doi:10.4149/neo_2010_06_595

Prevalence of high-risk human papillomavirus types (HPV-16, HPV-18) and their physical status in primary laryngeal squamous cell carcinoma

B. LIU[2,4], Z. LU[1,4], P. WANG[2,4], Z. BASANG[1], X. RAO[3]

[1]Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Genetics, Peking University School of Oncology, Beijing Cancer Hospital & Institute, Beijing 100142, China,e-mail: lbg29@sohu.com; *[2]Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Head and Neck Surgery, Peking University School of Oncology, Beijing Cancer Hospital & Institute, Beijing 100142, China;* *[3]Department of Pathology, Peking University Shougang Hospital, Beijing 100144, China;* *[4]These authors contributed equally.*

Received May 5, 2010

Recently oncogenetic human papilloma virus(HPV) infection has been suggested to promote laryngeal squamous cell carcinoma(LSCC). To determine the prevalence and genotypes of HPV infection in laryngeal cancer specimens, 84 specimens from pathologically confirmed LSCC patients were studied for the presence of viral DNA and possible virus integration into the cellular genome. HPV genotyping was assayed prior to the integration analysis by using two PCR-based assays, including HPV-16 and-18 E7 type-specific and L1 general primers (GP5+/GP6+). Additionally, a quantitative real-time PCR (qRT-PCR) was used to examine the physical status of HPV-16 or-18 in HPV positive LSCC. Using HPV L1 general primer amplification, HPV DNA was detected in 23 (27.4%) of the 84 LSCC samples. When PCR products were cloned and sequenced, HPV16 were found in all 23 L1 positive samples. However, when specific primers for HPV- 16 or -18 were used to amplify E6 and E7 in all samples, 29 cases (34.5%) were positive for HPV-16, while 6 cases (7.1%) were positive for HPV 18. Coinfection of HPV-16 and -18 were found in 4 cases (4.8%). In addition, qRT-PCR assay found that HPV-16 was characterized as episomal in 51.7% of cases, mixed (i.e., episomal and integrated) in 34.5%, and integrated in 13.8%, while HPV-18 was similarly characterized as episomal in 83.3% of cases and mixed in 16.7%. In the present study, about 36.9% of patients with LSCC were found to be infected with HPV-16 and -18 and integration of HPV-16 and -18 DNA into the host genome was found.

Keywords**:** human papillomavirus(HPV); laryngeal squamous cell carcinoma (LSCC);

The etiology of laryngeal squamous cell carcinoma (LSCC), the most common oropharyngeal cancer, is considered to be multifactorial. Two well-known risk factors for developing LSCC are tobacco smoking and alcohol consumption. However, during the past two decades, epidemiologic and biomolecular evidence have indicated that human papillomavirus (HPV) infection, known to cause cervical and other anogenital cancers, may be associated with the development of LSCC and serve as a distinct pathway for the oncogenesis of LSCC. Therefore, at least two distinct possible pathways exist for the carcinogenesis of LSCC, one driven primarily by the mutagenic effects of tobacco and alcohol and the other driven by HPV-mediated transformation [1].

Some discrepancy exists in the percentage of LSCC harboring HPV and the reported frequencies of HPV infection in LSCC vary over a wide range (3.3%-58.8%)[2]. The possible reasons for this difference could be the result of disparate geographic regions, varying analytic methods, difference in type of specimen and tumor site, as well as the number of cases included. Therefore, it is of interest to investigate the type and the physical state of the HPV viral genome in samples of LSCC.

We have reviewed all published articles detailing the association of HPV infection and laryngeal cancer. Most PCR-based investigations to detect HPV have employed L1 primers that amplify the gene encoding the viral capsid protein. Based on evidence from cervical cancer[3,4] and our previous data in the carcinogenesis of esophageal cancer [5], HPV prevalence based on L1 gene detection frequently will be reported lower than a type specific analysis. Two plausible explanations for this may be HPV integration into the human genome and primers stringency affecting sensitivity. However, most studies so

far have not analyzed HPV integration in LSCC and no data are available about the HPV physical status and its impact on HPV detection in LSCC. So, in order to definitively evaluate HPV presence in laryngeal cancer and the overall relationship between HPV prevalence and the development of LSCC, the detection of HPV E6 and E7 genes, in addition to the L1 gene, is necessary.

