Chewable risk factors - Threatened oral cancer
HPV’s Looming Epidemic in Pakistan

Saeeda Baig, Zile Rubab, Muhammad Mustafa Arif, Mohammad Haris Lucky

Abstract
Background: In Pakistan oropharyngeal cancer is the second leading malignancy after breast cancer, attributed to extensive use of several precarious chewable tobacco formulations. The human papillomavirus (HPV), as proven, plays a pivotal role in oropharyngeal cancer. This study was designed to investigate the relationship between chewable tobacco usage with HPV and its oncogenic strains in this population.

Methods: Oral samples were collected from camps during awareness campaigns on oral hygiene/hazards of chewable tobacco. Subjects addicted to chewable tobacco formulations such as naswar, gutka, and pan, areca nut with or without oral lesions, having no febrile conditions were included. DNA was extracted and PCR were performed. SPSS version 20.0 was used for analysis. Frequencies and percentages were calculated for qualitative and mean & standard deviation for numerical variables. Pearson chi-square with 95% confidence level was used, p-value less than 0.05 was taken as significant.

Results: A total of 1000 subjects and 450 controls (age 28.90±12.163) were included in this study. Out of 1000 (831 males and 169 females), 204 (20%) were HPV positive whereas, 13, 6, and 6 subjects were positive for HPV16, HPV18 and both HPV16&18 respectively, compared to only 14 (6.42%) in the controls. Overall 631 were chewing a single and 369 multiple forms. Significant association was found between different forms of chewable tobacco with Leukoplakia, Erythroplakia, Rough mucosa and Trismus.

Conclusions: Usage of chewable tobacco formulations is associated with high frequency of HPV infection OR= 7.981 (CI 4.587-13.89), which is a threat for an epidemic of oropharyngeal cancer.

Keywords: Chewable Tobacco; HPV; Oral Disorders; Ethnicity.

1. Introduction
The epidemic of oral cancer is lurking in the lower socioeconomic bloc of our population. The popularity of chewable tobacco among the young irrespective of racial ethnicity has aggravated oral health malady [1, 2]. It has been estimated that 58% of the total worldwide head and neck cancers occur alone in South and Southeast Asia [3]. A study conducted in 2010 showed that in Pakistan oropharyngeal cancer is the second leading malignancy and this was attributed to the wide use of smokeless tobacco formulations [4]. This is probably, the underlying reason for the ranking of Karachi South as a place with highest incidence of oral cancer in both genders worldwide [5]. The human papillomavirus (HPV), as proven by studies, plays a pivotal role in oral squamous cell carcinoma (OSCC) [6]. Research on oral cancer in tobacco chewers in Pakistan has found involvement of HPV [7]. Our preliminary study on gutka chewers showed 18% positivity for HPV, compared to controls [8]. HPV is small (55 nm diameter) non-envelope virus carrying circular double-stranded DNA consisting of approximately 8Kbp. HPV’s protein E6 binds to p53 resulting in immortalization in the cells leading to chromosomal instability, whereas, E7 binds to Rb leading to unchecked cellular growth and chromosomal instability, increasing the probability of malignant change [9].

The chewable tobacco products, occasionally blended with betel, areca nut, lime and some with toxic artifacts (khat, blood, crushed glass etc.), added for taste and flavor, are the cause of worst observable oral lesions. The link between the use of these tobacco based compounds and head and neck malignancies is now well established [3, 4, 10, and 11]. However, some studies did not find any correlation of smokeless tobacco to oral cancer [12], due to possibly lower concentrations of tobacco specific nitrosamines [13].
Such findings indicate that differences in composition significantly influence their carcinogenicity. In this regard, among Indian population, studies have estimated that 49% of oral cancers among males and 90% among females are attributed to chewing habits [14]. Another study conducted in Pakistan reported that betel, areca nut and tobacco chewing leads to 8.5 to 10 times increased risk of oral cancers, after adjusting the other co-variates [15]. The objective of the study was to investigate the relationship of chewable tobacco products with HPV and to find out the frequency of its oncogenic strains HPV 16 and 18 in this group.

