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Study on PIK3CA Gene Mutations in Oral Squamous Cell Carcinoma among South Indian populations

Jayalalitha Sathiyamoorthy[1]*, Vidyarani ShyamSundar[2], N.Aravindha Babu[2], Subbaih Shanmugam[3], Jagadeesan.G.Mani[3], Ponnuraja Chinnaiyan[4], Aparna Kalyanaraman[1] and Rajeswary Hari[1]

[1]Department of Biotechnology, Dr. M.G.R Educational and Research Institute, Maduravoyal, Chennai-95, India.
[2]Centre of Oral Cancer Prevention and Research, Sree Balaji Dental College & Hospital, Bharath Institute of Higher Education (Bharath University), Pallikaranai, Chennai-99, India.
[3]Centre of Oncology, Government Royapettah Hospital & Kilpauk Medical College, Chennai, India.
[4]Department of Statistics,National Institute of Research in Tuberculosis, No:1, Sathiyamoorthy Road, Chetpet, Chennai – 600031, India.
*Correponding author E-mail: jayalalitha.vit@gmail.com

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The present investigation was performed in South Indian Populations to determine the hotspot mutation frequency in Oral Squamous Cell Carcinoma (OSCC) patients with PIK3CA gene Exon 9 and Exon 20 and its correlations with help of their clinical characteristics leading to these mutations. PI3KCA belongs to a group of regulatory heterodimeric lipid kinase which is involved in proliferation of cells, apoptosis and as well in metastasis which is controlled by PIK3CA gene is subjected to high frequency of somatic mutation in various tumors including OSCC. Methods: **Total of 25 OSCC patients samples comprising of male and female subjects from Government tertiary care Centre were included in this study. Tumor specimen samples were collected and amplified for PIK3CA gene by PCR and subjected to genomic DNA Sequencing.** Results: **Our findings showed total of 20% of oncogenic frequency in PIK3CA gene. We also observed two hot spot mutations (E545K) in exon 9 gene and three hot spot mutations (H1047Q, H1047Y, H1048Q) in exon 20 gene in our study populations.** Conclusion: **Based on our findings it may be concluded that PIK3CA gene Exon 9 and Exon 20 contributes to a major role in pathogenesis on OSCC among South Indian populations may act as therapeutic target for a anticancer drug for the treatment OSCC. .**

Keywords: OSCC, PIK3CA, tumor, hotspot.

The cancer that affects the mouth and pharynx involving cancers of tongue, lips, mouth floor, gingiva, palate, alveolar mucosa, tonsils, uvula and salivary glands refers to oral cancer. Oral cancer, a malignant neoplasm ranks third among the cancer types with a prevalence of 45% in India. Oral Squamous Cell Carcinoma (OSCC) is an aggressive malignant epithelial cancer affecting the oral cavity which occupies the sixth most common neoplasm with an increasing incidence

and mortality globally every year[1]. It accounts for 80 to 90% of oral cavity malignancy[2]. In India, OSCC ranks third among the frequent cancers with an annual incidence of 52,000 patients and a mortality of 77% in developing nations.

Inspite of drastic improvement in therapeutic treatment, the five year survival rate is reported to be poor comprising of about 50-60% specifically due to the fact that most of patients are resistant to chemotherapy [3-5]. In addition, survival and prognosis of the OSCC patients are affected mainly by TNM (tumor node Metastasis) staging which is the clinical classification of cancer patients based on the severity and the extent of cancer proliferation. This classification contributes sufficiently in the survival of the patients since there will a less chance for the patients survival when they are diagnosed at TNM stage III and IV[6,7]. Further, improper clinical management at diagnosis and delayed clinical presentation of patients might be an important factor caused to the tongue lesion and lesion diameter[8].

The Phosphatidylinositol 3 Kinase (PIK3CA), gene is located on chromosome 3q26.32 which codes for the catalytic subunit, p110á, of class IA PI3K, which plays a very important role in cell proliferation, growth and apoptosis[9]. When activated by growth factor tyrosine kinases, the PI3K complex is assembled on the inner cell membrane, where this enzyme phosphorylates the substrate phosphatidylinositol (4,5) biphosphate (PIP2) generating its own second messenger phosphatidylinositol (3,4,5) triphosphate (PIP3), which in turn recruits the serine-threonine kinase AKT and 3-phosphoinositide- dependant kinase (PDK) to the plasma membrane[10]. PDK phosphorylates and activates AKT, which then regulates a range of target downstream proteins that control a variety of intracellular proteins.

