

# Association between human papillomavirus and *EGFR* mutations in advanced lung adenocarcinoma

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**Abstract.** Previous studies have demonstrated an association between human papillomavirus (HPV) and mutations in the epidermal growth factor receptor (*EGFR*) gene in lung cancer patients; however, few studies have investigated this association in advanced lung adenocarcinoma patients undergoing gefitinib treatment. The present study investigated the association between HPV and *EGFR* mutations in advanced lung adenocarcinoma patients. A total of 95 advanced lung adenocarcinoma patients were enrolled in the study. The HPV infection status and presence of *EGFR* mutations in tumor tissue was evaluated. Patient clinical characteristics were also determined and compared with HPV infection and *EGFR* mutation status to analyze their impact on progression-free survival. HPV DNA was identified in 27/95 (28.4%) lung adenocarcinoma tumors and was most common in patients with lymph node metastasis ( $P=0.016$ ). A total of 44/95 (46.3%) cases exhibited *EGFR* mutations, which were predominantly observed in female patients and non-smokers. The presence of HPV DNA was significantly associated with *EGFR* mutations ( $P=0.012$ ) and multivariate analysis also revealed that HPV DNA was significantly associated with *EGFR* mutations (odds ratio=3.971) in advanced lung adenocarcinoma. Patients with both HPV infections and *EGFR* mutations exhibit a marked decrease in the risk of lung cancer progression when compared with those without HPV infection or *EGFR* mutations (adjusted HR=0.640; 95% confidence interval: 0.488-0.840;  $P=0.001$ ). HPV infection was significantly associated with *EGFR* mutations in advanced lung adenocarcinoma patients. Furthermore, patients with HPV infections

exhibited the longest progression-free survival times, which may be due to good response to tyrosine kinase inhibitor- or platinum-based-adjuvant therapy in these patients. Patients with *EGFR* mutations exhibited a better prognosis when compared with those exhibiting wild-type *EGFR*, regardless of HPV status.

## Introduction

Lung cancer is the leading cause of cancer mortality, worldwide (1). Although cigarette smoking is the primary risk factor for lung cancer, the epidemiology of lung cancer remains partially unresolved, as the majority of cigarette smokers do not develop lung tumors. Furthermore, lung cancer is currently the seventh leading cause of cancer-related mortality in non-smokers (2). Human papillomavirus (HPV) infection has been postulated as a possible risk factor for the disease (3).

Recent studies have identified differences in the prevalence of HPV infection in lung cancer between different ethnicities and geographical variations (4,5). A lack of consensus with regard to detection methods, as well as methodological flaws may have been responsible for the large discrepancies reported, particularly between studies reporting conflicting results with regard to the prevalence of HPV in areas within the same geographical locations (6). Due to the variability in HPV prevalence reported, the involvement of HPV in lung cancer, specifically in non-small cell lung cancer (NSCLC), which accounts for 80% of all lung cancer cases, remains to be elucidated (7,8).

Previous studies in Taiwanese and Japanese populations have reported that female non-smokers with lung adenocarcinoma exhibit an elevated incidence of HPV infections (9) and observed that lung cancer patients with HPV infections were responsive to gefitinib treatment (10). Recently, two studies demonstrated that mutations in the epidermal growth factor receptor (*EGFR*) gene were associated with the presence of HPV infection in Taiwanese and Japanese patients (11,12). However, these studies did not evaluate the response rate to gefitinib as all subjects were diagnosed with primary lung cancer and subsequently underwent surgery rather than gefitinib administration.

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In the present study, 95 patients with advanced lung adenocarcinoma were enrolled to retrospectively investigate the association between HPV status, *EGFR* mutations and clinical characteristics, as well as their impact on progression-free survival (PFS).

## Materials and methods

**Clinical specimens.** A total of 95 paraffin-embedded tissue samples were obtained from advanced lung adenocarcinoma patients (stage III-IV) (13) prior to adjuvant therapy at the Department of Respiratory Oncology, Anhui Provincial Hospital (Hefei, China) between August 2011 and December 2013. Patients with complete clinicopathological and follow-up data were included. The tissues included 20 surgical specimens, 18 lung biopsy specimens, 18 bronchoscopic biopsy specimens, 23 pleural effusion specimens, 13 lymph node biopsy specimens and 3 bone biopsy specimens. All patients provided written informed consent was provided and the study was approved by the Institutional Review Board of Anhui Provincial Hospital.