Material and methods

Study Subjects
This study was carried out from 2010 to 2013 in Ziauddin University, Karachi. One thousand four hundred and fifty (1000 samples and 450 controls) oral rinse samples were collected, after filling a questionnaire from subjects gathered in the camps during the screening and awareness campaign regarding oral hygiene and hazards of chewable tobacco. The study was approved by Ziauddin Ethics Review Committee prior to samples collection. A written informed consent and approval was taken from the subjects. All chewable tobacco products were considered as risk factors. Subjects exposed to risk factors (with or without oral lesions such as trismus, ulcers, submucosal fibrosis (SMF), rough mucosa (with white or red patches or warts) but otherwise normal having no febrile condition were included. Subjects addicted to stuff other than chewable tobacco were excluded. Controls comprised area and age matched subjects, not exposed to risk factors and without oral lesions.

Data collection
Questionnaires were completed by trained medical volunteers who obtained information about demographic characteristics, oral hygiene, medical history, family history of cancer, and lifetime history of tobacco chewable products such as Gutka, Pan with tobacco and Naswar. Pathology trained professionals examined the oral lesions and recorded them.

Sample collection
Sample size was calculated using Epi Info version 6, frequency was taken as 50% with confidence interval 95% and power 90. Oral-mucosal Samples were collected in 50 ml corning tubes and stored at 4°C. Subjects were given 20 ml distilled water to roll in their mouth for one minute which was collected in the 50 ml corning tubes followed by gentle combing over the oral mucosa inside the cheeks and over the gums with the help of a brush at the other end of dental floss. This floss was left in the corning tube.

PCR: General HPV
Bench work was performed in the postgraduate laboratory of Ziauddin University. All primers, Gp5+/Gp6+, HPV16 and HPV18, were purchased from Gene Link, USA. DNA extraction and PCR was performed as previously reported.

HPV 16, 18 Genotyping
The genotyping was carried out in a volume of 25μl containing 12.5μl of master mix (Promega), 10μl DNA (1μg) and one μmol of HPV16, HPV18 specific primers each. The first DNA denaturation was for 2 minutes at 94°C; 35 cycles of PCR consisting of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 51°C, and extension for 30 seconds at 74°C, followed by a final extension for 5 minutes at 74°C. Data was entered on SPSS version 16.0. Frequencies and percentages for the qualitative data, mean and standard deviation for the numerical variables were taken out and association between the qualitative variables was calculated using Pearson chi-square.

Results
Two hundred and four (20.4%) out of 1000 subjects tested positive for HPV compared to only 14 (3.1%) out of 450 in the controls group. Table 1 shows the distribution of Subjects when compared to different variables. Odds ratio was calculated and was found highly significant (7.98(95% CI 4.587-13.89). The subjects were divided into two groups on the basis of chewing habit into single form chewers or multiple forms chewers. Demographic variables along with HPV status were compared in both groups (Table 2). People adhering to single form of tobacco belonged to older age group in contrast multiple forms were younger. No significant difference in HPV status among both groups was observed. Seropositive for HPV 16 were 13, for HPV 18 were 6 and for both HPV 16 and 18 were 6 out of total of 204 HPV positive whereas, HPV genotypes for rest of 179 subjects could not be determined. Analysis of the single chewable tobacco user showed 123 people positive for HPV; highest (26%) was among individuals using Naswar. Association of chewable tobacco with lesion is given in Figure 1. Rough mucosa of the oral cavity was significantly (p<0.000) associated with all forms of chewable tobacco. Naswar was also significantly associated with other forms of oral lesions such as Trismus (p<0.004) and Erythroplakia (p<0.001). Gutka was significantly associated with Leukoplakia (p<0.012) and Erythroplakia (p<0.000). Areca nut was found to be significant with Trismus (p<0.001) and Leukoplakia (p<0.005).

