

Human Papilloma Virus and Oral Lesions in *Gutka* Eating Subjects in Karachi

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ABSTRACT

Objective: To determine the frequency of HPV in eaters of *Gutka* (betel, areca, lime and tobacco concoction), presenting with oral lesions.

Study Design: A descriptive study.

Place and Duration of Study: Ziauddin University Research Laboratory, from February to July 2010.

Methodology: Subjects munching *Gutka* fulfilled the inclusion criteria. Samples were collected in the form of 20 - 40 ml oral rinse from 262 subjects who were habitual eaters of *Gutka* after an informed consent. Gentle brushings from the lesion were taken from subjects with the help of a brush at the other end of dental floss and the oral rinse was stored at 4°C until DNA extraction. DNA was extracted and PCR was performed using HPV consensus primers Gp5+/Gp6+. Oral cavity was examined for the presence of ulcer, trismus, sub-mucosal fibrosis, leukoplakia and/or warts.

Results: Out of 262 subjects, 42 were females and 220 males with an average age of 27± 10 years. HPV was positive in 47 subjects (17.9%). HPV frequency was 2.7% greater in chewers with more than 10 years of habit compared to less than 10 years. Examination of oral cavity showed 78% presenting with more than one complaint including oral ulcers (25%), rough mucosa (62%), sub-mucosal fibrosis (24%), leukoplakia (20%) and erythroplakia (10.6%). Highest frequency of HPV was observed in erythroplakia (25%). Association between presence of symptoms and HPV shows an ODDS RATIO: ad/bc= 4982/430=11.6

Conclusion: Oral lesions caused by constant exposure to *Gutka* are associated with high frequency of HPV infection, which may be a risk factor for squamous cell carcinoma of the oral cavity. People should be educated about the consequences of *Gutka* abuse.

Key words: Human papilloma virus. Squamous cell carcinoma. Erythroplakia. Tobacco concoction abuse. Chewable tobacco.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is a significant cause of cancer worldwide and ranks sixth among all malignancies worldwide.^{1,2} In Pakistan, an 8.5 to 10 times increase in the risk of oral cancers has been reported due to an increase in tobacco chewers in the recent years, after adjusting the other co-variables.³ *Gutka* (betel, areca, lime and tobacco concoction), marketed locally since 1975, is one of several smokeless tobacco formulations, and the cause of worst observable oral lesions. This habit has now integrated into the cultural setup of South Asians, especially the strong inclination of children toward chewable tobacco products, which warrants urgent action.⁴ Several studies have shown a link between the use of *Gutka* and oral submucosal fibrosis, oral cavity cancers, leukoplakias and other

head and neck malignancies.^{3,5,6} It has been estimated that 58% of the total worldwide head and neck cancers occur alone in South and Southeast Asia.⁶ In Pakistan, 40% of the adolescent and adult population of squatter settlement in Karachi has been reported to be using at least one product of chewable tobacco on a daily basis.⁷

The involvement of human papilloma virus (HPV) in oral and oropharyngeal carcinogenesis is well established.⁸ The epithelial areas of the upper aero-digestive tract display greatest susceptibility to HPV due to the easy exposure of the basal cells to HPV infection.⁹ The continuous exposure of oral mucosa to chewable tobacco causes abrasions making the mucosal surface susceptible to HPV thus making it easier for the virus to gain entry into the basal layer of squamous epithelium, with HPV high-risk types 16 and 18.

The objective of the present study was to determine the frequency of HPV in subjects who are habitual eaters of *Gutka*, and have developed oral lesions (trismus, sub-mucosal fibrosis, leukoplakia, or warts) in a population of squatter settlements in Karachi.

METHODOLOGY

This descriptive study was conducted from February to July 2010 and was a part of project funded by HEC (no.20-1469 R&D/2009) to Ziauddin University, Karachi.