Clinical characteristics including patient age, gender, smoking history, histological differentiation, lymph node metastasis, distant metastasis and treatment type [tyrosine kinase inhibitors (TKIs) (gefitinib or erlotinib) and platinum-based chemotherapy (cisplatin or carboplatin)] were collected for subsequent analyses. PFS was defined as the time from treatment to recurrence or the date of censorship (the last date of follow-up).

**DNA extraction.** DNA was deparaffinized and extracted from tissue samples using the QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, 5-6 formalin-fixed, paraffin-embedded 8- $\mu$ m tissue sections were soaked in xylene and vortexed vigorously. A tissue pellet was obtained and purified according to the manufacturer's instructions. DNA samples were quantified spectrophotometrically and normalized aliquots were obtained for each sample.

**HPV DNA detection.** HPV testing of lung cancer samples was performed using polymerase chain reaction (PCR) amplification of a fragment of the HPV *L1* gene. The presence of HPV infection was determined using a Tellgenplex™ HPV DNA Test kit (Tellgen Life Science Co., Ltd., Shanghai, China) using the Luminex® technique, which enables the detection of 26 HPV genotypes, including those of 19 high-risk HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 55, 56, 58, 59, 66, 68, 82 and 83) and 7 low-risk HPV types (6, 11, 40, 42, 44, 61 and 73). The experiments were performed in a Bio-Plex® 200 system (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Briefly, 2  $\mu$ g extracted DNA was amplified by PCR under the following conditions: 5 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. PCR products were subjected to rapid hybridization and the data were analyzed using the software provided by the manufacturer (Tellgen Software, version IS2.3; Tellgen Life Science Co., Ltd.) (14).

Mutations in *EGFR* exons 18-21 were detected using previously described methods (15).

**Statistical analysis.** Statistical analyses were performed using SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA).  $\chi^2$  tests were used to compare the associations between HPV status, *EGFR* mutation status and clinical characteristics. To investigate the combined effect of HPV infection and *EGFR* mutation on PFS, four subgroups were determined (HPV+/mutant+, HPV+/mutant-, HPV-/mutant+, and HPV-/mutant-). The survival curves were estimated by Kaplan-Meier methods and the differences in survival distributions were compared using the log-rank test. To evaluate the mortality risk, the hazard ratio (HR) and corresponding 95% confidence interval (CI) were estimated using Cox proportional hazards models to identify potential prognostic factors. All statistical tests were two-sided and  $P < 0.05$  was considered to indicate statistically significant differences.

## Results

**High-risk HPV infection in advanced lung adenocarcinoma patients.** Of the 95 DNA samples tested, 27 (28.4%) were positive for high-risk HPV. The most common high-risk HPV types identified in lung adenocarcinoma patients were HPV16 and HPV18; however, other genotypes including HPV33 and 58 were also detected.

**Association between *EGFR* mutation and HPV infection.** HPV infection was associated with lymph node metastasis ( $P = 0.016$ ) (Table I). A total of 44/95 cases (46.3%) exhibited *EGFR* mutations (in exons 19/21). *EGFR* mutations were more common in females ( $P < 0.006$ ) and never-smokers ( $P < 0.019$ ) when compared with males and ever-smokers, respectively. The presence of HPV DNA was significantly associated with *EGFR* mutations ( $P = 0.012$ ) (Table I). Multivariate analysis also revealed that HPV DNA was significantly associated with *EGFR* mutations (odds ratio=3.971) in advanced lung adenocarcinoma (Table II).

**Impact of HPV infection and *EGFR* mutations on PFS.** To determine the clinical significance of HPV infection status and *EGFR* mutations in advanced lung adenocarcinoma, the association between HPV infection, *EGFR* mutations and PFS was investigated in the present study. The Kaplan-Meier survival curve estimates are shown in Fig. 1. Patients with HPV infections exhibited a longer median PFS time than those without (13 vs. 9 months;  $P = 0.013$ ).

To evaluate the clinicopathological characteristics of patients and the impact of such on PFS in advanced lung adenocarcinoma, the HR and corresponding 95% CI were estimated using the Cox proportional hazards analysis, which was adjusted for smoking (never vs. ever), lymph node metastasis (yes vs. no), *EGFR* (mutated vs. wild-type) and HPV infection (positive vs. negative). The presence of *EGFR* mutations and lymph node metastasis were independent prognostic factors for survival, however, this trend was not observed in patients with HPV infection (Table III).

