ORIGINAL ARTICLE

Association of *MTR* A2756G Gene Polymorphism with Risk of Head and Neck Cancer

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ABSTRACT

Objective: To perform genotyping for *MTR* A2756G polymorphism and identification of risk factors associated with head and neck squamous cell carcinoma (HNSCC).

Study Design: Cross section, comparative study.

Place and Duration of Study: The study was carried out at the Department of Biochemistry of Quaid-i- Azam University, Islamabad from October 2014 to August 2015.

Materials and Methods: In this study, 292 diagnosed patients HNSCC and 324 normal individuals without any history of cancer were enrolled. Blood samples of patients and controls were collected in ethylenediamine tetra acetic acid (EDTA) and DNA was extracted using conventional method. All samples were genotyped for the *MTR* A2756G polymorphism using PCR-RFLP. Frequency of polymorphism was compared between HNSCC patients and controls. Multiple Logistic Regression (MLR) and chi-square test was performed to examine the association of *MTR* A2756G polymorphism with risk factor.

Results: Chi-square test of independence showed statistically significant difference among the variables of age, smoking and *MTR* A2756G genotype (*p*-value<0.05). Multivariate analysis showed that smoking (adjusted OR, 3.7; 95% CI, 2.3 – 6.0), age groups 41 – 50 years (adjusted OR, 3.6; 95% CI, .9 – 6.7) and > 60 years (adjusted OR, 3.5; 95% CI, 1.7 – 7.3), *MTR* 2756 AG genotype (adjusted OR, 2.1; 95% CI, 1.3 – 3.5) is associated with increased risk of HNSCC.

Conclusion: The results suggest that the genetic polymorphism *MTR* A2756G is associated with the occurrence of HNSCC in the Pakistani population while the individuals between 40 to 50 years of age and those who are smokers are at a greater risk of developing HNSCC.

Key Words: *Genetic Polymorphism, Head and Neck Cancer, Head and Neck Squamous Cell Carcinoma, MTR A2756G, PCR-RFLP.*

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Introduction

Cancers that are commonly recognized as head and neck carcinoma (HNC) typically begin in the moist, mucosal surfaces inside the head and neck lining of squamous cells which cover the oral cavity, pharynx, hypopharynx, and larynx. The most frequent type of HNC is head and neck squamous cell carcinoma (HNSCC) which affects the mucosal lining of head and neck regions.[1] HNC is ranked as the sixth most common malignancy in the world.[2] In 2013, the number of new cases and deaths reported in the US were 53,640 and 11,520, respectively.[3] HNC accounts for 40.1% of all malignant neoplasms and is reported as the second most common malignancy in Pakistan.[4] HNC is a complex disorder involving multiple factors including genetic, environmental and mental stress.[5] Alcohol and tobacco usage, human papilloma virus infection, folic acid and vitamin deficiencies also contributes to the occurrence of HNC.[6-8] The exact mechanism of pathogenesis of HNC is not yet fully known.[9]

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Folates in different forms are essential for cell division because of their role in purine and thymidine synthesis and DNA methylation. Hence folate deficiency or abnormal folate metabolism results in carcinogenesis.[10,11] In purine and thymidine synthesis folate acts as a one-carbon donor. DNA methylation regulates gene expression.[12] Epigenetic modifications in the DNA have functional roles from regulation of gene expression to chromatin structure stabilization.[13] Four enzymes involved in control of folate metabolism are methionine synthase (MTR), methylenetetrahydrofolate reductase (MTHFR), thymidylate synthase (TS) and methionine synthase reductase (MTRR).[14] In folate metabolism, 5, 10-methylenetetrahydrofolate is irreversibly converted to 5-methyltetrahydrofolate by the action of MTHFR. Remethylation of homocysteine to methionine is catalysed by MTR, methyl donor in this reaction is 5-methyltetrahydrofolate. (MTRR causes reductive methylation of vitamin B12 and activates MTR. In another reaction, TS uses the 5, 10-methylenetetrahydrofolate and converts deoxyuridylate to thymidylate (nucleotide synthesis). Polymorphisms in these genes involved in folate metabolism, are believed to increase the risk of cancer by altering methylation and DNA synthesis, consequently affecting the chromosomal structure and stability.[6]