It is observed that 75% PIK3CA mutations are found to occur frequently in various cancers such as OSCC ,colorectal, breast, brain, gastric, ovarian, oral, and lung respectively[11]. Though several studies have showed various hotspot mutations in PIK3CA gene, mutations such as E542K, E545K and H1047R have significant oncogenic frequency in majority of cancers which in turn elevate the lipid kinase activity and thereby leading to the subsequent activation of the downstream Akt signalling mechanism[12].With the above scenario the present investigation was undertaken to analyse the the hotspot mutation frequency of PIK3CA Exon 9 and Exon 20 gene in Oral Squamous Cell Carcinoma (OSCC) patients belonging to South India.

MATERIALS AND METHODS

Subject Characteristics

A total Number of (n = 25) inclusive of both males (n =17) and female population (n =8) Oral Squamous cell carcinoma (OSCC) samples were collected from Government tertiary care Centre, Department of Surgical Oncology, Chennai. All the individuals were provided prior informed consent for participation of study and patient's demographic details, habits were personally interviewed and recorded for study purpose. Tumor specimens were obtained at the time of surgery in RNA Later solution (Sigma, USA) and subjected to our further study. The study was approved by Ethics Committee of A.C.S Medical College & Hospital, Chennai.

Genomic DNA Isolation

Genomic DNA was extracted using Nucleospin Genomic Tissue DNA Isolation kit (Takara, Clontech, India) following the manufacturer's instruction protocol. DNA yield and purity were quantitated and assessed using the Nanodrop lite. Samples with an OD260/OD280 ratio e"1.8 were used for further downstream process, including PCR amplification and genomic DNA sequencing analysis.

PIK3CA Mutational screening

The amplification of PIK3CA gene exon 9 and exon 20 was performed by PCR using oligonucleotides reported (Shown in table 1). Primers were designed in order to produce a fragment of 261bp for b-actin, 340bp and 34bp (Amplicon size) for PIK3CA exon 9 and 20 gene. PCR products were run in 2% agarose gel electrophoresis containing 1.5% Ethidium Bromide. Total volume of 50ul reaction mixture was prepared which contained 10-50ng template DNA, 10 pmol/ml of each primer and 2X solution of Emerald Amp® GT PCR Master Mix (Clonetech, US).The PCR Condition for PIK3CA Exon 9 were set at the initial denaturation at 95°C for 7 min, followed by 37 cycles at 95°C for 45 sec, 55°C for 30 seconds and extension at 72°C

for 45 seconds with the final extension of 72° C for 5 min and for PIK3CA Exon 20 were set at the initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 60°C for 1.15 seconds and extension at 72°C for 1.30 seconds with the final extension of 72°C for 2 min in Eppendorf PCR Thermo cycler (Eppendorf, USA). PCR products were separated with 2% agarose gel electrophoresis in order to ensure the integrity of the targeted amplification before sequencing. The amplified PCR Products were purified and were sent to Scigenomics, Cochin for DNA sequencing. The samples were sequenced using ABI - 96 capillary 3730 XL DNA Sequencer.

Statistical Analysis

Statistical Analysis was performed for the study population with the help of IBM SPSS Software V.20.The analysis was performed with chi square and fisher exact test. A *P*-value less than 0.05 was considered to be as statistically significant.