Figure 2 shows the relationship of age compared to different forms of chewable tobacco. The study included all the major ethnicities living in Karachi, highest number of subjects were Sindhis (n = 267). In the rest of ethnicities included in the study, HPV was positive in 20.2% (n=28) Balochis, 24% (55) Pathans, 19% (23) Punjabis. Table 3 shows distribution of HPV related oral disorders and forms of chewable tobacco used by different ethnicities. Some subjects had overlapping lesions presentation.

Table 1: Distribution of Subjects and Controls according to Age Gender and HPV Status

<table>
<thead>
<tr>
<th>Age</th>
<th>Gender</th>
<th>HPV status</th>
<th>Odds Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>Subjects (n=1000)</td>
<td>28.90±12.163</td>
<td>831 (83.1%)</td>
<td>169 (16.9%)</td>
</tr>
<tr>
<td>Controls (n=450)</td>
<td>29.60±13.26</td>
<td>210 (46.7%)</td>
<td>240 (53.3%)</td>
</tr>
<tr>
<td>Grand total (n=1450)</td>
<td>12.51±9.11</td>
<td>1041</td>
<td>409</td>
</tr>
</tbody>
</table>
Table 2: HPV distribution according to number of tobacco forms used

<table>
<thead>
<tr>
<th></th>
<th>Single n=631 (63.1%)</th>
<th>Multiple n=369 (36.9%)</th>
<th>P- Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Mean±SD)</td>
<td>29.57±13.04</td>
<td>27.78±10.39</td>
<td>0.024</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n=831)</td>
<td>505 (80.0%)</td>
<td>326 (88.3%)</td>
<td>.001</td>
</tr>
<tr>
<td>Female (n=169)</td>
<td>126 (20.0%)</td>
<td>43 (11.7%)</td>
<td></td>
</tr>
<tr>
<td>HPV Positive (n=204)</td>
<td>123 (60.3%)</td>
<td>81 (39.7%)</td>
<td>.341</td>
</tr>
<tr>
<td>HPV 16 Positive (n=13)</td>
<td>6 (3.0%)</td>
<td>7 (3.4%)</td>
<td>.202</td>
</tr>
<tr>
<td>HPV 18 Positive (n=6)</td>
<td>3 (1.4%)</td>
<td>3 (1.4%)</td>
<td>.505</td>
</tr>
<tr>
<td>HPV 16 &amp; 18 Positive (n=6)</td>
<td>2 (1%)</td>
<td>4 (2.0%)</td>
<td>.130</td>
</tr>
</tbody>
</table>

Table 2: HPV distribution according to number of tobacco forms used

<table>
<thead>
<tr>
<th></th>
<th>Niswar</th>
<th>Pan</th>
<th>Gutka</th>
<th>Areca Nut</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Subjects (N=631)</td>
<td>154</td>
<td>81</td>
<td>254</td>
<td>142</td>
<td></td>
</tr>
<tr>
<td>HPV Positive (n=123)</td>
<td>40 (26.0%)</td>
<td>13 (16.0%)</td>
<td>45 (17.7%)</td>
<td>24 (17.0%)</td>
<td>0.118</td>
</tr>
<tr>
<td>HPV 16 Positive (n=6)</td>
<td>1 (0.6%)</td>
<td>2 (2.5%)</td>
<td>1 (0.4%)</td>
<td>2 (1.4%)</td>
<td>0.350</td>
</tr>
<tr>
<td>HPV 18 Positive (n=3)</td>
<td>1 (0.6%)</td>
<td>1 (1.2%)</td>
<td>1 (0.4%)</td>
<td>0 (0.0%)</td>
<td>0.615</td>
</tr>
<tr>
<td>HPV 16 &amp; 18 Positive (n=2)</td>
<td>0 (0.0%)</td>
<td>1 (1.2%)</td>
<td>1 (0.4%)</td>
<td>0 (0.0%)</td>
<td>0.369</td>
</tr>
</tbody>
</table>

Table 3: Distribution of HPV status, Oral Lesions and Chewable tobacco usage in different Ethnicities.

