ORIGINAL ARTICLE

Tobacco Associated Oral Cancer amongst Pakistani Group

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ABSTRACT

Objective: The aim of this study is to evaluate molecular progression of smokeless tobacco lesions to oral cancer among chewers in Pakistani population.
Materials & Methods: This rendomized control experimental study was carried out between September 2011 to August 2012, Department of Biotechnology, University of Karachi. All chemicals and reagents and instrumentation were obtained from the Biotechnology Department. High Speed Centrifugation was performed at the Centralized Science Laboratory, University of Karachi. Two hundred grams of fifty biopsy proven Oral Squamous Cell Carcinomas, were obtained from Jinnah Postgraduate Medical Centre and Civil Hospital Karachi. All samples were obtained directly from surgical specimen, placed in plastic labeled bottles with patient data and stored in normal saline at -20°C. DNA Extraction Kits from Fermentas^{R} were utilized for extraction of genomic DNA, with amplification for Exon-5 of p53 protein was carried out.
Results: Nineteen positive of fifty patients had 12 of 30 male and 7 of 20 females, with a single band and dimmers. Age predilection showed 7 cases from the fourth decade, 2 each from third, fifth, sixth and seventh decades, while one positive from the second decade. Buccal mucosa was most prevalent site with 9 of 19 cases positive. Alveolus and tongue had 4 of 19 each, while lip had 2 case positives. Betel quid was commonly used, as compared to moist snuff.
Conclusion: 186 bp PCR product were sequenced for mutational analysis of p53 in our local community, with no mutations seen.

Key words: Oral cancer, snuff, p53, mutation.

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INTRODUCTION

In Pakistan tobacco is smoked such as cigarettes, pipe, bidi or chewable form such as paan with or without tobacco. Naswar is another form of smokeless tobacco which is almost always in moist form. Its main contents are tobacco and lime wrapped in cellophane paper or a container. It is placed as moist ground leaf of tobacco in loose small portions in the lower labial sulcus area to absorb slowly. Paan with or without tobacco (using radicals), could be paan with paper leaf with slaked lime.[1] Loose leaf ground tobacco with or without paan (betel leaf vine) as part of quid, or paan with paper leaf, areca nut tobacco condiments. Keeping moist, maintains its pH, being vital for its absorption in mucosa. This is called Snuff in Scandanivia and USA Areca nut alone in pure dry roasted and dyed with chemicals. Gutka presents as chopped areca nut with tobacco lime. Tobacco leaves added are of variety of types i.e. dry, moist with varying nicotine and nitrosamine content at variable pH, affecting carcinogenity of quid. Also dose quantity, day and duration of year also effect carcinogenic potential.[2]

Snuff has been reported to be used from Sweden.[1] Naswar in Pakistan and Afghanistan is available as loose or portion moist. It contains lime, tobacco, bicarbonate. Since naswar is produced in cottage industry. No quality is same for nicotine or TSNA, as it varies with pH and lime content.[3] Naswar containing nicotine, nitrosamines and other non combustable carcinogens can produce dysplasia. Epithelial dysplasia matures to cause a malignant change amongst the oral epithelium, seen oral squamous cell carcinoma.

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Oral and pharyngeal cancer has developed as the fifth most common malignancy globally. Asian subcontinent has a higher cancer incidence amongst betel quid chewers, compared to Europeans alcohol users. Asymptomatic oral cancer presents with lymph node involvement and metastasis at an early stage. A 5 year of cancer survival rate of 50% in Europe Clinically bilateral fibrosis of buccal mucosa presents, loss of epithelial and connective tissue elasticity.[4] Oral cancer has been regarded as the sixth most common cancer worldwide. Tobacco and its carcinogens i.e. nornicotine (4 types) are the prime contributors to carcinogenesis. The use of smokeless tobacco in the form of Snuff in USA, Snus in Sweden, Toombak in Sudan, Shammah in Saudi Arabia, Naswar in Pakistan and Afghanistan, Shisha, bidi etc., in India and South East Asia are the known varieties.[1] Smokeless tobacco may present as portion bag and dry tobacco forms. Tobacco is roasted and air dried or as a portion moist in cellophane bag. Snuff placement in the oral cavity varies worldwide i.e. among the labial, buccal sulcus, floor of mouth. Oral white lesions associated to tobacco chewing progress from nicotinic patches i.e. nicotinic stomatitis, chewers mucosa commonly among buccal and labial mucosae. Progressive clinical red areas i.e. erytrhoplakia, oral ulcers to submucous fibrosis or eventually oral squamous cell carcinomas.[5] Genetic profile among precursor tobacco associated lesions has been experimented and global understanding of Swedish and British databases is fundamental. p53 is an early marker for progressive oral precancers. It is the tumor suppressor gene hindering tumor cell cycle regulation at the G1 Phase of cell cycle. Common mutations are amongst exons 5 at 271, 279 loci, exon 7 at loci, exon 8,9 at loci.[6]