In cervical cancers, it has been shown that the integration of the HPV DNA into the host genome marks the transition from dysplasia to invasive cancer [6.7]^{1}, yet few studies have been performed to analyze HPV's physical status in LSCC. HPV integration is usually accompanied by disruption of the viral genes, and a portion of the E2 open reading frame (ORF) has been identified as the preferential site of integration as it has been found to be disrupted or deleted more frequently than other sites [8]. Recently, real-time PCR targeting E6 and E2 was shown to reliably characterize HPV DNA's physical state (episomal or integrated) [9,10]. Therefore, it seemed of interest to investigate whether this succession of events occurring in cervical cancer might also take place during carcinogenesis in the larynx. To this end, the types and the physical state of the viral genome were evaluated in samples of LSCC.

In this study, we first employed a general primer-mediated PCR to investigate the presence and type of HPV in a series of primary LSCC. Next, type-specific HPV detection was employed which, as expected, increased the overall positive rate of HPV. Finally, we analyzed the physical state of HPV-16 and HPV-18 (episomal vs integrated) in LSCC.

Materials and methods

Patients and tumor samples. A total of 84 paraffin-embedded tissue samples of primary laryngeal carcinoma (diagnosed during the period between 2000 and 2008) were obtained from Beijing Cancer Hospital and Shougang Hospital for this study. All tumor specimens were reviewed independently by two pathologists for verification of the diagnosis. The entire study was conducted according to ethical permissions from the Ethical Committee of the Hospital.

Patients' ages ranged from 38 to74 years (median age, 64 years). Sixty-one patients were males (72.6%) and twenty-three were females (27.4%). Tumor staging was done according to TNM classification after histological studies. By histologic analysis on haematoxylin-eosin-stained sections, 36 tumors were graded as well differentiated, 33 as moderately differentiated, and 15 as poorly differentiated/undifferentiated. Special care was taken not to contaminate the samples. Disposable or sterile instruments were used to harvest and store the samples.

DNA extraction. Formalin-fixed, paraffin-embedded samples were cut into 10 μm slices and prepared according to the method previously described [11]. Briefly, paraffin sections were deparaffinized in xylene and rehydrated in graded ethanols, followed by digestion with proteinase K (200ug/ml) at 55 overnight. The presence of DNA was confirmed by PCR with beta-actin (75 bp) to determine that the extracted DNA was amplifiable. Only samples whose beta -actin PCR were positive were considered to have sufficient DNA quality and were therefore used for subsequent analysis. To prevent the carryover of DNA and cross-contamination between samples, a disposable blade was used for each tissue block and replaced with a new blade for the next block.

Detection of HPV and typing. The presence of HPV DNA was evaluated by PCR using GP5+/GP6+ primers (150 bp), a consensus primer set for the HPV L1 gene [12] with minor modification, which incorporates degenerated bases at positions where nucleotides differ among the types, especially close to the 3' end of the primers (table 1). PCR was performed in a total volume of 25ul containing 1unit of HotStar Taq (QIAGEN, Hilden, Germany), 0.5uM of each primer, 0.2mM of each dNTP and 10XHotStar Taq PCR buffer as supplied by the enzyme manufacturer (QIAGEN) (contains 1.5mM MgCl2, Tris-Cl, KCl, $(NH4)_2SO_4$ pH 8.7). The amplification was carried out with touch down PCR as the optimized program described [13]. As a positive control for amplification, full genomes of HPV-16 and -18 were used (kindly donated by Professor Harald zur Hausen, German Cancer Research Centre, Heidelberg, Germany), and water was used as a template for all negative control. PCR products were visualised on 2% agarose gel with ethidium bromide staining by electrophoresis.

For HPV typing, GP5+/GP6+ amplicons were subsequently cloned into PCR-Blunt vector (Invitrogen, Carls- bad, CA), and at least 10 clones for each sample were sequenced for determination of HPV type. Sequences obtained from the cloned amplicons were compared with sequences available in Genbank using the BLAST program. Classification was done according to the following criteria: (1) if the identity between the query sequence and the closest related papillomavirus sequence in the databank is below 90%, but above 80%, the query sequence is defined as related to the next closest papillomavirus type, and therefore probably defines a putative new papillomavirus type, (2) if identity of the L1 ORF is 90% to 98%, it constitutes a subtype, and (3) if higher than 98%, it is considered a variant of a known papillomavirus type [14].

