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Genotype Analysis of MLH1 (-93G/A) Polymorphism In Alcohol And Tobacco Consumers Associated With Risk Of Head And Neck Squamous Cell Carcinoma (HNSCC).

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ABSTRACT

Head and Neck cancer is a devastating disease and is the sixth most prevalent cancer accounting for 3% of all cancer. Majority of head and neck cancer originated from mucosal lining of the upper aero-digestive tract part especially in larynx, pharynx, oral cavity and nasal cavity collectively termed head and neck squamous cell carcinoma. Tobacco and alcohol are crucial risk factors of head and neck cancer. Additional etiologies that cause head and neck cancer are viral infection and genetic susceptibility factors and its association with head and neck cancer is still to be understood. Genetic instability is one of the prevalent features of all types of cancers. Genetic blueprint (DNA) is usually damaged by exogenous, endogenous mutant agents and genetic variant in combination with environmental exposure to exogenous/endogenous carcinogens is the main factor responsible for differences between individuals. Single nucleotide polymorphisms in DDR (DNA damage repair) genes are accountable for multiple cancers including head and neck cancer. MLH1 DNA repair gene plays an important role in BER pathway. DNA repair gene single nucleotide polymorphism (SNPs) can lead to changes in individual accountability to larynx cancer. In this time line article, we focus on the correlation between polymorphisms in the genomic stability pathway and emergence of head and neck cancer.

Keywords: SNP (Single Nucleotide polymorphism), DNA repair, MLH1 **(**MutL homolog 1)

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INTRODUCTION

Worldwide approximately about five lakhs individuals have diagnosed annually with squamous cell carcinoma of head and neck region (HNSCC). Increase hazard of HNSCC is seen with ecological factors like alcohol intake and tobacco consuming habits. Metabolites produced by consumption of alcohol and tobacco usage leads to an increase in the oxidative stress and DNA Strand interruption [1]. Three most important process of DNA repair is base excision repair (BER) pathway, nucleotide excision repair (NER) pathway, and double-strand break (DSB) repair. MLH1 DNA repair gene plays an important role in BER pathway. DNA repair gene single nucleotide polymorphism (SNPs) can lead to changes in individual accountability to larynx cancer [2]. MLH 1The gene is located on chromosome 3. The MLH1 gene protein plays an important role in repairing DNA damage. MLH1 (-93G/A) polymorphic site present in 5' UTR region of the gene. The MLH1 gene plays a crucial part in DNA damage resolution following the platinum-based CCRT. Variation in responsiveness of tumor cells toward CCRT is seen, when there is an alteration in MLH-1 DNA resolution gene and its protein expression [3, 4].

MATERIAL AND TECHNIQUE

A tertiary hospital-based prospective case-control research study was performed. Between August 2018 and July 2019, 100 male patients were incorporated into the study group with pathologically diagnosed HNSCC. The admittance basis for patients (cases) were the age of the patient ≥18 years, the performance score (Kernofsky Performance score) of ≥80, adequate Complete blood count test (C.B.C) and Differential count (D.C) test with neutrophils > 1.5 × 109 cells per liter, Platelets > 100 × 109 cells per liter, adequate kidney function test (K.F.T) with Creatinine Clearance (C.C) of ≥50 ml per minute, locoregionally progressed HNSCC (Stage III & IV), and with no distant metastasis to lung or liver[5]. The control group comprises an equal number of healthy males. The following information was taken from the cases, chief complaints, history of presenting illness, followed by the general and systemic examination, local examination of oral cavity and neck and direct laryngoscopy [6]. Data were collected regarding the family history of carcinoma, personal history of substance use (alcohol consumption, tobacco smoking, and tobacco chewing habits). Investigations to know the general health and organ function was done which include Complete blood count (C.B.C), Liver function test (L.F.T), Kidney function test (K.F.T), pre-treatment dental check-up[7]. To know the local and regional disease status, investigations like Head and neck Computed Tomography (C.T) scan and Magnetic resonance imaging (M.R.I) was done. Chest roentgenogram (Posterior anterior view) and Ultrasonography (U.S.G) whole Abdomen of the cases was done to rule out the lung metastasis, and liver metastasis. In this research, the American Joint Committee on cancer (A.J.C.C 2010) manual was used for the staging of head and neck cancer patients [8].

This research study convention was accepted by the human ethics committee. For enrollment in this research study group and before initiating the whole blood sample (2 milliliters) collection from cases and control, assent was taken. A questionnaire sheet was filled up by all the enrolled cases and controls regarding their medical history, tobacco smoking and chewing habits, family history of cancer, alcohol intake[9]. Cases and controls were categorized as tobacco smokers who had regular tobacco smoking habits and with smoking index (S.I) ≥ 730 (S.I = cigarettes smoked/day x 365 days). Cases and control were categorized as tobacco chewers who had the chewing year (C.Y) ≥ 365 (C.Y is defined as the frequency of tobacco chewed day for a time span of 3 years).10 Cases and controls with the habit of either tobacco smoking or chewing were categorized as tobacco substance users. Cases and controls with approximately total consumption of alcohol of ≥90 liters/year were categorized as alcohol substance users [10, 11].