Snuff Dippers Mucosa presents as a superficial lesion with degree 1, wrinkled whitish to yellowish lesion. Degree 2, yellowish to brown furrows of normal mucosa and degree 3 as whitish yellow to brown heavily wrinkled deep and reddish furrows.[5] Dysplasia among snuff dippers presented with abnormal mitosis; stratum spinosum to basalis, loss of basal layer polarity, nuclear hyperchromatism, russel bodies (lamina propria), nuclear pleomorphism, intraepithelial keratinization and loss of cellular cohesion.[7] Loose or portion bag snuff had hyperplasia, pyknosis, nuclear pleomorphism among 252 regular Snuff users.[8] Histologic changes among snuff dippers include cellular vacuolation (koilocytosis), chevron type of keratinization, amorphous changes, sialadenitis. Ovoid basophilic epithelium 'hydrosum layer'(decrease density) of cytoplasm and nuclear pyknosis. Atrophic, hyperplastic epithelium (1-2 thickness fold). Loss of rete pegs and basilar hyperplasia.[9] p53 located at 17p belong to p63 and p73 (transgene), controls cell proliferation at G1 checkpoint triggers apoptosis among irreparable cells. p53 presents as C deletion exon 4 codon 63 among 63% oral squmaous cell carcinomas.[1] Cell cycle progression by CDK inhibitor p21 (Waf 1/Cip1), Bax, Bcl2, GADD45 and MDM2. GADD45 binds PCNA and inhibits S-phase entry to stop cell cycle. Over expression of p53 and Bax inhibits bcl2, suppresses radiation mediated apoptosis. MDM2 binds and inactivate p53, bax and IGF-BP3 (insulin like growth factor) promoting apoptosis.[10]

Chromosomal deletions at 3p, 9p and 17p associate normal to dysplastic mucosa. Carcinoma has further aberrations at 4q, 6p, 8p, 11q, 13q and 14q. Fragile sites are FRA3B at 3p14.2 (85%) loss among HNSCC. 9p and 13q are mutations of p16 and pRb at loci 9p 21.1 and 13q14. Chromosomal short arm (p) and long arm (q) have aberrations i.e. primary (initiating carcinoma) and secondary (progressing carcinoma). Clustering observed at 1, 9 and 4 at positions 1p22, $11q^{13}$, $1p^{36}$, $9q^{32}$ and $11q^{32}$ (Scully). Chromosome 9 has allelic loss with multiple tumor suppressor gene (MTS). p53 Australian mutation at exons 4-9 at codon 205 and 248. India and South East Asia has H-ras (35%), LoH of H-ras (30%), N-ras (28%) and N-myc (29%) amplification. Over expression of Bcl2 and and suppression of FAS inhibit tumor cell apoptosis. Exon 7 is the most affected mutation among USA and exon 5 and 8 among Japanese.[10] p53 loci presents at the short arm of chromosome 17. p53 mutations present at the central portion of 200 amino acid protein sequence. Wild type p53 is a 53 kDa nuclear phosphoprotein (t-half); 20-30 minutes. Tumor progression with p53 aberrations include (i) p53 mutations (ii) deletion of wild type allele (iii) Increased dosage of mutant type (aneuploid) (iv) p53 gene amplification. Srilankans showed site prevalence with buccal mucosa (68%), and well differentiated carcinomas (79%) for p53 oncogene expression. Deletion of p53 alleles at basal cells showed carcinoma positive with (11%) reactive cells.[11]

