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Association of MTR, MTRR, MTHFD1 and RFC-1 gene polymorphisms and head and neck squamous cell carcinoma in north Coastal Andhra Pradesh

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Abstract

Aim: Head and neck cancer is identified by diverse group of malignant tumors that can develop in or all around the mouth, throat, nose and sinuses. It is a rather general multifactorial malignancy. The aim of the present study was to investigate the association of MTR (A2756G); MTRR (A66G); MTHFD1 (G1958) and RFC-1(G80A) gene polymorphisms in head and neck squamous cell carcinoma (HNSCC) patients and controls in North Coastal Andhra Pradesh.

Materials and methods: A total of 220 samples (110 HNSCC patients and 110 controls) were included in the present study and genotyping was accomplished by using PCR - RFLP technique. Data was analyzed by SPSS 19 software.

Results: The chi square p values revealed that MTR (A2756G) and RFC-1(G80A) gene polymorphisms has shows association with HNSCC and MTRR (A66G) and MTHFD1 (G1958)gene polymorphisms does not shows association with HNSCC. For MTR (A2756G) polymorphism the odds ratio p values of genotypes AG and GG were statistically significant, whereas AA genotype was found to be statistically insignificant. For MTRR (A66G) polymorphism the odds ratio p values of genotypes AA, AG and GG were found to be statistically insignificant. For MTHFD1 (G1958) polymorphism the odds ratio p values of genotypes GG, AG and AA were found to be statistically insignificant. For RFC-1(G80A) polymorphism the odds ratio p values of genotypes AG and GG were statistically significant, whereas AA genotype was found to be statistically insignificant.

Conclusions: In conclusion, the MTR (A2756G) and RFC-1(G80A) gene polymorphisms shows association with HNSCC, whereas MTRR (A66G) and MTHFD1 (G1958) gene polymorphisms does not shows association with HNSCC.

Keywords: HNSCC, MTR, MTRR, MTHFD1, RFC, Gene Polymorphism, Polymerase Chain Reaction, Restriction Fragment Length Polymorphism, Statistical Package of Social Sciences Software program

Introduction

The process of cancer development has been referred in numerous studies reporting its molecular basis and proposing genetic progression models for various tumour types. It is now well established that an accumulation of genetic and epigenetic alterations forms the basis of the change from a normal cell to a cancer cell, and is referred to as the process of multi step carcinogenesis [1]. "Head and neck cancer" (HNC) is a collective term defined on anatomical topographical basis to describe the malignant tumours of the upper aero digestive tract. This anatomical region includes the larynx, pharynx and oral cavity. A major sub-group of the head and neck carcinomas are the one referred to as "oral cancers" arising in the mucous membranes of the mouths (i.e. lip, tongue, the base of tongue, floor of mouth, gum and palate) and pharynx (comprising the nasopharynx, hypopharynx and oropharynx).

Head and neck carcinoma is the fifth most frequent cancers; its prevalence worldwide has been estimated at 50,000 new cases each year [3, 4]. According to World Health Organization - Union for International Cancer Control (WHO - UICC, 2003), globally the total burden of cancer is estimated to be around 22 million**.** Approximately 10 million new cases of cancer are diagnosed every year across the globe. The prevalence of head and neck cancer increases with age, particularly after 50 years of age. Even though most patients are between 50 and 70 years old, younger patients (age 18-45 years) can develop head and neck cancer. Head and neck cancers account for approximately 4% of all cancers in the United States [4]. These cancers are more than twice as common among men as they are among women [5].

In recent years, the joint effects of environmental, lifestyle and genetic factors are receiving increased attention for the occurrence of the cancer. Primary candidates for such interaction studies are those genes encoding enzymes related to the metabolism of established carcinogens. Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in folate metabolism and the potential protective effect of folate on cancer risk has been of research interest in the last decade [6].

The methylenetetrahydrofolate reductase (MTHFR) enzyme catalyzes the change of 5,10 methylenetetrahydrofolate into 5- methyltetrahydrofolate (5-MTHFR), which is the main circulating form of folate; it operates as a methyl group donor for the remethylation of homocysteine (Hcy) into methionine. This reaction is catalyzed by the methionine synthase enzyme (MTR), which requires vitamin B12 (methylcobalamin) as a cofactor, and which forms SAM.

