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# Human papillomavirus and the risk of oral potentially malignant disorders of betel-quid chewers in samosir island, Indonesia: an integrated epidemiologic and molecular study

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**Abstract**
Human Papillomavirus (HPV) is known to be associated with oral cancer and several types of oral potentially malignant disorders. Recent studies have shown that HPV oncogenes act synergistically with a common risk factor of chemical carcinogens in betel-quid chewing, resulting in oral malignant transformation. The aim of the present study was to investigate the association between HPV with the risk of oral potentially malignant disorders of betel-quid chewers. This case-control study was performed on habitual chewers of betel-quid, surrounded the working area of the local government clinic of Ambarita, regency of Samosir province of North Sumatra, Indonesia. As case group, 28 subjects with oral potentially malignant disorders and as control group, 23 subjects without oral potentially malignant disorders, were assigned into study. All HPV DNA samples were collected by swabing over the oral lesions and mucosa, thereafter performed using nested-polymerase chain reaction. Fisher exact test and multiple logistic regression were used to investigate associations between variables. The present study noted a high prevalence of positive HPV, both in case group (96.4%) and control group (82.6%). Positive HPV increased the risk of oral potentially malignant disorders of betel-quid chewers, but the association was not statistically significant (OR= 5.684; p= 0.162). As the conclusion, the present study highlighted a high prevalence of positive HPV on betel quid chewers in Samosir island Indonesia. Larger studies in this population with both evaluation of low risk and high risk HPV types, were recommended to detect HPV types with more valid empirical associations.

**Keywords:** Human Papillomavirus, betel-quid chewers, oral potentially malignant disorders

## 1. Introduction
Human papillomavirus (HPV) is a small, non-enveloped, double-stranded deoxyribonucleic acid (DNA) virus of 52 to 55 nm in a diameter, and highly epitheliotropic. Papillomaviruses belong to Papillomaviridae family and have been classified by types [1, 2] Infection of HPV are found in various body sites, including the anogenital tract, urethra, skin, larynx, tracheobronchial, nasal cavity, paranasal sinus, and oral cavity [3, 4] The role of HPV in the causation of cervical cancers of uteri has been established [2]. Meanwhile, the specific role of HPV in oral carcinogenesis have received considerable attention in recent years [5-7].
The meta-analysis studies had showed the association of HPV with oral cancer and potentially malignant disorders [5, 8]. The following oral potentially malignant disorders were included oral leukoplakia, lichen planus, and epithelial dysplasia [5]. Malignant transformation rates for these lesions have been reported by several studies [9-11]. Nevertheless, the etiologies and risk factors of oral carcinogenesis remain to be elucidated [12].
Several integrated epidemiologic and molecular studies in Asia region suggested that habitual chewing of betel-quid might interact with HPV infection to increase the risk of oral cancer and potentially malignant disorders [12-16]. Carcinogenesis is a multi-step process, therefore, in addition to insult by chewing of betel-quid associated intra oral carcinogens, several additional factors, such as external agent of viruses, may play a synergistic role in oral tumorigenesis [17, 18] The continuous exposure of oral mucosa to the habitual chewing of betel quid, causes abrasions thus making the mucosal surface susceptible to HPV infection [14, 15] The virus will entry into the basal layer of squamous epithelium and synergistically play a role with chemical

Carcinogens in betel quid chewing, resulting in the transformation of oral epithelium [7, 19].

In several parts of Indonesia, the epidemiologic studies had reported that habitual chewing of betel-quid were the important risk factor for oral cancer and potentially malignant disorders, [20-22] but did not find its link with HPV. Meanwhile in a molecular study, HPV was also known to play an important role as a risk factor for pathogenesis of oral squamous cell carcinoma and benign oral squamous cells [23]. North Sumatra is a province in Indonesia, where the habitual of betel-quid chewing is still practised by the communities [22, 24]. The integrated epidemiologic and molecular study to investigate the risk factors for the development of oral potentially malignant disorders, have not been available in this region. Thus, the present study was conducted to analyze the association between HPV with the risk of oral potentially malignant disorders of betel-quid chewers in Samosir island, Indonesia.

## Material and Methods

### Study Participants

This case-control study was performed on all habitual chewers of betel-quid, surrounded the working area of the local government clinic of Ambarita, regency of Samosir province of North Sumatra, Indonesia, between August to December 2016. A total of 51 subjects with a history of betel quid chewing and without a habit of smoking and alcohol consumption were consecutively selected. As case group, 28 betel-chewers with oral potentially malignant disorders i.e submucous fibrosis and/or leukoplakia, and as control group, 23 betel-chewers without oral potentially malignant disorders, were assigned into study.

