

# The Presence of Human Papillomavirus Type 16/18 DNA in Blood Circulation May Act as a Risk Marker of Lung Cancer in Taiwan

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**BACKGROUND.** Lung cancer is the leading cause of cancer death in Taiwan, and the paucity of dependable risk markers has impeded the early management of lung cancer. An association of human papillomavirus (HPV) 16/18 infection with lung cancer among nonsmoking Taiwanese women was revealed in our previous study. **METHODS.** Nested PCR was employed to detect HPV 16/18 DNA in the blood circulation of 149 lung cancer patients and 174 noncancer controls. In addition, correlation of prevalence of HPV DNA between the blood circulation and lung tumor tissue was compared from 70 sets of paired tumor tissues and peripheral blood samples available.

**RESULTS.** The results showed that the prevalence rate of HPV 16/18 in the blood circulation of lung cancer cases was significantly higher than that of noncancer controls (47.7% vs. 12.6% for HPV 16,  $P < 0.0001$ ; 30.9% vs. 5.2% for HPV 18,  $P < 0.0001$ ). A significantly higher HPV 16 prevalence was detected in female lung cancer patients than that of male (57.6% vs. 41.1%,  $P = 0.048$ ), as well as in cases with tumor Stages III/IV than those with tumor Stages I/II (54.6% vs. 29.3%,  $P = 0.006$ ). After adjusting the effects of age, gender, and smoking status, a 6.5-fold greater risk of lung cancer was demonstrated for those subjects with HPV Type 16 positive (95% CI 3.7–11.3,  $P < 0.0001$ ), a 9.2-fold for HPV Type 18 positive (95% CI 4.2–20.2,  $P < 0.0001$ ), and a 75.7-fold greatest risk for those with both HPV Type 16 and 18 positive (95% CI 9.8–582.1,  $P < 0.0001$ ).

**CONCLUSIONS.** These results suggested that the presence of HPV DNA in the blood circulation may serve as a feasible risk marker of lung cancer. *Cancer* 2003;97:1558–63. © 2003 American Cancer Society.

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**KEYWORDS:** human papillomavirus, blood circulation, cancerous tissue, lung cancer.

Lung cancer is a highly notable health problem in Taiwan that is the leading cause of cancer death. Its incidence rate has been increasing at a steady rate every year. This fact is partly due to that the tumor has often become disseminated by the time of lung cancer diagnosis. The lack of dependable risk markers has impeded the management of lung cancer and has called for the emerging studies. Previously, much of the attention of research for cancer markers has been focused on the analysis of cancer-related genetic alternations, particularly for oncogenes, tumor suppressor genes, and microsatellite DNA sequence.<sup>1,2</sup> All these mentioned techniques utilize peripheral blood as the studied subject on the basis of its convenience and noninvasiveness. Furthermore, a previous study has employed real-time quantitative polymerase chain reaction (PCR) to quantitatively detect the

cell-free Epstein-Barr virus (EBV) DNA in the plasma of more than 90% of nasopharyngeal carcinoma (NPC),<sup>3</sup> which have been proven to be quite reliable in detecting and monitoring such neoplasm.<sup>4,5</sup> Thus, circulating viral DNA might be feasible for the establishment of a noninvasive marker for cancer and be applied clinically.

From the intensive epidemiologic studies worldwide, cigarette smoking is believed to be the principal, but not the only, factor involved in lung carcinogenesis. However, other related factors must be considered based on that most of the female lung cancer patients in Taiwan were nonsmokers, and therefore, warrant further exploration. In our previous study, by looking at the prevalence rate of human papillomavirus (HPV), a powerful inactivator of p53 and close ally to cervical cancer, in lung cancer patients, we revealed the association of HPV 16/18 infection with lung cancer among nonsmoking Taiwanese women.<sup>6</sup> Because the major infected target of HPV 16/18 is the cervix and the bloodstream is the only connection between lung tissue and cervix in the above-mentioned study, another noticed event was the localization of HPV DNA in infiltrating lymphocytes within tumor areas. Therefore, we assumed that HPV 16/18 infection detected in lung cancer tissues may be originated at the cervix and spread to lung tissue via bloodstream. Afterwards, in our preliminary study by employing *in situ* PCR on the white blood cells being fixed on slides by cytospin, signals of HPV DNA were localized in the nucleus of circulating white blood cells. In light of these findings, we hypothesized that HPV DNA might be detectable in the circulation of lung cancer patients, who were HPV positive in their lung tissue, and may represent evidence for HPV-related pulmonary carcinogenesis. Therefore, the detection of HPV in the blood circulation of lung cancer patients is of clinical importance.