The methionine synthase reductase (MTRR) enzyme maintains the active state of the MTR enzyme [7]. The methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) enzyme catalyzes the oxidation of 5, 10-methylene-THF into 5,10-methynyl-THF, which is then changed into 10-formyl-THF [8].
Another enzyme is the reduced folate carrier 1 (RFC1) enzyme, which is established on the membrane of intestinal mucosa cells, and which is involved in folate absorption [7].
Folate (a vitamin that may be found in fruit and vegetables) deficiency in the body has been associated with an increased risk of several types of cancers, including head and neck cancer [9, 10, 11, 12, 13, 14]. Several enzymes, including Methionine Synthase (MTR); Methionine Synthase Reductase (MTRR); Methylenetetrahydrofolate Dehydrogenase 1 (MTHFD1) and The Reduced Folate Carrie 1 gene (RFC1) are among the key enzymes involved in folate metabolism (15,16). Functional polymorphisms of these genes may regulate folate metabolism, which may ultimately affect genetic susceptibility to cancer. Based on the above evidence, the present study was aimed to identify the association between MTR (A2756G); MTRR (A66G); MTHFD1 (G1958A) and RFC1 (A80G) gene polymorphisms and head and neck cancer patients in North Coastal Andhra Pradesh.

Materials and methods

The present case control study was carried out with 110 head and neck cancer patients (72 males and 38 females) from Pinnacle cancer hospital and Mahatma Gandhi cancer hospital, Visakhapatnam and 110 age and sex matched controls (70 males and 40 females) above 30 years from North Coastal Andhra Pradesh during the period 2017-2018. The study was approved by the institutional ethical committee for blood sample collection from the patients. The informed consent was obtained from each and every participant before collecting blood sample for the evaluation of MTR (A2756G); MTRR (A66G); MTHFD1 (G1958) and RFC-1(G80A) gene polymorphisms.

Molecular analysis

DNA extraction

The genomic DNA was extracted from peripheral blood by using salting out method. Genotyping was performed by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) technique.

Genotyping of MTR A2756G (rs1805087)

One set of forward "5'- CCA GGG TGC CAG GTA TAC AG -3' reverse: 5'- GCC TTT TAC ACT CCT CAA AAC C -3'" primers were used for amplification of fragments of 498 base pairs and then amplified fragments was digested with HaeIII enzyme. The PCR profile was: initial denaturation at 94°C, 5 minutes, denaturation: 94°C, 1 minute, annealing: 56°C, 1 minute followed by 30 cycles each of 1 minute, and 72°C, then final extension at 72°C for 5 minutes. The amplification product was visualized in a 2% agarose gel under UV light.
For MTR A2756G, the PCR yielded a 498 bp product, which on digestion with MboII enzyme produced a 290bp, 123bp and 85bp fragments for GG condition (homozygous polymorphic) and 498bp, 290bp and 85bp fragments for AG condition (heterozygous polymorphic) condition. An undigested product length of 498 bp was retained by the wild types.

Genotyping of MTRR A66G (rs1801394):

One set of forward 5'-CAGGCAAAGGCCATCGCAGAAGACAT-3′ reverse 5′-CACTTCCCAACCAAAATTCTTCAAAG-3′ primers were used for amplification of fragments of 66 base pairs and then amplified fragments was digested with NdeI enzyme. The PCR profile was: initial denaturation at 94°C, 5 minutes, denaturation: 94°C, 1 minute, annealing: 56°C, 1 minute followed by 30 cycles each of 1 minute, and 72°C, then final extension at 72°C for 5 minutes. The amplification product was visualized in a 2% agarose gel under UV light.
For MTRR A66G, the PCR yielded a 66bp product, which on digestion with NdeI enzyme produced a 66bp, 44bp and 22bp fragments for GG condition(Homozygous polymorphic) whereas the 66bp and 22bp fragments for AG condition (Heterozygous polymorphic).

Genotyping of MTHFD1 G1958A (rs2236225)

One set of forward 5'-CACTCCAGTGTTTGTCCATG-3' reverse 5'GCATCTTGAGAGCCCTGAC-3 'primers were used for amplification of fragments of 331 base pairs and then amplified fragments was digested with MspI enzyme. The PCR profile was: initial denaturation at 94°C, 5 minutes, denaturation: 94°C, 1 minute, annealing: 55°C, 1 minute followed by 30 cycles each of 1 minute, and 72°C, then final extension at 72°C for 5 minutes. The amplification product was visualized in a 2% agarose gel under UV light.
For MTHFD1 G1958A, the PCR yielded a 331 bp product, which on digestion with MspI enzyme produced 331bp, 266bp, 166bp and 70 bp fragment for GG condition (homozygous polymorphic) whereas the 331bp and 266bp fragments for AG condition (heterozygous polymorphic). 166bp and 70bp fragments for AG condition (heterozygous polymorphic) whereas the undigested product length of 331 bp was retained by the wild types.

