



# Prevalence of Human Papillomavirus in Patients With Oral Squamous Cell Carcinoma in Tabriz, Iran

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## Abstract

**Objectives:** Oral squamous cell carcinoma (OSCC) is one of the commonest malignant neoplasms in the oral cavity. Although human papillomavirus has been considered as one of the risk factors for oral cancer, the extent of its involvement in squamous cell carcinoma (SCC) has remained unclear. The present study aimed to detect the prevalence of HPV in the patients with OSCC in Tabriz, northwest of Iran.

**Materials and Methods:** Genomic DNAs of 30 normal non-smoking cases and 30 cases with tongue SCC who underwent surgery in Tabriz, Iran, were extracted and used for amplification of HPV L1 fragment using MY09/MY11 and GP5/GP6 primers by polymerase chain reaction (PCR) and nested PCR. The positive PCR products were sequenced to confirm the presence of HPV.

**Results:** Among 30 OSCC samples analyzed, two cases (6.6% of 30) were positive for HPV in PCR performed by MY09/MY11 primer, and none of them showed a positive result with GP5/GP6 primers. Nested PCR by MY/GP primers showed higher sensitivity in three positive cases (10% of 30) of SCC subjects.

**Conclusions:** According to these findings, HPV infection may not be a causative agent in tissue samples obtained from OSCC patients in Tabriz.

**Keywords:** Human papillomavirus, Nested PCR, Oral squamous cell carcinoma, Iran

## Introduction

Based on recent studies, SCC is known as the commonest malignant neoplasms in the oral cavity. It is reported that SCC accounts for 90% to 95% of all oral cavity cancers (1). Although alcohol and tobacco consumption is the most common risk factors for the onset of oral tongue SCC (OTSCC), there are reports about the prevalence of OTSCC in the group of never smokers and drinkers (2). Based on recent reports, about 60% of oral cancer patients are HPV positive (3). HPV is a virus with circular double-stranded DNA (8 kb) which encodes 8 genes including genes involved in the viral replication (E1, E2, E4, - E7) and two genes (L1 and L2) encoding capsid proteins (4,5). The results of recent studies indicated a direct association between HPV and head and neck cancers particularly with oral squamous cell carcinoma (OSCC) (6). Until now, more than 200 species of HPV have been identified including high risk and low risk HPV types, 24 of which are related to head and neck lesions (7,8). There are several methods for screening and molecular diagnosis of HPV infection including hybrid capture (9), in situ hybridization (10), and Polymerase chain reaction (PCR) (11). Among them, PCR technique with the ability to detect small amount of

DNA is a preferred technique with high sensitivity (12). This study was aimed to determine the frequency of HPV DNA in OSCC using PCR in Tabriz, Iran.

## Materials and Methods

### Samples and Population

Thirty paraffin-embedded and formalin-fixed specimens were obtained from patients with tongue SCC who had undergone surgery in Imam Reza Hospital of Tabriz, Iran. The samples were collected from June 2012 to May 2014. In addition, saliva samples from 30 normal cases without a history of head and neck cancer were utilized as control. They had no smoking and alcohol consumption.

### DNA Extraction

The paraffin-embedded and formalin-fixed specimens were sectioned using a microtome. For the extraction of DNA, paraffin-embedded specimens and tissue blocks were deparaffinized in xylene, washed with ethanol and then species were lysed in 400 µL of lysis buffer (2 mM EDTA, 10 mM Tris, and 100 mM NaCl) and 10 µL of proteinase K. Afterwards, DNAs were extracted by phenol-chloroform method and dissolved in TE buffer (1

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mM EDTA, 10 mM Tris, pH 8.0). The quality of extracted DNAs was examined by NanoDrop by determining A260/A280 ratio. The extracted DNAs were stored at  $-20^{\circ}\text{C}$  until use.