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Inclusion criteria comprised all persons who were exposed to risk factors defined as habitual eaters of *Gutka* and had developed lesions or trismus, ulcers, sub-mucosal fibrosis (SMF), rough mucosa with white or red patches or warts. Subjects addicted to stuff other than chewable tobacco were excluded. Controls comprised area and age matched subjects who were not exposed to risk factors and had no lesions.

Sample size was calculated using Epi Info version 6, expected frequency was taken as 50% with confidence interval 95% and power 90. Oral samples were collected from 262 subjects, after receiving informed consent, a questionnaire was completed after examination of the visible oral lesions of the subjects. Ethical approval was obtained from The Ziauddin Ethics Research Committee. All experiments were performed in the postgraduate laboratory of Ziauddin University.

Sample collection was done by setting up camps in five different areas of Karachi. All samples were collected as 20 - 40 ml oral rinse with gentle brushing over the lesion with the help of a brush at the other end of dental floss and were stored at 4°C until DNA extraction.

DNA was extracted with Omni-Pure™ DNA Purification System kit. Ten ml of oral rinse was centrifuged for 15 minutes at 10,000 rpm. Supernatant was discarded, leaving 100 µl at the bottom containing cells. This was transferred to 1.5 ml Eppendorf tube containing 500 µl lysis solution. Protein precipitation solution was added and it was vortexed for 30 seconds and centrifuged for 10 minutes at 10,000 g. Supernatant was transferred to a fresh tube and 500 µl of isopropanol was added. Contents were mixed by inversion (30-40 times). DNA pellet was retrieved after centrifugation at 4°C at 10,000 rpm for 10 minutes. Supernatant was discarded and pellet was washed with 75% ethanol. Pellet was dried and reconstituted with 50 µl TE buffer.

The PCR of all samples was performed with L1 gene-specific consensus primers Gp5+/Gp6+.¹⁰ Primers sequence for HPV - Gp5+ was 5'-TTTGTACTGTGGTAGATACTAC-

3', and HPV-Gp6+ was 5'-GAAAAATAAAGTAAATCATATTC-3' (Gene Link, NY, USA). DNA was amplified in a conventional thermocycler (BIOFLUX). A HPV positive control, human β-globin gene and a blank were used with every reaction. The PCR amplification was carried out in a volume of 25 µl containing 12.5 µl of master mix (Promega) containing as final concentrations 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM deoxynucleoside triphosphates (dNTPs), and 1 unit of Taq DNA polymerase (Promega) and 10 µl DNA (1 µg). One µmol of primers Gp5+ and Gp6+, The first DNA denaturation was performed for 5 minutes at 94°C; and then 35 cycles of PCR consisting of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 55°C, and extension for 1 minute at 72°C were run, followed by a final extension for 5 minutes at 72°C.

Data was entered on Statistical Package for Social Sciences (SPSS) version 16.0. Frequencies and percentages were taken out for the qualitative data; mean and standard deviation were taken out for the numerical variable. Association between the qualitative variables were taken out using Pearson chi-square. At 95% confidence level, p-value less than 0.05 was taken as significant.

RESULTS

A total of 262 *Gutka* eating subjects were enrolled which included 42 females and 220 males with an average age of 27 ± 10 (range 14-40) years. HPV was positive in 47 subjects (18%). Total subjects were 262, including teenagers (n = 63, 24%), age 20 - 29 years (n = 110, 42%), age 30 - 39 years (n = 58, 22%) and above 40 years (n = 31, 12%).

They were divided into six ethnic groups: Sindhi [72 (27.5%)], Punjabi [27 (10.3%)], Pathan [24 (9.2%)], Balochi [49 (18.7%)], Mohajir [55 (21%)] and Hindu [35 (13.3%)] were the main ethnicities. Among the ethnic group, HPV was positive in 25.5% of Sindhis, 12.8% of

Table I: Frequency of lesions and HPV in different oral examination findings seen in different ethnic *Gutka* chewing groups.

