Translational Oncology
www.transonc.com

Volume 11 Number xx Month 2018 pp. 593–598 593

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.

Translational Oncology (2018) 11, 593–598

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 *HPV* 16, 18, 31, 33, 35, 39, 45, 51, 52, 56,

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Received 15 January 2018; Revised 20 February 2018; Accepted 20 February 2018

1936-5233
https://doi.org/10.1016/j.tranon.2018.02.017

58, 59, 68, 73, and 82, and LR types are 6, 11, 40, 43, 44, and 54. Some other types such as 26, 53, and 66 are defined as probable high-risk types, and 69, 71, and 74 are defined as unknown-risk types [8,9]. Over 40 *HPV* types have been detected in genital tract infections, and 12 of them are related to carcinogenesis. *HPV-16* is responsible for about 60% of cervical cancers [10].

It seems that *HPV* DNA of some HR types such as 16, 18, and 31 in cancerous tissue has been integrated to host genome and causes higher production of cancerous cells [11,12]. In *HPV* oncogenesis, it is highly accepted that, in the neoplastic lesions, *HPV* genome is in the integrated form, and in the vegetative cycle inside differentiated epithelial cells of benign lesions, it is in an episomal form. In the integrated form, the viral genome self-replication has been interrupted [13–15]. 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 *HPVs*.

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 embedment, 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 CGT TTA AAT GTG TG-3′ (2767-2786), reverse primer was 5′-CGC ATG AAC TTC CCA TAC TT-3′ (3298-3318), and the amplicon length was 551 base pairs; *HPV16-E7* gene forward primer was 5′-TGC AAC CAG AGA CAA CTG 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.

Table 1. Descriptive Summary of HPV Survey Studies (*n*=672)

	HNSCC		CrCa		PeCa		CxCa	
	HPV16+	Total	HPV16+	Total	HPV16+	Total	HPV16+	Total
Total (%)	2 (1.3)	156 (23.7)	3 (4.5)	66 (10)	6 (54)	11	65 (14.9)	436 (66.2)
Men (%)	2 (1.6)	121 (77.6)	2 (66.7)	38 (57.6)	6 ()	11	0	0
Women (%)	0	35 (22.4)	1 (33.3)	28 (42.4)	0	0	65 (14.9)	436 (100)
Mean age±SD	65.5±12.6	60.5±12.6	64.3±13.2	59.3±14.4	35.0±5.5	35.8±5.7	38.4±13.6	35.3±10.1
Range	54-77	28-86	50-76	27-85	28-40	26-44	23-62	18-73
Other HPVs[a]	-	-	-	-	-	-	25 (38.5)	198 (45.4)

[a] Co-infected with other HPVs. *HNSCC*, head and neck squamous cell carcinoma; *CrCa*, colorectal cancer; *PeCa*, penile cancer; *CxCa*, cervical cancer; *SD*, standard deviation.

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. *HPV-16* physical genome status investigation details by pathologic status of specimens are shown in Table 3.

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

	HNSCC (%)	CrCa (%)	PeCa (%)	CxCa (%)
High grade	1 (50)	1 (33.3)	2 (33.3)	-
Low grade	1 (50)	2 (66.7)	4 (66.7)	-
ASCUS	-	-	-	8 (23.5)
LSIL	-	-	-	6 (17.6)
HSIL	-	-	-	11 (32.3)
Cancerous	-	-	-	9 (26.5)
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.

Statistical Analysis

The analysis of the different variables showed that there was a significant association between the integration of *HPV-16* 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).

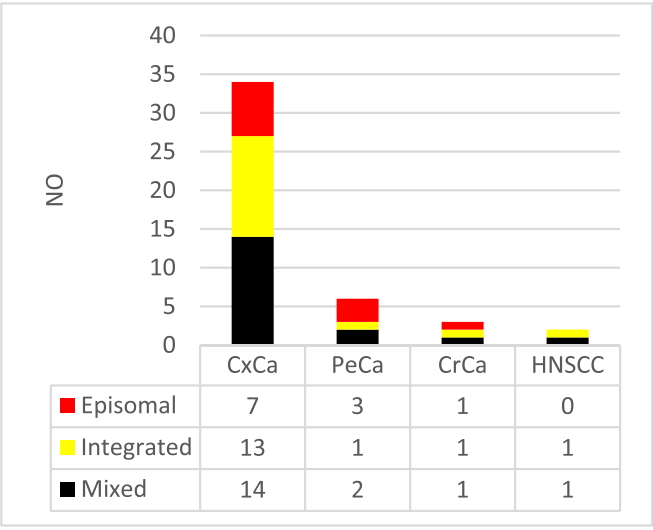

Figure 1. Frequency of HPV-16 genome status in our 45 cancerous patients. HNSCC, head and neck squamous cell carcinoma; CrCa, colorectal cancer; PeCa, penile cancer; CxCa, cervical cancer.

