Human Papillomavirus Type 16 Integration Analysis by Real-time PCR Assay in Associated Cancers

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Abstract

Human papillomavirus (HPV) is a common viral infection worldwide associated with a variety of cancers. The integration of the HPV genome in these patients causes chromosomal instability and triggers carcinogenesis. The aim of this study was to investigate the HPV-16 genome physical status in four major cancers related to HPV infection. Formalin-fixed paraffin-embedded blocks from our previous projects on head and neck, colorectal, penile, and cervical cancers were collected, and HPV-16–positive specimens were used for further analysis. The DNA extraction copy number of E2 and E7 genes was calculated by qualitative real-time PCR method. Serially diluted standards that were cloned in PUC57 plasmid were used. Standard curve and melting curve analysis was used for quantification. Of the 672 specimens studied, 76 (11.3%) were HPV-16 positive. We found that 35.6% (16/45) were integrated. Statistical analysis showed that there were significant correlations between integration of HPV-16 and cervical cancer end-stage carcinogenesis (P < .0001), episomal form, and ASCUS lesions (P = .045). Significant correlation in penile cancer patients was seen between the episomal form and high-grade cancer stage (P = .037). Integration is a major factor in the carcinogenesis mechanism of HPV and has different prevalence in various cancers with a higher rate in progression except in penile cancer.

Introduction

Cancer is one of the major public health challenges with a high mortality worldwide. Human papillomavirus (HPV) is a major cause of some human cancers such as cervical, penile, colorectal, esophagus, oropharyngeal, and head and neck cancers [1–4]. The risk of malignancy in HPV-positive cases is related to infection with specific types of HPVs [1,5]. HPVs are of numerous types (≥100), and they are divided into two major groups: high risk (HR) and low risk (LR) [6,7]. The HR types are more often detected in association with cancers such as cervical cancer. The major HR types include HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56,
HPV types have been detected in genital tract infections, and 12 of higher production of cancerous cells\[11,12\]. In cancerous tissue has been integrated to host genome and causes disrupted \[13\]. In the integrated form, the viral genome self-replication has been interrupted \[14\]. In the cancerous cells, HPV genes with E6, E7, and LCR (long control region) have been reported to integrate with the host genome, and the other viral genes are removed. One of the roles of these viral regions is the disruption in the functioning of the major cellular tumor suppressor genes such as Rb and p53; consequently, uncontrolled cell growth occurs \[13,14\].

The frequency of HPV genome integration varies based on several risk factors; the HPV types, specific cancers, and other personal or geographical factors could influence the genome integration \[16–19\]. For instance, HPV-16 integration has been reported to vary from 21% to 43% in oropharyngeal cancers, with the occurrence in 50%, 56%, and 100% of the Japanese, Pakistani, and Colombian esophageal cancer populations, respectively \[17,20\].

The determination of HPV genome status in cancerous and precancerous lesions is very important in tumor progress prognosis and estimation of survival or response to therapy in these patients \[15,17,21,22\]. Furthermore, in the HPV mix infection, there are no adequate data about the HPV genome status.

In this regard, we aimed to design a preliminary study of HPV-16 genome status investigation in Iranian patients suffering from head and neck squamous cell carcinoma (HNSCC) and colorectal, cervical, and penile cancers.

Materials and Methods

Patients

Patients were selected from our previous projects on HPV survey in different cancers including cervical cancer \[23\], HNSCC \[24\], and colorectal and penile cancer (not published) (n=672). The patients were referred to the referral hospitals affiliated to Iran University of Medical Sciences, Tehran, Iran. For the HPV detection, INNOLiPA HPV Genotyping assay and PCR-sequencing methods were used. The included patients had a positive result for HPV-16, and some had a mixed infection by other types of HPV's.

Ethics Statement

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in the prior approval by the institution’s human research committee. A written, informed consent was obtained from each patient included in the study. All human subjects were adult, and Institutional Review Board approval code was IR.IUMS.FMD.REC.1396.92215402.

Sampling

In this study with a retrospective design, the archived formalin-fixed paraffin-embedded (FFPE) blocks were collected from HNSCC and cervical, colorectal, and penile cancer populations \[23,24\]. Clinical databases and patient health records were used for retrieving pathological and clinical data from March 2011 to January 2017. The FFPE blocks were fixed in 10% buffered formalin before paraffin embedding, according to the documentation of the tissue repository.