Table 1. Primer Sequence for PIK3CA and β actin

	Gene Sequence	Amplicon size(bp)
PIK3CA Exon 9		
Forward Primer	5'-TGAAAATGTATTTGCTTTTTCTGT-3'	340
Reverse Primer	5'-TGTAAATTCTGCTTTATTTATTCC-3'	
	PIK3CA Exon 20	
Forward Primer	5'-GCTCCAAACTGACCAAACTGTTC-3'	346
Reverse Primer	5'-TGGAATCCAGAGTGAGCTTTC-3'	
	β actin	
Forward Primer	5' -CTCCATCCTGGCCTCGCTGT-3'	261
Reverse Primer	5' -GCTGTCACCTTCACCGTTCC-3'	

Table 2. Baseline Clinical cohort of our study populations

Patients clinical details	OSCC (n=25)	OSCC (%)
Age (Mean ± SD)	48.84±8.98	-
Age (range in yrs)	30-67	-
Gender		
Male	17	68%
Female	8	32%
Chewing		
Yes	22	88%
No	3	12%
Smoking		
Yes	14	56%
No	11	44%
Alcohol		
Yes	11	44%
No	14	56%
T- Stage		
T2	9	36%
T3	3	12%
T4	13	52%
Pathological Differentiation		
MDSCC	9	36%
WDSCC	14	56%
PDSCC	2	8%

RESULTS

Clinical characteristics of the study cohort

The base line of the patient's clinical characteristics such as age, gender, chewing or smoking tobacco, alcohol consumption among the study population were studied and tabulated in the Table - 2. The mean age of the study cohort was ranged from 30-67 years (mean 48.84 years) and among them 17(68%) were male and 8(32%) were females. It is observed 88% of them had history of chewing habits, 56% with smoking habits, 44% with alcoholic habits in the study populations. In addition oral cavity cancer diagnosis is done by the TNM staging [Tumor Node Metastasis) of malignant tumours system International Union against Cancer (UICC). Based on TNM staging in the present investigation 36% of the patients were belonging to T2 stage 12% were among T3 stage and 52% among T4 group. However the pathological identification, 56% of the study populations showed WDSCC, 36% with MDSCC and 8% with PDSCC respectively. In the present

Table 3. Mutations observed in PIK3CA gene of Exon 9 and Exon 20

S.NO	Cosmic database Number	Nucleotide changes	Amino acids	Mutation observed	No: of patients observed with mutations	Frequency (%)	Domain
PIK3CA Gene (Exon 9)							
1	COSM763	c.1633G>A	p.E545K	Missense mutation	2	8%	Helical Domain
PIK3CA Gene (Exon 20)							
2	COSM774	c.3139C>T	p.H1047Y	Missense mutation	1	4%	Kinase Domain
3	COSM1041525	c.3141T>A	p.H1047Q	Missense mutation	1	4%	Kinase Domain
4	COSM27157	c.3144T>G	p.H1048Q	Missense mutation	1	4%	Kinase Domain

Table 4. Clinico-pathological characteristics of study population in correlation with PIK3CA gene

Patients clinical details	Mutation observed in our study cohort	Absence of mutation in our study cohort	p value
Total number of Study cohort	5(20%)	20(80%)	
Age			
≤45	1(20%)	7(35%)	1.000
>45	4(80%)	13(65%)	
Gender			
Male	2(40%)	15(75%)	0.283
Female	3(60%)	5(25%)	
Cancer Location			
Buccal Mucosa	3(60%)	10(50%)	1.000
Tongue	2(40%)	10(50%)	
Chewing			
Yes	5(100.0%)	17(85%)	1.000
No	0(0.0%)	3(15%)	
Alcohol			
Yes	1(20%)	10(50%)	0.341
No	4(80%)	10(50%)	
Smoking			
Yes	2(40%)	12(60%)	0.623
No	3(60%)	8(40%)	
T Stage			
T2	1(20%)	8(40%)	0.344
T3	0(0.0%)	3(15%)	
T4	4(80.0%)	9(45%)	
Pathological Grading			
Well Differentiated	1(20%)	13(65%)	0.070
Moderately Differentiated	4(80%)	5(25%)	
Poorly Differentiated	0(0%)	2(10%)	