Table 3. Demographic and Pathologic Genome Status of Our Included Patients (n=45)

	Genome Status	No (%)	Sex	Age Mean ± SD	Grade		Pathologic Feature			
					High	Low	ASCUS	LSIL	HSIL	Cancerous
HNSCC	Integrated	1 (50)	Man	77	0	1	-	-	-	-
	Mixed	1 (50)	Man	54	1	0	-	-	-	-
CrCa	Episomal	1 (33)	Man	76	1	0	-	-	-	-
	Integrated	1 (33)	Woman	67	0	1	-	-	-	-
	Mixed	1 (33)	Man	50	1	0	-	-	-	-
PeCa	Episomal	3 (50)	Men	32.0±6.9	3	0	-	-	-	-
	Integrated	1 (17)	Man	40	1	0	-	-	-	-
	Mixed	2 (33)	Men	35.5±2.1	1	1	-	-	-	-
CxCa	Episomal	7 (21)	Women	30.7±3.4	-	-	4	2	0	1
	Integrated	13 (38)	Women	29.6±3.9	-	-	1	3	5	4
	Mixed	14 (41)	Women	31.1±10.5	-	-	3	1	6	4

Discussion

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 *E6*, *E7*, and LCR regions of the *HPV* genome have been reported to be integrated into the cancerous cells, and other genes such as *E2* and *E1* are eliminated [9,13,27]. The viral DNA of HR-*HPVs* such as *HPV-16* and *HPV-18* often integrates into the host cell genome and causes overexpression of *E6* and *E7* regions that disrupt cellular proto-oncogenes such as *pRb* and *p53*; consequently, it causes malignancy [21,39]. The *HPV* genome status can be used as a prognostic marker in HPV-associated cancers [15,17,21,22]. In this regard, we aimed to investigate the *HPV-16* genome status in different *HPV*-related cancers including HNSCC, colorectal, penile, and cervical cancer specimens which were positive for *HPV-16* genome detected by using INNO-LiPA Genotyping and PCR-sequencing methods from the previous studies [23,24]. Our study was designed to count the *HPV-16 E2* and *E7* gene viral load using qRT-PCR [13]. Among the 45 *HPV-16* infected patient samples analyzed, we found that 11 (24.4%) were episomal, 16 (35.6%) were integrated, and 18 (40%) were mixed form.

A study by Cheung et al. [13] showed that there were integrated, episomal, and mixed forms in 32.4% (11/34), 32.4% (11/34), and 35.3% (12/34) in European and 47% (31/66), 28.8% (19/66), and 24.2% (16/66) in Asian *HPV-16*–positive cervical specimens, respectively. They found the episomal form in 33.3% of CIN3 and 28% of SCC lesions. They emphasized the challenges in the use of the integration status as an adjunctive marker in the management of *HPV*-positive patients. In the current study, qRT-PCR was designed for viral load evaluation, and we found that 35.6% of our total specimens and 38% of our cervical specimens had integrated form of *HPV-16* genome; furthermore, integration in HSIL lesions was 45.4% (5/11), and in cancerous mass, it was 44.4% (4/9). The statistics showed that there was a significant relationship between the integration of *HPV-16* DNA in HSIL and cancerous vs. ASCUS and LSIL) that indicates a higher risk for these patients for cancer progression and chromosomal instability. Our study showed differences between the frequencies of *HPV* episomal form in cervical specimens similar to that described by Cheung et al. [13] (33% and 28% vs. 5%). *HPV* episomal forms were seen in only 5% (1/20) of the high-grade cervical cancer specimens (HSIL and cancerous) in our study, which is different from that reported in other studies. This could 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]. This association has not been proven by our study data. It may be due to the cancer therapies that were used to suppress the tumor progression. Studies have also shown a substantial proportion of *HPV* genome integration in low grade and primary stage of *HPV* infection [13,42]. We detected 28.6% (4/14) integrated form in ASCUS/LSIL lesions that were in the primary stages. The integration in early stages shows a probable role of other risk factors such as smoking, alcohol consumption, or UV radiation.

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). These findings could be related to the different populations or detection methods. It is simple to understand the higher prevalence of integration in the

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 Olthof 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 Djajadiningrat 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.

Acknowledgement

We are all thankful for the kind assistance of Keyvan Laboratory personnel, especially Mrs. Zohrebandian, and the kind cooperation of Dr. Mahshid Panahi and Dr. Seyed Dawood Mousavi Nasab. This study was a part of the Ph.D. dissertation by the grant number 922154020 in Iran University of Medical Sciences, Tehran, Iran.

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