To understand the feasibility of using circulating DNA as a sensitive marker for lung cancer, we examined the prevalence of HPV in the blood circulation of lung cancer patient and noncancer control subjects to assess the clinical significance of HPV infection in lung carcinogenesis.

## MATERIALS AND METHODS

### Study Subjects

A total of 149 primary lung cancer patients (International Classification of Diseases, 9th revision; ICD code 162), including 59 females and 90 males, were recruited from Veterans General Hospital-Taichung and Chung Shan Medical University Hospital into this study. All cases also underwent a series of examination of pathologic stages by board-certified pathologists. Meanwhile, 174 potential controls were randomly se-

lected from consecutive patients with no history of cancer. Demographic data were collected on each of these individuals, which included age, gender, and smoking status from the patient interview and a review of the hospital charts with informed consent. Smokers in this study were defined as those who smoked up to the day of pulmonary surgery.

### Nested Polymerase Chain Reaction (Nested PCR)

Venous blood was collected into heparinized tubes from all subjects. Surgical specimens of tumorous samples also were available for 70 lung cancer patients, since cases in this study included inoperable patients at later stage. Furthermore, DNA were extracted from whole blood or tissue samples by protease digestion, conventional phenol-chloroform extraction, ethanol precipitation, and finally dissolved in 20  $\mu$ l of sterile distilled water. HPV viral DNA was first amplified with type consensus primers MY09 and MY11<sup>7</sup> followed by a second round of amplification with type-specific primers flanking the L1 region to identify the subtype (sequences of Type 16 primers: 5'-TAC TAA CTT TAA GGA GTA CC-3' and 5'-GTG TAT GTT TTT GAC AAG CAA TT-3'; sequences of Type 18 primers: 5'-CCA AAT TTA AGC AGT ATA GC-3' and 5'-TTG TAC AAA ACG ATA TGT ATC CA-3'). Ten microliters of the final PCR product was loaded onto a 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet illumination. Appropriate negative and positive controls were included in each PCR reaction. DNA preparations from the SiHa cell (containing HPV 16) and the HeLa cell (containing HPV 18) were used as positive controls. A part of  $\beta$ -actin gene in all samples also was amplified to exclude false-negative results.

### Statistical Analysis

Comparisons between case and control groups for the status of HPV 16 and 18 infections were made using a chi-square test. The relation between the HPV 16/18 prevalence rate and clinical-pathologic parameters, including age, gender, smoking status, tumor type, and tumor stage, were calculated by a chi-square test. Adjusted odd ratios (ORs) and a 95% confidence interval (95% CI) on lung cancer were evaluated for age (< 67 and  $\geq$  67 years), gender (male and female), smoking status (yes and no), HPV 16 (positive and negative), and HPV 18 (positive and negative) using a multiple logistic regression model. Furthermore, a Kappa test was used to evaluate the correlation of HPV detection in blood circulation and cancer tissues of lung cancer patients.

**TABLE 1**  
The Characteristics and Prevalence of HPV DNA in Blood Circulation of Lung Cancer Patients and Noncancer Controls

Parameter	Cases (n = 149)	Controls (n = 175)	P value
Age			
< 67	73 (49%)	82 (46.9%)	0.701
≥ 67	76 (51%)	93 (53.1%)	
Gender			
Female	59 (40%)	79 (45.1%)	0.314
Male	90 (60%)	96 (54.9%)	
Smoking habit			
No	74 (49.7%)	107 (61.1%)	0.038
Yes	75 (50.3%)	68 (38.9%)	
HPV 16			
Positive	71 (47.7%)	22 (12.6%)	< 0.0001
Negative	78 (52.3%)	152 (87.4%)	
HPV 18			
Positive	46 (30.9%)	9 (5.2%)	< 0.0001
Negative	103 (69.1%)	165 (94.8%)	

## RESULTS

### Descriptive Data

A total of 323 individuals, including 149 nonsmall cell lung cancer (NSCLC) patients and 174 noncancer controls, were recruited into this study. The mean age of both groups were  $64.7 \pm 12.2$  (SD) and  $65.7 \pm 9.7$  years, respectively. The gender distribution in case group was comparable with that for the control groups. Statistical difference was observed in the parameter of smoking status between these two groups (50.3% in cases vs. 38.5% in controls,  $P = 0.038$ ; chi-square test).

