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RESEARCH ARTICLE

EVALUATION OF MUTATION OF BAX SNPS WITH RISK OF SQUAMOUS CELL CARCINOMA HEAD AND NECK (HNSCC) IN KASHMIRI POPULATION.

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Abstract

Objective: To find relationship between Bax SNPs and squamous cell carcinoma head and neck (SCCHN) in patients from Kashmir
Study design: Case control study.
Setting: Tertiary care hospital (SMHS associated Medical College, Srinagar, Kashmir, India)
Participants: 50 cases and 50 controls of squamous cell carcinoma head and neck reported our hospital from 2013-2016.
Results: In the present study no evidence of associations between BAX (-248 G>A) and squamous cell carcinoma head and neck , further analyses showed that among BAX heterozygotes after adjustment for age, sex, and smoking status, the BAX GC (Arg-Pro) genotype was associated with an elevated risk of SCCHN [OR=1.354, 95% CI= 0.8629-2.124)compared with the BAX GG genotype and OR=1.962, 95% CI=0.9129-2.465) compared with the combined genotypes (GG+CC)],. Our data suggest that the risk of SCCHN may be associated with these SNPs of BAX. Larger studies are needed to validate these findings.

Background:-

Head and neck cancer is the eighth leading cause of cancer death worldwide[1]. In 2007, 40566 new cases of squamous cell carcinoma of the head and neck (SCCHN) were diagnosed, and the poor survival of patients with SCCHN has not been improved for several decades[2]. Whereas tobacco and alcohol are the primary risk factors for SCCHN development[3] epidemiological studies suggest an association of SCCHN with genetic susceptibility in the general population[4], and recent studies suggest that genetic polymorphisms in cell-cycle control and apoptosis genes may attribute partly to this predisposition[5].

Cancer is a disease resulting from the accumulation of genetic alterations in the cell. Exposure to environmental carcinogens (chemicals, viruses, radiations) contributes to the genesis of at least 80% of all human cancers [6].

There are three possible outcomes for a cell following exposure to DNA damage: (a) the cell could repair the damage; (b) the cell could die; or (c) survive with a permanent mutation resulted by replication of the damaged DNA. DNA damage elicits cell cycle arrest at G1/S or G2/M transitions allowing to repair the errors occurred during DNA replication or chromosome segregation[7].The p53 protein acts as a checkpoint control to permit the repair of damaged DNA by cell cycle arrest in G1[8].Genes involved in the maintenance of genomic integrity - such as DNA-mismatch repair genes, p53 and BRCA1 – are also called as “care taker” genes. Defects in the cellular mechanisms protecting against DNA damages increase the genetic instability therefore lead to the accumulation of genetic errors, which may eventually result in tumor development. The inactivation mechanisms may be inherited alterations, acquired mutations and epigenetic events.

Apoptosis is a process of programmed cell death under both normal physiological and pathological conditions. Deficiency in cell apoptosis alters cell homeostasis and leads to carcinogenesis and tumor progression[9]. Apoptosis is regulated by different pathways involving a number of genes that either promote or inhibit apoptosis. The best-characterized apoptosis regulators include the anti-apoptotic B-cell lymphoma 2 (BCL2) gene and the pro-apoptotic B-cell lymphoma 2-associated X protein (BAX) gene. The protein products of these two genes physically interact with each other and the relative levels of the two proteins are important determinants of the apoptosis rate[10]. Therefore, the relative expression of these two genes plays a key role in cellular homeostasis and cancer development. Another master regulator of cellular apoptosis is the tumor suppressor tumor protein 53 (TP53) gene. Research from the last decade has shown that TP53, which is a transcriptional factor, functions to regulate expression of many apoptosis and cell-cycle regulatory genes. The TP53 protein directly binds to the TP53-binding element in the promoter of BAX gene and induces BAX expression[11] and TP53 inhibits BCL2 promoter-driven transcription[12]. Therefore, the interaction between TP53 and the promoters of this pair of apoptosis-regulatory genes is probably important for cellular apoptosis. However, what factors affect this interaction are less clear except that TP53 mutations that abolish the DNA-binding ability may render TP53 incapable of regulating apoptosis. TP53 mutations have been observed in >50% of all human cancers[13] and there is a high frequency of TP53 mutations in many cancer types including SCCHN[14]. It has been recognized that during tumor development, one copy of chromosome 17p (the second allele), where TP53 resides, is frequently lost [loss of heterozygosis (LOH)], and the other allele is mutated. Thus, in tumor cells, where two copies of the TP53 gene are present, the gene is probably a wild-type, which provides the opportunity to study the role of variants of the TP53 gene in interaction with other genes involved in the carcinogenesis.