Lesions N (%)	Punjabi N (%)	Pathan N (%)	Sindhi N (%)	Balochi N (%)	Mohajir N (%)	Hindu N (%)	HPV Positive N (%)	HPV Negative N (%)
Rough mucosa* 163 (62%)	20 (12.3)	13 (8)	50 (30.7)	24 (14.7)	28 (17.2)	28 (17.2)	27 (16.6)	136 (83.4)
Oral ulcers ** 66 (25%)	04 (6.1)	09 (13.6)	24 (36.4)	06 (9.1)	13 (19.7)	10 (15.2)	15 (19.20)	63 (80.8)
Erythroplakia* 28 (10.6%)	02 (7.1)	00 (0)	08 (28.6)	05 (17.9)	04 (14.3)	09 (32.1)	07 (25)	21 (75)
Leukoplakia 52 (20%)	07 (13.5)	04 (7.7)	10 (19.2)	13 (25)	11 (21.2)	07 (13.5)	10 (19.2)	42 (80.8)
Sub-mucosal fibrosis 63 (24%)	05 (7.9)	07 (11.1)	20 (31.7)	06 (9.5)	12 (19.1)	13 (20.6)	11 (17.5)	52 (82.5)
Burning sensation* 48 (18%)	02 (4.2)	06 (12.5)	22 (45.8)	03 (6.3)	09 (18.8)	06 (12.5)	09 (18.8)	39 (81.3)
Total	40	39	134	57	77	73	47	215

* p value < 0.05; ** p value < 0.005

Punjabis, 8.5% of Pathans, 31.9% of Balochis, 12.8% of Mohajirs and 8.5% of Hindus.

HPV frequency was 2.7% greater in chewers consuming *Gutka* more than 10 years (10.3%) compared to those consuming for less than 10 years (7.6%).

Examination of oral cavity showed 78% presenting with more than one complaint including oral ulcers (25%), rough mucosa (62%), Sub Mucosal Fibrosis (24%), Leukoplakia (20%) and Erythroplakia (10.6%). Highest frequency of HPV was observed in Erythroplakia (25%), (Table I). Association between presence of symptoms and HPV shows an ODDS RATIO: $ad/bc = 4982/430 = 11.6$.

DISCUSSION

In this study, all 262 subjects were habitual eaters of *Gutka* with more than 3 years of habit with 10 minutes to 16 hours of exposure. The habitual eaters place the *Gutka* in the mandibular or labial groove and suck on it slowly for 10-15 minutes and then either throw it away or simply let it sit in the mouth till it slowly dissolves.⁵ That is probably the reason that cancer development in our environment is mostly in the buccal mucosa (cheek) compared to the Western countries where tongue and the floor of the mouth are the primary sites. Less common sites include the gingiva, labial mucosa, and hard palate.¹¹ It is well established now that habitual *Gutka* usage is associated with severe oral mucosal disorders, and the consequences may extend beyond the oral cavity.¹² Overall, the frequency of HPV in *Gutka* eaters was found to be 47 (18%). The analysis of cancer cases recorded for Karachi South, at Karachi Cancer Registry during January 1995 to December 2002 demonstrate that cancer of the oral cavity ranked second in Karachi in both genders, whereas cancer of the pharynx ranked 7th in males and 14th in females.¹³

On the whole, HPV was positive approximately in 18% to 25% of subjects with ulceration, leukoplakia, burning sensation and erythroplakia respectively. It is well known that oral lesions may often precede oral carcinoma but fail to be detected.¹⁴ Some symptoms such as rough mucosa were found to have the highest frequency, 62% of subjects had them at the time of sample collection, whereas, 24% complained of occasional ulceration. Abrasions like white patches, swelling, Sub-mucosal fibrosis (SMF), pain, trismus, cuts, sepsis, etc. are caused due to continuous exposure of oral mucosa to *Gutka*, making this mucosal surface more vulnerable to viruses like HPV.