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>HPV Positive (n=204)</th>
<th>Oral disorders present</th>
<th>Chewable tobacco used</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leukoplakia (n=143)</td>
<td>Erythroplakia (n=60)</td>
<td>Rough Mucosa (n=463)</td>
</tr>
<tr>
<td>Balochi (n=138)</td>
<td>28 (20.2%)</td>
<td>31 (22.5%)</td>
<td>8 (5.8%)</td>
</tr>
<tr>
<td>Bengali (n=117)</td>
<td>29 (24.8%)</td>
<td>19 (16.2%)</td>
<td>5 (4.3%)</td>
</tr>
<tr>
<td>Pathan (n=225)</td>
<td>55 (24.4%)</td>
<td>29 (12.9%)</td>
<td>6 (2.7%)</td>
</tr>
<tr>
<td>Sindhi (n=267)</td>
<td>44 (16.5%)</td>
<td>31 (11.6%)</td>
<td>31 (11.6%)</td>
</tr>
<tr>
<td>Punjabi (n=119)</td>
<td>23 (19.3%)</td>
<td>12 (10.1%)</td>
<td>2 (1.7%)</td>
</tr>
<tr>
<td>Urdu Speaking (n=125)</td>
<td>23 (18.4%)</td>
<td>20 (16.0%)</td>
<td>7 (5.6%)</td>
</tr>
<tr>
<td>Others (n=9)</td>
<td>2 (22.2%)</td>
<td>1 (11.1%)</td>
<td>1 (11.1%)</td>
</tr>
</tbody>
</table>

Values marked in * are significant (P < 0.05)

Fig 1: Association of oral lesions with different forms of chewable tobacco.
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It is the major cause of trismus (45.5%) compared to other tobacco formulations. Widespread Gutka was the most popular having highest addiction rate (55.8%) compared to other socio-economic status and sexual behaviors.

Discussion
Several epidemiological studies verified chewable tobacco as cause of head and neck cancer but did not find its link with HPV. This study provides evidence that HPV transmission can also occur in the oral squamous cells in addition to defined risk factors, through a variety of habitual and culturally based activities of chewing tobacco, that are less commonly seen in the western world. Subjects habitual of chewable tobacco consumption regardless of different forms were found to be 8 times more likely to develop HPV infection. Currently, it is agreed in general, that OSCC due to HPV infection is likely to be acquired through smoking, alcohol and oral sexual contact.

This study denies this overly emphasized consensus in the west that oral HPV is transmitted through oral sexual contact. These habitual eaters are least concerned about oral health. Their poor dentition, infrequent tooth brushing and infrequent dental visits result in the form of oral lesions which may serve as a surrogate for chronic infection, inflammation, precancerous lesions and ultimately squamous cell carcinoma.

The main chewable tobacco formulations evaluated for possible cause of HPV transmission, were Gutka, Niswar, Pan (Betel Quid) and Areca Nut (chalia). HPV was found positive in 20% (204) subjects addicted to these chewing tobacco products. The population selected belonged to lower socio-economic sector, blue collar workers including laborers, daily wagers, cleaners, maids, drivers, fishermen, handymen, venders, janitors etc. having odd working hours and stressful milieu especially for young workers. Low level of education and ignorance about product carcinogenicity force these people ultimately to bow to substance abuse under lot of peer pressure. The risk factors for HPV in the West that match our findings are male gender and younger age at presentation whereas, in contrast to our findings are higher socio-economic status and sexual behaviors.

Gutka
Gutka was the most popular having highest addiction rate (55.8%) compared to other tobacco formulation. Widespread popularity of Gutka is due to its frequent availability and a very low-price. It is the major cause of trismus (45.5%). Ranganathan pronounce trismus as "Gutka syndrome or Areca Nut Chewer's syndrome". The oral cavity of chronic users, constantly corroded by gutka chewing, results in fibrosis of the sub-mucosal layers and characteristic facial abnormalities, following ultimately into trismus with a distinct Gutka face and Gutka speech. Its strong flavor and no health warning sign compared to cigarettes is cause of worst observable oral lesions including OSF, oral cancers, leukoplakias and other head and neck malignancies in Southeast Asia. Earlier study on gutka showed 18% HPV frequency which is consistent with this study, showing lesions such as ulcers, rough mucosa, SMF, Leukoplakia (20%) and Erythroplakia (10.6%) with highest frequency of HPV in Erythroplakia (25%).