### Data Collection Tool

A questionnaire-based interview regarding the habitual of betel-quid chewing was applied to the participants. Following the questionnaire data collection, the participants were subjected to performed a visual oral soft tissue examination. The clinical diagnosis of oral potentially malignant disorders was established based on the criteria as provided by the epidemiology guide for the diagnosis of oral mucosal diseases [25, 26] The participants were asked to rinse the entire mouth before collection of HPV DNA samples. Using a cotton tipped swab, the cytological swabs were taken from the lesional site of oral potentially malignant disorders and buccal mucosa of controls. All of the samples were stored at a thermoelectric-cooler (Mobicool®) until HPV DNA isolation in Laboratorium Terpadu, Faculty of Medicine Universitas Sumatera Utara.

### HPV DNA Isolation and Detection

Genomic DNA was extracted using the Presto™ Buccal Swab gDNA Extraction Kit (Geneaid) according to the instructions of the manufacturer [27] The DNA products were stored at –20 °C until further use. The β-globin gene was used to assess the quality of DNA [28] (Table 1). The presence of HPV was detected by nested-polymerase chain reaction (PCR), using outer primers MY09 and MY11 and inner primers $GP5^{+}$/ and $GP6^{+}$ from the consensus L1 region [29, 30] (Table 1). PCR was performed using the KAPA Taq™ EXtra HotStart ReadyMix with dye (KAPABIOSYSTEMS) according to the instructions of the manufacturer [31] Amplification was carried out in thermal cycler (Applied Biosystems Veriti®) with an initial denaturation step at 95 ºC for 3 minutes, followed by 35 cycles each at 95 ºC for 15 seconds, annealing at 48 ºC for 15 seconds and extension at 72 ºC for 60 seconds. The final extension was at 4 ºC for 60 seconds. Then a second PCR protocol was carried out using GP5+/GP6+ primer that will produce an amplicon size of 142 bp. The annealing temperature was at 42 ºC. The PCR products were analyzed on 2% agarose gels and detected using gel-doc imaging (Uvitec®) (Figure 1).

**Table 1:** Primer sequences and PCR products length (bp) of primers used in HPV DNA PCR amplification [29, 30]

| | Primer | Primer Sequences (5' – 3') | Products Length (bp) |
|---|---|---|---|
| β-globin gene | β-globin forward | GAA GAG CCA AGG ACA GGT AC | |
| | β-globin reverse | CAA CTT CAT CCA CGT TCA CC | 250 |
| HPV | MY09 | CGT CCM ARR GGA WA C TGA TC | |
| | MY11 | GCM CAG GGW CAT AAY AAT GG | 450 |
| | $GP5^{+}$ | TTTGTTACTGTGGTAGATACTAC | |
| | $GP6^{+}$ | GAAAAATAAACTGTAAATCATATTC | 142 |

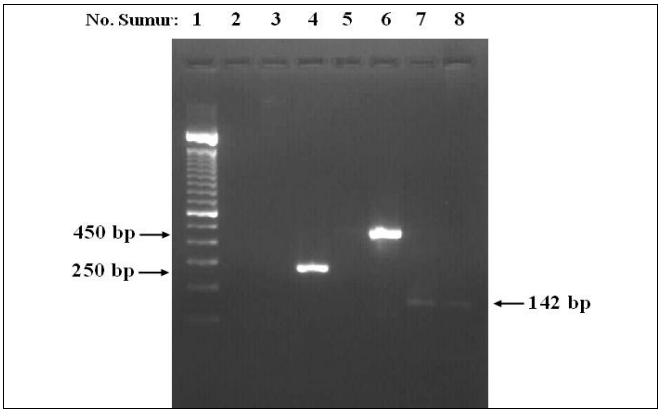


**Fig 1:** Agarose gel electrophoresis of PCR products after amplification with β-globin primers and nested-PCR products after amplification with MY09/MY11 primers followed by $GP5^{+}$/$GP6^{+}$ primers:

Lane 1: DNA ladder marker (100 bp ladder).
Lane 2: No template PCR control.
Lane 3: Blank control for β-globin PCR products.
Lane 4: Clinical samples. Expected size of β-globin PCR

product was 250 bp, indicating good DNA integrity.
Lane 5: Clinical samples. Expected amplicon of MY/GP$^+$ nested-PCR product was absence, indicating HPV negative samples.
Lane 6: Clinical samples. Expected size of MY09/11 PCR product was 450 bp.
Lane 7: and 8: Clinical samples. Expected size of MY/GP$^+$ nested-PCR product was 142 bp, indicating HPV positive samples. (Doc.)

