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PREVALENCE OF KRAS CODON 12 MUTATION IN PATIENTS WITH ORAL SQUAMOUS CELL CARCINOMA (OSCC) FROM SOUTH INDIAN POPULATION

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ABSTRACT

Background: *The RAS gene family is the most studied and best characterized of the known cancer-related genes KRAS is the most frequently altered gene, with mutations occurring in 17%–25% of all cancers. This study was designed to investigate the impact of Kras12 mutation in oral squamous cell Carcinoma*

Method: *We genotyped 100 cases with oral squamous cell carcinoma (OSCC)and 100 controls, using a Polymerase Chain Reaction-Restriction Fragment Length Polymorphism method.*

Results: *We found 13 cases harboring K-RAS G12D mutations & 9 cases harboring G12V among 100 OSCC cases. K-RAS codon 12 mutations was significantly different between OSCC patients and controls (P = 0.0007 & P=0.31) (Table 2). The G12D & G12V mutations were predominantly more in patients with chewing as the lone risk factor*

Conclusion: *Prevalence of K-RAS codon 12 mutations G12D and G12V was 13% & 9% in the study group. The frequency of KRAS mutations appears higher in the south Indian population indicating that this testing is very crucial for targeted therapy management. Further studies on K-RAS mutation associations among south Indian OSCC patients are needed.*

Keywords: KRAS, Oral Squmaous Cell Carcinoma (OSCC), Polymorphism, RFLP. K-RAS

Introduction

Oncogenes of the RAS family are strongly implicated in the pathogenesis of cancer. The Kristen Rat Sarcoma (*KRAS*) gene encodes a signal transduction protein, which in its active state forms a complex with a guanosine triphosphate (GTP) group. This complex is inactivated by the hydrolysis of GTP to guanosinediphosphate (GDP) (Schubbert, et al 2007). KRAS is a small G protein that acts as a transducer in the epidermal growth factor receptor (EGFR) pathway. K-RAS gene mutations have been reported in approximately 15–30% of human solid tumours (Spencer, et al 1995). This mutation is the most common abnormality of dominant oncogenes in human tumors and is a common event in the development and progression of adenocarcinomas of the pancreas (90%), colon (50%), thyroid (50%), bladder (50%), and lung (30%). The presence of a *KRAS* mutation is predictive for resistance to anti-EFGR monoclonal antibodies (mAbs) in advanced colon cancer (Perkins, et al 2014).

Two codons in the KRAS gene are mainly known to generate alternated proteins that are constitutively activated without the signal of a ligand bound to the EGFR. The most regularly found kinds of mutations are G>A and G>T transitions (*Palmirotta, et al 2009*) .The most frequent K-RAS alterations are detected in codon 12 (approximately 82%) and codon 13 (approximately 17%). K-RAS mutations in other positions, such as codons 61 and 146 are reported in minor proportion (1–4%). Both codons encode the amino acid glycine in the wild type protein. Replacement of one of the first two bases leads in both codons to an amino acid exchange in the KRAS protein, resulting in resistance of the tumor to the above-described treatment. Replacement of glycine leads to resistance of the GAPs, which are proteins causing the hydrolysis of KRAS bound GTP to GDP. The inability of GAPs to effect the GTP hydrolysis in mutated KRAS leads to a constitutive active protein

There are only few studies about the frequency of K-RAS codon 12 mutation in head and neck squamous cell carcinoma. Some suggested that mutational activation of RAS was not associated (Ruíz-Godoy, et al 2006 & Rizos, et al 1999) others found that K-RAS mutations had a direct causal role in the development of these cancers (Caulin, et al 2014 & Das, et al 2000). We have analyzed K-RAS codon 12 mutation to determine the exact frequency of this mutation. The aim

of the current study is to evaluate the prevalence of *KRAS* mutation in oral squamous cell carcinoma among the south Indian population treated at the MNJ Cancer hospital and try to correlate it with other clinicopathological factors.

Materials and Methods:

Sample Collection

The study population comprised 100 (45 males and 55 females) patients and 100 controls (55 males and 45 females). Patients were consecutively recruited from the MNJ cancer hospital, Hyderabad, Telangana, India, between January 2015 and August 2016. All patients were diagnosed with cancer. The controls were recruited from general population from Hyderabad during the same time period. Each control was matched to each case by age and sex. All subjects were given a questionnaire to investigate the demographic characteristics, history of cancer and alcohol and tobacco use. The clinical characteristics were collected from medical records, including tumor differentiation, tumor size, and chemotherapy. This study was approved by the MNJ cancer hospital Ethnical Committee, Hyderabad, and informed consent was obtained from all participants.

