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Research paper

ACE I/D sequence variants but not *MTHFR* C677T, is strongly linked to malignant glioma risk and its variant DD genotype may act as a promising predictive biomarker for overall survival of glioma patients

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ABSTRACT

Objective: ACE I/D and *MTHFR* C677T gene polymorphisms can be seen as candidate genes for glioma on the basis of their biological functions and their involvement in different cancers. The aim of this study was to analyze potential association and overall survival between *MTHFR* C677T and ACE I/D polymorphism in glioma patients in our population.

Materials and methods: We tested genotype distribution of 112 glioma patients against 141 cancer-free controls from the same region. Kaplan-Meier survival analysis was performed to evaluate overall survival of patients for both genes.

Results: No significant differences were found among *MTHFR* C677T wild type C and variant genotypes CT/TT with glioma patients. In ACE, the distribution of variant ID and DD was found to be significantly higher in glioma cases as compared to controls ($p < 0.0001$). ACE DD genotypes were highly presented in glioma cases 26.8% versus 10.6% in controls ($p < 0.0001$) and conferred 5-fold risk for predisposition in glioma cases. Per copy D allele frequency was found higher in cases than in controls (0.54 versus 0.25: $p < 0.0001$). Interestingly we found a significant overall survival (with log rank $p < 0.01$) in patients who presented with ACE DD genotypes had the least estimated overall survival of 13.4 months in comparison to 21. 7 and 17.6 months for ACE II and I/D genotypes respectively.

Conclusion: We conclude ACE I/D polymorphism plays a vital role in predisposition of higher risk for glioma. We also suggest that ACE DD genotypes may act as an important predictive biomarker for overall survival of glioma patients.

1. Introduction

Malignant gliomas, the foremost common intracranial malignant tumors in humans, create a singular threat as a result of their aggressive behavior and tissue invasion. Since a decade researchers have gained huge knowledge of the biological science of those tumors and open to a good extent glioma oncogenesis, proliferation, and invasion (Jansen et al., 2010). Globally on an average there are 5 cases of malignant glioma/10^5 people. It could develop in the late ages, the height incidence being within the 5th and 6th decades of life. Glioma is the commonest primary tumors of the central nervous system (Stupp et al., 2010) but despite marked advances within the characterization of their molecular pathological process, these tumors stay incurable. 5-Year survival varies which has been observed as 60% for biopsy coupled with watchful waiting and 74% for those with early resection in low-grade gliomas respectively (Jemal et al., 2009; Ohgaki and Kleihues, 2005). Among various risk factors, several occupations (Samkange-Zeeb et al., 2010; De Roos et al., 2003) environmental carcinogens (Maker et al., 1976) and diet have been found to be related with an increased risk of glioma (Kabat et al., 2011) as well as genetic factors such as

Abbreviations: ACE I/D, angiotensin-converting enzyme; MTHFR, methylenetetrahydrofolate reductase; C, cytosine; T, thiamine; SNPs, single nucleotide plymorphism; SAM, *S*-adenosylmethionine; SKIMS, Sheri-I-Kashmir Institute of Medical Sciences; DNA, deoxyribonucletide; Tris–HCl, tris-hydrocholric acid; KCl, potassium chloride; $MgCl_2$, magnesium chloride; dGTP, deoxyriboguanine triphosphate; dTTP, deoxyribothiamine triphosphate; PM, picomoles; Val, valine; OR, odds ratio; I/D, insertion/deletion; OS, Overall survival; PFS, progress free survival

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polymorphic variations (SNPs) Although SNPs may confer a small absolute cancer risk, they could be, individually or in combination, reason for the imbalance of crucial metabolism involved in cancer predisposition(Liu et al., 2010). Among these angiotensin-converting enzyme (*ACE*) gene and Methylenetetrahydrofolate reductase (*MTHFR*) have been found to have an impact on risk with various cancers(Li et al., 2003), (da Costa et al., 2012). Association between *ACE* I/D sequence variation and *MTHFR* C677T with glioma is biologically plausible but there are negligible evidences to support it.

MTHFR, an enzyme in folate metabolism whose main product *S*-adenosylmethionine (SAM) is the main source for provision of methyl groups during DNA methylation. Certain *THFR* gene polymorphisms usually result in protein changes in particular *MTHFR* gene (C677T) where cytosine changes to thymine at site 677 (Schaich et al., 2009). This change leads to a reduced activity of MTHFR enzyme and the net result is the impact on the production of SAM in addition to amount of methylation of the genome which includes DNA repair genes. Therefore evidence suggests that the individual polymorphic variation in these enzymes could prominently affect the general balance between DNA synthesis, repair and methylation. The sequence of these events aid in many cancers including gliomagenesis due to the genetic instability and owing to methylated genes (Gonzalez-Gomez et al., 2003).

The human ACE gene has either an insertion or deletion of a 287 nucleotide sequence in intron 15 called insertion/deletion (ID) polymorphism (Rigat et al., 1990). It's been confirmed by some studies that the ID polymorphism is in sturdy linkage state of affairs with a significant sequence impact at the ACE sequence locus, that controls up to 16–24% of the variability in ACE levels (Tiret et al., 1992), (Danilov et al., 1996). Individuals with genotype II have lowest serum ACE levels in comparison to those with ID or DD genotype (Castellon and Hamdi, 2007). Ample proof suggests that the ACE genotype insertion/deletion (I/D) polymorphisms might play a task within the susceptibleness for development of certain malignancies, as well as the propensity of certain tumors to metastasize (Chung et al., 2005).