Sub-mucosal fibrosis was present in 24% of *Gutka* eaters. A similar study from India in 2009 reported risk of developing oral sub-mucosal fibrosis as highest with *Gutka*-chewing (relative risk, 1,142.4), which was significant ($p < 0.01$) at 95% confidence interval.¹⁵ The study further observed that the next highest relative risk

for oral sub-mucosal fibrosis was with combination of *Gutka* with other chewing habits. The association of betel nut with sub-mucosal fibrosis (SMF) is well established.¹⁶ In this study, 24% of *Gutka* eaters had SMF out of which 17.5% were HPV positive. The rate of malignant transformation of SMF has been estimated to be 3% to 19%.¹⁷ A literature review reports an extension of oral sub-mucous fibrosis into the hypopharynx and esophagus in *Gutka* users which may lead to malignant transformations in oral sub-mucous fibrosis with the increase in frequency of *Gutka* usage.¹²

The natural progression of a pre-cancerous lesion is usually transformation from an *in situ* lesion to an invasive one. Luo *et al.* reported a prevalence of 30.4% HPV in precancerous lesions.¹⁸ Early cases of these precancerous lesions may often appear as precancerous leukoplakia and erythroplakia. The prevalence of HPV infection in oral leukoplakia in comparison with healthy oral mucosa has been reported to be 17.6%.¹⁹ HPV prevalence has been reported to be twice as high in pre-malignant lesions as in normal mucosa and is nearly five times higher in oral squamous cell carcinoma.²⁰ Its prevalence increases from normal to dysplasia and finally to cancer.

The ethnicity-related eating habits were found to be consistent with local perceptions that immigrants from different parts of the country practice chewing habits as part of the culture they have brought from the area of their origin. Many people of Sindh province adopted a *Paan* culture brought by Muhajirs and one finds the red spittle of *Paan* on sidewalks there more frequently than any other province of Pakistan. *Gutka*, another advanced variety of chewable tobacco products has further aggravated the scenario. In this study, Sindhis have now been found to be more (27%) addicted to *Gutka* than muhajirs (20%). Alternatively, it is possible that the number of Muhajirs living in these squatter settlements is low. It was interesting to observe that Pathan ethnicity, which is well known for *Naswar* habit (9.2%), has developed a craving for *Gutka* and other varieties of tobacco while living in Karachi. Similarly, Punjabis (10.3%) and Balochis (18.3%) have also developed this chewing habit which it is not part of their culture.

HPV has been shown to exhibit ethnic health disparities.²¹ Pathans had the highest prevalence (24%), compared to Sindhis (17%), Punjabis (23%), Muhajirs (18%) and Balochis (2.5%). Balochis, having a very high HPV frequency 31.9%, are particularly at a higher risk of head and neck cancers.

Age group indicates the higher prevalence of *Gutka* use among younger generation as reported earlier.²² This may be because in lower socioeconomic strata teenagers and youngsters in early twenties are the earning members of the family having access to free money for liberal spending.

HPV prevalence has been reported to be twice as high in pre-malignant lesions as in normal mucosa and is nearly five times higher in oral squamous cell carcinoma.²² Its prevalence increases from normal to dysplasia and finally to cancer due to scraping and scratching caused by continuous chewing on *Gutka*. The epithelium which lines the upper aero-digestive tract is the main area from where the majority of the head and neck malignancies originate from, which includes the oral cavity, pharynx and larynx.²³ The epithelial areas of the upper aero-digestive tract display greatest susceptibility to HPV due to the easy exposure of the basal cells to HPV infection.⁹

CONCLUSION

Oral lesions caused by constant exposure to *Gutka* are associated with high frequency of HPV infection, which may be a risk factor for squamous cell carcinoma of the oral cavity. People should be educated about the consequences of *Gutka* abuse.

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