DNA Extraction and Genotyping

DNA from each sample of whole blood was extracted with the epicenter DNA mini Kit (Macherey – Nahel, Germany), as directed by the manufacturer's instructions. The concentration of DNA and the purity of each sample were measured by Nano drop 2000c (Thermo Scientific, USA). The K-RAS Codon 12 polymorphism was identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique.

PCR Amplification & RFLP Analysis

PCR amplification was carried out using the given primers, 5' ACTGAATATAAACTTGTGGTAGTTGGACCT3' and 5'- CCAGGTCCTGGTAAGAACT3' synthesized at Bioserve Biotechnologies (Hyderabad, India). PCR was carried out in a total volume of 50 μl. The PCR mixture contained 2.5 μl of 25 mM $MgCl_2$, 10 mM dNTP mixture,

160 pmol of each primer (forward and reverse primers), , 0.2 μl of *Taq* (5 U/μl)(Bioserve) and a DNA template. The reaction volume was made up to 50 μl with sterile water. The PCR reaction was carried out in an IQ5 thermocycler (Bio-Rad, Hercules, CA, USA) using the following optimal conditions. Initial denaturation was carried out at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 56°C for 30 sec and extension at 72°C for 30 sec. After completion of 35 cycles, a final extension step was carried out at 72°C for 10 min. The optimized PCR conditions given were used throughout the study. PCR amplicon size was identified by electrophoresis using 2% agarose gel containing ethidium bromide under standard electrophoretic conditions. The bands were visualized under UV light, and the gel was imaged using the Gel Dock System (BIO-RAD Gel-Imaging System). The amplified PCR product was checked on a 2 % agarose gel. 10µl PCR reaction mix was subsequently digested with 0.5µl *BstN1 restriction* enzyme (Fermatas, USA) and separated on 3% agarose gel electrophoresis. Homozygous genotype allele for Kras had a fragment size of 114 bp whereas heterozygous genotype had fragment sizes of 143bp and 14 bp

Statistical Analysis

Statistical analyses were performed using SPSS 7.0 version. To examine the association of K-RAS codon 12 polymorphisms between cases and controls and different clinical and pathological parameters. The difference between the groups was considered significant if the p value was less than 0.05. The odds ratio was used as an estimate of relative risk.

Results

Biological Characteristics of the study population

In the present study 100 blood samples from Oral squamous cell carcinoma (OSCC) patients and 100 blood samples from healthy controls were used. Distribution of the selected demographic characteristics and risk factors in control subjects and OSCC patients is shown in Table 1. The demographic profile included sex, age, and various habitual risk factors involved in the progression of OSCC. Out of 100 OSCC cases, 45 (45%) patients were male and 55 (55%) patients were female. In the control group 55(55%) were male and 45(45%) were female. Ages ranged from 9-87 years for cases and 21-76 years for controls. The mean age of OSCC patients

and healthy controls at the time of diagnosis was 50.53 and 55.27 years, respectively. OSCC patients were divided into four groups according to age at diagnosis; these were < 25, 26-45, 46-65, and >66 years. Incidence of OSCC cases and a control group was higher in the age groups 46-65 (60% and 70%) years when compared to other age groups. In this study we observed that tobacco chewing (32%, 30%), alcohol + tobacco chewing (17% & 13%) alcohol smoking (12%, 6%) and alcohol + smoking + tobacco chewing (10%, 6%) in OSCC patients and controls respectively. According to histological differentiation of tumor grades, 27%, 39% and 34%, patients were classified in three grades, poor, moderate, or well grade, respectively. In our study we found that well and moderate groups were high than poor.

Analysis of Genotype frequency of K-RAS (Codon 12)

The genotype frequencies of the K-RAS Codon 12 polymorphism among the controls and OSCC patients are shown in Table 2. The wild type, GGT-GAT and GGT-GTT genotypes were found in 78(78%), 20(20%) and 2(2%) of 100 OSCC patients and in 97(97%), 2(2%) and 0(0%) of 100 controls. We found 13 cases harboring K-RAS G12D mutations & 9 cases harboring G12V among 100 OSCC cases. K-RAS codon 12 mutations was significantly different between OSCC patients and controls (P = 0.0007 & P=0.31) (Table 2). At codon 12, a G>A & G>T transition was found which changed the amino acid from glycine to Asparitic (GGT/GAT) & glycine to valine (GGT /GTT). K-RAS codon 12 mutations was significantly different between OSCC patients and controls (P = 0.0007 & P=0.31) (Table 2).