For advanced lung adenocarcinoma, treatment modality is one of the most important determinants of clinical outcome, thus, the PFS of patients treated with TKIs and platinum-based chemotherapy was analyzed using the Cox proportional hazards model. No significant difference in survival was

Table I. Clinicopathological characteristics, HPV infection status and EGFR mutation status of advanced lung adenocarcinoma patients.

Characteristics	HPV		P-value	EGFR		P-value
	Positive, n	Negative, n		Mutated, n	Wild-type, n	
Age, years			0.415			0.448
<64	16	34		25	25	
≥64	11	34		19	26	
Gender			0.255			0.006
Male	12	39		17	34	
Female	15	29		27	17	
Smoking status			0.078			0.019
Never	20	37		32	25	
Ever	7	31		12	26	
Histological differentiation			0.109			0.110
Well	4	21		10	15	
Poor	23	47		29	41	
Lymph node metastasis			0.016			0.801
Yes	11	46		27	30	
No	16	22		17	21	
Distant metastasis			0.255			0.326
Yes	12	39		26	25	
No	15	29		18	26	
EGFR mutations			0.012			
Mutated	18	26				
Wild-type	9	42				

HPV, human papillomavirus; EGFR, epidermal growth factor receptor.

Table II. Multivariate analysis of the association between human papillomavirus infection status and the clinicopathological characteristics of patients with advanced lung adenocarcinoma.

Variables	Multivariate logistic regression		
	Odds ratio	95% confidence interval	P-value
Gender (male vs. female)	0.920	0.252-3.358	0.899
Age, years (<64 vs. ≥64)	0.779	0.279-2.179	0.635
Smoking status (never vs. ever)	0.652	0.165-2.579	0.542
Histological differentiation (well vs. poor)	0.321	0.087-1.192	0.090
Lymph node metastasis (yes vs. no)	0.361	0.125-1.039	0.059
Distant metastasis (yes vs. no)	0.645	0.220-1.892	0.424
EGFR (mutated vs. wild-type)	3.971	1.364-11.562	0.011

EGFR, epidermal growth factor receptor.

identified between patients with HPV infections and those without in the subgroups receiving TKI and platinum-based chemotherapy (Table IV). The results indicated that the longest PFS times, which were observed in patients with positive HPV infection status, may have been due to a good response to TKI- or platinum-based-adjuvant therapy.

*HPV infection and EGFR mutation exhibit a synergistic effect on PFS time.* Multivariate analysis demonstrated that HPV DNA was significantly associated with EGFR mutations in lung adenocarcinoma and thus, the combined effects of these two biomarkers on PFS was evaluated, which revealed that they exhibited a synergistic benefit on PFS time. Patients with

Table III. Univariate and multivariate analyses of progression-free survival.

Variables	Univariate			Multivariate		
	HR	95% CI	P-value	HR	95% CI	P-value
Gender (male vs. female)	0.888	0.569-1.387	0.602			
Age, years (<64 vs. ≥64)	1.034	0.665-1.608	0.881			
Smoking status (never vs. ever)	1.709	1.086-2.690	0.020	1.174	0.738-1.866	0.498
Histological differentiation (well vs. poor)	0.829	0.494-1.391	0.478			
Lymph node metastasis (yes vs. no)	2.756	1.618-4.696	<0.001	2.750	1.566-4.829	<0.001
Distant metastasis (yes vs. no)	1.441	0.922-2.254	0.109			
<i>EGFR</i> status (mutated vs. wild-type)	0.229	0.134-0.392	<0.001	0.229	0.132-0.397	<0.001
HPV infection (positive vs. negative)	0.556	0.337-0.915	0.021	0.893	0.518-1.539	0.683

CI, confidence interval; HR, hazard ratio; *EGFR*, epidermal growth factor receptor; HPV, human papillomavirus.

Table IV. Subgroup analyses for PFS stratified by treatments.

