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Variation of Human papillomavirus 16 in cervical and lung cancers in Sichuan, China

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Summary. – Although the crucial role of human papillomaviruses (HPVs), especially HPV-16 in various cancers has been confirmed, the variation of HPV-16 among different cancers have not been investigated in a specific geographic location. In order to elucidate whether similar HPV-16 variants are involved in different kinds of cancers in the same geographic location, the analysis of sequence variants of E6 and E7 oncogenes and L1 gene of HPV-16 in cervical and lung cancers in Sichuan, China, was carried out. Tissue samples from 122 cervical cancers, 104 lung cancers, and 138 controls were subjected to RT-PCR or PCR, sequencing, and sequence analysis. The infection rates of HPV-16 in cervical, lung cancers, and non-malignant controls were 68.9%, 17.3%, and 37.0%, respectively. Asian prototype variants prevailed in cervical and lung cancers, while European prototype variants in non-malignant controls. In comparison to the lung cancer, cervical cancer showed a much higher diversity of HPV-16 oncogenes. These results indicate that in Sichuan, China, Asian prototype variants of HPV-16 are more pathogenic than their European counterparts.

Keywords: Human papillomavirus 16; cervical cancer; lung cancer; E6, E7, L1 genes; sequence variation

Introduction

HPV has been linked to the carcinogenesis of several types of cancers including cervical, lung, skin, and others types of cancer (zur Hausen, 2009). The genome of HPV16 consists of 7,904 bp including six early genes (E1, E2, E4, E5, E6, and E7), two late genes (L1 and L2), and a long control region (LCR) that regulates the expression of early genes (zur Hausen, 2009). The distribution of HPV genotypes in different geographic locations and populations is variable. Identification of a new HPV type requires that the nucleotide sequences of E6, E7, and L1 genes should be less than 90% identical to the known types. Furthermore, HPVs are classified into "subtypes" or "variants", if they share 90–98% identity or more than 98% identity to a prototype, respectively (de Villiers, 1994).

On the basis of sequence variations in E6, L1, L2 genes, and LCR, HPV-16 variants have been identified and grouped into six distinct phylogenetic branches (Yamada *et al.*, 1995): European (E), Asian (As), African 1 (Af1), African 2 (Af2), North American (NA), and Asian-American (AA). These variants have been found to have different geographic distributions with various carcinogenic potential (Yamada *et al.*, 1995). The E6 sequence patterns for this classification purpose are as follows (Seedorf *et al.*, 1985; Chan *et al.*, 2002): (i) European - are identical to or 1–2 nts different from HPV-16R, but with no signature patterns of other classes identified (Seedorf *et al.*, 1985), (ii) Asian – T178G, (iii) African 1 – G132C, C143G, G145T, T286A, A289G, C335T, (iv) African 2 – T109C, G132T, C143G, G145T, T286A, A289G, C335T, A403G, (v) North American – G145T, T286A,

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Abbreviations: AA = Asian-American; Af1 = African 1; Af2 = African 2; As = Asian; E = European; HPV = Human papillomavirus; LCR = long control region; NA = North American

A289G, C335T, T350G, and (vi) Asian-American – G145T, T286A, A289G, C335T, T350G, A532G (Ho *et al.*, 1991; Yamada *et al.*, 1995; Wheeler *et al.*, 1997).

Worldwide, cervical cancer is the second most common cancer in women. More than 500,000 new cases and approximately 250,000 deaths are reported annually with 95% of them occurring in developing countries. In West China, the average mortality of cervical cancer patients is moderately high approx. 36 per 100,000 women (Xiao *et al.*, 1988). HPVs are involved in the etiology of cervical cancer and its precursor lesions. HPV infection on a global scale accounts for more than 50% of HPV-linked cancers in females, while barely 5% in males (zur Hausen, 2009). HPV DNA is found in the tumor tissues of more than 93% of cervical cancer patients, of which HPV-16 is the most prevalent type accountable for approx. 50% of infections. Remarkably, HPV-16 positive rate in cervical cancer patients in Southern China was 79.6% (Lo *et al.*, 2002).