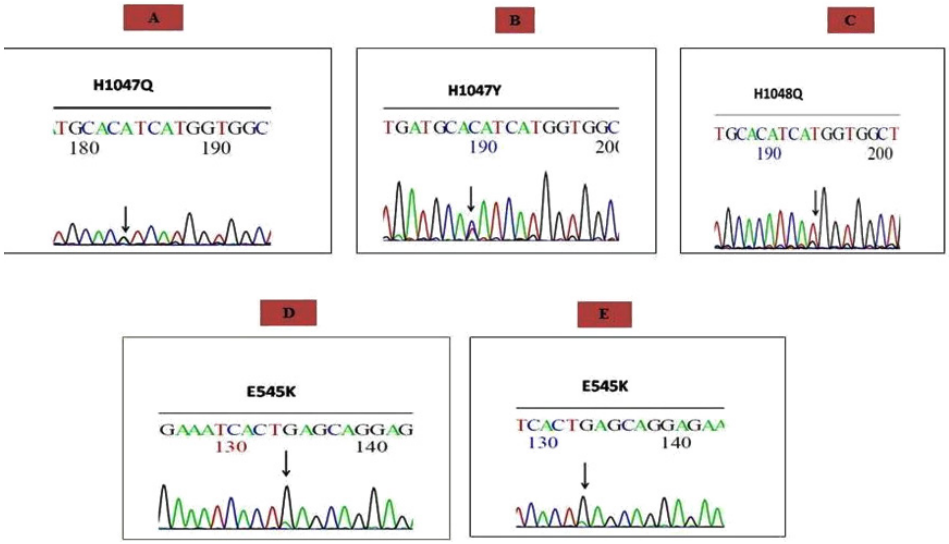

Fig.1. Sequence analysis of observed PIK3CA mutations in our samples: a) H1047Q(c.3141T>A), (b) H1047Y (c.3139C>T), c) H1048Q (c.3144T>G) of PIK3CA exon 20 geneand (d and E) E545K (c.1633G>A) of PIK3CA exon 9 gene

study we therefore investigated the genomic DNA of 25 OSCC tumours with direct DNA sequencing of the PCR products of PIK3CA gene of exon 9 and exon 20 and we found five somatic oncogenic hotspot mutations in exon 9 and exon 20 of PIK3CA gene.

PIK3CA mutations types and frequency

PIK3CA gene is responsible for the synthesis of the PIK3CA lipid kinase enzyme. The PIK3CA gene exon 9 and exon 20, encodes for helical domain and as well for kinase domain respectively. We observed two oncogenic hotspot mutations of PIK3CA gene with exon 9 and three oncogenic hot spot mutations in exon 20 of PIK3CA gene with total oncogenic frequency of 20%. Figure 1 represents the gene sequence analysis of exon 9 and exon 20 of the PIK3CA gene of the study population. Among the OSCC samples analyzed, missense mutations were reported in all these 20% patients. An average of 8% revealed the mutation of E545K in exon 9 in which the nucleotide guanine (G) is replaced by adenine (A). This reflects a change in the amino acid sequence that is glutamic acid (E) is replaced by lysine (K) due to the missense mutation in the helical domain of the phosphoinositol kinase enzyme. In the case of exon 20 gene which codes for the kinase domain of the same enzyme three patients (12%) found to have three different types of mutations H1047Q, H1047Y, H1048Q respectively which corresponds to the nucleotide replacement such as cytosine (C) to thymine, thymine (T) to adenine (A) and thymine (T) to guanine (G). As expected the original amino acid residue will also changes from histidine to glutamine in two positions and histidine to tyrosine in one position of the kinase domain of the PIK3CA enzyme (Summarised in Table 3).

Correlation between PIK3CA mutations with clinicopathological characteristiscs

We also attempted to study the correlations between mutations and as well as clinicopathological characteristics of the patients inducted for the present study. But we found no significant association between PIK3CA mutations with age, gender, cancer location, disease stage, habits and pathological grading (Shown in Table. 4)

DISCUSSION

Different varieties of somatic mutations have also been reported among the PIK3CA gene involving pancreatic, breast, gastric, hepatocellular, lung, esophageal, ovarian, and as well as more importantly even among the head and neck squamous cell carcinoma[13-17]. It is a well known

concept that somatic mutations contributes sufficiently in the pathogenesis of the cancer. So we made an attempt to identify the presence of the mutations in the PIK3CA gene in OSCC patients belonging South Indian populations. However, we observed a 20% oncogenic frequency in PIK3CA gene. Interestingly study conducted in American populations reported 20.8% mutation frequency among OSCC patients[18]. A 12.5% mutational frequency was observed in gall bladder carcinoma for the same gene[19]**.** Recently Chang et al has reported a 13.93% mutational frequency of PIK3CA gene in the OSCC patients with a rare mutation of Q546[20].