Naswar
Naswar chewers had the highest HPV positivity (40 (26.0%)). It was the second [154 (24.4%)] most common form of smokeless tobacco addiction among low socioeconomic subjects. Naswar is part of Pathans' culture consumed mostly by people of Khyber Pakhtoon Khaw (KPK). This province is in the north west of Pakistan where tobacco is grown on approximately 30800 hectares according to report published in 2006. There are the two types of tobacco varieties flue-cured Virginia and White Patta, the later contains more nicotine and about 80 percent of it is used in Naswar manufacture according to local dealers. Naswar is a kind of raw form of crushed moist tobacco leaves, slaked lime, indigo, flavored with cardamom and menthol, marketed in a very low cost package and can be homemade. It is not chewed rather stuffed in the mouth either under the lower lip or tongue, or inside the cheek from few minutes to extended periods of time where over the time an oral wart may develop. There are no studies available on Naswar regarding its contents, health hazards or carcinogenicity. Nass, a mixture similar to Naswar is available in northern Pakistan, Iran and the Central Asian Republics, containing tobacco, partially cured, ash, cotton or sesame oil and lime with the similar mode of dipping, marketed under the name of Toombak, popular in Sudan, is similar to Naswar in formulation and mode of use. Toombak dipping and infection with high risk Human Papilloma Virus (HPV) has been extensively investigated and linked to the etiology of oral cancer in Sudan.

Areca Nut
Areca nut ranked third [142(22.5%)] in popularity among the chewable tobacco preparations with HPV positivity in 17% (24) of the chewers. Earlier in the 70s', Areca nut mixed with aniseed formulations were marketed as safe mouth fresheners. Steady tobacco crept into areca nut packets and the deadly concoction sneaked into our society and became one of the

Fig 2: Chewable tobacco use according to age compares to seropositive HPV.
major addictive products. Oral submucous fibrosis is increasing due to the use of processed areca nut products, many containing tobacco [31]. Looking at lifestyle habits and high risk of mouth cancer among South Asian women in UK31, the researchers concluded that betel nut (or areca nut) with or without tobacco should be avoided. In our study we observed that youngsters (< 15 years) were more keen on areca nut, than later on as they grow older they switch to Gutka (16 to 25 years). Mainly Areca nut is the first form and not considered as chewable tobacco by the masses because of low tobacco content than others (Gutka and Naswar). Development of tolerance towards the effects of tobacco may result in subjects employing formulation with much higher tobacco content.

**Paan or Betel Quid**
Paan or betel quid was found the least popular [81(12.8%)] among all formulations, whereas, a couple of years back a study from Karachi reported Paan with tobacco as the second most common form of chewable consumption [32]. Paan or betel quid has been part of central and South Indian culture for hundreds of years. Karachi portrays an ethnically and linguistically assorted population of the four provinces as well as settlers (Muhajirs) from India and Afghanistan and hence its name mini Pakistan. These ethnicities differ on the basis of history, heritage, and culture. The eating habits including addictions are consistent with their area of origin. These addictions over the years, have evolved into many formulations with worst observable consequences. The Paan (betel quid) culture was brought by Muhajirs from India and now has been adopted by Punjabis, Sindhis, Balochis and Pathans residing. Today, studies around the world have identified betel quid an independent factor with or without tobacco in the causation of oral cancer, predominantly oropharyngeal cancer around places where south Asians and paan (betel quid) is used and sold freely [33,34]. Meta analysis of various studies suggest that betel-chewing-cessation programs should be developed to help prevent oral diseases [35].

**Single vs Multiple Forms of Chewable Habit**
Findings indicate that ‘dual users’ have distinct individuality such as they are significantly younger, have less income and less intention to quit tobacco [36]. Subjects indulged in chewing multiple forms 37% (n=369) in this study had 2.5% higher nicotine, 0.9% more arecoline, 0.5% more PAH, 0.5% more nitrosamine, 0.5% more mutagenic content than others (Gutka and Naswar). Development of tolerance towards the effects of tobacco may result in subjects employing formulation with much higher tobacco content.