### Prevalence of High-Risk HPV Was Higher in Circulation of Lung Cancer Patients than of Noncancer Controls

From the detection of HPV 16 and 18 by nested PCR, it was shown that the prevalence rates of both types of HPV in lung cancer patients were significantly higher than those in the control group (for HPV 16, 47.7% vs. 12.6%,  $P < 0.0001$ ; for HPV 18, 30.9% vs. 5.2%,  $P < 0.0001$ ; Table 1). As further shown in Table 2, for HPV Type 16 in peripheral blood, the higher prevalence rate was 57.6% of female cases compared with 41.1% of male cases ( $P = 0.048$ ). Such infection also was more frequent in adenocarcinoma (55.6%) than in squamous carcinomas (35.6%;  $P = 0.017$ ). Meanwhile, cases with tumor Stages I and II had a significantly lower prevalence rate of HPV 16 compared with those with Stages III and IV (29.3% vs. 54.6%,  $P = 0.006$ ). For HPV 18, cases age  $\geq 67$  years (the medium age of study subjects) had a more elevated prevalence rate than that of cases age  $< 67$  years. This discrimination was statistically significant (42.4% vs. 16.4%,  $P = 0.001$ ).

**TABLE 2**  
Relationships between the Presence of HPV DNA Detected by Nested PCR in Blood Circulation and Clinical-pathologic Parameters

Variable	HPV 16		HPV 18		Total
	Positive	Negative	Positive	Negative	
Number	71	78	46	103	149 (100%)
Age					
< 67	33	40	12	61	73 (49.0%)
≥ 67	38	38	34	42	76 (51.0%)
P value	0.558		< 0.001		
Gender					
Female	34	25	15	44	59 (39.6%)
Male	37	53	31	59	90 (60.4%)
P value	0.048		0.244		
Smoking					
No	40	34	21	53	74 (49.7%)
Yes	31	44	25	50	75 (50.3%)
P value	0.120		0.513		
Tumor type					
AD	50	40	29	61	90 (60.4%)
SQ	21	38	17	42	59 (39.6%)
P value	0.017		0.660		
Tumor stage					
I and II	12	29	11	30	41 (27.5%)
III and IV	59	49	35	73	108 (72.5%)
P value	0.006		0.510		

AD: adenocarcinoma lung cancer; SQ: squamous cell lung cancer.

Regarding to the prevalence of both types, prevalence of HPV 16 was higher than that of HPV 18 in female (34/59, 57.6% vs. 15/59, 25.4%) and adenocarcinoma (50/90, 55.6% vs. 29/90, 32.4%).

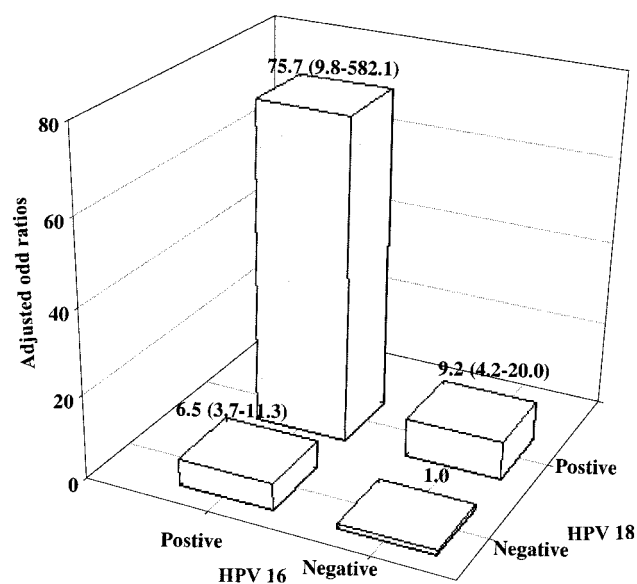
### Presence of High-Risk HPV DNA in the Blood Circulation Had a Significantly Higher OR of Lung Cancer Incidence