Other kinds of HPV related cancers have also been reported including lung, skin, head and neck, anal, and vulvar cancer (zur Hausen, 2009). HPV has been supposed to infect epithelial basal cells at micro-lesion or trauma, such as the squamo-columnar transitional zone of the uterine cervix and the bronchial mucosa of cigarette smokers (Syrjänen, 1980; Burger *et al.*, 1993). It was suggested that cigarette smoking and the presence of oncogenic HPV had a direct causal relationship (Burger *et al.*, 1993). Furthermore, HPV18 DNA and E6/E7 mRNAs were detected in lung cancer (Kinoshita *et al.*, 1995). The results revealed not only the presence of the HPV genome, but also its expression showed that HPV played a causative role in the development and progression of lung cancer.

Although the role of HPV-16 as a carcinogenic factor in various cancers has been confirmed, its variations among different cancers have not been properly evaluated in the specific geographic locations. Considering that the majority of cancers are derived from epithelial cells, we hypothesize that HPV in different cancer cells or in different individuals with the same type of cancer would be similar with limited variation in the certain location such as Sichuan, a province of China with 88 million people. Further examination of this hypothesis is essential to our understanding of the carcinogenic role of HPV and will help us to develop a vaccination strategy for the prevention of HPV-induced cancers.

In the present study, we attempted to reveal the variation of HPV-16 in its E6, E7, and L1 genes in cervical and lung cancers in Sichuan, China.

Materials and Methods

Clinical specimens. Cervical specimens from 122 cervical cancer patients and 138 patients with non-malignant uterine diseases were obtained from the Department of Obstetrics & Gynecology, West China Second University Hospital. Paraffin-embedded lung cancer tissues from 104 patients were obtained from the Department of Pathology of Affiliated Hospital, Luzhou Medical College, and the Department of Thoracic and Cardiovascular Surgery, West China Hospital. All patients were from Sichuan province and gave us informed consent. The study was approved by the institutional ethical committee. The diagnosis for all patients was confirmed by two histopathologists. If there was disagreement, a third pathologist was consulted until a consensus was reached. Among the 122 cervical cancer patients, 104 were diagnosed as squamous cell carcinoma, 6 as adenocarcinoma, 8 as adeno-squamous carcinoma, 2 as minimal deviation adenocarcinoma, 1 as small cell carcinoma, and 1 as metastatic cervical cancer derived from endometrial carcinoma. Among the 104 lung cancer patients, 51 were diagnosed as squamous cell carcinoma, 30 as adenocarcinoma, 4 as adeno-squamous carcinoma, 1 as pulmonary abscess, and 18 as other carcinomas including large cell carcinoma and bronchoalveolar carcinoma.

RT-PCR. Total RNA was extracted from the fresh cervical tissue samples immediately after surgery using TRIzol (Invitrogen). Reverse transcription was carried out according to the instruction of cDNA synthesis kit ReverTra Ace-α-*TM* (Toyobo).

PCR. Due to the institutional arrangement of the surgery department, we could not obtain the fresh lung tissues immediately after the surgery, therefore paraffin embedded blocks were used for extraction of DNA using a Nucleospin® Tissue kit (Macherey-Nagel). HPV was detected using consensus primers MY11/MY09 targeting L1 gene as previously reported (amplicon 452 bp) (Resnick *et al.*, 1990). GAPDH was amplified as an internal control using primers 5'-ACC ACA GTC CAT GCC ATC AC-3' and 5'-TCC ACC ACC CTG TTG CTG TA-3' (amplicon 450 bp). For L1 positive samples, HPV16 E6 or E7 specific PCR was further performed with the following primers: E6 (nt 46–572): 5'-TTG AAC CGA AAC CGG TTA GT-3' and 5'-TCT CCA TGC ATG ATT ACA GC-3' (amplicon 527 bp); E7 (nt 562–858): 5'-GCC GGA TCC ATG CAT GGA GAT ACA CCT AC-3' and 5'-GCT CTC GAG TTA TGG TTT CTG AGA ACA GAT G-3' (amplicon 297 bp). The oligonucleotides were synthesized and purified by TaKaRa Biotechnology (Dalian). PCR was conducted using a 25 µl reaction mix containing 100–200 ng DNA or cDNA, 10 mmol/l Tris-HCl (pH 8.4), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 12.5 µmol/l dNTP mix, 10 pmoles primers, and 1 U *Taq* DNA polymerase (Bioteke). The temperature parameters were denaturation at 95°C for 5 mins followed by 36 cycles with denaturation at 95°C for 30 secs, annealing at 52°C for 30 secs for L1 (or 58°C for E6, or 60°C for E7 and GAPDH) and extension at 72°C for 45 secs, and final extension for 10 mins on Eppendorf Mastercycler.