In addition, all samples were tested using type specific PCR, including HPV-16 E6, E7 and HPV-18E6. All primer sequences and length of amplified products are shown in table 1.

HPV physical status in LSCC. The integration of HPV-16 and -18 DNA was evaluated using real-time PCR according to the report by Huang et al [7]. Based on the assumption that E2 and E6 gene segments are present in equivalent proportions within each episomal HPV genome and that integrated HPV genome forms would have the E2 target deleted or absent, the integrity of E2 was taken as a marker that HPV had integrated into the host genome. Thus, using the E2/E6 gene copy number ratio, the physical status of the HPV genome for each sample was determined. Viral genomes were regarded as completely integrated when the E2/E6 gene copy number ratio was 0, mixed (both integrated and episomal) form when samples had

Table 1: Primer sequences of E2 and E6 in HPV type 16, 18, detection and E2/E6 ratio determination

Oligo name	Sequence	Product size (bp)	Reference
GP5+	TTTGTTACTGTGGTAGATAC(TC)AC	150	12
GP6+	GAAAAATAAACTGTAAATCATA(TC)TC		
16E2	F: AATTATTAGGCAGCACTTGGCCA	101	7
	R: ATCTTGGTCGCTGGAT-AGTCGTCT		
16E6	F: GAGCGACCCAGAAAGTTACCAC	107	7
	R: ACCTCACGTCGCAGTAACTGTTG		
16E7	F:AGCTCAGAGGAGGAGGATGA	203	5
	R:GGTTTCTGAGAACAGATGGG		
18E2	F: CCGCTACTCAGCTTGT-TAAACAGCT	97	7
	R: GCCGACGTCTGGCCGTAG		
18E6	F :CGGCGACCCTACAAGCTACC	107	7
	R: ACCTCTGTAAGTTCCAATACT-GTCTTGC		
beta-actin	F: TCCACCTTCCAGCAGATGTG	75	This article
	R:GCATTTGCGGTGGACGAT		

an E2/E6 ratio between 0 and 1, and episomal form when the E2/E6 ratio was equal to 1.

Real-time PCR was carried out in a 96-well reaction plate format in an ABI Prism 5700 Sequence Detection System (PE Biosystems, Foster City, California, USA). Amplification and quantification of the E2 and E6 genes were carried out simultaneously in separate reaction tubes. All reactions were carried out in triplicate. The sequences of all primers are shown in Table 1 [7]. A standard PCR was carried out in a 25ul reaction volume, SYBR Green containing reaction buffer and 100 nmol of primers for E2 or E6, 10-50 ng of DNA and 1 unit of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA). The cycling conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 58°C for 15 s. The specificity of each amplification was confirmed by checking the dissociation curve against the expected melting temperature of the amplification product. A standard curve for E6 and for each of the E2 gene targets was obtained by plotting threshold cycle values against 10-fold serial dilutions of plasmids containing the full genome of HPV type 16 and 18, and all samples showed high amplification efficiency. The number of threshold cycles obtained from E2 PCR and E6 PCR was equivalent in each run. Linear plots of the log of copy numbers versus number of threshold cycles were obtained for both genes, and their correlation coefficient was between 0.97 and 1.00 in each run. The slopes of the standard curves indicate that amplification efficiencies of E2 and E6 PCR systems were very similar. The validity of the assay was further confirmed by the accurate determination of the physical status of HPV-16 in SiHa cervical carcinoma cell line, which harbors pure integrated forms of HPV-16 genes.

Statistical analysis. Statistical analysis was performed using SPSS statistical software. The association between HPV presence and other clinical and pathological features was compared using a X^2 test. The statistical relationship was considered as significant if the derived p-value was <0.05.