1q43 is the location of *MTR* gene.[15,16] This enzyme maintains normal intracellular methionine and homocystine concentrations.[17] Molecular studies revealed that change from A to G (rs1805087) at position 2756 of *MTR* gene results in an amino acid substitution of aspartic acid to glycine (D919G). This substitution lowers MTR efficiency which in turn results in hyperhomocysteinemia.[16,18]

Many studies have explored the link between *MTR* A2756G polymorphism with breast cancer[19], colon cancer[20], lung cancer[21] and role of polymorphism in tumour development and treatment responses against cancer.[22] Several studies have reported association of *MTR* A2756G polymorphism with head and neck cancer, where genotypic frequencies of HNC patients and control individuals were compared. These studies concluded that the *MTR* 2756AG genotype significantly increases the risk of HNC.[23,24] In addition *MTR* 2756 AG or GG genotype is shown to be significantly associated with increased risk of laryngeal cancer.[25] Contrary to these findings, another research group showed that *MTR* A2756G polymorphism is not associated with increased risk of head and neck cancer.[26]

Based on this contradictory literature, this study was designed to compare genotypes of *MTR* gene in HNSCC patients and normal individuals to investigate the association of this polymorphism in Pakistan.

Materials and Methods

Patient Selection

In this study 616 individuals were recruited, 292 HNSCC diagnosed patients and 324 control individuals without any history of cancer. Nuclear Medicine Oncology and Radiotherapy Institute (NORI), was the site for sample collection. Institutional ethical approval was obtained from Quaid-i-Azam University and NORI before starting the study. Blood samples (5 ml/individual) of HNSCC patients and controls were collected with the informed consent of the participants. Blood samples were transferred to the research lab at Quaid-i-Azam University, Islamabad for further processing.

DNA Extraction

The standard phenol protocol already demonstrated was used for DNA isolation from blood.[27] DNA samples were run on to 1% agarose gel electrophoresis for 30 min at 120V in 10 X Tris-Borate-EDTA running buffer and visualizing under ultraviolet trans-illuminator.

Genotyping

To study the *MTR* A2756G polymorphism (rs1805087), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used. Primer 3 software was used to design primers for amplifying exon 26 of *MTR* gene. The primers used in this study were sense 5' GCCCACTGAGTTTACCTTTTCC 3' and anti-sense 5' CCTGCCTCATGTCTCCATTT 3'. To amplify target DNA, a reaction mixture of 25 µL was prepared containing 1.5 µL MgCl2 (25 mM), dNTPs (10 mM), forward and reverse primers 0.5 µL (0.1 µM), 0.5 µL *Taq* DNA polymerase (5 U/µL) 0.5 µL, 1X (10 mM) 2.5 µL PCR buffer (10x), 1 µL DNA template and 18 µL PCR water. The conditions for thermal cycler were as follows: an initial denaturation at 96 °C for 5 min, followed by 35 rounds of 96°C at 1 min for denaturation, annealing at 60°C for 45 seconds, extension at 72°C for 1 min and a final extension of 5 min at 72°C. PCR amplified

product of 664 bp was confirmed on 2% agarose gel. The PCR product bp was digested with HaeIII enzyme for 10 min at 37°C following manufacturer's instruction.

Statistical Analysis

Percentages of the total number of alleles and genotypes were calculated to determine frequencies. Pearson's chi-square test was applied to compare allelic frequencies of patients and controls with *P* value<0.05 documented as significant. Genetic polymorphism and variables related interaction effect with HNSCC was determined using multiple logistic regression model. Models included genotype (reference: A/A), sex (reference: female), smoking (reference: non-smokers) and age group (reference: < 40 Years), using the Statistical Package for the Social Sciences (SPSS) Version 6.0. Odds ratio (OR) and at 95% confidence intervals (95% CI) were calculated and results were documented.