Reaction mixture containing no DNA or cDNA served as the negative control and cDNA synthesized from RNA isolated from HPV16-positive cell line (Caski) was used as the positive control. PCR product was subjected to the electrophoresis on 1.5% agarose gels with GreenView staining (Bioteke) and visualized under ultraviolet light using a Molecular Imager Gel Doc XR System (Bio-Rad). PCR was repeated at least three times for each sample. HPV infection was designated when the sample was positive for L1 and confirmed as HPV-16-positive when the sample was positive for L1, E6, and E7 genes.

Sequencing and sequence analysis. For variant analysis, the positive PCR products were sequenced by TaKaRa Biotechnol-

ogy (Dalian). The prototype sequence HPV-16R was used for the comparison and nucleotide position numbering and referred as the "European prototype" (Seedorf *et al.*, 1985). All sequences were analyzed by NCBI Blast and Mutation Surveyor software (version 2.28) (SoftGenetics).

Statistical analysis. Statistical analysis was performed using SPSS version 13.0. A chi-square test was used to determine differences in HPV infection and HPV-16 variants among different cancers. Fisher's exact test was applied to compare the small numbers, when the minimum expected count was less than 5. A difference with $P \leq 0.05$ was considered as significant.

Results

HPV infection in cervical and lung cancer

Among the 122 cervical cancer cases, 95 (77.9%) were positive for HPV and 84 (68.9%) were positive for HPV-16. Totally 18 (17.3%) lung tissues (including a case of lung abscess) were positive for HPV-16. Of the 138 control samples, 51 (37.0%) were positive for HPV-16 (Table 1).

Table 1. HPV infection in cervical and lung cancer and controls

Patient group	No. of patients	Age range (mean)	No. (%) of HPV positives	No. (%) of HPV-16 positives
Cervical cancer	122	20–71 (43.8)	95(77.9)	84(68.9)
Controls	138	29–69 (46.8)	51(37.0) [a]	51(37.0) [a]
Lung cancer	104	19–78 (57.3)	18 (17.3) [a]	18 (17.3) [a]
Males	76	19–76 (57.2)	16 (21.1) [a]	16 (21.1) [a]
Females	28	38–78 (57.7)	2 (7.1) [a]	2 (7.1) [a]

[a]Significantly different from the cervical cancer group.

L1 variants

A partial sequence of L1 gene was screened using a 452 bp amplicon for 52 cervical cancer samples, 18 lung tissues, and 16 benign cervical tissues. We found eleven L1 variants in total and most of these variants were found in the cervical cancer samples. Compared with E6 and E7 variants, L1 had more synonymous mutations including: A6665C, G6719A, C6824T, A6869G, T6887C, A6950G, and A6989G in the codons for S369, K387, S422, G437, T443, E464, and L477, respectively. For all samples, most of their L1 sequences were identical to the prototype. One of the missense mutations was G6818C found exclusively in a pulmonary abscess tissue in the codon for M420I (Fig. 1a, Table 2).