Results

Human papillomavirus genotyping and prevalence. The GP5+/GP6+ primers were used for PCR amplification. Of the 84 samples tested, 23 (27.4%) were positive. For all HPV-positive samples, the amplicon was cloned into pCR-Blunt vector, and at least ten clones for each sample were randomly picked and sequenced. Sequence comparisons and the interpretation of DNA sequence identities revealed that all HPV-positive clones showed 99-100% homology with the HPV-16 prototype described by Seedorf et al [15]. In addition, two variants of HPV-16 L1 sequences were identified among the 23 sequenced samples. Such variations indicate that the presence of HPV-16 sequences is not due to contamination. We and others have reported that the prevalence of E6 and E7 genes are always higher than L1 gene [3-5]. Hence, primers for HPV-16 type-specific E6 and E7 were used for PCR amplification. As expected, HPV-16 type-specific E6 and E7 revealed the presence of HPV-16 in 29 (34.5%) cases. Moreover, it should be noted that E6 and E7 are always present together and are always positive in L1 positive samples, resulting in six HPV E6 and E7 positive samples identified without L1 amplification.

HPV-16 and HPV-18 are the predominant types in cancerous lesions accounting for about 74% of all HNSCC worldwide [2]. Using the general primer and HPV-16 type-specific primer in this study, we demonstrated the high prevalence of HPV

type 16 in LSCC. In order to further evaluate the presence of HPV-18 in LSCC, HPV-18 type-specific E6 primers were used for PCR amplification and HPV-18 E6 was found in 6 cases (7.1%), of which 4 cases were co-infected with HPV-16. Overall, HPV type 16 and 18 infections were present in 36.9% of the LSCC samples.

HPV genome physical status. Besides demonstrating that primers for E6 and E7 gene are more sensitive than consensus primers for the L1 gene, these results also imply the possibility of viral gene integration in LSCC, which causes the loss of the L1 gene while retaining both E6 and E7 genes.

In general, an intact E2 gene is disrupted upon HPV integration, thus distinguishing the episomal form of HPV DNA from the integrated form by detection of an intact E2 gene. To determine the physical status of HPV DNA detected in LSCC, the HPV E2 gene from all 31 HPV DNA-positive LSCC was examined. As shown in Table 2: of the 29 HPV-16 positive samples, fifteen (51.7%) showed equivalence in gene copy number for the E2 gene and E6 gene indicating a HPV DNA episomal form. Four (13.8%) showed no detectable amplification for the E2 gene indicating the integrated HPV form. The remaining 10 (34.5%) samples contained both the episomal and integrated forms of HPV, which were thus considered to represent mixed forms. In 5 of 6 HPV-18 DNA-positive LSCC no detectable E2 gene was found indicating the presence of integrated HPV-18 with the remaining case being mixed form. The episomal and mixed forms of HPV DNA were the most prevalent physical state for HPV-16, while the integrated form was the most prevalent physical state for HPV-18. The frequencies of DNA integration for HPV-16 and -18 were significantly different in LSCC ($p<0.01$).

HPV status and tumor clinical/pathological parameters. The association between prevalence of HPV infection and clinical/pathological parameters is shown in Table 3. No statistical correlation was observed between HPV positivity and age, gender, tumor status, tumor localization, differentiation, tumor stage, lymph node involvement, alcohol, or tobacco use.

Discussion

Epidemiologic and molecular studies have suggested that HPV infection may be associated with LSCC. In a recent comprehensive review, Kreimer reported that the overall prevalence of HPV positivity assessed by PCR was 24.0% [2]. In the reviewed studies, the number of tumors ranged from two to 103, and the HPV prevalence varied from 3.3% to 58.8%. In the present study, HPV DNA was detected in 36.9% of cases of laryngeal carcinoma in a population of 84 patients. This finding is consistent with the average prevalence of HPV positivity found in several studies on a large number of LSCC cases.

Table 2: Physical state of HPV 16 and HPV 18 in LSCC

	HPV 16 (n=29)	HPV 18 (n=6)
episome	15 (51.7%)	0
mix	10 (34.5%)	1 (16.7%)
integration	4 (13.8%)	5 (83.3%)

Presently, L1 ORF remains the definition of a papillomavirus type. The GP5+/GP6+ primers amplify a shorter region within the region of the L1 ORF[12,13].Several consensus PCR primer sets have been developed, including broad-spectrum GP5+/6+, which have been shown to permit adequate detection of the ever-expanding spectrum of HPV genotypes. The uses of these sensitive consensus primer sets, followed by cloning and sequencing of the amplicons, allow for highly effective detection of HPV types. Using this approach, HPV DNA was determined in 27.4% of 84 paraffin embedded tissue samples of LSCC with a genotyping assay showing all of them to be HPV-16. This result is consistent with other reports that HPV-16 is the most frequently detected type, where, among positive samples, approximately 90-100% are positive for HPV-16 [1,16-18]. Economically, it is not feasible to identify multiple HPV type infections via our method of analysis, especially considering the large numbers of colonies/inserts employed, but most studies detect multiple type infections, so it is appropriate to include HPV-18, the second most frequent type, for the purposes of this study.

