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Original Research Article

Methionine synthase A2756G polymorphism associated with an increased risk of head and neck cancer

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ABSTRACT

Background: The 5-methyltetrahydrofolate-homocysteine methyltransferase gene (*MTR*) encodes the methionine synthase enzyme (OMIM 156570). Methionine synthase synthesizes methionine by re-methylation of homocysteine. A single nucleotide variation *MTR*-A2756G may affects the function of methionine synthase enzyme, which could lead to the development of head and neck squamous cell carcinoma (HNSCC).
Methods: In current study, 292 HNSCC patients and 324 normal individuals without any history of cancer (control) were enrolled. EDTA whole blood samples of patients and control individuals were collected, and DNA was extracted. All samples were genotyped for *MTR*-A2756G polymorphism using polymerase chain reaction-restriction fragment length polymorphism. Frequency of polymorphism was compared between HNSCC patients and control individuals. The association of *MTR*-A2756G polymorphism with risk factors was statistically analysed through multivariate analysis (multiple logistic regression) whereas univariate analysis (chi square) was performed for group comparisons.
Results: Univariate analysis revealed that the frequency of groups like age, smoking and *MTR*-A2756G genotype was different in HNC patients and controls (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*-A2756G genotype (adjusted OR, 2.1; 95% CI, 1.3-3.5) is associated with increased risk of HNSCC.
Conclusions: Our data suggests that the *MTR*-A2756G polymorphism is associated with the occurrence of HNSCC in Pakistani population while the individuals between 40 to 50 years of age and tobacco smokers are at a greater risk of developing HNSCC.

Keywords: Methionine synthase, *MTR*-A2756G, Genetic polymorphism, PCR-RFLP, HNSCC

INTRODUCTION

The cancers that 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 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 ranked as sixth most common malignancy in the world.[2] In 2019, 65 410 new cases of head and neck cancers reported in the US, which accounted for about 3.7% of new cancers.[3] HNC accounts for 40.1% of all malignant neoplasms and reported as the second most common malignancy in Pakistan.[4] Cancer is a group of heterogenous disorders.[5,6] Environmental factors, genetic factors and mental stress

mainly contribute to arising cancer including HNCs.[7] Alcohol and tobacco usage, human papilloma virus infection, folic acid and vitamins deficiency also considered key factors in the occurrence of HNC.[8] The exact mechanism of pathogenesis of HNC is not yet fully known.[9]

Folates in different forms are essential for cell due to their important role in purines and thymidine synthesis and DNA methylation, hence folate deficiency or abnormal folate metabolism leads to carcinogenesis.[10] DNA methyltransferases mainly conduct the DNA methylation by transferring methyl group from 5-methyltetrahydrofolate to homocysteine, producing methionine and tetrahydrofolate. The resulting products play a vital role in DNA methylation and pyrimidine synthesis. DNA methylation regulates the gene expression.[11] Epigenetic modification in the DNA attribute to gene expression regulation and chromatin structure stabilization.[12] Several enzymes including methionine synthase (*MTR*), methylenetetrahydrofolate reductase (MTHFR), thymidylate synthase (TS) and methionine synthase reductase (MTRR) involved in regulating folate metabolism.[13]

In folate metabolism, 5, 10-methylenetetrahydrofolate is irreversibly converted to 5-methyltetrahydrofolate by the action of MTHFR. Re-methylation of homocysteine to methionine catalysed by *MTR*, methyl donor in this reaction is 5-methyltetrahydrofolate. MTRR causes reductive methylation of vitamin B12 and activates *MTR*. In other reaction TS uses the 5, 10-methylene-tetrahydrofolate and converts deoxy uridylate to thymidylate (nucleotide synthesis). Consequence of polymorphism in these genes that involve in folate metabolism, believed to increase the risk of cancer through altering DNA methylation and DNA synthesis, consequently affecting the chromosomal structure and their stability.[14]

MTR gene is located at 1q43 in chromosome, which expresses the methionine synthase enzyme. This enzyme has vital role in folate metabolism.[15] This enzyme maintains normal intracellular methionine and homocysteine concentrations.[16] Molecular studies revealed that change in nucleotide 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 the methionine synthase efficiency which in turn results in hyper-homocysteinemia.[17]

