotide position 857G/A, the G allele and the GG genotype were the most prevalent forms in both the glioma and normal samples. We did not find any homozygous AA genotypes in the

glioma patients. Based on this preliminary evidence, the *EGF* 5'-UTR at position 61 and the *NAT2* SNP at position 857 polymorphisms are associated with increased risk for glioma.

Key words: Genetic polymorphisms; EGF; *NAT2*; Glioma

INTRODUCTION

Glioma is a common primary brain tumor of the central nervous system, where the major histologic types are astrocytic, oligodendroglial, and mixed gliomas (e.g., oligoastrocytic). According to WHO, gliomas can be classified into four grades of malignancy (Kleihues and Cavanee, 2000). Despite advances in modern neurosurgery, radiosurgery and chemotherapy, the survival rate of glioma patients is dismal. Survival rate of glioblastoma multiforma (GBM) patients, the most aggressive form (WHO grade 4) of glioma in adults, is very poor, with median survival times ranging from 10 months in patients younger than 65 years to 3.5 months for those older than 65 years (Davis et al., 1998).

At our teaching hospital, University of Malaya Medical Center (UMMC) Malaysia, there is an average of 100 brain tumor cases surgically treated per year, which include glioma, pituitary tumors, metatastic lesions, etc. However, there are not many cases of glioma of various grades compared to other brain tumors. Due to this limitation, there has been no previous genetic correlation study in this disease reported so far from our hospital.

Generally, epidermal growth factor (EGF) is encoded by a 4.8-kb mRNA transcript from a gene that is 110 kb in length, which contains 24 exons and is located on human chromosome 4q25 (Salomon et al., 1995). Many previous studies have shown that among the different families of growth factors and growth factor receptors, EGF and its receptor (EGFR) play a central role in carcinogenesis. An adenine (A) to guanine (G) single nucleotide polymorphism (SNP) at position 61 in the 5'-untranslated region (5'-UTR) of the *EGF* gene has been reported to be associated with different levels of EGF production (Bhowmick et al., 2004).

N-acetyltransferase 2 (arylamine N-acetyltransferase) or NAT2 is polymorphic, metabolizes aromatic amines, and is involved in phase II metabolism of several compounds, such as caffeine and tobacco contents. This enzyme has been used in many studies relating acetylation capacity to disease, and a positive association between NAT2 activity and susceptibility to cancer (Ambrosone et al., 1996), Parkinson's disease (Agundez et al., 1998) and autoimmune diseases (von Schmiedeberg et al., 1999) has been reported. In particular, the mutations in the NAT2 isoenzyme are postulated to show a higher risk of cancers.

The *NAT2* gene has been well characterized with 13 disease susceptibility SNPs recognized. Mutation in one to a combination of four SNPs has been correlated with the slow, intermediate, and rapid acetylation phenotypes (Butcher et al., 2002). However in this study, we just focused on the investigation of the main disease predisposing SNPs, G to A transition at 5'-UTR position 61 of the *EGF* gene and of the *NAT2* SNP at position 857.

MATERIAL AND METHODS

Sample collection and genomic DNA extraction

There were a total of 12 glioma patient tissue samples and 13 normal controls included

in this study. This study was approved by the University of Malaya Medical Centre (UMMC) ethics approval board prior to sample collection (Ethics approval 696.13). All samples were collected from the volunteers with informed consent. Genomic DNA of the samples was extracted using the Gentra Puregene Tissue Kit (Qiagen, Germany). The purity and concentration of the extracted DNA were determined using a spectrophotometer.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis

Analysis of the EGF 5'-UTR polymorphism

Analysis of the EGF 5'-UTR polymorphism was carried out via the PCR-RFLP approach using the primers as reported by Bhowmick et al. (2004). The forward and reverse primers used in PCR are as follows: 5'-TGTCACTAAAGGAAAGGA-3' (forward); 5'-TTCA CAGAGTTTAACAGCCC-3' (reverse). *In silico* PCR has been reported in the previous research was performed to double confirm that the primers used were targeted to the regions of interest (Thong et al., 2011; Chua et al., 2011a,b; Ng et al., 2012). PCR was performed with the cycling conditions as reported previously (Chua et al., 2009a). Post-PCR, a 242-bp amplicon was expected and subjected to *Alu*I restriction enzyme digestion at 37°C overnight. The G allele was represented by a 193-bp RE fragment, while the A allele resulted in two digestion fragments of 91 and 102 bp. The post-PCR amplicons were visualized using 3% (w/v) agarose gel electrophoresis with ethidium bromide staining. Sequencing of a few samples was also carried out to confirm the results.