Aim of the study:-

To find the association between SNPs of BAX with risk of squamous cell carcinoma head and neck (SCCHN).

Materials and methods:-

Study subjects:-

This study was done in tertiary health centre of kashmir in Department of ENT and Head & Neck surgery SMHS and included patients with histologically confirmed squamous cell carcinoma Head and neck (SCCHN) over a period of 18 months. Patients with squamous cell carcinoma of oral cavity, oropharynx, nasal cavity, nasopharynx, hypopharynx and larynx, identified at the Department of ENT and head&neck surgery SMHS Hospital were included. The patients with secondary SCCHN, Thyriod malignancies, primaries outside the upper aero digestive tract, cervical metastases of unknown origin or histopathologic diagnoses other than squamous cell carcinoma were excluded. All cases were from Kashmir and had not received any treatment at the time of recruitment. Controls were also taken from Kashmiri population who were admitted to our hospital for some other non-neoplastic disease. After verbal and written consent 2ml of blood sample was taken from controls and analysed for genetic mutation.

From Histologically proven patients of squamous cell carcinoma. 2 ml of blood was taken from each patient after proper consent and was analysed at the Department of Biochemistry (DNA extraction and genotyping). Samples were stored at temperature -80 degrees Celsius.

Genotyping:-

From each blood sample of case and control, a leukocyte cell pellet was obtained from the buffy coat by centrifugation of 2 ml of whole blood for DNA extraction. Genomic DNA was isolated with the help of DNA extraction kit (Biotools Spain).

After the isolation of DNA from the samples, Polymerase Chain Reaction (PCR) using Thermal cycler (Eppendorf) was performed to amplify BAX gene to see whether there is any mutation in these genes using specific primers listed in table 1

Table - 1

BAX	**5′-CATTAGAGCTGC-GATTGGACCG-3′**	**5′-GCTCCCTCGGG-AGGTTTGGT-3′**

The reaction volume was 25 μL containing the reagents listed in table 2 and the PCR conditions for the above three genes are listed in table 3

Table 2 : Shows Reagents Used For Polymerase Chain Reaction of the above three different genes in Head & Neck Carcinoma cases and controls

Condition	BAX gene	
Initial Denaturation	94 ^{o}C, 5min	
Denaturation	94^{o}C, 30sec	30 cycles
Annealing	55^{o}C,30sec	
Extension	72^{o}C,30sec	
Final elongation	72^{o}C,5min	

After the PCR was over, 10 µL of the PCR products were run on 2.5% agarose gel using electrophoresis apparatus and PCR products were verified 109bp for Bax gene.

For analysing BAX (−248 G>A) polymorphism, the PCR product was digested by MspI (New England BioLabs, Beverly, MA) overnight at 37°C. The digested product was separated on 2.5% agarose gel with ethidium bromide and was photographed with the help of gel documentation system present in Biochemistry lab. The wild-type allele (GG) produced two bands (89 and 20 bp); wild-type/variant allele (GA) produced 20, 89 and 109 bp and the variant allele (AA) which lacks the MspI restriction site produced a single 109 bp band.

Results and observations:-

Results:-

This study was taken to understand the affect of Bax gene polymorphism in Head and Neck squamous cell Carcinoma patients. Genomic DNA was isolated from all Samples by using kit provided by Zymo Research.