Many studies investigated the link between *MTR*-A2756G polymorphism and various cancers including breast cancer, colon cancer and lung cancer.[18,19] Furthermore, association of *MTR*-A2756G polymorphism with severity of disease and variable treatment responses in cancer patients has also been determined.[20] Several studies investigated the role of *MTR*-A2756G polymorphism in the onset of head and neck cancer in different ethnic populations. Findings of these studies indicate strong association of *MTR*-A2756G genotype with risk of the onset of HNC.[21] Moreover, a study reported *MTR*-A2756G or G2756G genotypes increase the risk of laryngeal cancer.[22] Contrary to these findings a research group reported no significant association of *MTR*-A2756G polymorphism with increased risk of head and neck cancer.[23] Given the evidence, the current study designed to perform genotyping of *MTR* gene in head and neck cancer patients and normal individuals (control) in Pakistani population. Frequencies of the *MTR*-A2756G polymorphism measured and compared to determine its association with HNC.

METHODS

Patient selection

In this case-control study 616 individuals including 292 HNSCC diagnosed patients (mean age 45±10) and 324 control individuals (mean age 40±8) without any history of cancer were recruited between March 2018 to September 2019. Retrospective EDTA blood sampling carried out at nuclear medicine oncology and radiotherapy institute (NORI), Islamabad, Pakistan. After taking approval from NORI ethical committee, venous blood samples of HNC patients (5ml/individual) were collected in 5ml vacutainers (BD, USA) containing EDTA. Whole blood samples of HNSCC patients and control individuals were collected and analysed after taking proper informed consent from patents/guardians.

DNA extraction

DNA from blood samples isolated using classic phenol-chloroform DNA isolation protocol as previously described.[24] DNA samples were subjected to 1% agarose gel electrophoresis for 30 minutes at 120V in 10X Tris-borate-EDTA running buffer followed by visualizing under ultraviolet trans-illuminator.

Genotyping

To detect *MTR*-A2756G polymorphism (rs1805087), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) molecular tool was used. The primer 3 software was used to design PCR primers. The primers used in this study were: sense 5'GCCCACTGAGTTTACCTTTTCC'3 and anti-sense 5'CCTGCCTCATGTCTCCATTT'3. DNA amplified through PCR using above mentioned primers and PCR amplified product was electrophoresed on to 2% agarose gel electrophoresis at 100V for 1 hour for confirmation. Following confirmation, PCR product of 664 bp was treated with HAEIII enzyme (Invitrogen, Massachusetts, USA) for 10 min at 37°C following manufacturer instructions. HAEIII treated restricted DNA fragments were analysed through agarose gel electrophoresis for presence of polymorphism. 664 bp DNA fragment is representative of AA genotype. 415 bp, 229 bp DNA

fragments are representative of GG genotype while 664 bp, 415 bp, 229 bp DNA fragments are representative of AG genotype in electropherogram.

Statistical analysis

Percentages and numbers were used to express the data. Percentages of the total number of alleles and genotypes were calculated to determine respective frequencies. Pearson's chi-square ($\chi 2$) test was applied to compare genotypes of patients and controls. Probability (p) calculated and p value <0.05 was documented as significant. Genetic polymorphisms and variables related interaction effect with HNSCC was determined through multiple logistic regression (MLR) models using SPSS version 16.0. Models included genotype (reference: A/A), sex (reference: female), smoking (reference: non-smokers) and age group (reference: <40 years. Results showed as 95% confidence intervals (95% CI) and odds ratio (OR).

Ethical approval

Study was approved by ethical committee of NORI hospital (ethical approval number: IRB 1207).

RESULTS

Demographic findings of participants

Among 292 HNC patients and 324 control individuals, male to female ratio found out to be 2:1 and 3:1, respectively. Mean age of HNC patients and control revealed 46±12 and 35±10 years, respectively. Among HNC patients 57% (n=166) had oral cavity cancer, 23% (n=68) had cancer of larynx and 20% (n=58) had pharynx cancer. Significantly (p<0.01) higher number of patients belong to 41-50 years age group. Significantly (p<0.01) higher percentage 66% of HNC patients (n=194) were tobacco smokers compared to control individuals 35% (n=112) (Table 1).