## Results

The study population comprised of 28 betel-chewers with oral potentially malignant disorders (case group) and 23 betel-chewers without oral potentially malignant disorders (control group). The distribution of HPV in both groups with different oral lesions are presented in Table 2. Positive HPV was identified in all oral potentially malignant disorders, including oral submucous fibrosis (50.0%), leukoplakia (7.1%) and more than one lesion (39.3%). Positive HPV was also seen in control group (82.6%).

**Table 2:** Distribution of HPV for various oral lesions in subjects with oral potentially malignant disorders and control group.

| HPV | Oral potentially malignant disorders (Case group) n= 28 | | | | | | Non-oral potentially malignant disorders (Control group) n= 23 | |
|---|---|---|---|---|---|---|---|---|
| | Submucous fibrosis | | Leukoplakia | | >1 oral lesions | | | |
| | n | % | n | % | n | % | n | % |
| HPV (+) | 14 | 50.0 | 2 | 7.1 | 11 | 39.3 | 19 | 82.6 |
| HPV (-) | 0 | 0.0 | 1 | 3.6 | 0 | 0.0 | 4 | 17.4 |

The analyses of HPV versus oral potentially malignant disorders of betel-quid chewers by the Fisher exact test, OR, and 95% CI are listed in Table 3. Positive HPV was present in oral potentially malignant disorders (96.4%), compared with control group (82.6%). HPV had OR of 5.684 (Cl, 0.588-54.940) in oral potentially malignant disorders, but the OR was not statistically significant (p= 0.162).

**Table 3:** Analyses of HPV versus oral potentially malignant disorders of betel-quid chewers.

| HPV | Oral potentially malignant disorders (Case group) n= 28 | | Non-oral potentially malignant disorders (Control group) n= 23 | | P value | OR | 95% Cl | |
|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | | | Min | Max |
| HPV (+) | 27 | 96.4 | 19 | 82.6 | 0.162 | 5.684 | 0.588 | 54.940 |
| HPV (-) | 1 | 3.6 | 4 | 17.4 | | | | |

Fisher exact test

Table 4 shows that in a multivariate analyses of all suggested risk factors by the multiple logistic regression, only the lifetime exposure more than $1.8x10^4$ hours of betel-quid chewing remained an independent risk factor for oral potentially malignant disorders of betel-quid chewers (p= 0.162). HPV was not a significant independent risk factor for oral potentially malignant disorders of betel-quid chewers, but the OR was 10.561 (Cl, 0.891-125.122) compared with the lifetime exposure more than $1.8x10^4$ hours of betel-quid chewing which had OR of 6.741 (Cl, 1.049-25.561).

**Table 4:** Multivariate analyses of suggested various risk factors versus oral potentially malignant disorders of betel-quid chewers.

| Risk Factors | P value | OR | 95% Cl | |
|---|---|---|---|---|
| | | | Min | Max |
| HPV (+) | 0.062 | 10.561 | 0.891 | 125.122 |
| Lifetime exposure >$1.8x10^4$ hours | 0.005 | 6.741 | 1.049 | 25.561 |

## Discussion

Habitual chewing of betel-quid is still commonly practiced in many parts of Indonesia. North Sumatra is one of the province in Indonesia where the habitual chewing of betel-quid has been viewed as publicly acceptable among all strata of its community due to long-standing cultural perspectives [22, 24, 32] As the study population of the present study was derived from the same habit, it can be claimed to be representative of all habitual betel-quid chewers in this study location.
HPV DNA could be detected in subjects with oral submucous fibrosis and leukoplakia in the present study. The previous several studies in Asia regions have reported that HPV was identified in oral potentially malignant disorders of betel-quid chewers. Nevertheless, the risk estimates for the association of HPV with oral lesions and conditions varied widely in the range from 5.0% to 34.6% [12, 15, 33]. Thus, this case-control study was performed to investigate the presence of HPV DNA in oral potentially malignant disorders of betel-chewers, compared with non-oral potentially malignant disorders of betel-chewers.
The prevalence of positive HPV in the present study was higher than other reported studies [12, 15, 33] HPV detection method, polymerase chain reaction (PCR) is considered to be of the highest sensitivity and can detect even a single copy of viral DNA per-infected cell samples [6]. In the present study, the use of nested-PCR could decrease the risk of false negatives because this method can amplify small amounts of PCR product of the first round of PCR in a case of low concentration of viral DNA in clinical samples [34] The previous study has reported that the technique of nested-PCR MY09/11 followed by GP5+/6+ increased in HPV DNA detection from 6.8% to 29.9% [35]. Besides of the technique, the use of MY and GP consensus primers could allow for a broad number detection of HPV genotype [30].
The present study noted HPV is quite common in the oral cavity of subjects without oral potentially malignant disorders (control group) in comparison with that of case group. The infection rate of HPV in subjects without oral potentially malignant disorders and normal mucosa have been reported to range from 4.5%-81.0% [12, 36, 37]. The oral route of HPV transmission in normal oral mucosa is not fully understood. It has been suggested that HPV prevalence in the normal oral mucosa includes subclinical and/or latent infections, and that the infection with a low number of virus copies in the oral cavity [37]. In the latent HPV infection, the replication of viral DNA is synchronized with the cell cycle but in which none of the cytopathogenic effects of HPV can be detected [2]. Latent infection can be as a result of low immune clearance of the