Correlation K-RAS (Codon 12) genotypes and clinicopathological characteristics

We correlated the genotypes with demographic factors to understand the influence of Kras on the OSCC risk. The combined effect of gender age distribution and risk factors are summarized in table-I. The frequency of *KRAS* gene G12D & G12V mutations observed in men (7/13 & 5/9; 54% & 55%) was higher than that observed in women (6/13 & 4/9; 46% & 44%). the difference was statistically significant ($P = 0.05$) The frequency of G12D & G12V mutations in the median age group 46-65 was significantly high (15 cases out of 22 cases) (68.18%), compared to other age group in this study (Table-III). We also revealed the association of allele with the site of occurrence. In this study, the most predominant tumour location was Buccal mucosa (35%) followed by the tongue (23%). We observed that both stage 2 and stage 3 were associated with K-RAS polymorphism, clinical stage 3 being the most common stage (39%). The G12D & G12V

mutations were predominantly more in patients with chewing as the lone risk factor. Alcohol and smoking combination risk factor was also observed to be higher than other risk factors. (Table-3).

Discussion

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein, plays an important role in tumorigenesis and tumor progression of in cancer. EGFR has evolved as a relevant target in the treatment of cancer. *K-ras* serves as a mediator between extracellular ligand binding and intracellular transduction of signals from the EGFR to the nucleus (*Heinemann, et al 2009*). K-RAS is a proto-oncogene that normally relays signals from a variety of transmembrane receptors to intracellular effectors that regulate processes such as proliferation, survival, and migration. K-RAS mutations contribute to tumour formation; they are common in many epithelial tumours' including cancers of the pancreas, colon, and lung (Friday and Adjei, 2005). Mutation status of RAS in the tumor has important clinical implications as it may affect the response to treatment and has treatment-independent prognostic value (*Karapetis CS, et al 2008, Schunnert S, et al 2007 & Amado RG, et al 2008*). Human tumors very frequently express Ras proteins that have been activated by point mutation. Therefore, mutation detection of RAS is of clinical importance in cancer prognosis and treatment.

In the present study we have evaluated *KRAS* codon-12 mutations. In our study, we have observed a rate of 13% G12D (G>A) & 9% G12V (G>T) mutations in K-RAS codon 12 & in oral squamous cell carcinoma (OSCC). K-RAS codon 12 Mutations were significantly associated with OSCC (p=0.005). It seems that the G to A & G to T in K-RAS codon12 mutations may be important in the carcinogenesis of south Indian oral squamous cell carcinoma. Thus, results of present study provide further evidence of the important role of KRAS in oral carcinoma. The Gly>Val mutation is actually the most frequent *K-ras* mutation in pancreatic cancer (Janeik, S et al 2010). In colorectal carcinoma, it has been associated with poor prognosis and metastasis (*Al Mulla F, et al 1998*). Several studies reported that K-RAS codon 12 mutations, was associated with multiple kind of cancers, such as colorectal cancer. Our results support findings by *Eric Bissada et al 2013* reported 3.5% K-RAS codon 12 mutations in patients with locally advanced HNSCC, and they did not show an increased metastatic potential compared to their nonmutated

counterparts. It is interesting to note that variability in the frequency of *KRAS* mutations has been linked to ethnicity and certain environmental factors, such as use of chewing tobacco. RAS gene activation, usually by point mutation, may be an important event in the transformation of glandular tissue to adenocarcinoma (Rumsby, et al 1990).

The association between genotypes and clinicopathological parameters of OSCC was also analyzed in our study. It was found that there was significant association between K-RAS codon 12 mutations and age, gender. In our study we found that the frequency of K-RAS codon 12 mutations showed higher frequency in advanced stages (III & IV tumors) when compared to early stage. Naguib et al, 2011 also reported positive association between K-RAS mutations and the advanced stage of the tumor. We found significant association between K-RAS codon 12 mutations and buccal mucosa, tongue. Further our study also showed that K-RAS codon 12 mutations were present in 70% of cases with history of Chewers in OSCC. Our results indicate that K-RAS oncogene activation may play a role in the oncogenesis of chewing-related human oral squamous cell carcinomas. Saranath et al 1989 reported that 50% of chewing tobacco related OSCC cases in India has 5 to 10 folds DNA amplification K-RAS oncogene. In Taiwan, 18% of oral cancer patients investigated were found to have a Kras mutation, and these patients associated with chew betel quid (*Kuo et al., 1994*).

Conclusion:

We conclude that, K-RAS gene polymorphism is thought to function and modulate cancer risk. The findings of the present study indicate that K-RAS codon 12 mutations may play a crucial role in the development of OSCC in south Indian population. The sample size of our study is relatively small, which may reduce the statistical power Therefore, Further studies on *K-RAS* mutation associations among south Indian OSCC patients are needed to investigate the association between K-RAS codon 12 mutations and the risk of Oral squmaous cell carcinoma (OSCC).