Based on the high prevalence of both types of HPV, the ORs of HPV in blood circulation and several other parameters of lung cancer patients were evaluated with multivariate logistic regression analysis. As shown in Table 3, after adjusted by age, gender, and smoking status, the co-infection of HPV Types 16 and 18 had an extremely high OR of 38.5 (95% CI 5.2–288.2;  $P = 0.0004$ ) using those with either one type of HPV infection or no infection as a reference (OR 1.0). Meanwhile, individuals with the presence of HPV Types 16 and 18 DNA in the blood circulation had a significantly high OR of lung cancer incidence as 6.5 (95% CI 3.7–11.3;  $P < 0.0001$ ) and 9.2 (95% CI 4.2–20.0;  $P < 0.0001$ ), respectively, compared with 1.9 (95% CI 1.00–3.7) and 2.1 (95% CI 1.1–4.1;  $P = 0.0351$ ) of smoking status (Table 3). When using those with neither history of HPV Type 16 infection nor HPV Type 18 infection as a reference (OR 1.0), those with both HPV Types 16 and 18 positive experienced a 75.7-fold

**TABLE 3**  
Multivariate Logistic Regression Analysis of the Risk of HPV DNA Detected in Blood Circulation of Controls and Cases and the Association with Age, Gender, and Smoking Status

Variable	Groups unfavorable/favorable	OR (95% CI)	P value
Co-infection of Types 16 and 18	+/-	38.5 (5.2-288.2)	0.0004
Age	≥ 67/< 67	0.7 (0.5-1.2)	0.1808
Gender	Male/female	1.0 (0.3-2.0)	0.9318
Smoking status	Smoking/nonsmoking	1.5 (0.8-3.0)	0.1898

The ORs of presence of HPV 16 DNA alone in peripheral circulation of lung cancer patients and smoking status were 6.5 (95% CI 3.7-11.4) and 1.9 (95% CI 1.00-3.7), respectively. The ORs of presence of HPV 18 DNA alone in peripheral circulation of lung cancer patients and smoking status were 9.2 (95% CI 4.2-20.2) and 2.1 (95% CI 1.1-4.1), respectively.



**FIGURE 1.** The adjusted odd ratios (ORs) of lung cancer by presence of HPV types 16 and 18 DNA in the blood circulation after adjusting for potential confounders.

greater risk of lung cancer (95% CI 9.8-582.1,  $P < 0.0001$ ; Fig. 1). In more detail, after adjusting the effect of age, the OR of lung cancer for HPV 16 infection compared with non-HPV-16 infection in non-smoking females was 13.6 (95% CI 5.3-35.3), followed by 4.0 (95% CI 1.1-15.3) in nonsmoking males and 3.6 (95% CI 1.6-8.4) in smoking males. Meanwhile, the OR of lung cancer for HPV 18 infection compared with non-HPV-18 infection was 7.1 (95% CI 1.9-26.5) in nonsmoking women, 5.1 (95% CI 1.2-20.6) for non-smoking men, and 18.9 (95% CI 4.1-87.2) for smoking men. An additional multivariate analysis using logistic regression to relate HPV (and HPV 18) in the blood to age, gender, tumor type, and tumor stage to just those

**TABLE 4**  
Multivariate Logistic Regression Analysis of the Risk of HPV DNA Detected in Blood Circulation of Lung Cancer Patients Only and the Association with Age, Gender, Tumor Type, Tumor Stage and Smoking Status

Variable	Groups unfavorable/favorable	OR (95% CI)	P value
HPV 16			
Age	≥ 67/< 67	1.4 (0.7-2.8)	0.358
Gender	Male/female	0.6 (0.2-1.6)	0.294
Tumor type	AD/SQ	0.6 (0.3-1.2)	0.148
Tumor stage	III & IV/I & II	2.5 (1.1-5.7)	0.025
Smoking status	Smoking/nonsmoking	1.0 (0.4-2.6)	0.982
HPV 18			
Age	≥ 67/< 67	4.3 (2.0-9.5)	0.000
Gender	Male/female	1.6 (0.6-4.5)	0.381
Tumor type	AD/SQ	1.0 (0.4-2.2)	0.973
Tumor stage	III & IV/I & II	1.7 (0.7-4.1)	0.224
Smoking status	Smoking/nonsmoking	0.9 (0.3-2.4)	0.802

AD: adenocarcinoma lung cancer; SQ: squamous cell lung cancer.