Results

The 292 HNSCC patients had a male to female ratio of 2:1, mean age 46 ± 12 . The 324 controls had male to female ratio of 3:1, mean age of 35 ± 10. Among the HNSCC patients 57% (n=166) had oral cavity cancer, 23% (n=68) had cancer of the larynx and 20% (n=58) had cancer of the pharynx. Patient from age group of 41-50 years had higher frequency of HNSCC (n= 194) and many participants among controls were tobacco users (n=112). The electropherogram of digested products of the PCR-RFLP assay revealed three DNA band patterns as depicted in Figure 1, representing AA, AG, GG genotypes (Figure 1).

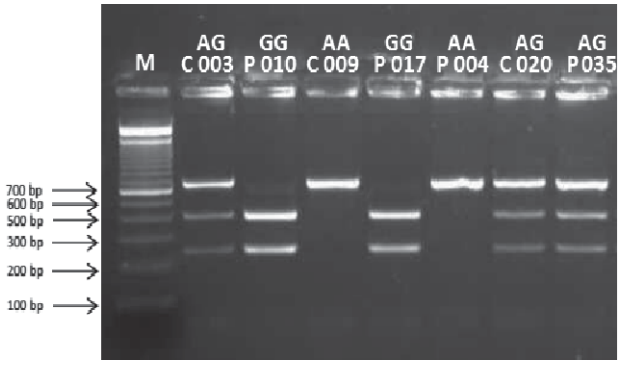

Fig 1: RFLP analysis of 664 bp fragment of *MTR* gene. C represents normal controls while P indicates HNSCC patient DNA. M is a 100 bp ladder. Presence of one DNA band on gel indicates AA genotype, three DNA bands indicate AG genotype and two DNA bands indicate GG genotype.

In HNSCC patients AA, AG and GG genotype frequencies for *MTR* A2756G polymorphism were 59%, 37% and 4% respectively and in control individuals AA, AG and GG genotype frequencies for *MTR* A2756G polymorphism were 76%, 22% and 2% respectively. The genotype frequencies for all investigated polymorphisms among controls (χ^2 test: $P = 0.846$) and P patients (χ^2 test: $P = 0.490$) were in Hardy–Weinberg equilibrium.

Table 1. Frequency Distribution of Demographic Details, Risk Factors and Genotypes in HNSCC Patients and Normal Controls

Variables	Patients (%) (n=292)	Controls (%) (n=324)	*p*-value
Gender			
Male	204 (69.9)	248 (76.5)	0.185
Female	88 (30.1)	76 (23.5)	
Age			
< 40 years	54 (18.5)	124 (38.3)	
41 – 50 years	124 (42.5)	78 (24.1)	< 0.001*
51 – 60 years	56 (19.1)	84 (25.9)	
>60 years	58 (19.9)	38 (11.7)	
Smoking			
Smokers	194 (66.4)	112 (34.6)	< 0.001*
Non -Smokers	98 (33.6)	212 (65.4)	
***MTR* 2756 Genotypes**			
AA	172 (58.9)	246 (75.8)	
AG	108 (36.9)	72 (22.1)	0.003*
GG	12 (4.2)	06 (2.1)	

*P-value <0.05 (Two sided χ^2 test

Chi-square test of independence analysis showed significant association (*p*-value < 0.05) of patients age (41-50 years), smoking status and *MTR* 2756 AG genotype with HNSCC, (Table 1). Further these variables were adjusted by performing multivariate analysis.

Multivariate analysis showed that smoking (adjusted OR, 3.7; 95% CI, 2.3 – 6.0), age group 41–50 years (adjusted OR, 3.6; 95% CI, 0.9–6.7) and > 60 years (adjusted OR, 3.5; 95% CI, 1.7–7.3) and *MTR* 2756AG (adjusted OR, 2.1; 95% CI, 1.3 – 3.5) genotype is associated with increased risk of HNSCC (Table 2).