We and others have previously noted that HPV E6 and E7 primers are more sensitive than L1 consensus primers [3/5], so all samples were analyzed with HPV 16 and 18 type-specific primers. To the best of our knowledge, the current study is the first combining L1 consensus primer and type-specific primers to detect HPV-DNA in a large series of primary LSCC. In most studies, type-specific PCR is used only for HPV typing in positive samples amplified with consensus primers [19,20]. According to our results, this method inevitably results in an underestimation of the true HPV prevalence. Our data also support the hypothesis that primer choice in part accounts for the variation in reported HPV prevalence in LSCC. HPV-16 and -18 type-specific primers revealed the presence of HPV in 31 cases (36.9%) versus 23 cases (27.4%) by consensus primer. Moreover, it is imperative to note that E6 and E7 are always present at the same time and positive in all L1 positive samples. These data confirm that the presence of HPV type 16 and 18 are highly correlated with LSCC. Although most studies suggested that HPV-16 and -18 are two of the most predominant HPV types in all HPV-related cancers, including LSCC, other oncogenic HPV types may also be involved in the oncogenesis of LSCC [2], so a slight underestimation of HPV-infected LSCC may be present in this study because we only focused on HPV types 16 and 18.

Studies on the presence of HPV DNA in cervical samples showed that 10% or more of clinical lesions contain at least two different HPV genotypes [22,23], and double infections in LSCC have also been reported in a study of head and neck carcinomas [21]. In the present study, we observed co-infection of HPV-16 and -18 in 12.5% (4/32) of the genotyped samples,

Table 3: Risk factors associated with HPV positive and negative LSCC

	Total(n=84)	HPV-positive N=31(36.9%)	HPV-negative N=53(63.1%)	X2 test			
	No.	%	No.	%	No.	%	Pr<= P
Gender							
Male	61	72.6	22	70.9	39	73.6	0.7952
Female	23	27.4	9	29.1	14	26.4	
Age							
<60	35	41.7	13	41.9	22	41.5	0.9695
≧60	49	58.3	18	58.1	31	58.5	
Tumor Status							
T0	3	3.6	0	0	3	5.7	0.4013
T1	9	10.7	3	9.7	6	11.3	
T2	21	25	7	22.6	14	26.4	
T3	31	36.9	15	48.4	16	30.2	
T4	20	23.8	6	19.4	14	26.4	
Localization							
Supraglottic	28	33.3	11	35.5	17	32.1	0.8719
Glottic	49	58.3	18	58.1	31	58.5	
Subglottic	7	8.3	2	6.5	5	9.4	
Grade							
Well	36	42.9	11	35.5	25	47.2	0.3625
Moderate	33	39.2	15	48.4	17	32.1	
Poor/un	15	17.9	5	16.1	10	18.9	
Stage							
I	5	5.9	1	3.2	4	7.5	0.7412
II	12	14.3	4	12.9	8	25.8	
III	24	28.6	8	25.8	16	30.2	
IV	43	51.2	18	58.1	25	47.2	
Lymph nodes							
Yes	55	65.5	24	77.4	31	58.5	0.078
No	29	34.5	7	22.6	22	41.5	
Alcohol Use							
Yes	28	33.3	9	29.0	19	35.8	0.5225
No	56	66.7	22	71.0	34	64.2	
Tobacco use							
Yes	19	22.6	6	19.4	13	24.5	0.5844
No	65	77.4	25	80.6	40	75.5	

and it is of note that 4 out of 6 of HPV-18 positive samples in our study show double infection with HPV-16. Further studies to examine the synergy of HPV coinfection in LSCC are warranted to examine the possible additive effects of multiple infections.