**TABLE 5**  
Correlation of Prevalence of HPV DNA between the Blood Circulation and Tumor Tissue for Lung Cancer Patients

	Tissue	Peripheral blood		Total
		Positive	Negative	
HPV 16	Positive	22	8	30 (42.9%)
	Negative	13	27	40 (57.1%)
HPV 18	Positive	2	11	13 (18.6%)
	Negative	23	34	57 (81.4%)

with lung cancer has shown that HPV 16 was significantly related to later tumor stage with an OR of 2.537 (95% CI 1.1-5.7; Stages III and IV vs. Stages I and II). Meanwhile, HPV 18 infection was significantly related to older age with an OR of 4.3 (95% CI 1.9-9.5; age ≥ 67 vs. < 67 years) (Table 4).

#### Significant Correlation of Prevalence of HPV DNA between the Blood Circulation and Lung Tumor Tissue

To evaluate the correlation of HPV DNA present in the blood circulation and tumor tissue, DNAs were extracted from 70 sets of paired tumor tissues and peripheral blood samples available and subjected to HPV type-specific nested PCR. The presence of HPV 16 and 18 in both types of samples were found to be correlated (Table 5). However, the statistical agreement reached significance in HPV 16 only (Kappa = 0.40, 95% CI 0.19-0.61,  $P < 0.001$ ) but was not observed in HPV 18 (Kappa = -0.18, 95% CI 0.004-0.364,  $P = 0.09$ ), which could be due to that cases with HPV 18 positive in both types of samples was small.

## DISCUSSION

From our previous study, the association of HPV Types 16/18 infections with lung cancer among non-smoking Taiwanese women has been established by looking at the prevalence of HPV DNA in tumor tissues.<sup>6</sup> The subsequent vital question will be how HPV gets into lung tissue.

For virus-associated cancer, detection of viral DNA in individual circulation presents a noninvasive and easier sampling way for clinical application. Shotelersuk et al.<sup>8</sup> in 2000 reported a higher incidence, 59% (98/167), of EBV DNA in sera/plasma of NPC patients compared with 13% (10/77) of healthy blood donors. Lo et al. in 1999 also indicated a higher viral load in patients was associated with tumor recurrence.<sup>5</sup> These results indicated that the circulating EBV DNA has become a reliable determinant for NPC. Thus, we further hypothesized that blood cells are the transmission for HPV between the cervix, the presumed target organ for HPV infection, and the lung.

Previously, the low detection frequencies of HPV DNA in sera/plasma of cervical cancer patients were reported as low as 6.3% (11/175) by PCR and 20% (8/40 samples) by PCR followed by hybridization.<sup>9,10</sup> One explanation may be the integration of viral DNA into a host chromosome, which is commonly found in cervical cancer cases and therefore prevents the presence of cell-free viral DNA in sera/plasma. Another explanation could be due to the low copy number of HPV DNA in the circulation, which has been reported as 183.5 copies/ml,<sup>10</sup> far less than 32,350 copies/ml of EBV DNA.<sup>5</sup> Therefore, in this case-control study, we employed nested PCR, an extreme sensitive technique, to conduct HPV 16/18 DNA detection in peripheral blood cells instead of sera/plasma samples. In addition, considering the extreme sensitivity of nested PCR, all possible precautions were taken to eliminate any false-positive result in the current study.

Consistent with that of our previous study with lung tumor tissues,<sup>6</sup> the prevalence of HPV 16/18 in the blood circulation of lung cancer patients was significantly higher than that of the control group. Moreover, female lung cancer cases were more prone to HPV 16 infection than males and the prevalence in adenocarcinoma was higher than that of squamous carcinomas. For this difference in tumor type, although a previous study had detected HPV in up to 79% of squamous carcinoma of the lung,<sup>11</sup> Miyagi et al.<sup>12</sup> had revealed a decreasing trend in the prevalence of HPV in lung squamous carcinoma from 79% in 1993 to 24% in 1998. Furthermore, HPV DNA was detected in a high proportion (78%) of lung adenocarcinoma in a previous study.<sup>13</sup> However, the higher HPV preva-

lence in lung adenocarcinomas of this study also could be due to that > 70% of female patients were with adenocarcinoma and most HPV-positive cases were female patients.