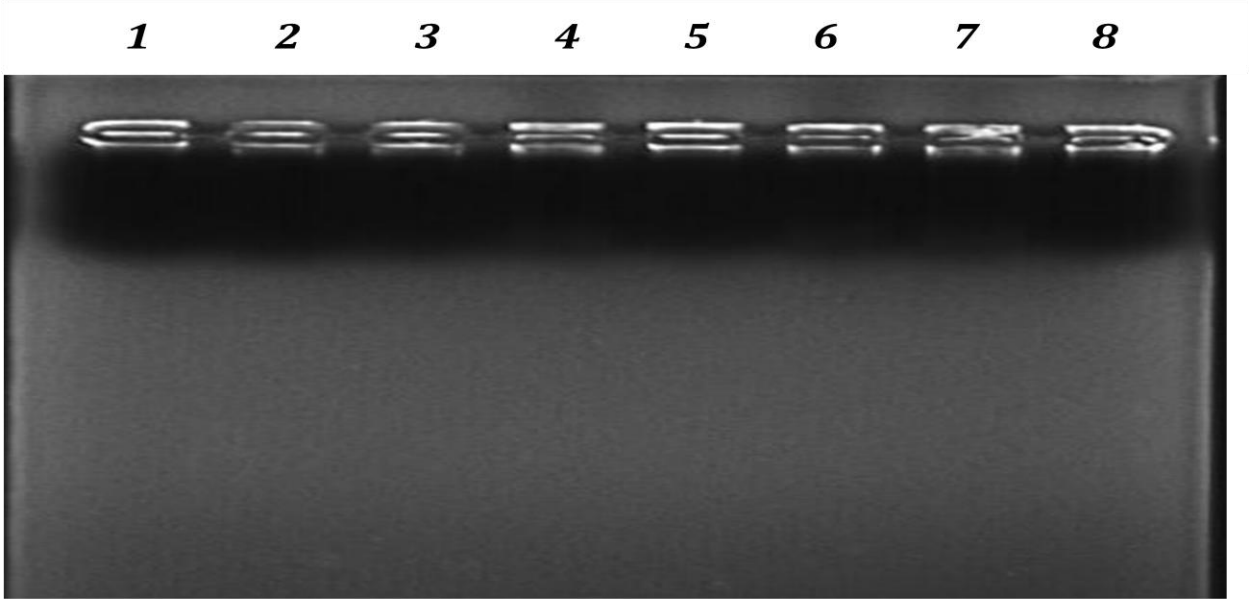


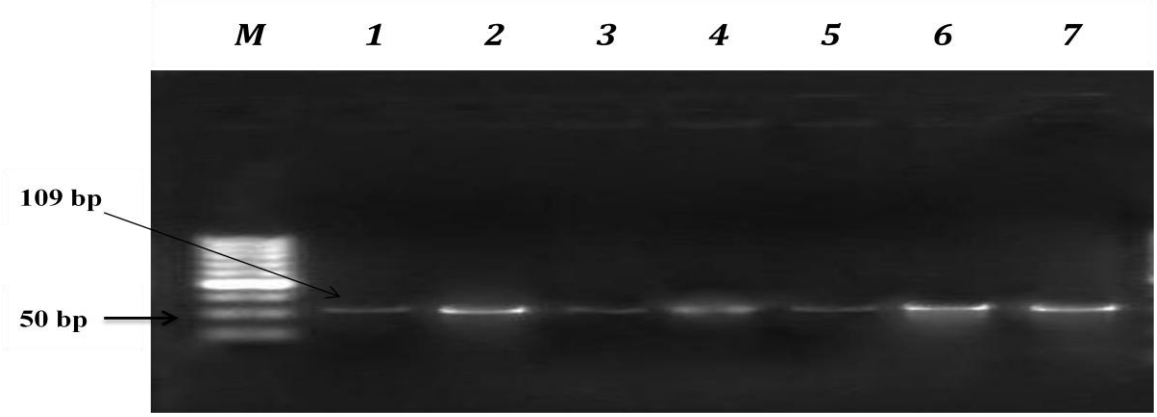
Figure 1: Representative gel picture run on 0.8% agarose showing isolated Genomic DNA
L1-L5= Cases of Head & Neck carcinoma
L6-L8= Controls

10 µl PCR products were subsequently digested with MspI restriction enzyme (Biotools, USA). A single 109bp fragment for homozygous AA genotype, two fragments of 89bp and 20bp for homozygous GG genotype and three fragments of 109bp, 89bp and 20bp for the heterozygous GA genotype (Figure 5). Negative control with ddH2O instead of DNA template was included in each PCR run. Data was analyzed and the frequency

Distribution of Bax gene genotypes/alleles in Head & Neck carcinoma cases and Controls:-

In the present study, HNSCC patients showed 17 (34%) GG, 24 (48%) GA and 9 (18%) AA genotypes and controls shows 21 (42%) GG, 16(32%) GA and 13 (26%) AA. The frequency of GG genotype was elevated in HNC controls compared to patients p>0.05 (P value 0.5368, OR 0.7114,95% CI 0.3161-1.601), where as frequency of GA (OR.