Genotyping of RFC1 A80G (rs1051266)

One set of forward 5'-AGTGTCACCTTCGTCCC-3' reverse: 5'-TCCCGCGTGAAGTTCTTG-3'primers were used for amplification of fragments of 229 base pairs and then amplified fragments was digested with CfoI enzyme. The PCR profile was: initial denaturation at 94°C, 5 minutes, denaturation: 94°C, 1 minute, annealing: 50°C, 1 minute followed by 30 cycles each of 1 minute, and 72°C, then final extension at 72°C for 5 minutes. The amplification product was visualized in a 2% agarose gel under UV light.
For RFC1 A80G, the PCR yielded a 229 bp product, which on digestion with CfoI enzyme produced 162bp, 125bp, 68bp and 37bp fragment for GG condition (homozygous polymorphic) whereas the 162bp and 68bp fragments for AG condition (heterozygous polymorphic). The undigested product length of 229 bp was retained by the wild types.

Statistical analysis

The data was analyzed by using Statistical Package of Social Sciences Software program (SPSS) 19 was used for calculating genotype and allele frequencies. Chi-square analysis was used to test for allele frequencies and odds ratio analysis was used to test for genotype frequencies in

comparison of patients and healthy control groups. P value less than 0.05 considered statistically significant.

Results

Table 1: Genotype and allele frequencies of MTR (A2756G) MTRR (A66G), MTHFD1 (G1958A) and RFC1 (A80G) in HNSCC patients and controls

Genes	Genotypes and alleles	HNSCC Patients N=110(%)	Controls N=110(%)	Odds ratio	95% CI	P-value
MTR (A2756G)	AA	41 (37.27%)	57 (51.81%)	0.813	0.449-1.472	0.496
	AG	38 (34.54%)	43 (39.09%)	0.285	0.123-0.657	0.003**
	GG	31 (28.18%)	10 (9.09%)	4.309	1.901-9.766	0.000**
	A	120 (54.54%)	157 (71.36%)	-		
	G	100 (45.45%)	63 (28.63%)		13.341	0.000**
MTRR (A66G)	AA	36 (32.72%)	43 (39.09%)	0.926	0.511-1.670	0.800
	AG	47 (42.72%)	52 (47.27%)	0.502	0.238-1.057	0.069
	GG	27 (24.54%)	15 (13.63%)	2.015	0.942-4.313	0.070
	A	119 (54.09%)	138 (62.72%)			
	G	101 (45.90%)	82 (37.27%)	-	3.377	0.066
MTHFD1 (G1958A)	GG	39 (35.45%)	51 (46.36%)	0.6834	0.379-1.231	0.205
	AG	47 (42.72%)	42 (38.18%)	0.7927	0.375-1.674	0.542
	AA	24 (21.81%)	17 (15.45%)	1.8462	0.873-3.901	0.108
	G	125 (56.81%)	144 (65.45%)	-		
	A	95 (43.18%)	76 (34.54%)		3.453	0.063
RFC1 (A80G)	AA	51 (46.36%)	60 (54.54%)	0.831	0.479-1.444	0.513
	AG	41 (37.27%)	46 (41.81%)	0.198	0.061-0.633	0.006**
	GG	18 (16.36%)	04 (3.63%)	5.294	1.683-16.652	0.004
	G	143 (65%)	166 (75.45%)	-		
	A	77 (35%)	54 (24.54%)		5.75	0.016**

Table 1 describes the genotype frequencies of MTR A2756G in HNSCC patients and controls. The genotype frequencies of AA (51.81%) and AG (39.09%) were higher in controls than genotype frequencies of AA (37.27%) and AG (34.54%) of HNSCC patients, whereas the genotype frequency of GG (28.18%) was higher in HNSCC patients than controls (9.09%). The odds ratio of GG genotype was found to be showing higher risk when compared to AG and AA genotypes. The odds ratio p values of genotypes AG and GG were statistically significant whereas AA genotype was found to be statistically insignificant.

The frequency of A allele was (54.54%) in HNSCC patients and (71.36%) in controls. The G allele frequencies in HNSCC patients and controls were (45.45%) and (28.63%) respectively. The chi- square p value reveals the MTR A 2756G gene shows association with HNSCC.