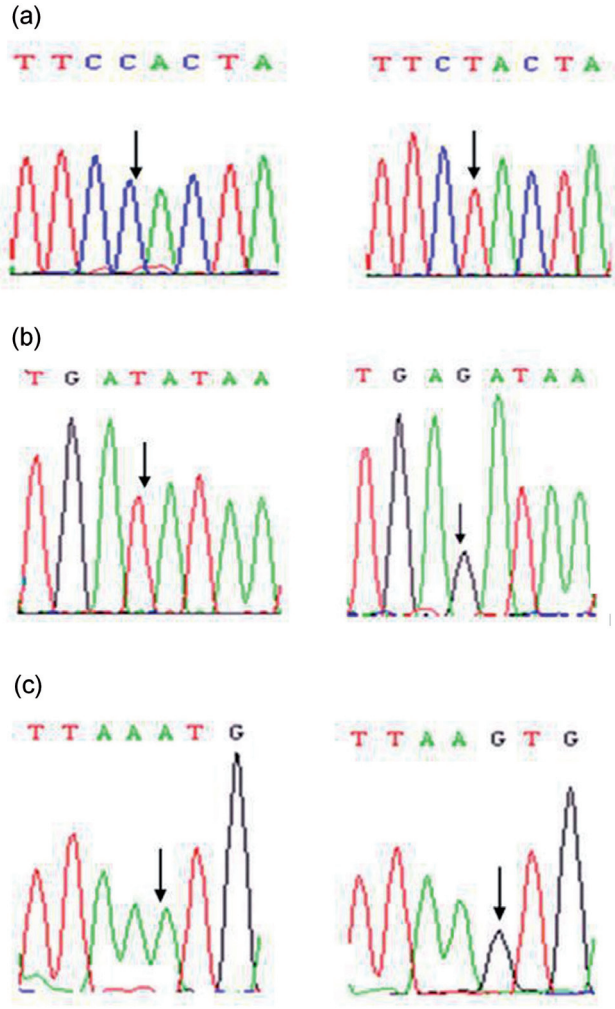

Fig. 1

Sequencing electropherogram showing frequently detected nucleotide variations in HPV16 L1, E6, and E7 genes

L1 C6824T (a), E6 T178G (b), and E7 A647G (c). Prototype sequences (left panel), variant sequences (right panel) are indicated by arrows.

E6 variants

We obtained the complete sequence of E6 ORF in 60 cervical cancer tissues, 4 lung tissues, and 16 non-malignant cervical tissues. Twelve different HPV-16 E6 variants were identified in the cervical cancer and only two of them were found in the lung tissues (Table 3). The variants showed base substitutions at different sites and most of them resulted in the corresponding amino acid changes. Only two of the variants T109C and A131C led to the synonymous mutations of phenylalanine at codon 2 and arginine at codon 10, respectively. The cervical cancer tissues contained the most variants with the Asian prototype prevailing (40.0%) (Fig. 1b). The Asian prototype and E-G168G

Table 2. HPV16 L1 variants identified in cervical and lung cancer and control samples

Variant No.	ORF nucleotides									Predicted amino acid substitution	No. (%) of identified sequences		
	6665	6687	6719	6818	6824	6869	6887	6950	6989		Cervical cancer (n=52)	Controls (n=16)	Lung cancer (n=18)
1	A	T	G	G	C	A	T	A	A	–	37 (71.2)	14 (87.5)	17
2	–[a]	–	–	–	T	–	–	–	–	S422S	3 (5.8)	0	0
3	–	G	–	–	T	–	–	–	–	S377A +S422S	1 (1.9)	0	0
4	–	–	–	–	–	–	–	–	G	L477L	1 (1.9)	0	0
5	–	–	–	–	–	G	–	–	–	G437G	2 (3.8)	0	0
6	C	–	–	–	–	–	–	–	–	S369S	4 (7.7)	0	0
7	–	–	A	–	–	–	–	–	–	K387K	1 (1.9)	2 (12.5)	0
8	–	–	–	–	T	–	–	G	–	S422S + E464E	1 (1.9)	0	0
9	–	–	–	–	–	–	–	G	–	E464E	1 (1.9)	0	0
10	–	–	–	–	–	–	C	–	–	T443T	1 (1.9)	0	0
11	–	–	–	C	–	–	–	–	–	M420I	0	0	1

[a](–) = no change.

variant were equally distributed in the lung tissues. Among the 16 non-malignant cervical tissues, the European and Asian prototypes were the most prevalent variants. The frequently reported T350G mutation was also observed.

E7 variants

We obtained the complete sequence of E7 ORF in 72 cervical cancer tissues, 4 lung tissues, and 37 non-malignant cervical tissues. Nine mutations and twelve variants were detected in total (Table 4). Two variants were exclusively found in the control group and only two variants were detected in the lung tissues. Among them, G663A and G666A were synonymous mutations. Variants exhibiting both A646C and A647G mutations deserve a special attention, since they lead to the N29R mutation. The frequently reported A647G mutation was found alone or in combination with other mutations (Fig. 1c) and this variation predominated in cervical cancer with a frequency of 62.5%.

