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RESEARCH ARTICLE

EVALUATION OF MUTATION OF BCL2 SNPS WITH RISK OF SQUAMOUS CELL CARCINOMA HEAD AND NECK (HNSCC) IN KASHMIRI POPULATION.

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Abstract

Objective:- To find relationship between Bcl2 SNPs and squamous cell carcinoma head and neck (SCCHN) in patients from Kashmir population.

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:- Results were obtained from cases and 50 controls with a mean age of 32(range 25-39).

Conclusion:- In the present study statistically insignificant association of Bcl2 and Squamous cell carcinoma Head & Neck was seen ,further analyses showed that among BCL2 heterozygotes after adjustment for age, sex, and smoking status, BCL2 A variant genotypes were associated with a decreased risk of SCCHN (adjusted OR=0.40, 95% CI=0.158-1.01 for CA and OR=0.4, 95% CI=0.36-0.95 for combined (GG +CC genotype. These altered risks appeared to be consistent with the anti-apoptotic role of BCL2 in the pathogenesis of Head and Neck Squamous cell carcinoma. Our data suggest that the risk of SCCHN may be associated with these these SNPs of BCL2 promoter regions in SCCHN. Larger studies are needed to validate these findings.

Background:-

Head and neck squamous cell carcinoma (HNSCC) is the sixth leading cancer by incidence worldwide and eighth by death.[1] There are 0.5 million new cases a year worldwide. Two-thirds occur in industrialized nations. HNSCC usually develops in males in the 6th and 7th decade. The five-year survival rate of patients with HNSCC is about 40-50%. Head and neck cancer is cancer that starts in the lip, oral cavity (mouth), nasal cavity (inside the nose), paranasal sinuses, pharynx, larynx or parotid glands.

Most head and neck cancers are biologically similar. 90% of head and neck cancers are squamous cell carcinomas, so they are called head and neck squamous cell carcinomas (HNSCC). These cancers commonly originate from the mucosal lining (epithelium) of these regions. Head and neck cancers often spread to the lymph nodes of the neck, and this is often the first (and sometimes only) sign of the disease at the time of diagnosis.

Head and neck cancer is strongly associated with certain environmental and lifestyle risk factors, including tobacco smoking, alcohol consumption, UV light, particular chemicals used in certain workplaces, and certain strains of viruses, such as human papillomavirus.

Apoptosis regulator Bcl-2 is a family of related proteins govern mitochondrial outer membrane permeabilization (MOMP) and can be either pro-apoptotic (Bax, BAD, Bak and Bok among others) or anti-apoptotic (including Bcl-2 proper, Bcl-xL, and Bcl-w, among an assortment of others).

Active cell suicide (apoptosis) is induced by events such as growth factor withdrawal and toxins. It is controlled by regulators, which have either an inhibitory effect on programmed cell death (anti-apoptotic) or block the protective effect of inhibitors (pro-apoptotic).[2][3] Many

viruses have found a way of countering defensive apoptosis by encoding their own anti-apoptosis genes preventing their target-cells from dying too soon.

There are a number of theories concerning how the Bcl-2 gene family exert their pro- or anti-apoptotic effect. An important one states that this is achieved by activation or inactivation of an inner mitochondrial permeability transition pore, which is involved in the regulation of matrix Ca2+, pH, and voltage. It is also thought that some

Bcl-2 family proteins can induce (pro-apoptotic members) or inhibit (anti-apoptotic members) the release of cytochrome c into the cytosol which, once there, activates caspase-9 and caspase-3, leading to apoptosis. Although Zamzami et al. suggest that the release of cytochrome c is indirectly mediated by the PT pore on the inner mitochondrial membrane,[4] strong evidence suggest an earlier implication of the MAC pore on the outer membrane.[5][6]

Another theory suggests that Rho proteins play a role in Bcl-2, Mcl-1 and Bid activation. Rho inhibition reduces the expression of anti-apoptotic Bcl-2 and Mcl-1 proteins and increases protein levels of pro-apoptotic Bid but had no effect on Bax or FLIP levels. Rho inhibition induces caspase-9 and caspase-3-dependent apoptosis of cultured human endothelial cells.[7]

The site of action for the Bcl-2 family is mostly on the outer mitochondrial membrane. Within the mitochondria are apoptogenic factors (cytochrome c, Smac/Diablo homolog, Omi) that if released activate the executioners of apoptosis, the caspases.[8] Depending on their function, once activated, Bcl-2 proteins either promote the release of these factors, or keep them sequestered in the mitochondria. Whereas the activated pro-apoptotic Bak and/or Bax would form MAC and mediate the release of cytochrome c, the anti-apoptotic Bcl-2 would block it, possibly through inhibition of Bax and/or Bak.[9] The Bcl-2 family has a general structure that consists of a hydrophobic helix surrounded by amphipathic helices.