virus and probably the site viral genome integration or lack integration [19].

Factors that contribute to increase the prevalence of HPV infection and its recurrence in the oral cavity are a reduction in the host's immune response for the virus, genetic predisposition, sexual contact, autoinoculation and self-transmission [19, 37-39] Notiwithstanding, the results seen in several studies bring about controversies which are attributed mainly to the sensitivity variation of the methods employed, as well as the diversity of the populations studied and the sample sizes. Thus, this present study indicates the needs to follow-up all of the subjects in the control group of this population to prevent the development of oral potentially malignant disorders.

The present study showed that positive HPV increased the risk of oral potentially malignant disorders, but the result was unable to provide support for the suggested association between the presence of HPV and oral potentially malignant disorders. The high detection rate, however, may overestimate the significance of HPV in oral lesions, because only a few copies of HPV DNA could be detected. Scope of the study was to use of MY and GP consensus primers which are allow for a broad number detection of HPV genotypes [30]. Various previous studies used type specific primers of high-risk subtypes of HPV16 and HPV18 which are the most common subtypes of HPV associated with oral cancer and potentially malignant disorders [2, 5, 40] To the best of our knowledge, a study of the association between HPV and oral potentially malignant disorders of betel chewers in this study location, has never been attempted before. These findings warrant further study by using type specific primers of high risk subtypes of HPV.

This study showed that in a multivariate analysis of the suggested risk factors, only the lifetime exposure of betel quid chewing remained an independent risk factor for the oral potentially malignant disorders, however positive HPV had the highest adjusted odd-ratios for the oral potentially malignant disorders. The data indicates that HPV is not as critical as betel-quid chewing in the development of oral potentially malignant disorders, similar with the previous study in Taiwan. It could be suggested that HPV is not a primary factor in the initiation of the oral potentially malignant disorders but, instead, may be a later event in the further carcinogenesis [12].

Oral mucosa are composed of squamous epithelium with a thin layer of keratin or with no keratin at all, where in both of areas the epithelium is subject to microtrauma of various types, as well as to chemical irritants and viruses [7, 14] Betel quid chewers were comparatively two times more prone to HPV positivity [14]. Continuous exposure of oral mucosa to chewing of betel-quid makes the mucosal surface more susceptible to HPV to enter epithelial basal cells.[40] The integration of HPV genome into the host cell genome results in the increased expression of E6 and E7 oncoproteins, thus the key functions of tumour suppressor genes such as p53 and pRb are rendered useless. This leads to abnormalities in apoptosis, DNA repair mechanisms, cell cycle regulation and finally to cellular immortalization, thus inducing and maintaining a malignant cell phenotype [6] This pathway had been suggested to act synergistically with the common risk factor of chemical carcinogens in betel-quid chewing, resulted in oral tumorigenesis [14, 40] The results of this study provide support for the suggested that the HPV oncogenes has a synergistic effect with carcinogens in betel-quid chewing, resulting oral potentially malignant disorders. However, further study were recommended to use subjects of non-habitual chewers of betel-quid as control group, to analyze the association between HPV with the oral potentially malignant disorders with more valid empirical associations.

## Conclusions

As the conclusion, the present study highlighted a high prevalence of positive HPV on betel quid chewers in Samosir island Indonesia, both in subjects with oral potentially malignant disorders and control group. In addition, HPV oncogenes has a synergistic effect with carcinogens in betel-quid chewing, resulting oral potentially malignant disorders. Larger studies in this population with both evaluation of low risk and high risk HPV types, were recommended to detect HPV types with more valid empirical associations. It is recommended to the local public health office to improve the public health programmes through the implementation of effective measures for the prevention of oral potentially malignant disorders and promotion of oral health for the habitual betel chewers since this habit is the long-standing cultural perspectives and wealth of nations.

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