Furthermore, our results are in line with the hypothesis that in HPV-positive tumors HPV types 16 and 18 are integrated in LSCC. We observed viral integration into the host genome occurred in 4 out of 29 HPV-16 positive tumors (13.8 %) and in 5 out of 6 HPV-18 tumors (83.3%). The episomal and mixed forms of HPV DNA were the most prevalent physical states for HPV-16, while the integrated form was the most prevalent physical state for HPV-18. The current data are consistent with previous evidence by Venuti and Badaracco, who detected integrated HPV-16 DNA in 35.4% of malignant cervical lesions containing HPV-16 DNA and 100% of HPV-18 types as pure integrated forms [19,24]. It is conceivable that the variable integration levels between the HPV-16 and HPV18 are responsible for viral prevalence and the somewhat different biological behavior in disease progression. It has often been suggested that HPV18 infection is clinically more aggressive in cervical cancer partly due to its high rate of integration [25. However, we cannot draw any such conclusions about integration or the physical state of the HPV genome in our samples, due to the small number of HPV positive cases investigated. Further research to assess the influence of HPV genome integration is therefore needed on a larger cohort of patients.

Although the biological significance awaits further research, the impact on HPV detection based on widely used L1 consensus primers is challenged by our data.

In addition to increased sensitivity, the uses of type-specific E6 and/or E7 primers to detect HPV and determine its physical status have two important implications regarding HPV pathogenesis and prognosis. First, molecular evidence has shown that E6 and E7 viral oncogenes are almost always actively expressed in HPV-associated cancers where they interact with the tumor suppressor gene products p53 and pRB pathways, promoting genomic instability and maintenance of the transformed state both in vivo and in vitro [26,27]. Second, HPV typing may play a potential role in clinical management of LSCC. HPV DNA positive tumors are associated with improved overall patient survival due to a higher sensitivity to chemoradiotherapy and are a significant predictor for disease free survival in a subgroup of head and neck cancers including LSCC [28-31]. Meanwhile, specific types of HPV show differential risk for the progression to cervical cancer [32]. Thus, accurate assay of the HPV prevalence in LSCC might become the basis of treatment decisions and prognostic indicator of tumor behavior in the future.

The association of HPV prevalence with various clinical/pathological parameters is shown in Table 2. We found that HPV presence was not significantly associated with age, gender, clinical stage, nodal status, alcohol or tobacco use. Previous studies have similarly reported clinical stage and TNM status not to be associated with HPV presence [16,21]. Presently, a follow-up study is on-going to observe the length of disease-free interval between HPV positive and negative patients.

One possible limitation of our study design was the inability to examine all HPV types. If such tests became cost-effective in the future, it is likely that we could examine as many as possible high-risk HPV types using type-specific primers to identify overall HPV infection. However, high-risk HPV types other than HPV-16 and 18 account for approximately less than 5% of cases in the literature [2,23]. Another limitation of our study is the intrinsic complexity of HPV integration. It is also possible to extensively map the deletion regions to exclude though integrated, still conserve intact copies E2 gene, which could mask the detection of integration in some samples [34].

In conclusion, our findings support the emerging consensus that HPV is involved in the etiology of a subset of LSCC and a large proportion of specimens harbor HPV genome in the integrated form. In the future, follow-up studies seem warranted to draw any conclusions between the relationships of the HPV genotype/integration to the main clinical/pathological features in LSCC. Finally, with the availability of efficacious vaccines against HPV types 16 and 18 used to prevent the development of HPV-related diseases of the cervix, it remains to be determined whether these vaccines could similarly prevent an increase in the incidence of LSCC.

References

[1] GILLISON ML, D'SOUZA G, WESTRA W, SUGAR E, XIAO W et al. Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. J Natl Cancer Inst 2008 ;100 :407-20.

[2] KREIMER AR, CLIERFFORD GM, BOYLE P, FRANCESCHI S . Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. Cancer Epidemiol Biomarkers Prev 2005; 14 : 467-475. doi:10.1158/1055-9965.EPI-04-0551

[3] Zur Hausen H. Papillomavirus infections-a major cause of human cancers. BBA 1996; 1288:55-78.

[4] HERRINGTON CS, ANGERSON SM, BAUER HM, TRONCONE B, DE ANGELIS ML et al. Comparative analysis of human papillomavirus detection by PCR and non-isotopic in situ hybridization. J Clin Pathol 1995; 48: 415-419. doi:10.1136/jcp.48.5.415

[5] LI T, LU Z, CHEN K, GUO M, XING H et al. Human papillomavirus type 16 is an important infectious factor in the high incidence of esophageal cancer in Anyang area of China. Carcinogenesis 2001; 22, 929-934. doi:10.1093/carcin/22.6.929

[6] CULLEN AP, REID R, CAMPION M, LÖRINCZ AT. Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasm. J Virol 1991; 65: 606-612.