The genotype frequencies of MTRR A66G in HNSCC patients and controls were represented in table 1. The genotype frequencies of AA (39.09%) and AG (47.27%) were higher in controls than genotype frequencies of AA (32.72%) and AG (42.72%) of HNSCC patients, whereas the genotype frequency of GG (24.54%) was higher in HNSCC patients than controls (13.63%). The odds ratio of GG genotype was found to be showing higher risk when compared to AG and AA genotypes. The odds ratio p values of genotypes AA, AG and GG were found to be statistically insignificant.

The frequency of A allele was (54.09%) in HNSCC patients and (62.72%) in controls. The G allele frequencies in HNSCC patients and controls were (45.90%) and (37.27%) respectively. The chi- square p value reveals that MTRR A66G gene does not shows association with HNSCC.

The genotype frequencies of MTHFD1 G1958A in HNSCC patients and controls were represented in table 1. The genotype frequencies of GG was higher in controls (46.36%) than HNSCC patients (35.45%), whereas the genotype frequencies of AG (42.72%) and AA (21.81%) were higher in HNSCC patients than genotype frequencies of AG (38.18%) and AA (15.45%) in controls. The odds ratio of AA genotype was found to be showing higher risk when compared to AG and GG genotypes. The odds ratio p values of genotypes GG, AG and AA were found to be statistically insignificant.

The frequency of G allele was (56.81%) in HNSCC patients and (65.45%) in controls. The A allele frequencies in HNSCC patients and controls were (43.18%) and (34.54%) respectively. The chi- square p value reveal the MTHFD1 G1958A gene does not shows association with HNSCC.

The genotype frequencies of RFC1 A80G in HNSCC patients and controls were represented in table 1. The genotype frequencies of AA (54.54%) and AG (41.81%) were higher in controls than genotype frequencies of AA (46.36%) and AG (37.27%) of HNSCC patients, whereas the genotype frequency of GG (16.36%) was higher in HNSCC patients than controls (3.63%). The odds ratio of GG genotype was found to be showing higher risk when compared to AG and AA genotypes. The odds ratio p values of genotypes AG and GG were statistically significant whereas AA genotype was found to be statistically insignificant.

The frequency of G allele was (65%) in HNSCC patients and (75.45%) in controls. The A allele frequencies in HNSCC patients and controls were (35%) and (24.54%) respectively. The chi-square p value reveals that RFC1 (A80G) gene shows association with HNSCC.

Table 2: Distribution of MTR (A2756G) genotype frequencies in relation to demographic variables in HNSCC patients and controls.

Variables	MTR (A2756G) geno type	HNSCC Patients N= 110 (%)	Controls N= 110 (%)	Chi –Square p- value
Smoking	AA	32 (29.09%)	05 (4.54%)	0.009**
	AG	27 (24.54%)	10 (9.09%)	

	GG	24 (21.81%)	06(5.45%)	
Non smoking	AA	09 (8.18%)	52 (47.2%)	0.001**
	AG	11 (10%)	33 (30%)	
	GG	07 (6.36%)	04 (3.63%)	
Tobacco chewing	AA	33 (30%)	09 (8.18%)	0.131
	AG	19 (17.27%)	13 (11.81%)	
	GG	25 (22.72%)	07 (6.36%)	
Tobacco non chewing	AA	08 (7.27%)	48 (43%)	0.000***
	AG	19 (17.27%)	30 (27.27%)	
	GG	06(5.45%)	03 (2.72%)	
Alcohol consumption	AA	29 (26.36%)	14 (12.72%)	0.197
	AG	16 (14.54%)	08 (7.27%)	
	GG	27 (24.54%)	05 (4.54%)	
Alcohol non consumption	AA	12 (10.90%)	43 (39.09%)	0.109
	AG	22 (20%)	35 (31.81%)	
	GG	04 (3.63%)	05 (4.54%)	

Table 2 explains the distribution of MTR (A2756G) genotype frequencies in relation to demographic variables in HNSCC patients and controls. In smoking, the genotype frequencies of HNSCC patients were AA (29.09%), AG (24.54%) and GG(21.81%) and in controls it was AA (4.54%), AG (9.09%) and GG (5.45%), whereas in non-smoking, the genotype frequencies of HNSCC patients were AA (8.18%), AG (10%) and GG (6.36%) and in controls it was AA (47.2%), AG (30%) and GG (3.63%).

In tobacco chewing, the genotype frequencies of HNSCC patients were AA (30%), AG (17.27%) and GG (22.72%) and in controls it was AA (8.18%), AG(11.81%) and GG (6.36%), where as in tobacco non chewing, the genotype frequencies of HNSCC patients were AA (7.27%), AG (17.27%) and GG (5.45%) and in controls it was AA (43%), AG (27.27%) and GG (2.72%).