Treatment	HPV status	Median PFS time, months	HR (95% CI)	P-value
TKI	Positive	16	0.689	0.304
	Negative	14	(0.338-1.402)	
Platinum	Positive	10	0.600	0.170
	Negative	7	(0.290-1.245)	

PFS, progression-free survival; HPV, human papillomavirus; HR, hazard ratio; TKI, tyrosine kinase inhibitor; CI, confidence interval.

both HPV infection and *EGFR* mutations exhibited a significantly reduced risk when compared with those exhibiting neither biomarker (adjusted HR=0.640; 95% CI: 0.488-0.840; P=0.001; Table V). The PFS curves are shown in Fig. 2. In addition, the results demonstrated that patients with *EGFR* mutations exhibited a better prognosis compared with those exhibiting wild-type *EGFR*, regardless of HPV status.

## Discussion

In the present study, 27/95 (28.4%) advanced lung adenocarcinoma patients were positive for high-risk HPV infection. Multivariate analysis revealed a significant association between HPV infection and *EGFR* mutations. Subgroup analyses for PFS stratified by treatments indicated that patients with HPV infections exhibited the longest PFS times in both the TKI and platinum-based-adjuvant treatment groups, which may be due to patients exhibiting a good response to TKI- or platinum-based-adjuvant therapy. Patients with both *EGFR* mutations and HPV infections (HPV<sup>+</sup>/mutant<sup>+</sup>) exhibited the longest survival time when compared with the other subgroups (HPV<sup>+</sup>/mutant<sup>-</sup>, HPV<sup>-</sup>/mutant<sup>+</sup> and HPV<sup>-</sup>/mutant<sup>-</sup>). However, patients with *EGFR* mutations exhibit a better prognosis compared with those exhibiting wild-type *EGFR*, regardless of HPV status.

The reported frequency of HPV infection in lung cancer patients varies worldwide. A comprehensive literature review

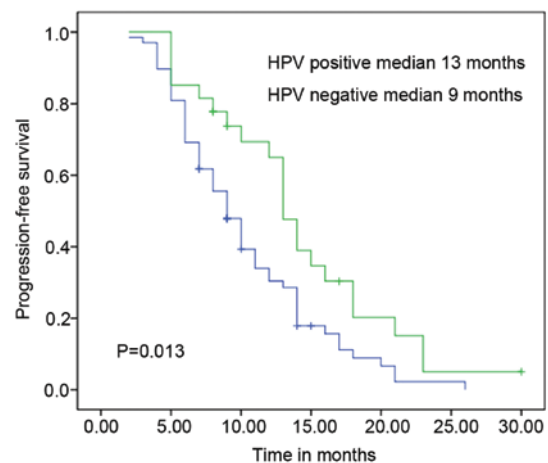


Figure 1. Progression-free survival curves according to HPV infection for advanced lung adenocarcinoma patients. HPV, human papillomavirus.

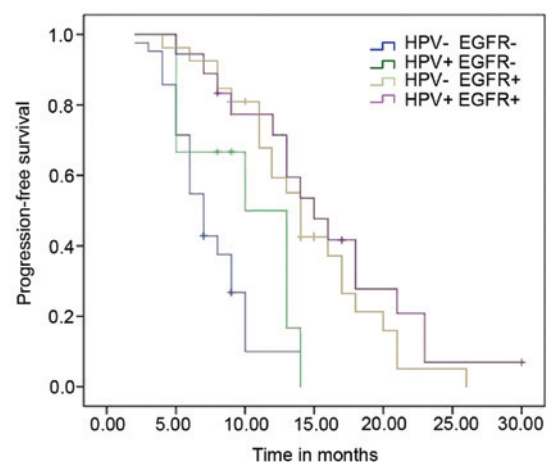


Figure 2. Progression-free survival curves for the joint assessment of HPV infection and *EGFR* mutation in advanced lung adenocarcinoma patients. HPV, human papillomavirus; *EGFR*, epidermal growth factor receptor.

revealed mean frequencies of 17 and 15% in Europe and the United States, respectively, with a higher frequency in Asia (35.7%) (16). The most common high-risk HPV types



Table V. HRs for PFS according to subgroups.