DNA Extraction

A thin 20-μm tissue section was obtained from the blocks, and deparaffinization was performed with xylene. Then, series of distilled water and graded ethanol solutions were used for rehydration, according to previous works \[2,24,25\]. The DNA purification was performed by using QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany), according to the manufacturer’s protocol. NanoDrop ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA) spectrophotometry was used for the evaluation of the isolated DNA. The purified DNA was kept at −20°C.

Physical Status of HPV DNA

In the integrated form of HPV DNA, the E6, E7, and LCR regions were found, and the other HPV genes were removed. E2 and E6 or E7 genes quantification is the way to evaluate the HPV integration \[13,14,26\]. In this regard, by the quantitative real-time PCR (qRT-PCR) method, the viral loads of these genes (E2 and E6/E7) can be counted separately to distinguish the episomal, integrated, or a mixture of the two forms. In this study, we used the E2/E7 ratio to define the HPV-16 genome status. If the E2/E7 ratio was 0, the genome was in an integrated form; if >1, it was episomal; and if >0 and <1, it was a mix of the two forms \[9,13,27\].

Real-Time PCR Assay

The Rotor-Gene-Q 6000 thermocycler (Corbett, Australia) was used for HPV-16 E2 and E7 quantification. The total volume of the reaction mix was 15 μl that was composed of 7.5 μl of 2× Amplicon III mix (Odense M, Denmark), 0.5 μl of each forward and reverse primers corresponding to 0.5-μM concentration, and 3 μl each sample or control corresponding to 0.2 to 0.5 μM concentration, and rest of the total volume was obtained by adding distilled water. HPV16-E2 gene forward primer was 5′-TGC CAA GTG TG-3′ (2767-2786), reverse primer was 5′-CAG TCA TTA CAT AT-3′ (3298-3318), and the amplicon length was 551 base pairs; HPV16-E7 gene forward primer was 5′-TGC AAC CAG AGA CAA CGT AT-3′ (605-624), reverse primer was 5′-TGT CTA CGT GTG TGC TTT GT-3′ (768-787), and the amplicon length was 183 base pairs \[13\]. The E7 gene was heated at 95°C for 5 minutes followed by 40 cycles at 95°C for 30 seconds and 60°C for 30 seconds, and acquiring was in the annealing/extension step. The E2 gene was heated at 95°C for 5 minutes followed by 45 cycles at 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, and acquiring was in the extension step. Melting curve program (50°C–95°C with a heating rate of 2°C per second and a continuous fluorescence measurement) was used.

RT-PCR standardization and Interpretation

Full-length HPV-16 E2 and E7 genes were cloned with PUC57 plasmid as a standard control for quantification. Cloning was done using a commercially available cloning system (TA Cloning kit of Invitrogen, San Diego, CA) according to manufacturer’s instructions. The serial dilution manner was used for each gene, and DNase RNase-free water was used as the solvent. Efficient standard curve (E = 0.95-1.05) of standards was used for further quantification.
Statistical Analysis

SPSS software version 22 was used for statistical analyses of anatomic and demographic variables. The P values more than .05 were not regarded as statistically significant.

Results

Participants

Based on the previous HPV surveys (n = 672) on patients with colorectal cancer (n = 66), HNSCC (n = 156), cervical cancer (n = 436), and penile cancer (n = 14), we detected 76 (11.3%) HPV-16 positive specimens by INNOLiPA HPV genotyping assay and PCR-sequencing–based methods. Brief details of the HPV investigations are shown in Table 1.

Totally, 45 HPV-16 positive specimens, based on the clinical status and availability of the samples, were randomly selected for genome status analysis. Brief results of the cytological and histological analysis of the 45 HPV-16 positive cases are shown in Table 2.

qRT-PCR

Standard curves were drawn for HPV E7 and E2 genes by using standards diluted five to seven times. The efficiency of each E7 and E2 standard curve was 97% and 117%, respectively. The melting curve analysis confirms our standard interpretations (Supplementary Figure 1). During 40 real-time PCR amplification cycles, there were no primer-dimer generation.

Then, a total of 45 HPV-16 specimens were subjected to qRT-PCR, and the viral load of each of the E2 and E7 genes was reported based on the melting analysis and appropriate positive (standard) and negative controls (Supplementary Figure 2). A negative control (water or DNA extracted from previous works that was negative for HPV) was used in each run, and it did not have nonspecific yielded fluorescence signals above the background.