Many members of the family have transmembrane domains. The members of the Bcl-2 family share one or more of the four characteristic domains of homology entitled the Bcl-2 homology (BH) domains (named BH1, BH2, BH3 and BH4). The BH domains are known to be crucial for function, as deletion of these domains via molecular cloning affects survival/apoptosis rates. The anti-apoptotic Bcl-2 proteins, such as Bcl-2 and Bcl-xL, conserve all four BH domains. The BH domains also serve to subdivide the pro-apoptotic Bcl-2 proteins into those with several BH domains (e.g. Bax and Bak) or those proteins that have only the BH3 domain (e.g. Bim Bid, and BAD).
Various apoptotic stimuli induce expression and/or activation of specific BH3-only family members, which translocate to the mitochondria and initiate Bax/Bak-dependent apoptosis.

Aim of the study:-

- To find the association between SNPs of BCL2 with risk of squamous cell carcinoma head & 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 Kahmiri 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 BCL2 gene to see whether there is any mutation in these genes using specific primers Listed in table 1

Table1:- Shows Forward and Reverse Primers for BCL2 Genes Used For Polymerase Chain Reaction of in Head & Neck Squamous cell carcinoma cases and controls

Gene	Forward primer	Reverse primer
BCL2	5'-CTGCCTTCATTTATCCCAGCA-3'	5'-GGCGGCAGATGAATTACAA-3'

The reaction volume was 25 µL containing the reagents and the PCR conditions for the above BCL2 gene is listed in table 2

Table 2:- Shows Reagents Used For Polymerase Chain Reaction of BCL2 gene in Head & Neck Carcinoma cases and controls.

Reagents			BCL2 gene				
Modified DNA			1.5µl				
dNTPs			2 µl				
Forward & Reverse Primer			1.5µl each				
Taq Buffer			2 µl				
DMSO			3 µl				
Taq DNA Polymerase			0.2 µl (5U/ µl)				
Deionised Water			14.8 µl				
Total Volume			25µl				
Initial Denaturation	94 ^{o}C, 5min		94^{o}C, 5min		96^{o}C, 5min		
Denaturation	94^{o}C, 30sec	30 cycles	94^{o}C, 30sec	40 cycles	96^{o}C,45sec	35 cycles	
Annealing	55^{o}C,30sec		58^{o}C, 30sec		56^{o}C,45sec		
Extension	72^{o}C,30sec		72oC, 30sec		72^{o}C,30sec		
Final elongation	72^{o}C,5min		72^{o}C, 5min		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 300bp for BCL2 gene.

For analysing BCL2 (-838 C >A) polymorphism, the PCR product was digested by MspI (Biotools Spain) overnight at 37°C. The digested product was separated on 2.5% agarose gel with ethidium bromide and photographed with the help of gel documentation system present in Biochemistry lab. The wild-type allele (CC) produced two bands

(189 and 111 bp); wild-type/variant allele (CA) produced 189,111 and 300 bp and the variant allele (AA) which lacks the MspI restriction site produced a single 300 bp band.