[7] HUANG L-W, CHAO S-L, LEEB-H. Integration of human papillomavirus type-16 and type-18 is a very early event in cervical carcinogenesis. Journal of Clinical Pathology 2008; 61: 627-631. doi:10.1136/jcp.2007.052027

[8] CHOO KB, PAN CC, HAN SH. Integration of human papillomavirus type 16 into cellular DNA of cervical carcinoma: preferential deletion of the E2 gene and invariable retention of the long control region and the E6/E7 open reading frames. Virology 1987; 161:259-261. doi:10.1016/0042-6822(87)90195-4

[9] SAUNIER M, MONNIER-BENOIT S, MAUNY F, DALSTEIN V, BRIOLAT J et al. Analysis of Human Papillomavirus Type 16 (HPV16) DNA Load and Physical State for Identification of HPV16-Infected Women with High-Grade Lesions or Cervical Carcinoma. J Clin Microbiol 2008; 46: 3678-3685. doi:10.1128/JCM.01212-08

[10] Briolat J, V DALSTEIN M, SAUNIER K, JOSEPH S, CAUDROY J et al. HPV prevalence, viral load and physical state of HPV-16 in cervical smears of patients with different grades of CIN Int J Cancer 2007; 121:2198-2204. doi:10.1002/ijc.22959

[11] LI T, LU Z, GUO M, WU Q, CHEN K et al. p53 Codon 72 Polymorphism (C/G) and the Risk of Human Papillomavirus-Associated Carcinomas in China. Cancer 2002;95: 2571-2576. doi:10.1002/cncr.11008

[12] DE RODA HUSMAN AM, WALBOOMERS JM, VAN DEN BRULE AJ, MEIJER CJ, SNIJDERS PJ. The use of general primers GP5 and GP6 elongated at their 3 ends with adjacents highly conserved sequences improves human papillomavirus detection by polymerase chain reaction. J Gen Virol 1995; 76:1057-1062.

[13] EVANS MF, ADAMSON CS, SIMMONS-ARNOLD L, COOPER K. Touchdown General Primer (GP5+/GP6+) PCR and optimized sample DNA concentration support the sensitive detection of human papillomavirus. BMC Clinical Pathology 2005; 5:10 doi:10.1186/1472-6890-5-10

[14] DE VILLERS EM, WHITLEY C, GUNST K. Identification of New Papillomavirus Types.In Methods in Molecular Medicine, Vol. 119: Human Papillomaviruses: Methods and Protocols(Davy C. and Doorbar J.)Humana Press Inc., Totowa, NJ,2005: 1–13

[15] SEEDORF K, KRAMMER G, DUERST M, SUHAI S, ROWEKAMP WG. Human papillomavirus type 16 DNA sequence. Virology 1985;145: 181-185. doi:10.1016/0042-6822(85)90214-4

[16] RINGSTRÖM E, PETERS E, HASEGAWA M, POSNER M, LIU M et al. Human papillomavirus type 16 and squamous cell carcinoma of the head and neck. Clin Cancer Res 2002;8:3187-92.

[17] GILLISON ML, KOCH WM, CAPONE RB, SPAFFORD M, WESTRA WH et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst 2000; 92:709-20. doi:10.1093/jnci/92.9.709

[18] HERRERO R, CASTELLSAGUE X, PAWLITA M, LISSOWSKA J, KEE F et al. Human papillomavirus and oral cancer: the International Agency for Research on Cancer multicenter study. J Natl Cancer Inst 2003; 95: 1772-83.

[19] VENUTI A, MANNI V, MORELLO R, DE MARCO F, MARZETTI F et al. Physical state and expression of human papillomavirus in laryngeal carcinoma and surrounding normal mucosa. J Med Virol 2000; 60:396-402. doi:10.1002/(SICI)1096-9071(200004)60:4<396::AID-JMV6>3.0.CO;2-V

[20] JUNG AC, BRIOLAT J, MILLON R,DE REYNIES A, RICKMAN D et al. Biological and clinical relevance of transcriptionnally active human papillomavirus (HPV) infection in oropharynx squamous cell carcinoma. Int J Cancer 2010; 126:1882-1894.