In a previous study, several head and neck squamous cell carcinoma (HNSCC) patients carrying HPV-positive sera have been found to develop distance metastasis.<sup>14</sup> In this study, the prevalence rate of HPV 16 for patients with more advanced stage also was significantly higher. However, the possibility for decreasing immunity that is prone to viral persistent infections during the tumor progression could not be ruled out. Furthermore, by using real-time quantitative PCR, Lo et al.<sup>5</sup> revealed a much higher concentration of EBV DNA in patients with tumor recurrence than that in patients in continuous remission, suggesting that viral copy number may be an important factor for monitoring the metastasis or cancer remission. Although the presence of HPV DNA acting as a risk biomarker also was verified in cervical cancers, conflicting conclusions in the application of HPV viral load have been reported.<sup>15-17</sup>

In our study, individuals with HPV Types 16 or 18 DNA alone in the blood circulation had a significantly higher risk of lung cancer at 6.5 and 9.2, respectively, whereas those with both HPV Types 16 and 18 positive had a prominently higher risk at 75.7 (95% CI 9.8–582.1) compared with those with neither history of HPV 16 nor HPV 18. This is the first study to demonstrate a synergistic effect for HPV Types 16 and 18 infection on lung cancer development through multivariate modeling.

Clearly, our results suggested that the presence of HPV 16/18 DNA in the blood circulation might be an applicable risk marker for lung cancer despite a couple of previous studies having reported conflicting results, including low incidence of HPV DNA in sera of patients with cervical cancer (20%) and HNSCC (6%).<sup>9,14</sup> The possible explanations for this inconsistency, as discussed above, may be on the issues of detection sensitivity and sample origins. In addition, since viral DNA in serum/plasma is expected to be only released from cell-free viral particles or shedding tumor cells, not as in previous studies,<sup>9,14</sup> we prepared DNA from peripheral cells instead of serum/plasma. Meanwhile, HPV DNA was detected in peripheral blood of more than 30% of patients at the early stage; the positive nested PCR results in this study were unlikely to indicate the shedding tumor cells. It is more likely to indicate that peripheral lymphocytes could harbor HPV particles and may be involved in the spreading of HPV viral particles. The preliminary results of an ongoing *in situ* PCR study conducted in our lab have shown the location of HPV 16 DNA in the nucleus of

peripheral blood cells (data not shown) and further confirmed the accuracy of this methodology, as well as eliminated the possibility that this HPV DNA detection in circulation resulted from the shedding tumor cells. However, the possible route of HPV entering the blood cells remains an enigma.

From the analysis of 70 sets of paired tissue and blood samples, we have observed a close correlation between the presence of HPV 16 and 18 in both types of samples, which further support the involvement of HPV in lung tumorigenesis. It should be pointed out that the dissected tumor tissues were used as biopsies in our previous study,<sup>6</sup> indicating that most of those patients were at the early stage and operable, whereas patients in this study included inoperable patients at later stage. Based on these two studies, we have observed a higher prevalence of HPV 16 than that of HPV 18 in blood circulation (47.7% vs. 30.9%) but lower in tumor tissue (35.5% vs. 41.1%). This tissue discrimination could be due to the different case constitution of these two studies, variations in the tissue tropisms of HPV 16 and 18 during the transmission from infected epithelial cells to internal organs, or the various viral load of HPV 16 and 18 existed in blood circulation. Furthermore, E7 of HPV 16 has been demonstrated to disturb the keratinocyte differentiation and stimulate the host DNA replication machinery and, therefore, promote HPV DNA synthesis and is essential for the productive stage of the HPV 16 life cycle.<sup>18</sup> In other studies conducted in this laboratory, there were good correlations between HPV 16/18 DNA and E6/E7 mRNA expressions in lung tumors, as well as in peripheral blood (data not shown). Therefore, the impacts of copy number and E6/E7 mRNA expression status could contribute to such different prevalence of HPV 16/18 in various tissue sites.

In conclusion, our present study clearly revealed the higher prevalence of HPV DNA in the blood circulation of lung cancer patients. Undoubtedly, the presence of circulating HPV 16/18 DNA may act as a risk marker of lung cancer and also further supports the involvement of HPV in lung tumorigenesis.

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