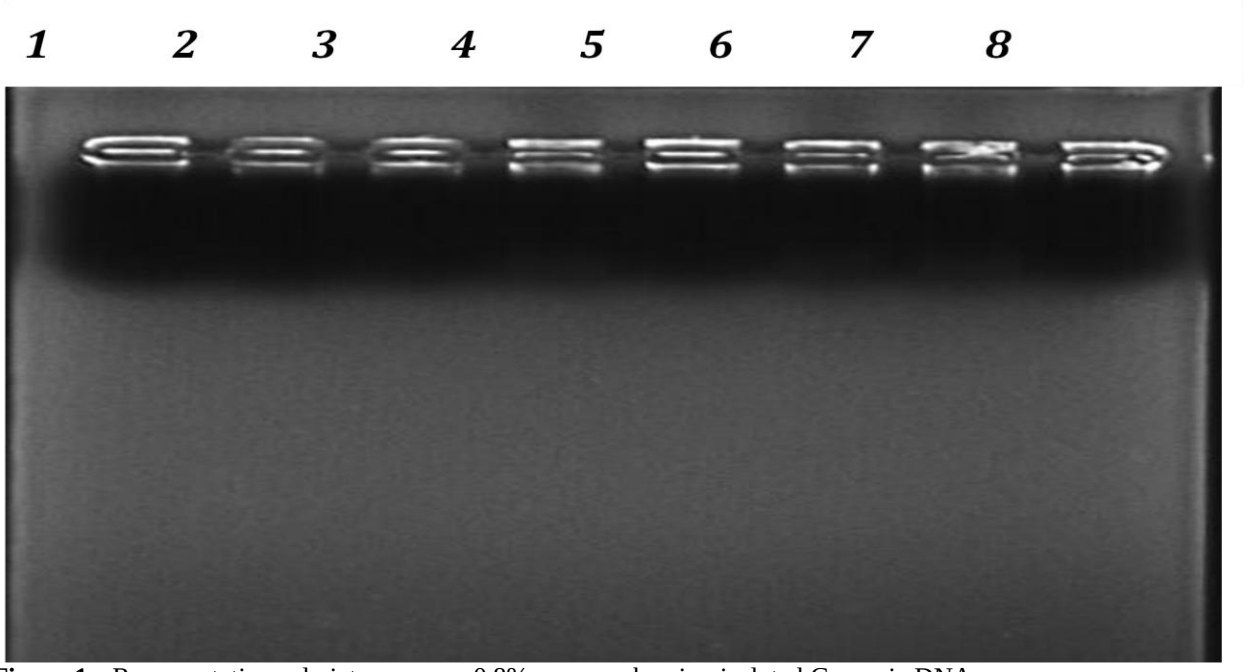

Figure 1:- Representative gel picture run on 0.8% agarose showing isolated Genomic DNA
L1-L5= Cases of Head & Neck carcinoma
L6-L8= Controls

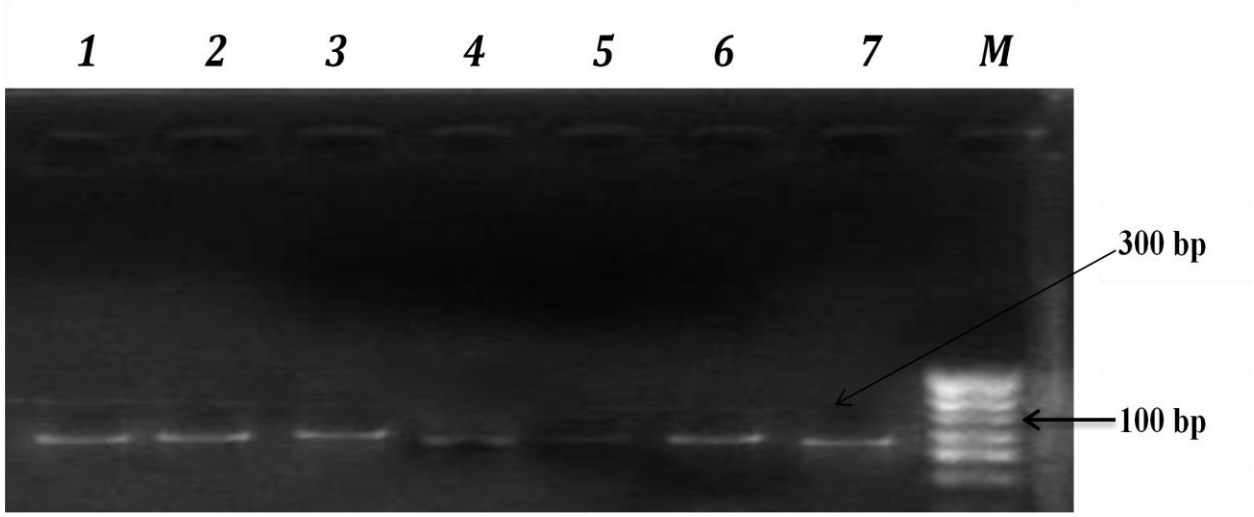

Figure 3:- PCR amplification of BCL2 (-838 C>A) gene showing band size of 300bp
L1-L5=Cases of Head & Neck Carcinoma
L6,L7=Controls,LM= 100bp ladder