**Mucosal corrosion and HPV invasion**
The detrimental effects generally observed in oral cavities due to the use of chewable tobacco were leukoplakia, erythroplakia, rough mucosa and submucous fibrosis. Studies [37], have established that the development of premalignant lesions leading to oral cancers are due to these oral habits. The leukoplakia and erythroplakia were found significantly (P < 0.05) associated with Gutka, Naswar and Areca nut. Rough mucosa was highly significant (P < 0.05) in chewers of Gutka, Naswar, Paan and Areca nut with highest prevalence in gutka chewers (53.5%). Arecoline, an alkaloid found in areca nut leads to fibrosis. Areca nut in this study was found to be significantly associated with Trisman (P< 0.001) and Leukoplakia (p<0.005) 38. Leukoplakia, a precancerous lesion, characterized by white plaque and hyperkeratosis was found in 143 subjects, with significant association with Gutka and Areca nut. Although erythroplakia was less frequent among all chewable consumers yet it is one condition among all oral precancerous lesions that carries the highest risk to malignant progression. The mechanism of development of erythroplakia is still under research.

**Postulated Mechanism of HPV Invasion in Desquamated Oral Epithelium**
A number of processes are reported in the literature but none is well recognized. However, one of the most likely mechanisms postulate that the HPV invasion is due action of constituents in these products leading to inflammatory changes. Arecoline, an active alkaloid found in areca nuts, stimulates fibroblasts which leads to increased production of soluble collagen. This soluble collagen becomes insoluble by up regulation of lysyl oxidase39. Continuous exposure of oral mucosa to chewable tobacco makes the mucosal surface more susceptible to viruses like HPV to enter epithelial basal cells. The viral DNA proteins E6 and E7 are expressed and HPV starts proliferating in desquamated epithelial cells due to insult by the chewable tobacco 40.

**Counseling**
Counseling done during the camps was quite effective. Almost 90% of participants agreed to quit the habit and 10% trashed the additional packets in their pockets. Almost 2% approached the researchers for further counseling when they found about their HPV positivity. This proves that proper counseling can lead to appropriate public responsiveness which can be helpful in curtailing the addiction to these substances and may result in averting the threat of epidemic of oral cancer and related mortality in this and similar settings [40].

Following recommendations are proposed:
1. Ban on the sale, manufacture and storage of all these tobacco based chewable products.
2. Large-scale educational interventions. Intervention can be the greatest tool if the target population is identified along with its tool.
3. Marketing candies with similar flavor or taste can be an alternative attraction for habitual teenagers.
4. Awareness campaigns through media and educational institutes regarding information on oral hygiene, awareness of risk factors and symptoms and the importance of seeking early professional help around the target populations should be the aim for healthy Pakistan.
5. Population screening would reduce the incidence of oral cancer, but requires careful planning, new research projects and monitory support.
6. Participation of family physicians and medical students for early detection of oral cancer is the need of time.
7. Enforce bans on tobacco advertising, promotion and sponsorship

**Conclusion**
In this era of globalization, nothing is restricted to geographical vicinity. Many of these habits have now crossed borders and appear around various areas throughout the world. All these products contain Nicotine and people with or without addictive personalities fall victim to its dependence. Nicotine preys on genetically, mentally and socially predisposed individuals. Once addiction is established the other carcinogens in Gutka, Naswar, Paan and Areca nut erode the oral mucosa making the environment conducive for HPV to
anchor in the basal epithelium, where the virus replicates in synchrony with the S-phase of the host. HPV-associated oral cancer is affecting a population younger than that typically is affected by HPV-independent oral cancer.

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Author Contributions
SB drafted, designed, executed, and supervised the project along with preparation and critical revision of the manuscript. ZR contributed in drafting of the manuscript, along with results interpretation, acquisition of data and bench work. MMA contributed in data entry, analysis and interpretation of data and drafting of manuscript along with critical review of the article. MHR contributed in acquisition of the data along with bench work.

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