[21] BADARACCO G, VENUTI A, MORELLO R, MULLER A, MARCANTE ML. Human papillomavirus in head and neck carcinomas: prevalence, physical status and relationship with clinical/pathological parameters. Anticancer Res 2000; 20(2B):1301-5.

[22] ROUSSEAU MC, ABRAHAMOWICZ M, VILLA L, COSTA MC, ROHAN TE et al. Predictors of cervical coinfection with multiple human papillomavirus types. Cancer Epidemiol Biomarkers Prev 2003; 12: 1029-1037.

[23] SCHELLEKENS M, DIJKMAN A, AZIZ M, SIREGAR B, CORNAIN S et al. Prevalence of single and multiple HPV types in cervical carcinomas in Jakarta, Indonesia. Gynecol Oncol 2003;93: 49-53. doi:10.1016/j.ygyno.2003.12.015

[24] BADARACCO G, VENUTI A, SEDATI A, MARCANTE ML. HPV16 and HPV18 in genital tumors: significantly different levels of viral integration and correlation to tumor invasiveness. J Med Virol 2002; 67:574-82. doi:10.1002/jmv.10141

[25] HUDELIST G, MANAVI M, PISCHINGER KI, WATKINSRIL T, SEDELINGER CF et al. Physical state and expression of HPV DNA in benign and dysplastic cervical tissue: different levels of viral integration are correlated with lesion grade. Gynecol Oncol 2004; 92:873-80. doi:10.1016/j.ygyno.2003.11.035

[26] JEON S, LAMBERT PF. Integration of human papillomavirus type 16 DNA into the human genome leads to increased stability of E6 and E7 mRNAs: implications for cervical carcinogenesis. Proc Natl Acad Sci U S A 1995; 92:1654-8. doi:10.1073/pnas.92.5.1654

[27] PARK TW, FUJIWARA H, WRIGHT TC. Molecular biology of cervical cancer and its precursors. Cancer 1995; 76:1902-13. doi:10.1002/1097-0142(19951115)76:10+<1902::AID-CNCR2820761306>3.0.CO;2-0

[28] FAKHRY C, WESTRA WH, SIGUI L et al. Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. J Natl Cancer Inst 2008; 100 261-269. doi:10.1093/jnci/djn011

[29] LI W, THOMPSON CH, O'BRIEN CJ, MCNEIL EB, SCOLYER RA et al. Human papillomavirus positivity predicts favorable outcome for squamous carcinoma of the tonsil. Int J Cancer 2003; 106:553-8. doi:10.1002/ijc.11261

[30] RITCHIE JM, SMITH EM, SUMMERSGILL KF, HOFFMAN HT, WANG D et al. Human papillomavirus infection as a prognostic factor in carcinomas of the oral cavity and oropharynx. Int J Cancer 2003; 104:336-44. doi:10.1002/ijc.10960

[31] LINDEL K, BEER KT, LAISUE J, GREINER RH, AEBERSOLD DM. Human papillomavirus positive squamous cell carcinoma of the oropharynx: a radiosensitive subgroup of head and neck carcinoma. Cancer.2001; 92:805-13. doi:10.1002/1097-0142(20010815)92:4<805::AID CNCR1386>3.0.CO;2-9

[32] KHAN MJ, CASTLE PE, LORINCZ AT, WACHOLDER S, SHERMANM et al. The Elevated 10-Year Risk of Cervical Precancer and Cancer in Women With Human Papillomavirus (HPV) Type 16 or 18 and the Possible Utility of Type-Specific HPV Testing in Clinical Practice. J Natl Cancer Inst 2005; 97: 1072-1079. doi:10.1093/jnci/dji187

[33] HERRERO R, CASTELLSAGUE X, PAWLITA M. Human papillomavirus and oral cancer: the International Agency for Research on Cancer multicenter study . J Natl Cancer Inst 2003; 95 :1772-1783.

[34] ARIAS-PULIDO H, PEYTON CL, JOSTE NE, VARGAS H, WHEELER CM. Human Papillomavirus Type 16 Integration in Cervical Carcinoma In Situ and in Invasive Cervical Cancer J Clin Microbiol 2006; 44: 1755-1762.