In alcohol consumption, the genotype frequencies of HNSCC patients were AA (26.36%), AG (14.54%) and GG (24.54%) and in controls it was AA (12.72%), AG (7.27%) and GG (4.54%), whereas in alcohol non consumption, the genotype frequencies of HNSCC patients were AA (10.90%), AG (20%) and GG (3.63%) and in controls it was AA (39.09%), AG (31.81%) and GG (4.54%).

The chi-square p value shows that smoking, non-smoking and tobacco non chewing variables were found to be significantly associated with MTR (A2756G) gene polymorphism.

Table 3: Distribution of MTRR (A66G) genotype frequencies in relation to demographic variables in HNSCC patients and controls

Variables	MTRR(A66G) genotype	HNSCC Patients N= 110 (%)	Controls N= 110 (%)	Chi – Square p- value
Smoking	AA	26 (23.63%)	07 (6.36%)	0.504
	AG	32 (29.09%)	12 (10.90%)	
	GG	19 (17.27%)	10 (9.09%)	
Non smoking	AA	10 (9.09%)	36 (32.72%)	0.018*
	AG	15 (13.63%)	40 (36.36%)	
	GG	08 (7.27%)	05 (4.54%)	
Tobacco chewing	AA	25 (22.72%)	06 (5.45%)	0.440
	AG	31 (28.18%)	15 (13.63%)	
	GG	21 (19.09%)	08 (7.27%)	
Tobacco non chewing	AA	11 (10%)	37 (33.63%)	0.251
	AG	16 (14.54%)	37 (33.63%)	
	GG	06 (5.45%)	07 (6.36%)	
Alcohol consumption	AA	21 (19.09%)	13 (11.81%)	0.119
	AG	27 (24.54%)	05 (4.54%)	
	GG	24 (21.81%)	09 (8.18%)	
Alcohol non consumption	AA	15 (13.63%)	30 (27.27%)	0.919
	AG	20 (18.18%)	47 (42.72%)	
	GG	03 (2.72%)	06 (5.45%)	

Table 3 shows the distribution of MTRR (A66G) genotype frequencies in relation to demographic variables in HNSCC patients and controls. In smoking, the genotype frequencies of HNSCC patients were AA (23.63%), AG (29.09%) and GG(17.27%) and in controls it was AA (6.36%), AG (10.90%) and GG (9.09%), whereas in non-smoking, the genotype frequencies of HNSCC patients were AA (9.09%), AG (13.63%) and GG (7.27%) and in controls it was AA (32.72%), AG (36.36%) and GG (4.54%).

In tobacco chewing, the genotype frequencies of HNSCC patients were AA (22.72%), AG (28.18%) and GG (19.09%) and in controls it was AA (5.45%), AG (13.63%) and GG (7.27%), where as in tobacco non chewing, the genotype frequencies of HNSCC patients were AA (10%), AG (14.54%) and GG (5.45%) and in controls it was AA (33.63%), AG (33.36%) and GG (6.36%).

In alcohol consumption, the genotype frequencies of HNSCC patients were AA (19.09%), AG (24.54%) and GG (21.81%) and in controls it was AA (11.81%), AG (4.54%) and GG (8.18%), whereas in alcohol non consumption, the genotype frequencies of HNSCC patients were AA (13.63%), AG (18.18%) and GG (2.72%) and in controls it was AA (27.27%), AG (42.72%) and GG (5.45%).

The chi-square p value shows the non-smoking variable was

found to be significantly associated with MTRR (A66G) gene polymorphism.

Table 4: Distribution of MTHFD1 (G1958A) genotype frequencies in relation to demographic variables in HNSCC patients and controls

Variables	MTHFD1(G1958A) genotype	HNSCC Patients N= 110 (%)	Controls N= 110 (%)	Chi – Square p- value
Smoking	GG	29 (26.36%)	40 (36.36%)	0.290
	AG	38 (34.54%)	38 (34.54%)	
	AA	16 (14.54%)	11 (10%)	
Non smoking	GG	10 (9.09%)	11 (10%)	0.465
	AG	09 (8.18%)	04 (3.36%)	
	AA	08 (7.27%)	06 (5.45%)	
Tobacco chewing	GG	28 (25.45%)	08 (7.27%)	0.240
	AG	30 (27.27%)	09 (8.18%)	
	AA	19 (17.27%)	12 (10.90%)	
Tobacco non chewing	GG	11 (10%)	43 (39.09%)	0.095
	AG	17 (15.45%)	33 (30%)	
	AA	05 (4.54%)	05 (4.54%)	
Alcohol consumption	GG	21 (19.09%)	05 (4.54%)	0.041*
	AG	35 (31.81%)	09 (8.18%)	
	AA	16 (14.54%)	13 (11.81%)	
Alcohol non consumption	GG	18 (16.36%)	46 (41.81%)	0.021*
	AG	12 (10.90%)	33 (30%)	
	AA	08 (7.27%)	04 (3.36%)	