1.962 , 95% CI 0.9427-2.034, P=0.1527) and AA (OR. 0.6248, 95% CI 0.4514-1.342, P=0.4695) genotypes were elevated in patients compared to controls (Table 3).



PCR amplification of BAX (-248 G > A) gene showing band size of 109bp LM=50bp Molecular Marker DNA ladder, L1-L4= Cases of Head & Neck carcinoma, L5-L7=Controls

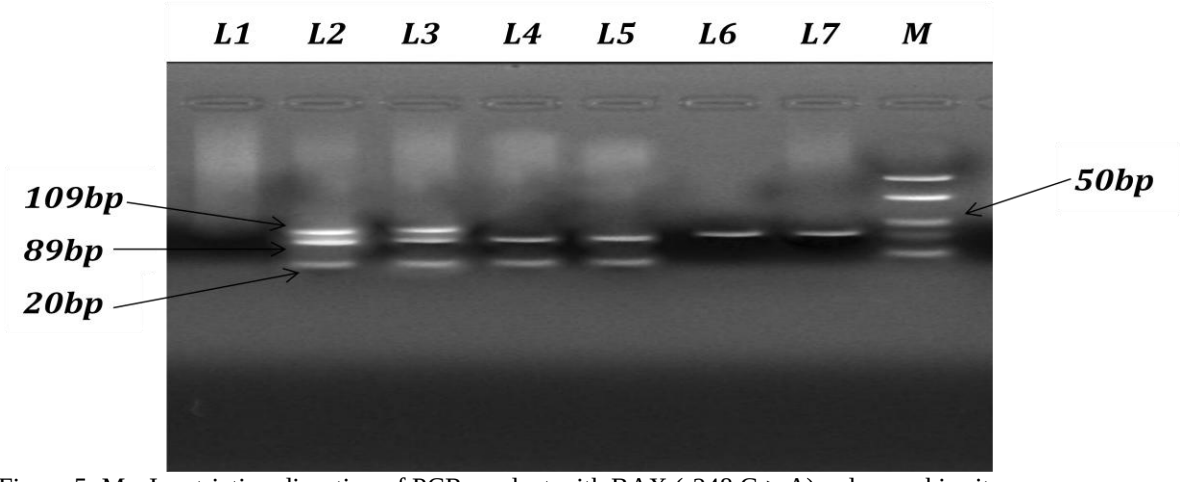


Figure 5: MspI restriction digestion of PCR product with BAX (-248 G > A) polymorphic sites,
L1= Negative Control (water)
L2, L3=Heterozygous GA genotype (89bp, 20bp & 109bp)
L4, L5= Homozygous GG genotype (89bp & 20bp)
L6, L7=Homozygous AA genotype (109bp) and
LM=Molecular Marker 50bp DNA ladder

Table 3 : Frequency of Bax gene genotypes

Results				
	Wild type GG	Heterozygous GA	Homozygous AA	Row Totals
CASES	17 (34 %)	24 (48%)	9 (18%)	50
CONTROLS	21 (42%)	16 (32%)	13 (26%)	50
Column Totals	38	40	22	100 (Total)

Data was analysed using Graph-pad prism softwear version-6 and the association was found insignificant in relation to BAX gene as P Value >0.05 (0.5368), RR = 0.8095, Odds ratio= 0.7114 and 95% CI= 0.4884 to 1.342

Discussion:-

This prospective study was conducted first time in the department of Otorhinolaryngology, Head and Neck Surgery, Government Medical College and Associated SMHS Hospital Srinagar in collaboration with department of Biochemistry and department of Pathology. The study included patients who were histopathologically documented cases of Squamous cell carcinoma Head and Neck visiting our OPD themselves or referred from some other

speciality or institution. A total of fifty patients were taken based on histopathological reports of biopsy. All the patients underwent surgery and Histopathology was done to confirm the malignancy and genetic analysis done to find out the Bax mutation if any.

Geographical distribution:-
Our study showed even district wise distribution in Kashmir valley. District Srinagar accounted for maximum number of 12 patients due to central Location of the Hospital and easy accessibility for the patients. Maximum no patients belonged to rural area where majority of the population resides.

Age and sex:-
The study group comprised of total 50 patients with majority of patients being males (82%) and maximum within age group of 40- 60 years. Mean age at diagnosis was 50.5±10 with youngest patient being age of 30 years male and the eldest was 85 years male. Females comprised only 18% of cases with youngest patient being 45 years and eldest was 70 years. The male: female ratio in our study was 4.56:1.