Electropherogram of Hae III treated DNA samples confirmed the presence of AA, AG and GG genotypes

DNA samples of both HNC patients and controls individuals were subjected to digestion with Hae III restriction enzyme. Electropherogram of digested products revealed three DNA band patterns; each represents AA, AG, GG genotype (Figure 1).

Samples were treated with Hae III enzyme for 10 min. Then digested product and 100 bp ladder were subjected to 2% gel electrophoresis and DNA bands of different sizes were obtained. With reference to the DNA bands of 100 bp ladder, DNA band size in samples noted. Presence of one DNA band on gel indicates AA genotype, three DNA bands indicate AG genotype and two DNA bands indicate GG genotype of *MTR* gene. M, C and P represent marker, normal controls and HNC patient's DNA samples, respectively.

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
	N (%)	N (%)	
Gender			
Male	204 (69.9)	248 (76.5)	0.185
Female	88 (30.1)	76 (23.5)	
Age in years			
<40	54 (18.5)	124 (38.3)	<0.01**
41-50	124 (42.5)	78 (24.1)	
51-60	56 (19.1)	84 (25.9)	
>60	58 (19.9)	38 (11.7)	
Smoking			
Smokers	194 (66.4)	112 (34.6)	<0.01**
Non-smokers	98 (33.6)	212 (65.4)	
***MTR*-2756 genotypes**			
AA	172 (58.9)	246 (75.8)	<0.005***
AG	108 (36.9)	72 (22.1)	
GG	12 (4.2)	06 (2.1)	

P value <0.01**, p value<0.005*** vs control (two sided χ^2 test).

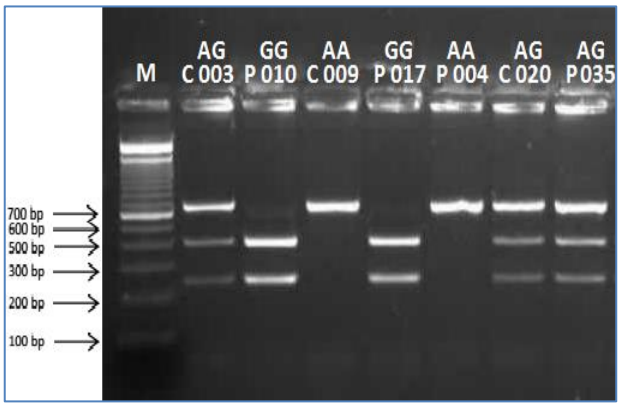

Figure 1: PCR-RFLP analysis of 664 bp fragment of *MTR* gene.

MTR A2756G polymorphism may increase the risk of HNC onset

In HNC patients AA, AG and GG genotype frequencies for *MTR*-A2756G polymorphisms were 59%, 37% and 4% respectively and in control individuals AA, AG and GG genotype frequencies for *MTR*-A2756G polymorphisms were 76%, 22% and 2% respectively. The genotype frequencies for all investigated polymorphisms in control individuals (χ^2 test: p=0.846) and patients (χ^2 test: p=0.490) were in accordance with the Hardy-Weinberg equilibrium. The frequency

distributions of selected characteristics of the cases and controls are summarized in (Table 1).

Univariate analysis (chi-square) on our data showed that the frequency of gender is well corresponding in HNC patients and controls but the frequency of groups like age, smoking and *MTR*-2756AG genotype were different in HNC patients and controls and these frequency differences were statistically significant (p value <0.05*, Table 1). Thus, further these variables were adjusted by performing multivariate analysis (multiple logistic regression).

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, 0.9-6.7) and >60 years age group (adjusted OR, 3.5; 95% CI, 1.7-7.3), *MTR*-A2756G (adjusted OR, 2.1; 95% CI, 1.3-3.5) genotype is associated with increased risk of HNSCC (Table 2).