Analysis of the NAT2 polymorphism at nucleotide position 857

PCR-RFLP analysis of the *NAT2* G/A polymorphism at nucleotide position 857 was performed with the following forward and reverse primers: 5'-GTCACACGAGGAAATCAA ATGC-3' and 5'-GTTTTCTAGCATGAATCACTCTGC-3', respectively (Cascorbi et al., 1995). The PCR was carried out for a total of 35 cycles with the same conditions as stated previously (Chua et al., 2009b). Subsequently, the resulting amplified product (1211 bp) was subjected to *Bam*HI RE digestion at 37°C for 15 h. The presence of a G allele would result in two digestion fragments of 925 and 286 bp, while the A allele would remain intact as a larger 1211-bp fragment. All fragments were visualized on a 2% (w/v) agarose gel stained with ethidium bromide. Sequencing of a few samples was also carried out to confirm the results.

RESULTS

Analysis of the *EGF* 5'-UTR 61G polymorphism

Figure 1 shows the different PCR-RFLP profiles of the glioma and normal tissues. A total of three genotypes were scored, i.e., homozygous GG, homozygous AA and heterozygous AG. Table 1 shows the summary of the allelic (G and A) and genotypic (GG, GA and AA) frequencies. Interestingly, the G allele was more common in the healthy controls, while

the A allele, in the glioma patients. Hence, the most frequently typed genotype was the homozygous GG in the normal samples, while the heterozygous GA was the dominant genotype in the glioma patients. It is noted that no AA homozygous genotypes were observed in the normal blood donor samples collected.

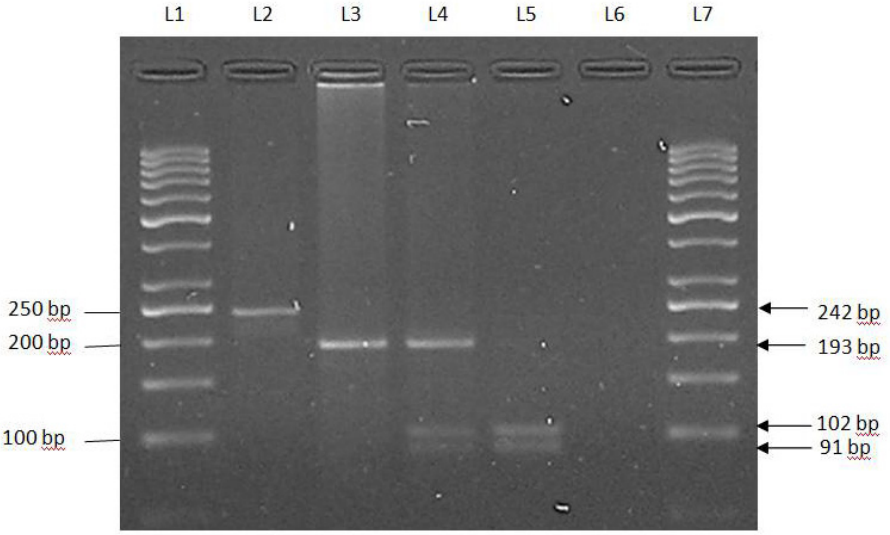

Figure 1. PCR-RFLP detection of the *EGF* 5'-UTR 61G polymorphism on agarose gel. *Lane 1* = 50-bp DNA ladder; *lane 2* = PCR product without restriction enzyme digestion; *lane 3* = homozygous G; *lane 4* = heterozygous G/A; *lane 5* = homozygous A; *lane 6* = DNA blank; *lane 7* = 50-bp DNA ladder.

Table 1. Allelic and genotypic frequencies of the *EGF* 5'-UTR 61G in normal healthy controls and patients with glioma.

EGF 5'-UTR	Glioma patients (%) (N = 12)	Healthy controls (%) (N = 13)
Allele		
G	14 (58.3)	20 (76.9)
A	10 (41.7)	6 (23.1)
Genotype		
G/G	4 (33.3)	7 (53.8)
G/A	6 (50.0)	6 (46.2)
A/A	2 (16.7)	0 (0.00)

Analysis of the *NAT2* polymorphism at nucleotide position 857G/A

Figure 2 shows the different PCR-RFLP profiles of the glioma and normal sampled tissues. A total of three genotypes were scored, i.e., homozygous GG, homozygous AA and heterozygous AG. Table 2 shows the summary of the allelic (G and A) and genotypic (GG, GA and AA) frequencies. The results showed that the G allele was the most prevalent form in both the glioma and normal samples. Similarly, the homozygous GG genotype was also the most common. We did not score any homozygous AA genotypes in the glioma patients, with a single observation coming from the healthy control group.