#### Analysis of Integrity of Extracted DNAs

To determine the integrity of extracted DNAs,  $\beta$ -actin PCR was performed as an internal control. The amplification of the human  $\beta$ -actin gene was performed using  $\beta$ -actin specific primers in a 25- $\mu\text{L}$  reaction for 35 cycles ( $94^{\circ}\text{C}$  for 1 minute,  $60^{\circ}\text{C}$  for 1 min,  $72^{\circ}\text{C}$  for 1 minute) after an initial denaturation at  $94^{\circ}\text{C}$  for 4 minutes. The final extension was performed at  $72^{\circ}\text{C}$  for 5 minutes. After the analysis of PCR products by electrophoresis on 1% agarose gel, the positive samples for the human  $\beta$ -actin gene were selected to amplify HPV DNA.

#### PCR Amplification

The prevalence of HPV among OTSCC patients were examined by three sets of PCR reactions. The first PCR reaction was performed using MYII/MY09 primers, Forward: 5'-CGTCCMARRGGAWACTGATC-3' and Reverse: 5'-GCMCAGGGWCTATAAYAATGG-3' in a 25- $\mu\text{L}$  reaction for 35 cycles ( $94^{\circ}\text{C}$  for 1 minute,  $52^{\circ}\text{C}$  for 1 min,  $72^{\circ}\text{C}$  for 1 minute) after an initial denaturation at  $94^{\circ}\text{C}$  for 4 minutes. The final extension was performed at  $72^{\circ}\text{C}$  for 5 minutes. The second PCR protocol was performed by GP5/GP6 primers; Forward: 5'-TTTGTTACTGTGGTAGATACAC-3', Reverse: 5'-GAAAAATAAACTGTAAATCATATTC-3'. The annealing temperature was  $42^{\circ}\text{C}$ . In each PCR, DNA extracted from Hela cells infected with HPV-18 was used as positive control.

The third PCR reaction was performed as a nested PCR using the combination of the above-mentioned primers (MYII/MY09 and GP5/GP6), both related to L1 region of the HPV genome. The first round PCR was performed using MYII/MY09 primers. Then, PCR products obtained from this reaction was used for the second PCR using GP5/GP6 primers. The PCR products were analyzed on 1.5% (w/v) agarose gels. DNA purified from HPV-18 positive Hela cells was used as positive control (13).

#### Statistical Analysis

To analyze the data, chi-square and Fisher exact tests were used ( $P$  value < 0.05).

#### Results

Thirty tissue samples from OSCC patients with an age range of 32 to 88 years (mean age: 61.26) were collected from June 2012 to May 2014. 55.6% of patients (15 cases) with the mean age of 61.53 years were men and 44.4% of them with the mean age of 60.92 years were women and 22% of patients including 2 women and 4 men were less than 45 years. After DNA extraction from the normal and patient specimens, integrity of extracted DNAs was

confirmed by the human  $\beta$ -actin PCR.

#### HPV Detection by PCR

The examination of the prevalence of HPV DNA in different samples showed that 2 cases (6.6% of 30) were positive in PCR using MYII/MY09 primers appeared as 450 bp band in electrophoresis (Figure 1). However, none of the samples showed positive result in PCR with GP5/GP6 primers (Figure 1).

#### HPV Detection by Nested PCR

Among 30 patient subjects confirmed by the human  $\beta$ -actin PCR, 3 cases (10% of 30) were positive in nested PCR using MYII/MY09 and GP5/GP6 primers, which displayed as 150 bp band. The positive PCR products were confirmed by sequencing (Figure 1).

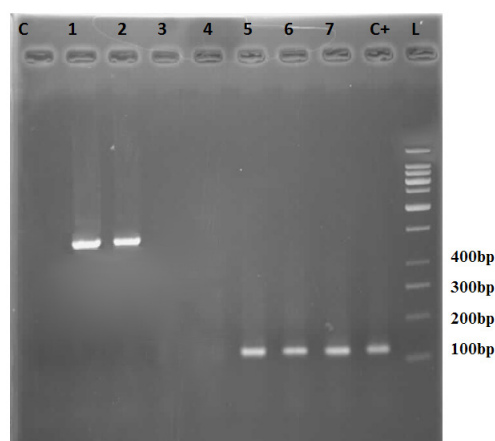
#### Discussion

During recent years, HPV infection has been identified as a causative factor of head and neck carcinomas (HNSCC) especially oral tongue SCC (OTSCC) (14,15).