We observed the mutation of 1633G>A (E545K) in the exon 9 of PIK3CA gene. The same mutational target was observed in the Caucasian OSCC populations with frequency of 5.7% by Zanaruddin et al apart from the mutations in Q546R, M1043I, and H1047R[21]. In our present study three different types of mutations 3139C>T(H1047Y), 3141T>A (H1047Q) and 3144T>G (H1048Q) in exon 20 gene were observed in the kinase domain of the enzyme. Though Chiosea et al have already reported H1047Y mutation in the kinase domain of the human papilloma virus positive oropharyngeal squamous cell carcinoma the other two mutations H1047Q and H1048Q were novel hot spot mutations, for the first time reported in the kinase domain of the enzyme among the South Indian OSCC patients[22]. These somatic missense mutations were thought to increase the kinase activity of PIK3CA. Based on several studies more than 75% of these mutations affect the helical (exon 9) and kinase domains (exon 20) of the gene[23]. Moreover hot spot" mutations occurring in E542K,E545K, and H1047R in the PI3K enzyme have the ability to increase the catalytic activity to phosphorylate AKT and thereby activate the PI3K-AKT signaling pathway leading to the increased cell survival by inhibiting apoptotic mechanism[24]

Genetic alterations PIK3CA gene have a significant effect in the p110á catalytic subunit of the enzyme. This alteration in the p110á catalytic subunit subsequently increases lipid kinase activity of the enzyme leading to the activation of Akt signaling. According to Kang et al these mutations have the tendency to promote aberrant cell growth *in vitro* and cause uncontrolled cell proliferation at a rate of 50% in newly hatched chicks. Though the exact mechanism of functional transformation of the enzyme towards these hot spot mutations were not clearly understood, substitutions in the amino acid residues in the helical and kinase domain may contribute towards these catalytic transformations[25]. Various biochemical studies have confirmed the change in the amino acid residue (glutamic acid to Lysine) as a result of mutation, may change the nature of the helical domain (acidic to basic) that can modify the catalytic activity of the enzyme. Further its interaction with kinase domain may also be increased due to the other modified amino acid residues due to the mutation observed in that domain[26].

We observed from our study population, that all the five patients had a history of chewing habits which might be one of the major causes for the mutation in them. A similar study reported on OSCC patients in South Indian population's showed 10.5% oncogenic hot spot mutation[27]. The observed somatic mutations of PIK3CA gene in our study was seen to have higher oncogenic frequency because of less sample size when compared to the above study reported. However, the correlation between the gene expression and clinic-pathological results were analysed it was found to be statistically less significant.

PIK3CA, important gene which plays an vital mechanism in the PI3K signaling pathway, but, knowledge towards PIK3CA mechanism is still considered to be critical. In addition, studying genetic alterations on the mechanism in PI3K/AKT signalling pathway acts as a very important role in the tumorigenesis as they are considered as vital factors in the pathogenesis of tumors. The prevalence of oncogenic mutations in the PIK3CA gene is not frequently observed among South Indian Population. Oncogenic mutations in PIK3CA gene showed a very minor role in the development of OSCC in South Indian populations; however, their role in personalized combination therapeutics cannot be ignored. With this insight we are the pioneers in the identification of the possible hot spot mutations in PIK3CA gene. So OSCC can be treated with specific kinase inhibitor pathway based combinational therapy instead of conventional treatment strategy.

CONCLUSION

Thus from our preliminary study, we showed the presence of gene mutations in PIK3CA gene in OSCC patients among South Indian populations, implying that PIK3CA pathway can play as an important targeted drug therapy in near future for Oral Squamous cell carcinoma patients. However, this study was performed in smaller sample size; it has to be evaluated in larger scale samples and complete follow up in near future for better outcome of the study.

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