Thus ACE I/D and *MTHFR* C677T polymorphism could contribute to the differences between individuals or racial groups in susceptibility and severity of disease. On basis of their plausible biological functions, ACE I/D and *MTHFR* C677T can be seen as promising candidate genes for malignant glioma. In the present study, a population based case -control study was conducted to examine the distribution and/or association between the ACE I/D and *MTHFR* C677T and malignant glioma in Kashmiri population (North India).

2. Material and methods

2.1. Study subjects

The present study was conducted at Advanced Centre for Human Genetics in Sheri-I-Kashmir Institute of Medical Sciences (SKIMS), Srinagar, India and was approved by the Regional Ethics Committee SKIMS (IEC-SKIMS). 112 patients diagnosed with malignant gliomas of different pathologies were included in this study. Blood samples were collected from 112 histologically proven brain tumors patients from Department of Neurosurgery (SKIMS) wherein tumor tissue was available for 70 patients. Biopsy samples were reviewed by two expert neuropathologists to confirm the diagnosis of malignant glioma and ensure uniformity of classification criteria. All the tumors resected by Neurosurgeons were confirmed histologically to be malignant gliomas. Besides blood samples were taken from 141 healthy controls free from any tumor or benign disease from the Out Patients Departments of SKIMS. The clinical information, including age, gender and survival data, was obtained from each patient after a written pre-informed consent was obtained from all cases and controls. The sample size of the study was calculated to be 80% using nMaster2.0 statistical software. The study was conducted as per the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans. An informed consent was taken from each patient as per the norms of SKIMS Ethics Committee before sample collection.

3. Extraction of genomic DNA

DNA was extracted from the blood and tumor tissues of glioma patients and healthy controls using DNA Extraction kit (Zymo Research Corporation, USA).

3.1. Polymerase chain reaction for amplification of MTHFR gene

To amplify *MTHFR* (C677T) and *ACE* I/D polymorphic regions, we used genomic DNA:250 ng/ml, 1 × PCR buffer: 100 mM Tris–HCl, pH 8.3; 500 mM KCl; 15 mM $MgCl_2$; deoxyribonucleotide triphosphate (Cinnagen Co., Tehran, Iran): 10 mM dATP; 10 mM dCTP; 10 mM dGTP; 10 mM dTTP, primers (Sigma–Aldrich, USA): 10 pM in sterile deionized water and Taq DNA polymerase 5 U/μl (Biotools, Madrid, Spain). The set of primers previously reported were utilised for the amplification of 494 bp region encompassing the SNP of the interest within the *MTHFR* gene with forward primer 5′-GGTCAGAAGCATATCAGT CA T GAG-3′ and the reverse primer 5′-CTGGGAAGAACTCAGCGAACTCAG-3′[20]. and for *ACE* I/D forward primer, 5′-CTG GAG ACC ACT CCC ATC CTT TCT-3′, and reverse primer, 5′-GAT GTG GCC ATC TTC GTC AGA T-3′. The thermal conditions used were one first denaturation step at 94 °C for 5 min, 40 cycles of denaturation at 94 °C, annealing at 58 °C, and extension at 72 °C for 30 s, followed by a final extension cycle at 72 °C for 5 min.

4. Genotyping

For *MTHFR* (C677T), Restriction Fragment Length Polymorphism (RFLP) was used. 10 μl PCR product of 490 bp was digested with *Hinf* I (5 U at 37 °C for 16 h) (Fermentas, USA). In the case of *MTHFR* C677T polymorphism, the wild-type CC (Ala/Ala) produced products of size 394-bp and 100-bp bands, while the TT (Val/Val) homozygous mutant was identified by 229-, 165- and 100-bp bands and the heterozygous CT (Ala/Val) variant displayed all four bands of sizes 394 bp, 229 bp, 165 bp, and 100 bp. For *ACE* I/D polymorphism, the PCR product is a 490-bp fragment in the presence of the insertion (I) allele. Thus, each DNA sample shall reveal one of three possible patterns after electrophoresis: a 490-bp band (genotype I/I), a 190-bp band (genotype D/D), or both 490-bp and 190-bp bands (genotype I/D). DNA amplicons in both the cases were subjected to electrophoresis on a 2–3% agarose gel and visualized with ethidium bromide in gel documentation system (Cell Bioscience FlourChem HD2).

For quality control, each PCR reaction used distilled water instead of DNA as a negative control, and > 10% of the samples were analyzed twice for reproducibility of results.

4.1. Statistical analysis

Statistical analysis was performed by using IBM Statistics SPSS software (Version-23).The cases and controls were compared using the chi square test for categoric variables, such as sex and age, of the demographic variables. A goodness-of-fit chi-square test was employed to evaluate whether the polymorphisms were in Hardy-Weinberg equilibrium between cases and controls. Odds ratios (OR) were used as estimates of the relative risk, and 95% confidence intervals (CI) were calculated to estimate the association between certain genotypes or other related risk factors of glioma. Different tests for homogeneity of proportions including Chi square and Kaplan Meier analysis to evaluate survival outcome probabilities were used to determine significance of the distribution patterns with respect to different clinico-analytical parameters.