Although smoking and alcohol consumption have been known as risk factors for SCC, there are reports regarding the enhancement of the percentage of HPV-positive OSCCs with no history of alcohol/tobacco consumption (16). Another study indicated enhancement of the percentage of HPV-positive OSCC from 20% to more than 70% in some of the European countries and the United States (15).

Among conventional methods for the detection of HPV infection, PCR method is a highly effective technique since it is capable of detecting low amount of DNA (12).

In clinical and histological studies on HPV infection, MY09/MY11 and GP5+/GP6+ primers are commonly used for amplification of the L1 region of viral genome (17). These primers are suitable for the amplification of



**Figure 1.** The Results of PCR and Nested PCR Using MY09/MY11 and GP5+/GP6+ Primers to Detect HPV-L1 Gene. C-: negative control, 1 and 2: positive samples amplified using MY09/MY11 primers as 450 bp band, 3 and 4: PCR products amplified using GP5+/GP6+ primers, 5-7: PCR products amplified using nested PCR method as 150 bp band; C+: positive control, L: ladder.

a broad range of HPV genotypes (18,19). Therefore, a 450-bp fragment from HPV genome is amplified using MY09/MY11 primers and the internal 150 bp fragment is amplified by GP5+/GP6+ primers (20).

In the present study, we collected 30 tissue samples of tongue SCC from Imam Reza Hospital of Tabriz, Iran. Among tissue samples, 22% belonged to patients under 45 years old, while based on the results of a study by Kantola et al, around 12% of their patients were under 45 years old (21).

In this study, MY09/MY11 and GP5+/GP6+ primers were used to detect HPV infection in OTSCC samples. Previous studies indicated that the detection rate of MY/GP primers depends on the types of the samples (frozen material, smears, and paraffin material), population and anatomical localization (22,23).

In the present study, 2 distinct methods were used to detect HPV DNA. The results of PCR using each of MY09/MY11 and GP5+/GP6+ primers on oral specimen were different. 6.6% of OTSCC samples showed positive result by MY09/MY11 primers, however, none of them indicated positive result with GP5+/GP6+ primers. However, 10% of the cases were positive for HPV in nested PCR with MY/GP primers. These results were not consistent with the reports of Zehbe and Wilander about the same sensitivity of MY09/MY11 and GP5+/GP6+ primers in PCR on cervical biopsies (24).

The results of our study were consistent with reports of Goot-Heah et al, regarding detection of HPV18 using nested PCR method in OSCC patients. They reported a prevalence rate of 3.3% for HPV18 serotype in saliva samples of Malaysian OSCC (25). Moreover, our findings were in line with results of the similar study done by Remmerbach et al, indicating low prevalence (2.8%) of HPV in OSCC patients (25).

In other similar study performed in China on 200 patients with oral cancer, 55 cases (27.5%) were HPV positive based on results of PCR with GP5+/GP6+ primers (26). Recently, in a study done in northwest region of the Philippines on HNSCC patients by PCR with GP5+/GP6+ primers, low prevalence of HPV (2.7%) has been reported (27).

### Conclusions

The results of this study confirmed the high specificity of nested PCR with MY/GP primers. On the other hand, these findings revealed low frequency of HPV in tissue samples obtained from OSCC patients in Tabriz, indicating that HPV infection may not have a causative role in these OSCC patients.

### Conflict of Interests

Authors have no conflict of interests.

### Ethical Issues

This study was reviewed and approved (no: 5.4.2830)

by Ethics Committee of Tabriz University of Medical Sciences.

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