MATERIALS AND METHODS

This research was carried out at the Department of Biotechnology, University of Karachi. All chemicals and reagents and instrumentation were obtained from the Biotechnology Department. High Speed Centrifugation was performed at the Centralized Science Laboratory, University of Karachi. Study was conducted from September 2011 to August 2012. Fifty samples of biopsy proven Oral Squamous Cell Carcinomas from tobacco chewers among all major Pakistani races

were included. Biopsied tissue were crushed and defragmented to be placed in a 25 mg of tissue in a 2 ml microcentrifuge tube. 200µl of samples were added with 400µl of lysis solution. Incubated at 65°C for 5 minutes. 600 µl of chloroform was added and inverted (3-5 times). Centrifuged for 2 minutes at 12000 g (room temperature). The upper aqueous phase containing DNA were transferred to a fresh tube. 800µl of precipitation solution (comprising of 720µl distilled water: 80µl of concentrated solution) was added. Mixed for 1-2 minutes discarding the supernatant completely. Pellets were completely dissolved in 100 µl of NaCl solution 300 µl of cold ethanol was added and the DNA was precipitated for (10 minutes, -20°C). Centrifuged for 4 minutes at 12000 g (room temperature). Carefully removed the ethanol by pipettin. The pellet was washed with 70% cold ethanol. The tissue pellets were resuspended in 100µl TE buffer.

Preparation of PCR mix

1. DNA 2 µl
2. Primer-1 2 µl
3. Primer-2 2 µl
4. H2O 4 µl
5. PCR Mix(2x) 10 µl

PCR Condition

1. Preheat amplification at 95°C.for 5 minutes
2. 40 Cycles of 95°C for 1 minute
3. 40 Cycles of 60°C for 2 minutes
4. 40 Cycles of 72°C for 1 minute
5. 72°C for 7 minutes chain extension at the end.

RESULTS

Tobacco associated fifty Oral Squamous Cell Carcinoma were included in this study. Three of 30 male and 1 of 20 females presented with a single band and dimmers. Age predilection showed 7 cases from the fourth decade, 2 each from third, fifth, sixth and seventh decades, while one positive from the second decade. Buccal mucosa was most prevalent site with 9 of 19 cases positive. Alveolus and tongue had 4 of 19 each, while lip had 2 case positives. Betel quid was commonly used, as compared to moist snuff (naswar) (Table-1, Table 2 & Table 3).

Table 1

Gender	Cases	Percentage
Male	30	60%
Female	20	40%

Table 2

Age Years	Age Distribution			
	Male	Percentage	Female	Percentage
11-20	2	4	0	0
21-30	5	10	2	4
31-40	7	14	6	12
41-50	5	10	7	14
51-60	5	10	4	8
61-70	4	8	1	2
71-80	2	4	0	0
Total	30	60	20	40

Table 3

Positive cases	Carcinoma (site)		Gender	Age (Years)
19	Buccal Mucosa	9	Male 12	11-20 = 1
	Alveolus	4	Female 7	21-30 = 2
	Tongue	4		31-40 = 8
	Lip	2		41-50 = 4
				51-60 = 2
				61-70 = 2

DISCUSSION

p53 mutations were seen as 53% among non smokers for oral squamous cell carcinoma from lip, compared to 38% intraorally. Transitions (G: C-A: T) were more prominent than transversions (G: C-T: A).[12] Thai group presented with 7 of 8 mutations being G:C to A:T transitions amongst betel quid related Oral Squamous cell Carcinoma.[13] G:C to A:T transitions were also predominant amongst mutations reported by.[13]

p53 protein induction is elementary for stimulating C-myc. Loss of pRb hyperproliferates oral keratinocytes and deactivates p53 and pRb carcinogenesis. Over expression of Bcl2 and suppression of FAS inhibit tumor cell apoptosis. Exon 7 is the most affected mutation among American and exon 5 and 8 among Japanese population. p53 loci presents at the short arm of chromosome 17. p53 mutations present at the central portion of 200 amino acid protein sequence. Wild type p53 is a 53kDa nuclear phosphoprotein. Tumor progression with p53 aberrations included (i) p53 mutations, (ii) deletion of wild type allele, (iii) Increased dosage of mutant type (aneuploid), (iv) p53 gene amplification.[14]

Exon 5 of p53 Gene for Oral squamous cell carcinomas, in our study showed 4 of 19 positive cases with dimers. Five of 22 mutations were reported for exon 5, sequenced from exons 2-9 for p53 protein amongst oral squamous cell carcinoma subjects from Pakistani Group.