Discussion

HNC is a group of cancers comprising of cancer of larynx, oral cavity and pharynx. This is the sixth most frequent cancer globally.[1,2] In the present study, among HNSCC patients male to female ratio was 2:1. The finding has also been reported in previous studies conducted in different populations[23,28,29], while one study reported that male and females were affected by this cancer.[30] In our study we found

Table 2: Multiple Logistic Regression Analysis for Association of Risk Factors with Onset of HNSCC

Variables	OR (95 % CI)	*p*-value
Smoking		
Non-smokers	Reference	
Smokers	3.7 (2.3 – 6.0)	< 0.001*
Gender		
Female	Reference	
Male	1.0 (0.5 - 1.8)	0.18
Age		
< 40 years	Reference	
41 - 50 years	3.6 (1.9 - 6.73)	< 0.001*
51 - 60 years	1.5 (0.7 – 2.9)	0.205
> 60 years	3.5 (1.7 – 7.3)	0.001*
***MTR* 2756 Genotypes**		
AA	Reference	
AG	2.1 (1.3 - 3.5)	0.003*
GG	1.1 (0.6 – 2.1)	0.081
***MTR* 2756 Alleles**		
A	Reference	
G	1.9 (1.2 - 3.5)	0.002*

P-value <0.05 (Multiple logistic regression)

that the incidence of HNSCC is more in age groups 41 – 50 years and > 60 years as previously reported by Northern Ireland Cancer Registry, 2010 and Welsh Cancer Intelligence and Surveillance Unit, 2014. This finding is suggestive that these age groups are a risk factor for onset of HNSCC.[31] Incidence of oral cancer has increased while frequency of pharyngeal cancer has reduced in the past few years in many populations worldwide. Oral cancer has been reported as the most frequent cancer among the head and neck regions in England[32] and Taiwan.[33] Similar results have been observed in our study where the incidence of oral cancer was high among HNC patients. Alcohol and tobacco usage, in majority of cases (90%), followed by human papilloma virus infection and nutrients deficiency are predisposing factors.[28,34]

Recent literature showed that low levels of folate play a role in the aetiology of HNSCC[8,35,36] Folate is a vital nutrient, which play an important role in many biological processes like DNA synthesis , DNA repair, and DNA methylation (epigenetics).[37] *MTR* gene which encodes MTR enzymeis responsible for catalysing the methylation of homocystine to methionine[38], an important reaction that regulates normal homeostasis of methionine and intracellular homocystine concentrations.[17] The functional activity of MTR in the presence of the *MTR* A2756G polymorphism has not been assessed *in vitro*. There is contradiction in studies related to changes in homocystine and folate levels.[39-43] Some studies indicated that polymorphic homozygous *MTR* 2756GG genotype is associated with low levels of homocystine and high levels of folate.[40,43] In some other studies it is reported that in the presence of this variant homocystine levels are high[41] while it has also been reported that in the presence of this polymorphism homocystine levels do not changed.[42] DNA methylation has been linked to *MTR* 2756AG or *MTR* 2756GG polymorphism with reduced level of S-adenosylmethionine which ultimately results in DNA hypomethylation[44] and several cancers to control tissues studies confirmed frequent overall genomic hypomethylation.[45-48] Few studies reported the association between the *MTR* 2756GG genotype and DNA hypomethylation also in colorectal, breast, lung and cervix cancers.[24,44,49,50]

Research studies have suggested the association between risk of head and neck cancer and *MTR* 2756AG polymorphism[23-25] while another study found no such association.[26] In this study, it has been found that the polymorphic variant of *MTR* 2756 AG and *MTR* 2756 GG are associated with HNC. Statistical analysis showed that association with increased risk of HNC and *MTR* 2756 AG genotype which supports the findings of many researchers who reported this association.[23-25]

Our findings suggest that *MTR* 2756AG genotype is associated with increased risk of head and neck cancer and we suggest *in vivo* studies in cell lines or animal models should be done to evaluate the functional activity of this enzyme in the presence of 2756 AG polymorphism. Further investigation of altered expression and function of other genes involved in folic acid metabolism is required for better understanding of etiopathogenesis of HNSCC and to develop better anticancer strategies to control and treat the HNC.

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