Table 4 demonstrates the distribution of MTHFD1 (G1958A) genotype frequencies in relation to demographic variables in HNSCC patients and controls. In smoking, the genotype frequencies of HNSCC patients were GG (26.36%), AG (34.54%) and AA (14.54%) and in controls it was GG (36.36%), AG (34.54%) and AA (10%), whereas in non-smoking, the genotype frequencies of HNSCC patients were GG (9.09%), AG (8.18%) and AA (7.27%) and in controls it was GG (10%), AG (3.36%) and AA (5.45%).
In tobacco chewing, the genotype frequencies of HNSCC patients were GG (25.45%), AG (27.27 %) and AA (17.27%) and in controls it was GG (7.27%), AG (8.18%) and AA (10.90%), where as in tobacco non chewing, the genotype frequencies of HNSCC patients were GG (10%), AG (15.45%) and AA (4.54%) and in controls it was AA (39.09%), AG (30%) and AA (4.54%).
In alcohol consumption, the genotype frequencies of HNSCC patients were GG (19.09%), AG (31.81%) and AA (14.54%) and in controls it was GG (4.54%), AG (8.18%) and AA (11.81%), whereas in alcohol non consumption, the genotype frequencies of HNSCC patients were GG (16.36%), AG (10.90%) and AA (7.27%) and in controls it was GG (41.81%), AG (30%) and AA (3.36%).
The chi-square p value shows that alcohol consumption and alcohol non consumption variables were found to be significantly associated with MTHFD1 (G1958A) gene polymorphism.

Table 5: Distribution of RFC1 (A80G) genotype frequencies in relation to demographic variables in HNSCC patients and controls

Variables	RFC1 (A80G) genotype	HNSCC Patients N= 110 (%)	Controls N= 110 (%)	Chi – Square p- value
Smoking	AA	40 (36.36%)	16 (14.54%)	0.062
	AG	32 (29.09%)	03 (2.72%)	
	GG	11 (10%)	02 (1.81%)	
Non smoking	AA	11 (10%)	44 (40%)	0.000***
	AG	09 (8.18%)	43 (39.09%)	
	GG	07 (6.36%)	02 (1.81%)	
Tobacco chewing	AA	35 (31.81%)	20 (18.18%)	0.072
	AG	26 (23.63%)	07 (6.36%)	
	GG	16 (14.54%)	02 (1.81%)	
Tobacco non chewing	AA	16 (14.54%)	40 (36.36%)	0.636
	AG	15 (13.63%)	39 (35.45%)	
	GG	02 (1.81%)	02 (1.81%)	
Alcohol consumption	AA	39 (35.45%)	16 (14.54%)	0.781
	AG	21 (19.09%)	08 (7.27%)	
	GG	12 (10.90%)	03 (2.72%)	
Alcohol non consumption	AA	12 (10.90%)	44 (40%)	0.002**
	AG	20 (18.18%)	38 (34.54%)	
	GG	06 (5.45%)	1 (0.90%)	

Table 5 shows the distribution of RFC1 (A80G) genotype frequencies in relation to demographic variables in HNSCC

patients and controls. In smoking, the genotype frequencies of HNSCC patients were AA (36.36%), AG (29.09%) and GG(10%) and in controls it was AA (14.54%), AG (2.72%) and GG (1.81%), whereas in non-smoking, the genotype frequencies of HNSCC patients were AA (10%), AG (8.18%) and GG (6.36%) and in controls it was AA (40%), AG (39.09%) and GG (1.81%).

In tobacco chewing, the genotype frequencies of HNSCC patients were AA (31.81%), AG (23.63%) and GG (14.54%) and in controls it was AA (18.18%), AG (6.36%) and GG (1.81%), where as in tobacco non chewing, the genotype frequencies of HNSCC patients were AA (14.54%), AG (13.63%) and GG (1.81%) and in controls it was AA (36.36%), AG (35.45%) and GG (1.81%).