We believe that male predilection is because of addiction to smoking and tobacco chewing compared to females. A number of studies have found this male predilection in head and neck malignancies like a study done by Atlana et al and Kwok Hung et al. The Kwok Hung et al in their study of 101 patients of head and neck malignancies with 48 controls. They found male : female ratio of 80:21 (4:1) for patients 16:32 (1:2) for controls. The mean age of patients was 64 years (range 32 -87 years). Atlanta et al in their study on head and neck squamous cell carcinomas found that it is twice more common in males than female ratio.

- We found that most of our patients presented to us in the 4th to 5th decade of life which is consistent with study done by AH Kamel et al who reported maximum no of cases of head and neck cancers in same age groups and consistent with National cancer Institute were more of the cases were found over age 50 than among young people. Early presentation in our study is because of rise in psychological trauma and secondary increase in involvement in high risk behavior viz. smoking, alcoholism, etc and also easy access to the tertiary care SMHS hospital.
- The present study has 68% of patients that were smokers while only 32% were non-smokers which is consistent with the study of Annah et al who in his study found that cigarette smokers had elevated odd ratios for SCC head and neck and extensive literature about smoking confirmed our observations
- It was seen that the most common malignancy in our study is Laryngeal carcinoma (50%) followed by sinonasal malignancy (9%).
  The most common carcinoma in our study was carcinoma larynx followed by sinonasal, Hypopharyngeal and oral cavity tumours which is somewhat similar to study conducted by Kwok Hung et al found Larynx (40 patients) as commonest primary site, followed by hypopharyngeal (16 patients) and oropharyngeal (14 patients).
  Another study done by Majid M et al who in his study found 20 out of 41 head and neck were laryngeal Carcinoma (48.78%) which is consistent with our study were 50% of patients are of laryngeal carcinomas.
- It is clear from our study that almost 52% of patients with Head and Neck squamous cell carcinoma presented within 3 months and almost 6% presented after 1 year period, this is because most of our patients (50%) are Laryngeal SCC.

Summary:-

This study conducted in the Department of ENT, HNS in collaboration with Department of Biochemistry and Department of Pathology, Govt. Medical College, Srinagar, which included 50 documented cases of squamous cell carcinoma Head and Neck and 50 controls is summarized as:
Out of the 50 patients 41 (82%) were males rest being females with sex ratio of 4.56:1.
80% (40) were from rural area while as 20% (10) were from urban area.
54 % (27) patients presented in the 4th-6th decade of life.
68% (34) patients were smokers as against 32% (16) non smokers.
Laryngeal squamous cell carcinoma were most common 50% (25) followed by sinonasal malignancy 18% (9) .

Out of 50 patients of head and neck squamous cell carcinoma, 33 BAX SNP mutations were seen which comprises 66% of total (Heterozygous GC 24 and Homozygous CC 9). Rest of 17 patients that comprises 34 % were found with wild BAX genotype which is statistically insignificant.

From this study it is clear that most of the patients with well differentiated Laryngeal squamous cell carcinoma are carrying non-mutated wild type BAX gene while in poorly differentiated Laryngeal squamous cell carcinoma most of the patients are having either Heterozygous or homozygous mutations with equal frequency.

For BAX statistically insignificant association was seen with Squamous cell carcinoma Head and Neck.
While there was no evidence of associations between BAX (-248 G>A), BCL2 (-938 C>A) and squamous cell carcinoma head and neck , further analyses showed that among BAX and BCL2 heterozygotes after adjustment for age, sex, and smoking status, the BAX GC (Arg-Pro) genotype was associated with an elevated risk of SCCHN [OR=1.354, 95% CI= 0.8629-2.124)compared with the BAX GG genotype and OR=1.962, 95% CI=0.9129-2.465) compared with the combined genotypes (GG+CC)], Larger studies are needed to validate these findings.

Conclusion:-

Pro-apoptotic BAX gene with heterozygous mutation are at increased risk of squamous cell carcinoma head and neck which is approximately twice as compared to homozygous wild and homozygous mutated.

Ethical Clearance: Sought from ethical committee GMC Srinagar.
Conflict of interest: Nil
Source of funding: Hospital
Acknowledgement - Nil

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