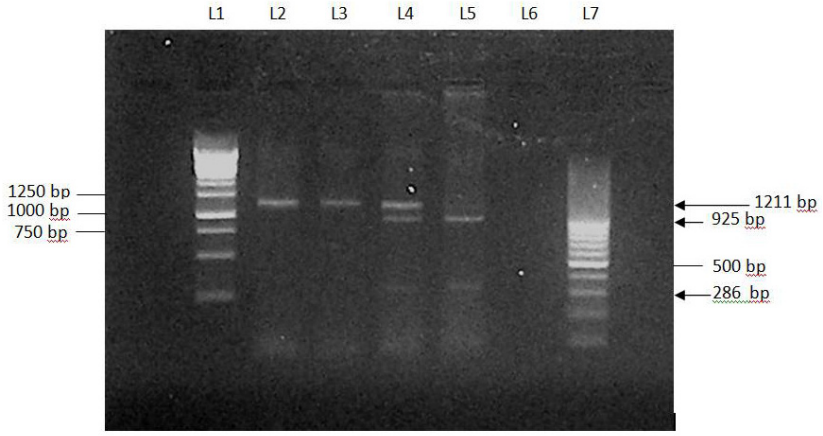

Figure 2. PCR-RFLP detection of the *NAT2* 857G/A polymorphism on agarose gel. *Lane 1* = 1-kbp DNA ladder; *lane 2* = PCR product; *lane 3* = homozygous A; *lane 4* = heterozygous G/A; *lane 5* = homozygous G; *lane 6* = DNA blank; *lane 7* = 100-bp DNA ladder.

Table 2. Allelic and genotypic frequencies of the *NAT* 857G/A polymorphism in normal healthy controls and patients with glioma.

NAT2 857G>A	Glioma patients (%) (N = 12)	Healthy controls (%) (N = 13)
Allele		
G	19 (79.2)	21 (80.8)
A	5 (20.8)	5 (19.2)
Genotype		
G/G	7 (58.3)	9 (69.2)
G/A	5 (38.5)	3 (23.1)
A/A	0 (0.0)	1 (7.7)

DISCUSSION

Glioma is a primary brain tumor found in adults and the risk increases in first degree relatives of patients and other primary brain tumours (Liu et al., 2010). Glioma such as GBM is uniformly lethal, with a mean survival time of 12 months post diagnosis. Various clinical parametersfor example age, level of function, radiological and histopathological evidence of tumor necrosis, tumor size, and extent of resection are known predictors of survival (Kleihues and Cavanee, 2000). Despite many on-going research, the main causes of this disease have been largely unknown, due to the lack of statistically powered studies. However, the effect or correlation of common genetic variances, evident as polymorphisms in the gliomas, have not been extensively investigated as factors that may have functional significance in predicting clinical outcome in glioma patient samples.

An adenine (A) to guanine (G) SNP at position 61 in the 5'-UTR of the *EGF* gene has been reported to be associated with different levels of EGF production (Bhowmick et al., 2004). Shahbazi et al. (2002) identified a single nucleotide polymorphism in the 5'-UTR of the *EGF* gene that appears to have functional consequences.

Many human cancers such as lung, colon, breast, head and neck, ovarian, and gliomas

highly express EGFR (Nicholson et al., 2001; Halatsch et al., 2006), and the expression of this receptor has been correlated with advanced tumor stages and poor prognosis (Nicholson et al., 2001). EGFRs are transmembrane glycoprotein receptors, and they share a similar structure characterized by an extracellular ligand-binding domain, an intracellular tyrosin kinase domain, and a transmembrane anchoring segment (Olayioye et al., 2000).

In this study, we found that A allele frequency was higher than that of G allele in *EGF* 5'-UTR at position 61, in the glioma patients compared to normal individuals. However, a previous study has shown that the G allele shows a higher risk of glioma (Costa et al., 2007). In our study, we found that heterozygous GA and homozygous AA genotypes were the dominant genotypes in the glioma patients, whereas the healthy controls had no AA homozygous genotype. Costa et al. (2007) found that both GG and combined AG+GG genotypes were associated with increased risk for glioma, while in oligodendroglioma cases, the AG, GG, and AG+GG genotypes were all associated with a higher risk. As a whole, due to the small sample size of our study, we were unable to conduct a univariate analysis.

The G allele was the most prevalent form of *NAT* 857G/A polymorphism in both glioma and normal samples. It is interesting to note that the homozygous GG genotype was also the most common and there were no homozygous AA genotypes in the glioma patients. This clearly shows that heterozygous genotyping plays an important role in glioma formation. Although the gene-gene interaction is based on very few subjects and must be interpreted with utmost caution, there is biological plausibility for synergism between the two risk genotypes. The results of this pilot study indicate the need for a more in-depth study of genetic polymorphisms, environmental factors, and brain tumor risk in larger, well-designed case-control studies.

CONCLUSION

We showed that the *EGF* 5'-UTR at position 61 and the *NAT2* SNP at position 857 are associated with an increased risk for glioma. In the future, more studies with bigger sample size are necessary to confirm the potential of these polymorphisms as predictors of glioma patient outcome and therapeutic responses to the newly approved anti-EGFR therapy.

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