In alcohol consumption, the genotype frequencies of HNSCC patients were AA (35.45%), AG (19.09%) and GG (10.90%) and in controls it was AA (14.54%), AG (7.27%) and GG (2.72%), whereas in alcohol non consumption, the genotype frequencies of HNSCC patients were AA (10.90%), AG (18.18%) and GG (5.45%) and in controls it was AA (40%), AG (34.54%) and GG (0.90%).

The chi-square p value shows that non-smoking and alcohol non consumption variables were found to be significantly associated with RFC1 (A80G) gene polymorphism

Table 6: MTR (A2756G) polymorphism and its interaction with other polymorphisms of MTRR (A66G), MTHFD1 (G1958A) and RFC1 (A80G) genes in HNSCC patients and controls. MTR (A2756G) AA v/s

Genes	Genotypes	HNSCC N=110	Control N=110	Odds ratio	95% CI	p- value
MTRR (A66G)	AA	07	05	0.311	0.045 – 2.110	0.231
	AG	09	02	6.300	0.928 – 42.730	0.059*
	GG	05	07	0.510	0.100 – 2.585	0.416
MTHFD1 (G1958A)	GG	09	06	6.000	1.171 – 30.725	0.031*
	GA	03	12	0.205	0.048 – 0.877	0.032*
	AA	17	14	0.809	0.231 – 2.831	0.740
RFC1 (A80G)	AA	14	15	3.577	1.125 – 11.374	0.030*
	AG	06	23	0.156	0.028 – 0.848	0.031*
	GG	05	03	1.785	0.358 – 8.898	0.479

Table 7: MTR (A2756G) AG + GG v/s

MTRR (A66G)	AA	29	38	1.004	0.528 – 1.907	0.989
	AG	38	50	0.276	0.111 – 0.688	0.005**
	GG	22	08	3.603	1.404 – 9.248	0.007**
MTHFD1 (G1958A)	GG	30	45	0.454	0.236 – 0.874	0.018 *
	GA	44	30	0.628	0.150 – 2.626	0.524
	AA	07	03	3.500	0.838 – 14.614	0.085
RFC1 (A80G)	AA	37	45	0.540	0.273 – 1.069	0.077
	AG	35	23	0.117	0.014 – 0.956	0.045*
	GG	13	01	15.810	1.975 – 126.556	0.009**

Table 6 represents the MTR (A2756G) polymorphism and its interaction with MTRR (A66G), MTHFD1 (G1958A) and RFC1 (A80G) gene polymorphisms in HNSCC patients and controls. Form the above table, it is evident that the AA genotype of MTR (A2756G) polymorphism was statistically significant with AG genotype of MTRR (A66G), GG and GA genotypes of MTHFD1 (G1958A) and AA and AG genotypes of RFC1 (A80G) polymorphisms, whereas it is insignificant with AA and GG genotypes of MTRR, AA genotype of MTHFD1 (G1958A) and GG genotype of RFC1 (A80G) polymorphisms.

The AG and GG genotypes of MTR (A2756G) polymorphism was statistically significant with AG and GG genotypes of MTRR (A66G), GG genotype of MTHFD1 (G1958A) and AG and GG genotypes of RFC1 (A80G) polymorphisms, whereas it is insignificant with AA genotype of MTRR (A66G), GA and AA genotypes of MTHFD1 (G1958A) and AA genotype of RFC1 (A80G) polymorphisms.

Discussion

Accumulated evidence propose a role for folate deficiency in the etiology of HNSCC [17, 18, 19], and the MTR and MTRR genes play an key role in folate metabolism [16]. Studies have revealed that the polymorphisms of these two genes, MTR A2756G and MTRR G66A, contributed to change of plasma levels of homocysteine and folate.

In the present study, the chi- square p value reveals the MTR A 2756G gene shows association with HNSCC. The odds ratio p values of genotypes AG and GG were statistically significant whereas AA genotype was found to be statistically insignificant. In the present study**,** the chi-square p value reveals that MTRR A66G gene does not shows association with HNSCC. The odds ratio p values of genotypes AA, AG and GG were found to be statistically insignificant.

The genotyping data of [20] indicate that the rarer (variant) MTR 2756 revealed G allele is a risk allele but the rarer (variant) MTRR 66A is a protective allele and that both the MTR A2756G and MTRR G66A polymorphisms had a main effect on HNSCC risk. Further analyses revealed that the MTR 2756AG but not the MTR 2756GG (because of small number of subjects) genotype was associated with a significantly increased risk of HNSCC compared with the 2756AA genotype. Consequently, the MTR A2756G and MTRR G66A polymorphisms that may cause folate deficiency could increase risk of HNSCC.