In general, 16 (35.6%) of the 45 specimens had undetectable levels for one or more of the E2 gene, and all of them had E7 viral load ranging from 1,270,709,529 to 1098 copies/μL. For the E2 gene, it was 13,193,565 copies/μL to undetectable levels. The E2:E7 ratio was calculated for the interpretation of the viral genome status (Figure 1). The HPV-16 integrated form was detected in 35.6% (16/45), episomal form in 24.4% (11/45), and mixed form in 40% (18/45) of the specimens.

Statistical Analysis

The analysis of the different variables showed that there was a significant association between HNSCC and end stages of carcinogenesis (higher-stage lesions (HSIL) and cancerous lesions [n = 9]) (P < .0001). This finding suggests that the integration of HPV-16 in high-grade lesions in cervical cancer is more common than the other types of lesions, about 69.2% (9/13) in our study. There was a significant association between HPV-16 episomal form and ASCUS lesions (P = .045). This highlights the primary stage of HPV infection in these patients in which the HPV genome is episomal in the vegetative stage. The analysis of variables from penile cancer patients showed a significant result only in the correlation of the episomal form with high-grade cancer stage (P = .037). The analysis of age with the genome status showed that there was no significant relationship between age and integration of HPV-16 genome. The results for the other variables were not significant (P > .05).

![Table 2](https://example.com/table2.png)

**Table 2. Pathologic Characteristics of our HPV-16–Positive Cases (n=45)**

|                | HNSSC (%) | CrCa (%) | PeCa (%) | CxCa (%) |
|----------------|-----------|----------|----------|----------|
| High grade     | 1 (50)    | 1 (33.3) | 2 (33.3) | -        |
| Low grade      | 1 (50)    | 2 (66.7) | 4 (66.7) | -        |
| ASCUS          | -         | -        | -        | -        |
| LSIL           | -         | -        | -        | -        |
| HSIL           | -         | -        | -        | -        |
| Cancerous      | -         | -        | -        | -        |
| Total          | 2 (4.4)   | 3 (6.7)  | 6 (13.3) | 34 (75.6) |

ASCUS, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Frequency of HPV-16 genome status in our 45 cancerous patients. HNSSC, head and neck squamous cell carcinoma; CrCa, colorectal cancer; PeCa, penile cancer; CxCa, cervical cancer.
HPV is one of the common viral infections, affecting nearly 80 million people currently in the United States (about 25% of the population) [28,29]. There are several HPV-related cancers including cancer of the cervix, vulva, vagina, penis, anus, rectum, tongue, and tonsils (oropharynx) as well as colorectal cancer [4,23,24,30–34]. The best described prevention method for HPV infection is vaccination [35–37]. Oropharyngeal and penile cancers are HPV-related cancers that are more prevalent in men and cervical cancer in women [18,28,29,38]. HPV genome integration plays a crucial role in HPV-related carcinogenesis and can trigger instability in the chromosomes [19,29]. The severity of the disease after integration of the HPV genome increases and may cause a reduction in the viral load. The severity of the disease after integration of the HPV genome has been reported to be associated with some invasive cervical cancers [13,40,41]. This association has not been proven by our study data. It may be due to the impact of other risk factors on the integration status of HPV, such as alcohol consumption or smoking. Meanwhile, there is evidence that indicates that the pure episomal form of HPV associates with some invasive cervical cancers [13,40,41].

A study by Liu et al. [43] of HPV-16–positive cervical carcinoma specimens showed integration in 63% (19/30). We found that the overall integration frequency was 35.6% (16/45), and in cervical specimens, it was 38%. In patients with only cervical cancer, the frequency was 44.4% (4/9), which is different to that mentioned in the report by Liu et al. [43]; this could be due to the environmental, viral, or host factor effects. A study by Bodelon et al. [10] of 38 HPV-16–positive CIN3 (17/38) and cervical cancer (21/38) specimens showed that there were 60.5% (23/38) integrated and 39.5% (15/38) episomal form, respectively. The episomal form was detected in 53.9% (9/17) and 28.6% (6/21) and the integrated form in 47.1% (8/17) and 71.4% (15/21) of CIN3 and cancer patients, respectively. The authors concluded that the instability of chromosomes in the absence of integration might play a crucial role in facilitating integration. Our study showed that 24.4% (11/34) were episomal, 35.6% (16/34) were integrated, and the rest of them were mixed form. These differences may be due to our pooled population of four cancers and the presence of mixed form including episomal and integrated forms. In the pure cervical cancer specimens, the episomal and integrated form frequency was 20.6% (7/34) and 38.2% (13/34), which by using pure HSIL and cancer patients became 5% (1/20) and 45% (9/20), respectively. Our current study showed that the integration occurred more in high-grade and cancerous cervical lesions, 45% (9/20), and it was significant in higher-grade malignant lesions (HSIL and cancerous vs. ASCUS and LSIL).
higher stages of cancer due to the higher chromosomal instability in these lesions.