HPV/EGFR mutant	Patients, n	Median PFS	Unadjusted HR (95% CI)	P-value	Adjusted HR <sup>a</sup> (95% CI)	P-value
		time, months				
HPV <sup>+</sup> mutant <sup>+</sup>	18	15	0.581 (0.445-0.758)	<0.001	0.640 (0.488-0.840)	0.001
HPV <sup>+</sup> mutant <sup>-</sup>	9	10	0.763 (0.507-1.148)	0.194	0.829 (0.538-1.275)	0.393
HPV <sup>-</sup> mutant <sup>+</sup>	26	14	0.216 (0.113-0.416)	<0.001	0.224 (0.117-0.428)	<0.001
HPV <sup>-</sup> mutant <sup>-</sup>	42	7	1.0		1.0	

<sup>a</sup>Adjusted hazard ratio for smoking and lymph node metastasis. HR, hazard ratio; PFS, progression-free survival; HPV, human papillomavirus; EGFR, epidermal growth factor receptor; CI, confidence interval.

identified in lung cancer patients are HPV16 and HPV18, however, other HPV types such as HPV6, 11, 31, 33, 39, 52, 58 and 82 have also been reported (17). In accordance with these reports, in the present study HPV16 was identified as the most common genotype and positivity for HPV18, 33, 58, 6 and 11 was also observed (data not shown). The overall frequency of HPV DNA in advanced NSCLC patients was 25.2% (data not shown). A previous study by Wang *et al* (18) demonstrated that HPV infections were present in localized and earlier stages of lung cancer, however, in the present study HPV infections were more commonly identified in patients with lymph node metastasis. The reason for these discrepancies remains unclear. We hypothesize that HPV infection may be associated with certain disease stages and metastasis in lung cancer.

Recently, NSCLC in never-smokers has emerged as a global public health issue. The cause remains unclear and few studies have focused on the prevalence of HPV in this population (19). A previous study revealed that Taiwanese never-smokers exhibited a high prevalence of HPV16/18 infection, indicating that this is a potential etiological agent of lung cancer (20). In accordance with these results, in the present study, HPV infections were observed in never-smokers (data not shown).

*EGFR* mutations have been demonstrated to be more common in never-smokers, women, individuals of Asian ethnicity and adenocarcinoma patients (21,22). Furthermore, lung adenocarcinoma patients harboring *EGFR* mutations are considered to exhibit an increased sensitivity to *EGFR* TKIs, such as gefitinib and erlotinib (23). Notably, these clinicopathological features of *EGFR* mutations were evident in the present study and a significant association was identified between HPV infections and *EGFR* mutations in advanced lung adenocarcinoma patients. However, the present study also indicated that patients with *EGFR* mutations exhibited a better prognosis compared with those exhibiting wild-type *EGFR*, regardless of HPV status, and those patients exhibited the longest PFS times, which may be due to good response to TKI- or platinum-based-adjuvant therapy. However, these findings contradict previous studies. For example, Hsu *et al* (24) reported survival benefits for stage I NSCLC patients that expressed the HPV16/18 E6 oncoprotein. Furthermore, Wang *et al* (18) demonstrated that patients with HPV infections exhibited a better survival than those without in a subgroup receiving platinum-based chemotherapy; however, this trend was not observed in subgroups receiving TKI and radiation. We hypothesize that

this difference may be related to the survival index using PFS but not overall survival, and the fact that the majority of patients with *EGFR* mutations received TKI therapy but not platinum-based chemotherapy.

Multivariate analysis in the present study revealed that HPV DNA was significantly associated with *EGFR* mutations in advanced lung adenocarcinoma. Furthermore, patients with both *EGFR* mutations and HPV infections exhibited the longest PFS times. Márquez-Medina *et al* (25) previously reported that HPV infections were more common in patients harboring *EGFR* mutations or those sensitive to erlotinib. In addition, Wu *et al* (26), Zhang *et al* (27) and Sung *et al* (28) demonstrated that HPV16 E6 upregulates cellular inhibitor of apoptosis 2 (cIAP2), which is located downstream of *EGFR* signaling, via the *EGFR*/phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) cascade, and that cIAP2 expression was positively correlated with *EGFR* mutations. Therefore, the *EGFR*/PI3K/AKT cascade may exhibit a pivotal function in HPV-associated lung carcinogenesis in individuals with *EGFR* mutations.

In conclusion, the present study indicates a significant association between HPV infections and *EGFR* mutations in advanced lung adenocarcinoma patients. However, further large-scale prospective studies are required to confirm this hypothesis. Currently, the authors of the present study are planning a basic study to elucidate the association between HPV infections and *EGFR* mutations using patient-derived tumor xenograft models, which may increase current understanding with regard to the pathogenesis of HPV infections in lung cancer.

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