Detection of MLH1 (-93G/A) polymorphism

MLH1 (-93G/A)polymorphic site present in 5' UTR region of the gene which was identified by the method of park et al. (2004). The primer sequences used for identifying -93G/A (rs1800734) polymorphism is as follows- FP 5'-CCGAGCTCCTAAAAACGAAC-3'and RP 5'-CTGGCCGCTGGATAACTTC-3'. The reaction mixture in 50µl contained 1X buffer (10 mMTris-Hcl, pH 8.3, 1.5 mM $MgCl_2$, 25mM KCl), 200 mM of each nucleotides. 1 unit of Tag polymerase (MBI Fermentas, Gennany), 100 ng of genomic DNA and sterile milliQ water. Amplification was performed on GeneAmp 9700 PCR thermal cycler using the following protocol: 94°C for 5 minutes for initial denaturation followed by 35 cycles of 94°C for 45 seconds, 56°C for 45 seconds and 72°C for 45 seconds and a final elongation step of 72°C for 10 minutes.

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PCR reaction resulted in a 387bpPCR product. 10µlof the PCR product was digested with 1 U of *PvuII*(New England Biolabs, US) in a final volume of 15 µl. This reaction mixture was incubated at 37°C for one hour. Electrophoresis was carried out in a 3.5 % gel containing ethidium bromide and analyzed on VERSA DOC Imaging System (Model 1000, Bio-Rad). Primers designed to amplify -93G/A(*PvuII)*containing region of the MLH1 gene produce a band of 387bp corresponding to the mutant genotype, heterozygous genotypes produced 387bp, 207bp & 180bp PCR products, while homozygous wild genotypes gave bands of 180bp, 207bp after *PvuII*digestion [12].

PCR-RFLP Analysis of MLH1 gene for *-93G/A (rs1800734 or Puv II)* polymorphic site

Digestion of PCR product with *Puv*II to identify-*93G/A (rs1800734 or Puv II)* polymorphism produced a fragment size of 180bp and 207bp for homozygous wild type allele and an undigested 387bp fragment for homozygous mutant allele [13]. In heterozygous genotype, all the three bands corresponding to 387bp, 207bp, 180bp were visible (Figure 1.1).

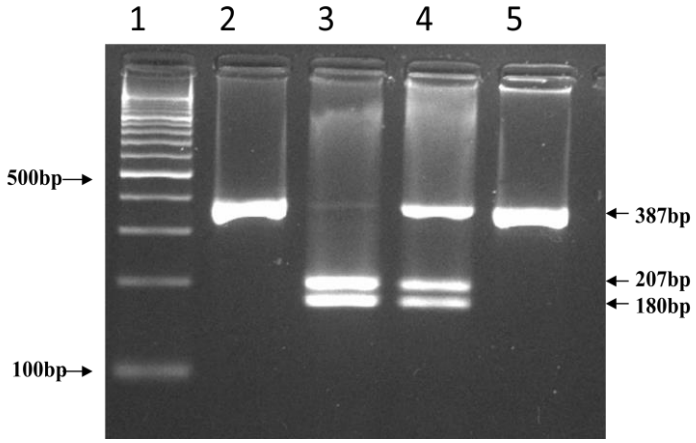

Figure 1.1: Genotyping of -93G/A polymorphism of MLH1 gene by RFLP (lane 1: 100bp DNA Ladder, lane 2: homozygous mutant genotype, lane 3: homozygouswild genotype, lane 4: heterozygous genotype, lane 5: PCR product).

Table1 Genotypes distribution of MLH1 gene for (-93G/A) polymorphism

SNP	Genotypes	Control (n=100)	Cases (n=100) (%)	OR (95%CI)	*P* Value
-93G/A	GG	82(63)	83(42)	1 (Ref.)	
(Puv II)	GA	42(32)	57(38)	1.34(0.81-2.21)	0.25
	AA	6(5)	10(20)	1.64(0.57-4.73)	0.35
Variant	GA+AA	48(37)	67(58)	1.37(0.85-2.22)	0.18
Allele	GG	206(79)	223(74)	1 (Ref)	
	AA	54(21)	77(26)	1.31(0.88-1.95)	0.17

OR: odds ratio; 95% CI: 95% confidence interval; Ref: reference category.
P<0.05 is considered statistically significant.

Table 1 Summarizes the genotypic distribution of MLH1 (-93G/A) polymorphism among controls and cases. The genotype and allele frequencies among controls were in Hardy-Weinberg equilibrium. The frequency of the heterozygous genotype of -93G/A was found to be higher in case (38%) when compared to the controls (32%). The frequency of the homozygous mutant genotype was also increased in case (20%) as compared to the controls (5%). The crude OR for heterozygous genotype was found to be 1.64 (95%CI: 0.57–4.73) while the crude OR for homozygous mutant genotype was 1.34 (95%CI: 0.81–2.21), which shows both genotypes of -93G/A polymorphism was non-significantly associated with increased risk to HNSCC. The frequency of AA/AA allele was also found to be higher in cases (26%) when compared to the controls (21%). The crude OR for mutant allele (AA/AA) was found to be 1.31 (95% CI: 0.88-1.95), which was found to be statistically non-significant (p=0.17) for the cases when compared to the controls.

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CONCLUSION

A correlation between theMLH1 (93G/A) polymorphism with smoking, alcohol consumption and HNSCC was identified in polymorphic homozygote (TT) genotype (O.R: 1.34; 95% C.I: 0.81–2.21). The main limitation of this investigational research was the small number of patients, and hence this data has a constraint to be summarized to all the loco-regionally progressed HNSCC patients. The additional investigational study is needed to exactly estimate and endorses the outcome of our investigational research which proposes that MLH-1 gene 93 G/A genotype variants have impinged the hazard in alcohol and tobacco users.

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