The study by Castillo et al. [20] in Japan, Pakistan, and Colombia of the HPV genome status in patients with tonsil, tongue, other oral cancers, and esophageal carcinoma reported HPV-16 genome integration in 60% (6/10), 80% (16/20), 95% (18/19), and 71% (17/24) and mixed form in 40% (4/10), 10% (2/20), 5% (1/19), and 21% (5/24), respectively; episomal form in tongue mass was 10% (2/20) and in esophagus was 8% (2/24). This emphasizes the frequent integration of HPV-16 genome in SCCs of the oral cavity, oropharynx, and esophagus. Our study showed that the frequency of integration of HPV-16 genome in HNSCC patients is 50% (1/2), this being seen in a patient with tonsil cancer. It confirms the findings of the study by Castillo et al. in three countries. Further study with a larger sample size is needed to obtain comprehensive results. A study by Othof et al. [44] in 75 patients with HPV-16–positive OSCC analyzed for the physical status of the viral genome showed integration in 39% (29/75) and episomal form in 61% (46/75). Our total integration frequency in four surveys is similar to this study, but in HNSCC patients, it was 50%, and its differences could be due to the limited population in the present study.

The association of HPV with different cancers has been reported frequently. Colorectal cancer is one of the challenging issues, and its association with HPV infection is under investigation [45,46]. In the study by Bernabe-Dones et al. [45] of 12 colorectal cancer patients infected with HPV-16, the E2 gene evaluation for genome status by nested PCR method found that the genome in all the specimens was in an integrated form. They conclude that the integration of HPV-16 genome into the host genome could be a proof of the probable role of HPV in colorectal carcinogenesis. In the present study, we used the previous study (data not published) specimens that were HPV-16 positive. In a preliminary study, we analyzed the three HPV-16 genomes in colorectal cancer for the physical status and found that 33% (1/3) of them were in an integrated form, 33% (1/3) were episomal, and 33% (1/3) were mixed. Based on the histological and pathological data, 67% (2/3) of the specimens were of high-grade cancerous stage with episomal and mixed forms of HPV-16. Interestingly, integration was seen in low-grade cancerous tissue. This indicates the probable role of HPV in the initial stages of chromosomal instability and its potential to trigger the neoplasia in colorectal cancer patients, although there was no association between the pathologic stage of cancer and the physical status of the HPV genome. Further studies are required to clarify the subject.

Penile cancer is one of the major genital cancers in men that could be multifactorial, with smoking, phimosis, and poor hygiene being some of them. Its association with HPV infection has been investigated, and its frequency has been reported to be 20% to 50% [33,47,48]. The study by Djadjiningrat et al. [47] in 212 patients with penile cancer showed that they were infected with the HR HPV types with a frequency of 25%, and of them, 79% were HPV-16 types. Our previous study (data not published) showed the presence of HPV-16 genome in 54% (6/11) of penile cancer patients (6/11). Few studies have reported the HPV genome status in these patients. In the present study, we found the prevalence of the integrated form of HPV-16 in these patients to be 17% (1/6), episomal form 50% (3/6), and mixed form 33% (2/6). It seems that the episomal form of HPV is common in penile cancer patients, and high grade of penile cancer lesions has lower chromosomal instability or insertion of a foreign gene (HPV) compared with that in cervical cancer patients. This finding is different from our understanding about carcinogenesis of HPV infection and needs further analysis for better comprehension.

Our study limitations were the lack of another confirmatory or more accurate method such as TaqMan qRT-PCR to calculate the copy numbers, although relative calculation facilitates our study. For further studies, we recommended using a larger number of specimens and studying the other major HR HPV types’ behavior in the host genome using more demographic data from the history and risk factors involved in the patients.

In conclusion, there is a significant risk of HPV integration in a higher level of carcinogenesis compared with lower stages, although in a group of penile cancer specimens in this study, we found the episomal form to be more common in higher grade of cancer. Higher replication of cancerous and precancerous cells causes chromosomal instability and could facilitate the deletion/insertion of foreign genes such as HPV genome. As we observed in a majority of higher grade of cancerous lesions, the risk of HPV genome integration is inevitable, although other environmental and personal risk factors should not be neglected. The mixed form of HPV genome at cancerous and precancerous levels declares the primary phase of pure integration in the subsequent progressive stages.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2018.02.017.

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