Cancers of oral cavity had elevated risks to occupational exposures. Chromates and snuff usage promote sinusitis, nose bleed and nasal polyps. Tobacco pouch snuff keratosis was 1%, with 7% leukoplakia highest at the

lip, followed by mandibular gingiva, tongue and floor of mouth.[14] Lower lip cancer risk associated with herpes labials among 61 cases, from 5th-9th decade, showed tumor initiation among diagnosed oral squamous cell carcinoma.[15] Oral cancer had higher prevalence for tongue, buccal mucosa, gingiva, is comparable to our study. Gingival mucosal epithelium being the most common site with seven positive cases seen. Our results presented Buccal mucosa as most prevalent site with 9 of 19 cases positive. Alveolus and tongue presented with 4 each from 19, while lip had 2 of 19 case positives. South Africans snuffers had 84% lower labial sulcus and 15% lower buccal sulcus lesions. Keratosis prevailed among 80% lesions at the snuff placement site.[16]

Eight of 16 OSCC presented on tongue, accounting for deletions amongst codons (135-176). Base change and amino acid shift was seen as.[17] Oral squamous cell carcinoma, p53 mutational hotspots for exon-5 were amongst codon 141, 175 and 179, from which codon 175 being common to most cancers.[18] Peltonen et al, presented with three mutations amongst codon 171, two among 155 and 172, while one each was reported amongst codon region 139, 148, 157, 159 and 184 respectively.[19] Metastatic oral squamous cell carcinomas showed codon 72 as the hotspot mutant region.[20] Shwe et al, presented with one mutation each in codon region 141, 147, 175 and 180 for exon 5 of p53 protein.

Hsieh et al, accounted for buccal mucosa as the most mutant site, comparable to 9 of 19 positive cases, with no mutations seen for exon 5 p53 gene in Oral Squamous Cell Carcinomas.[18]

p53 mutations was related to tumor metastasis with DNA-binding surface, instead of tumor core mass, documented by.[20] Hsieh et al, showed three p53 mutations from codon 135, two from region 175 and 176, while one each from region 143, 148, 160, 161, 173 and 177. Yamazaki et al, showed five cases positive for codon 176, three amongst codon 175, two each for codon region 135 and 152, while one mutation each 136, 141, 146 and 161 being prevalent for p53 exon 5 amongst oral cancer patients.[19] It is comparable to our nine positive cases, arising from buccal mucosa, with no mutations seen. Thongsuksai et al, reported three mutant cases amongst codon 152, two each from region 144 and 179, while one each for codon 145, 146, 173 and 184.[21] Six of 12 mutation positive cases consumed smokeless tobacco and betel quid, which was comparable to our snuff habit seen in 12 of 19 positive cases.

CONCLUSION

Oral lesions associated to tobacco chewing i.e. Oral Squamous Cell Carcinomas lesions, among individuals with genetic aberrations are advocated. p53 profile among 50 known Oral Squamous Cell Carcinoma cases were considered. Twelve of 30 male and 7 of 20 female were positive. Polymerase chain reaction (PCR) method implied presents Exon 5 mutations prevailed. Primers were standardized for p53 exons 5. DNA extraction was done using DNA extractions kits from Fermantas protocol. PCR amplication of Exon 5 of p53 gene were found to be positive in 19 samples. Thirty (60%) male and twenty (40%) female's were included. Seven cases were from the forth decade, 5 from the fifth, 2 each from the third, sixth and seventh decade. One case was from the second decade. Most prevalent site was buccal mucosa with 9, alveolus and tongue with 4 each and lip with 2 case positives. Their DNA sequencing and further characterization of various mutations showed no mutations in our sample.

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