Table 3. HPV16 E6 variants identified in cervical and lung cancer and control samples

Class/Subclass	ORF nucleotides											Predicted amino acid substitution	No. (%) of identified sequences		
	109	131	136	168	178	185	276	306	335	350	442		Cervical cancer (n=60)	Controls (n=16)	Lung cancer (n=4)
E prototype	T	A	G	C	T	T	A	A	C	T	A	–	10 (16.7)	7[b] (43.7)	0
As prototype	–[a]	–	–	–	G	–	–	–	–	–	–	D25E	24 (40.0)	6 (37.5)	2
E–G276T	–	–	–	–	–	–	G	–	–	–	–	N58S	5 (8.3)	0	0
E–G306T	–	–	–	–	–	–	–	C	–	–	–	K68T	1 (1.7)	0	0
E–350G	–		–	–	–	–	–	–	–	G	–	L83V	5 (8.3)	1 (6.3)	0
As–C109	C	–	–	–	G	–	–	–	–	–	–	F2F+D25E	2 (3.3)	0	0
As–C131	–	C		–	G	–	–	–	–	–	–	R10R+D25E	1 (1.7)	0	0
As–C136	–	–	C	–	G	–	–	–	–	–	–	K11N+D25E	1 (1.7)	0	0
E–G168G	–	–	–	G	–	–	–	–	–	G	–	T22S+L83V	4 (6.7)	0	2
As–G185	–	–	–	–	G	G	–	–	–	–	–	D25E+L28V	2 (3.3)	0	0
As–C442	–	–	–	–	G	–	–	–	–	–	C	D25E+E113D	3 (5.0)	0	0
E–T335C442T	–	–	–	–	–	–	–	–	T	–	C	H78Y+E113D	2 (3.3)	2 (12.5)	0

[a](–) = no change. [b]Significantly different from the cervical cancer group.

Table 4. HPV16 E7 variants identified in cervical and lung cancer and control samples

Variant No.	ORF nucleotides									Predicted amino acid substitution	No. (%) of identified sequences		
	623	646	647	663	666	675	700	712	758		Cervical cancer (n=72 [c])	Controls (n=37)	Lung cancer (n=4)
1	A	A	A	G	G	A	C	C	G	–	7 (9.7)	17 [b] (45.9)	0
2	G	– [a]	–	–	–	–	–	–	–	D21G	0	2(5.4)	0
3	–	C	–	–	–	–	–	–	–	N29H	9 (12.5)	4 (10.8)	2
4	–	–	G	–	–	–	–	–	–	N29S	30 (41.7)	7 (18.9)	2
5	–	–	–	–	A	–	–	–	–	E35E	10 (13.9)	0	0
6	–	–	–	–	–	–	–	T	–	H51Y	1(1.4)	0	
7	–	C	G	–	–	–	–	–	–	N29R	2 (2.8)	1(2.7)	0
8	–	–	G	A	–	–	–	–	–	N29S+E34E	1(1.4)	0	
9	–	–	G	–	A	–	–	–	–	N29S+E35E	2 (2.8)	0	0
10	–	–	G	–	–	G	–	–	–	N29S+I38M	10 [c] (13.9)	5 (13.5)	0
11	–	–	G	–	–	–	–	–	A	N29S+R66Q	1 [c] (1.4)	0	0
12	–	–	–	–	A	–	T	–	–	E35E+P47S	0	1 (2.7)	0

[a](–) = no change. [b]Significantly different from the cervical cancer group. [c]Two kinds of variants were found in one cervical cancer case.

Discussion

HPV-16 is considered as the most common high-risk type, which correlates closely with the cervical cancer especially with squamous cell carcinoma. In the present study, 68.9% (84/122) cervical cancer tissues were found to contain HPV-16, what is in accordance with previously reported 78.6% HPV-16 positive cases in Sichuan province (Qiu *et al.*, 2007). In lung tissues, HPV-16 positive rate was 17.3%, what was similar number to the 20% reported by Li *et al.* (1995).

In this study, we focused on the HPV-16 E6/E7 genes and the widely targeted MY09/11 region of L1 ORF in the cervical and lung cancers. Generally, most of the E6, E7, and L1 variants existed in poorly differentiated squamous cell carcinoma of the cervix. We also found HPV-16 variants in lung tissues. Compared with the diverse variants detected in cervical cancer, HPV-16 variants in lung tissues were relatively simple. Most of the variants found in lung tissues could be detected also in the cervical tissues except G6818C. The Asian and European prototypes predominated in Sichuan province, in accordance with the worldwide perspective study by Yamada *et al.* (1997), but we did not find the other four variants mentioned above or the Javanese variant reported by de Boer *et al.* (2004).