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)	<0.01**
Gender		
Female	Reference	
Male	1.0 (0.5-1.8)	0.18
Age in years		
<40	Reference	
41-50	3.6 (1.9-6.73)	<0.01**
51-60	1.5 (0.7-2.9)	0.205
>60	3.5 (1.7-7.3)	0.005***
***MTR*-2756 genotypes**		
AA	Reference	
AG	2.1 (1.3-3.5)	0.01**
GG	1.1 (0.6-2.1)	0.081
***MTR*-2756 alleles**		
A	Reference	
G	1.9 (1.2-3.5)	0.05*

P value <0.05*, p value<0.01**, p value <0.005*** vs control (multiple logistic regression).

DISCUSSION

HNC is group of cancers comprises of cancer of larynx, oral cavity and of pharynx. This is the sixth most frequent cancer globally.[2,25] In the present case-control study, among HNSCC patient male to female ratio was found out to be ~2:1, which means that males can be more affected by HNC than females. The similar finding has also been reported in previous studies conducted in different populations, but another study reported that male and females can equally be affected by this cancer.[21,26] In our study we found that the incidence of HNSCC is more in age groups 41-50 years and >60 years as previously reported.[27] This finding suggests aging factor should be considered as risk factor for onset of HNSCC. One study reported that incidence of oral cancer has been increased while frequency of pharyngeal cancer has been reduced for the past few years in many populations worldwide.[28] Oral cancer is most frequent cancer in head and neck cancer types in Taiwan.[29] Same results have been observed in our case-control study that the incidence of oral cancer is high among HNC patients in Pakistan. Alcohol and tobacco usage, human papilloma virus infection and nutrients deficiency are predisposing factors for HNC.[8] In literature we found that tobacco and alcohol consumption are important risk factors, in 90% of cases, responsible for the onset of head and neck cancer.[30]

Recent literature showed that low levels of folate play a role in the etiology of HNSCC.[31] Folate is a vital nutrient, which play an important role in many biological processes like DNA synthesis (genetics), DNA repair, and DNA methylation (epigenetics).[32] *MTR* gene which encodes methionine synthase enzyme, has significant role in folic acid metabolism.[33] Methionine synthase, an enzyme responsible for catalysing the methylation of homocysteine to methionine, a significantly important reaction that regulates normal homeostasis of methionine and intracellular homocysteine concentrations.[34,16] As far as we know, the functional activity of methionine synthase enzyme in the presence of the *MTR*-A2756G polymorphism has not been assessed *in vitro*. There is contradiction in studies related to changes in homocysteine and folate levels. Several studies indicated that polymorphic homozygous *MTR*-G2756G genotype is associated with low levels of homocysteine and high levels of folate.[35] Some studies reported that in the presence of this variant homocysteine levels are high.[36] In other study it is reported that in the presence of this polymorphism homocysteine levels remained unchanged.[37] DNA methylation has been linked to *MTR*-A2756G or *MTR*-G2756G polymorphism with reduced level of S-adenosylmethionine which ultimately results in DNA hypomethylation.[38] Several studies confirmed frequent overall genomic hypomethylation in several cancers harbouring *MTR*-A2756G polymorphism.[39] Some studies reported the association between the MTR-A2756G genotype and DNA hypomethylation also in colorectal, breast, lung, and cervix cancers.[40]

Literature review indicated that many studies have suggested the association of *MTR*-A2756G polymorphism and risk of HNC while a study found no such association.[21,22,23] In this study it has been found that the frequency of polymorphic variants of *MTR* gene, *MTR*-A2756G and *MTR*-G2756G is significantly higher compared to control individuals. Statistical analysis showed that *MTR*-A2756G genotype may be one the reasons of increased risk of HNC onset. Likewise findings have been reported previously.[21,22] In present study, the association of *MTR*-A2756G polymorphism with the site of onset, severity and progression of cancer in HNC patients is not evaluated. In our study, although

the incidence of HNC is more in males than female but statistical analysis on our data showed that gender is not associated with increased risk of head and neck cancer (p value >0.05).

CONCLUSION

In conclusion, the findings of our case control study suggest that smoking, aging and presence of *MTR*-A2756G genotype may increase the risk of head and neck cancer. We suggest further confirmation of this finding through cell culture techniques and *in vivo* studies in animal models to evaluate the functional activity of this enzyme in the presence of *MTR*-A2756G 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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