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Table 1: Clinical Characteristics of the OSCC Patient & Healthy controls

Clinical Characteristics	n = 100 (Cases)	n = 100 (Controls)
Gender		
Males	45(45%)	55(55%)
Females	55 (55%)	45 (55%)
Mean age & Range Males	50.53/9-87	
Mean age & Range Females	55.27/30-75	
Age Distribution		
26-45	28 (28%)	22(22%)
46-65	60 (60%)	70(70%)
66 and above	12 (12%)	8 (8%)
Habitual Risk		
Alcoholics	16 (16%)	3 (3%)
Smokers	32 (32%)	13 (13%)
Tobacco chewing	52 (52%)	30 (30%)
Alcohol + Smoking	12 (12%)	6 (6%)
Alcohol + Tobacco chewing	17 (17%)	13(13%)
Smoking + Tobacco chewing	1 (1%)	4 (4%)
Alcohol + Smoking + Tobacco chewing	10(10%)	6 (6%)
No Habits	10(10%)	25 (25%)
Site of Diagnosis		
Tongue	23(23%)	
Buccal mucosa (BM)	35 (35%)	
Mandible	12 (12%)	
Oral Cavity	10 (9%)	
Retromolartrigone	8(8%)	
Floor of mouth	4(4%)	
Lip	3(3%)	
Base of tongue	2 (2%)	
Maxilla	2 (2%)	
Palate	1 (1%)	
Staging		
Stage 1	6 (105)	
Stage 2	21 (19%)	
Stage 3	39 (50%)	
Stage 4	34(21%)	

Table 2: Distribution of Genotype of K-RAS Codon 12 in Oral squamous cell carcinoma (OSCC) and controls.

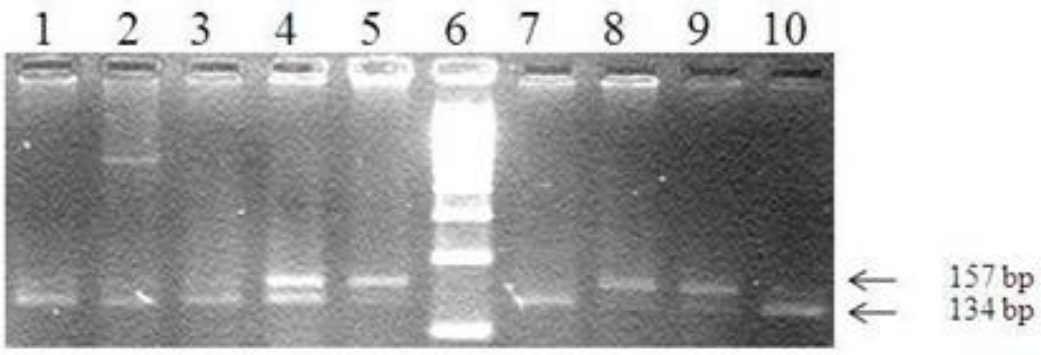
Genotyping	Cases n=100(%)	Controls n=100 (%)	Odds Ratio	95% CI	P-Value
Wild	78(78%)	97 (97%)	9.11	2.632-31.5	0.0005
GGT-GAT	13(13%)	2(2%)	8.08	1.77-36.89	0.0007
GGT-GTT	9 (9%)	1(1%)	11.19	1.38-90.25	0.02

Table 3: Correlation of OSCC patient's demographic factors and K-RAS codon 12 genotype frequencies.

Demographical Characteristics	Wild N= 78	Hetero N=13	Homo N=9	Chi square	CI Interval	Statistics P-value
Gender				1.07	0.73-0.4	0.005
Males (n=45)	33	7	5			
Females (n=55)	45	6	4			
Age Distribution						
<25	1	0	0			
26-45	22	2	3	0.84	1.34-1.69	0.000
46-65	45	9	6			
66 and above	10	2	0			
Site of Diagnosis						
BM	31	4	5			
Tongue	18	6	2			
BOT	2	0	0			
FOM	3	0	0			
LIP	3	0	0	0.47	1.91-3.14	0.000
Mandible	11	0	0			
Maxilla	2	0	0			
Palate	0	0	0			
RMT	3	0	0			
Oral Cavity	5	2	2			

Staging						
Stage 1	5	0	0			
Stage 2	13	2	0	0.35	1.46-1.93	0.000
Stage 3	30	8	6			
Stage 4	30	3	3			
Habitual Risk						
Alcoholics	0	0	0			
Smokers	5	3	1			
Chewing	25	7	5			
Alcohol+Smoking	8	0	0	0.61	2.02-2.81	0.000
Alcohol+Chewing	22	0	2			
Smoking+Chewing	1	0	0			
Alcohol+Smoking +Chewing	8	2	0			
No Habits	9	1	1			

Fig-I: K-Ras gel



Representation 2% agarose gel stained with ethidium bromide showing Lane 1-3, 7, 10: 134bp & 23bp (Wild type) Lane 4, 8, 9: 157bp, 134bp, & 23bp (Heterozygous mutant) Lane 5 & 8: 157bp (Homozygous mutant) and Lane 6 100 bp DNA Marker