We detected twelve E6 variants in the cervical cancer and most of them resulted in corresponding amino acid changes. We found that the Asian prototype was the major variant in Sichuan. In contrast, only two E6 variants were found in the lung tissues, since the number of samples was limited. This fact may reflect the relative simplicity of variants found in the lung tissues. Compared with other reported HPV-16 variants in major cities in China, we found three new E6 variants, G136C, T185G, and A306C, all occurring sporadically in the cervical cancer patients suggesting that more E6 variants may exist in Sichuan than in other districts of China (Stephen *et al.*, 2000; Chan *et al.*, 2002; Wu *et al.*, 2006; Qiu *et al.*, 2007). We also detected the frequently reported T350G variant, which was claimed to be associated with the increased risk of cervical disease progression (Zehbe *et al.*, 1998a, b; Kämmer *et al.*, 2002). In our study, T350G variant was detected with a frequency of 15% (9/60) in the cervical cancer tissues, what is much lower number than 100% (42/42) reported in North India (Pande *et al.*, 2008). Interestingly, we did not find any significant correlation between T350G mutation and the invasive cervical cancer, in good agreement with those reported by Nindl *et al.* (1999) and Hu *et al.* (2001). The N-terminus of the E6 protein is antigenic and can elicit a strong T cell response *in vivo* (Kast *et al.*, 1994). Therefore, mutations in nt 168–188 might cause amino acid alterations in this region. Our study observed three missense mutations in this region, which might have some effect on E6 oncogenicity. In addition, based on our current study, it appears that nts 120–130, 140–160, 190–260, 280–300, 310–330, and 360–440 are the most conserved regions of HPV-16 E6 and are suitable for the silencing of E6 expression using short interfering RNA and/or ribozyme treatment for HPV-16 related cancers.

HPV-16 E7 gene mutations are relatively rare in various geographic locations and ethnic populations around the world (Eschle *et al.*, 1992; Radhakrishna Pillai *et al.*, 2002; Pande *et al.*, 2008). Nevertheless, here we found twelve E7 variants similar to the number of E6 and L1 variants. However, only two E7 variants were detected in the lung tissues. We also found a hot spot of A647G with a frequency of 62.5% (45/72) in cervical cancer tissues and 35.1% (13/37)

in benign cervical tissues, what is in accordance with studies from Guangdong, China (33/47, 70.2%), Japan (9/15, 60%), and Korea (25/42, 59.5%) (Fujinaga *et al.*, 1994; Song *et al.*, 1997; Wu *et al.*, 2006). These rates were significantly higher than 0.9% in Germany indicating the high similarity of E7 variants in Asian populations (Nindl *et al.*, 1999). Accordingly, the frequently reported amino acid change N29S in E7 was the most frequent E7 variation in the cervical cancer, lung cancer, and control samples. A study from South Korea reported that this mutation was significantly more prevalent in the cervical carcinomas (70%) than in control group (33%) or in cervical intraepithelial neoplasia, grade III group (50%) (Song *et al.*, 1997). However, this variant was distributed equally among all subjects in our study, in accordance with other reports from Japan and India (Fujinaga *et al.*, 1994; Radhakrishna Pillai *et al.*, 2002). In this study, only one cervical cancer sample contained two different complex E7 variants. This could be due to either methodological bias or the factual infections reported by Yamada *et al.* (1997).

Examinations of the partial L1 gene showed that most of the samples exhibited sequences identical to the prototype with frequencies of 71.2% for the cervical cancer, 94.4% for lung diseases, and 87.5% for the controls. Most of the variants were detected in the cervical cancer samples sporadically with the synonymous mutations. One missense mutation (G6818C) occurred exclusively in the pulmonary abscess.

In summary, we found that variants of HPV-16 E6, E7, and L1 occurred more frequently in cervical cancer patients than in patients with lung diseases. Future case/control and longitudinal studies will be necessary to estimate the risk of each variant, especially E6 and E7 variants, and establish the prevalence of HPV variants in the particular populations. Undoubtedly, further analysis of HPV variants will be critical for the development of reliable diagnostic and therapeutic approaches for the HPV infections.

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