A high plasma level of folate has been found to decrease the risk of HNSCC in some case control studies [17, 19]. Functional polymorphisms of genes playing an key role in folate metabolism contribute to changes in plasma levels of folate [16]. It is therefore plausible that they have a

substantial impact on risk; though, few studies have previously examined the relationships between these polymorphisms and HNSCC risk. Hospital based case control studies in the USA showed a decreased risk with MTRR AA genotype, an increased risk with MTR AG/GG genotypes [21, 22, 23].
The study of [24] shows association of MTR polymorphisms and esophageal cancer risk. The observations of [25] suggest that MTR 2756G alleles may contribute to the risk of laryngeal cancer in Polish patients. To date, it has been found that the MTR 2756A>G polymorphism contributes to the risk of squamous cell carcinomas of the head and neck, esophagus, lung cancer, colorectal carcinoma, glioblastoma multiforme, and multiple myeloma [26, 27, 28, 29, 30, 31 32, 33, 23].
The study of [34] revealed differences in the MTR A2756G genotype frequencies between head and neck cancer patients and controls and between larynx cancer patients and controls, respectively, while [33] did not found any association between the presence of the polymorphism and the risk for the disease. The studies of [35, 36] did not find an association between MTRR A66G polymorphic variant with the HNSCC cases.
The study of [37] represents an updated comprehensive meta-analysis of the association between the MTRR A66G polymorphism and cancer risk and included 85 studies with 32,272 cases and 37,427 controls. The results revealed that the MTRR A66G polymorphism was significantly associated with an increased overall cancer risk. In the subgroup analysis, the association was more evident for head and neck cancer, Caucasians, Africans and high quality studies.
The presented discrepancies between different studies may be a result of population differences, genetic heterogeneity and distinct habitual cigarette smoking and alcohol drinking in the investigated cohorts.
In the present study**,** the chi- square p-value reveal the MTHFD1 (G1958A) gene does not shows association with HNSCC. The odds ratio p values of genotypes GG, AG and AA were found to be statistically insignificant.
Consequently, deficiency in MTHFD1 gene may exacerbate the disruption of folate metabolism (38). The study of (39) did not find any association between MTHFD1 G1958A polymorphism and head and neck cancer as (34) study that also did not find association between laryngeal cancer and this polymorphism. The present study correlates with the above studies.
In the present study, the chi-square p value reveals that RFC1 (A80G) gene shows association with HNSCC. The odds ratio p values of genotypes AG and GG were statistically significant whereas AA genotype was found to be statistically insignificant.
According to HWE analysis of [35] showed that RFC1 gene is not in equilibrium. The deviation from the HWE equilibrium may result from the random selection of the studied individuals, the disease model adopted, and evolutionary factors which can influence changes in the genotype frequencies [40, 41]. On the other hand, this disequilibrium should be expected, considering that it reflects biologic and genetic characteristics in complex disease models [42].
The study of [43] observed that RFC1 80AG/GG genotypes are related with an increase of HNSCC risk. The study of [35] revealed that RFC1 80AG/GG genotypes are associated significantly with an increase of HNSCC risk corroborating with his previous study. The present study correlates with the study of [43, 35].

Conclusions

In conclusion, the accumulated evidence has indicated an associated between MTR (A2756G) and RFC1 (A80G) gene polymorphisms and HNSCC and lack of association found with MTRR (A66G) and MTHFD1 (G1958A) gene polymorphisms.
Although, genetic and environmental risk factors are experientially recognized as sharing a role in increasing the risk for certain types of cancers, there is very limited data on gene-environment interactions. Therefore an attempt was made to evaluate the risk factors with the genes. But in the present study, the impact of gene environment interaction has not been observed, as the risk factors of smoking and tobacco chewing shows statistical significance and association without gene environment interaction also.
As the detection of Gene-Gene interaction is an important approach to understand the etiology of HNSCC, an attempt was made to evaluate the risk of Gene-Gene interaction. But in the present study the impact of gene-gene interaction has not been observed in the etiology of HNSCC.
Large-scale case-control studies are required to evaluate the risks and to investigate the potential gene-gene and gene-environment interactions between MTR (A2756), MTRR (A66G), MTHFD1 (G1958A) and RFC-1 (A80G) gene polymorphisms and head and neck squamous cell carcinoma.

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