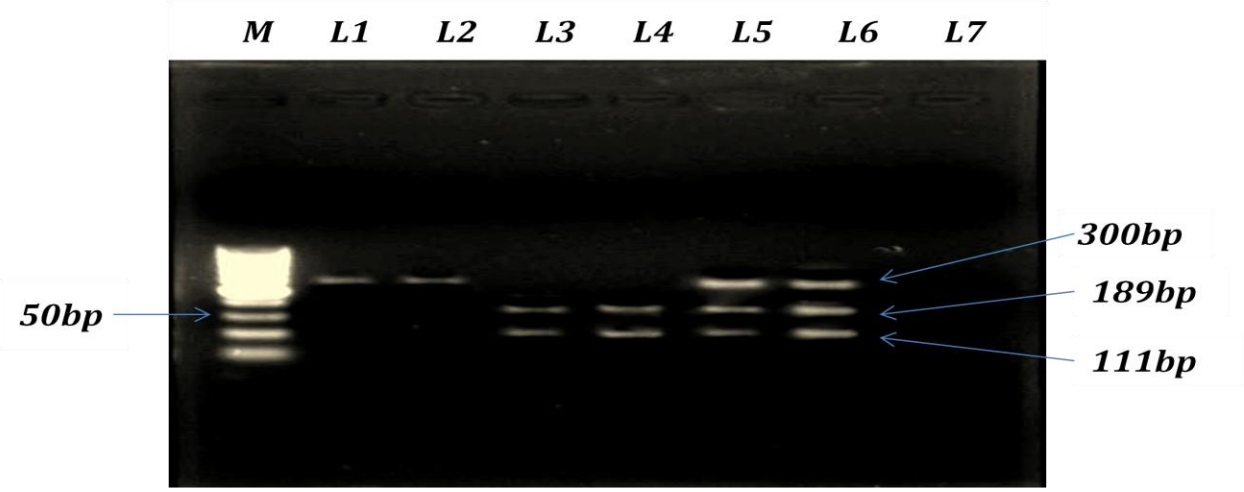

Figure 6:- BccI Restriction digestion of PCR product with Bcl2 (-838 C>A) polymorphic sites,
LM=Molecular Marker 50bp DNA ladder
L1, L2= homozygous CC genotype (300bp)
L3, L4-=homozygous GG genotype (189bp & 111bp)
L5, L6= heterozygous GC genotype (189bp, 111bp & 300bp) and
L7= Control (Water)

Results:-

In the present study, HNSCC patients showed 19(38)% CC, 16(32)% CA and 15(30%) AA genotypes and controls shows 13(26%) CC, 27(54%) CA and 10(20%)AA. The frequency of CC (P value 0.355, RR 1.286,OR 1.744,95% CI 0.683– 4.302)and AA genotype(p value 0.2837,RR 0.7678,OR 0.5733, 95% CI 0.2445-1.344)was elevated in HNSCC patients compared to controls which is statistically insignificant. The heterozygous CA (P value 0.04,RR 0.623,OR 0.4009, 95% CI 0.1776-0.9048) genotypes were elevated in controls compared to patients,which is Statistically significant (Table 3).

Table 3:- Frequency of Bcl2gene genotypes.

Results				
	Wild type CC	Heterozygous CA	Homozygous AA	Row Totals
CASES	19 (38%)	16 (32%)	15 (30%)	50
CONTROLS	13(26%)	27 (54%)	10 (20%)	50
Column Totals	32	43	25	100 (Grand Total)

Data was analysed using Graph-pad prism softwear version-6 and the association was found insignificant inrelation to BCL2 gene as P Value > 0.05 (0.2837) ,RR = 0.767, Odds ratio= 0.5733 ,95% CI= 0.8129 to 2.628

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, 31 BCL2 SNP mutations were seen which comprises 62% of total (Heterozygous GC- 16 and Homozygous CC- 15). Rest of 19 patients that comprises 38 % were found with wild BCL2 genotype which is statistically insignificant.

Conclusion:-

Anti-apoptotic BCL2 A variant genotypes were associated with a decreased risk of SCCHN (adjusted OR=0.40, 95% CI=0.158-1.01 for CA and OR=0.4, 95% CI=0.36-0.95 for combined (GG +CC genotype. These altered risks appeared to be consistent with the roles of the anti-apoptotic. Our data suggest that the risk of SCCHN may be associated with SNPs BCL2 promoter regions in SCCHN.

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