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# No Association Found between Lung Cancer and HPV in a German Collective

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## Introduction

In 1979 Syrjanen et al. [1] first described a possible association between Human Papillomavirus (HPV) and lung cancer. Since then multiple studies on this topic have been conducted.

Since the HPV was first identified more than 200 subtypes have been differentiated. They are most commonly classified into high-risk HPV types (16,18,31,33,39,45,51,52,58) and low-risk HPV types (6,11,42,43,44) [2].

The Human Papillomavirus is small non-enveloped with a diameter between 50 to 60 nanometers and belongs to the *Papillomaviridae* family. It contains one circular, double-stranded DNA genome consisting of approximately 7,000 bp to 8,000 bp [3].

HPV has first been detected in cutaneous warts and has first been proven to have oncogenic potential in cervical cancer [4]. Since then HPV has been detected in numerous other organ sites such as anogenital cancer, head and neck cancer and breast cancer [5-8].

The GLOBOCAN 2018 report estimated 18.1 million new cancer cases and 9.6 million cancer deaths in 2018 worldwide [9]. The incidence of lung cancer is rising not only in smokers but also in never-smokers. The prevalence of different subtypes of lung cancer shows large geographic variation depending on ethnicity, lifestyle factors, virus infections and possibly other still unknown factors.

Around 30% of all lung cancers are classified as Squamous Cell Carcinomas (SCC) [10]. SCC is defined as a malignant epithelial tumor expressing squamous differentiation. Smokers have a 10- to 20-fold higher risk of SCC of the lung than non-smokers.

In many cases B-symptomatic is the only early symptom of lung cancer or the patients display other unspecific symptoms such as coughing or hemoptysis. The prognosis of lung cancer patients can be determined according to the TNM stage at primary diagnosis [11].

The main risk factor for developing lung cancer is smoking [12]. Between 15% and 25% of lung cancer cases are diagnosed in never-smokers [13].

Therefore, HPV infection as a potentially modifiable risk factor in both smokers and never-smokers has been getting more and more attention in recent years. In our systematic review convincing evidence was found that HPV infection might increase the risk of developing lung cancer [14]. Furthermore, on a global observation HPV prevalence in SCC was significantly higher than in lung cancer patients with non-squamous cell histology.

The possible transmission route of HPV into lung tissue has not yet been proven. Both a hematogenous infection as well as oral sexual activity has been discussed [15].

Many of the published studies have relied on the use of polymerase chain reaction because of its high level of sensitivity.

The aim of this study was to analyze a possible association between SCC and HPV using Multiplex PCR in German patients.

## Materials and Methods

After creating an initial list of 16 patients diagnosed with primary SCC of the lung between 2017 and 2018 the material was obtained from the tissue bank of the department of pathology at the University of Regensburg. From 14 cases enough material for multiplex PCR was still available. In two patients two separate tumor biopsies were available and were used for analysis. In all

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**Table 1:** Patient characteristics.

| | Total number | Percentage |
|---|---|---|
| **Total number of patients** | 14 | |
| **Mean age** | 69.6 | |
| **Sex** | | |
| Male | 8 | 57.0 |
| Female | 6 | 43.0 |
| **Tumor histology** | | |
| SCC | 14 | 100 |
| **Smoking behavior** | | |
| Current smokers | 5 | 35.7 |
| Former smokers | 8 | 57.2 |
| No information | 1 | 7.1 |

cases formalin-fixed paraffin-embedded tissue from the resected lung cancer was used. All lung resections were performed by the Department of Thoracic Surgery at the University Clinic Regensburg between May 2017 and March 2018 (Table 1).

HPV detection was carried out according to the method of nested multiplex PCR described by Sotlar et al. [16].

In this protocol both consensus and type-specific primers are used. In the first round of amplification 18 different HPV subtypes including all high-risk HPV types are tested. These are combined with type-specific primers in the following rounds of amplification. The advantage of this approach lies in its ability to test for multiple HPV types while also typing the exact HPV subtype. With the applied method HPV subtypes 6,11,16,18,31,33,35,39,42,43,44,45,51,52,56,58 ,59,66 and 68 can be detected. It also allows to detect multiple HPV infections in a tissue sample. As controls pre-characterized HPV-positive samples were used. Nested PCRs were performed acc. to Sotlar et al. [5] with 10 µl DNA and GP-Primer-Mix containing GP-E6-3F, GP-E6-5B and GP-E6-6Bas outer primers and primer-pool-1, -2, -3 und -4 as inner primers. PCR conditions from first PCR were: 94°C for 1 min, 40°C for 1 min, and 72°C for 2 min for a total of 40 amplification cycles. 0.5 µl were directly used for the inner PCR with following conditions: 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s. Before the first cycle, there was a 4-min denaturation step was and the last cycle was followed by a 4-min elongation step. All PCRs were performed in a final volume of 50 l with 0.3 µM GP-Primer-Mix (outer PCR) and primer-pools 1-4 (inner PCR), respectively as well as 1x RedTaq-Mix (Promega, Germany). 5 µl of the outer PCR were loaded and electrophoretically analyzed on a 2.5% agarose gel and stained with ethidium bromide.

### Statistical analysis

For the patient characteristics the average and standard deviation were calculated using Excel 2013 and afterward the calculations were repeated using SPSS Version 22.

## Results

From 14 patients a total of 16 tissue samples were included in the presented study. The average age was 69.6 years (SD: 9.6). Of those 57% are male and 43% female. For all but one patient information on smoking behavior was available. Five of the patients were smoking at the time of the operation while eight were former smokers. For one patient the information was not available. The average number of pack years was 65 for the current smokers at time of diagnosis and 39 for the former smokers, respectively. 43% had a previous diagnosis of COPD. All of the patients were diagnosed with primary lung cancer between 2017 and 2018 at the University Clinic Regensburg. They all received a partial lung resection by the Department of Thoracic Surgery at the University Clinic Regensburg.

All of the tumors were histologically determined to be squamous cell carcinomas.

None of the tested tumor samples tested positive for HPV by nested PCR. Therefore, the calculated HPV prevalence is zero.

## Discussion

In the published literature, there is a huge variety within the detected HPV prevalence in primary lung cancer.

To our knowledge only one study conducted in Germany detected HPV DNA in primary lung cancer tissue [17]. In 1992 Eberlein-Gonska et al. [17] used PCR to test tissue of 50 patients with primary lung cancer and 15 biopsies of normal bronchial mucosa. In two cases of SCC and one case of AC the results were positive. None of the tested controls were positive. There are two other published studies that included tissue from German patients but neither of them detected HPV DNA in the analyzed lung cancer tissue [18,19].

These results are in contrast to many results from Asia that report high HPV detection rates [20,21]. A possible geographic or ethnic correlation between HPV infections in lung cancer has been discussed but has not yet been proven.

Some authors have discussed a possible association between certain histological subtypes and HPV infection. One of the connected theories is that chronic smoke inhalation like in the case of smokers might make the bronchial epithelium more fragile and therefore more susceptible to a possible virus infection. In the published studies higher HPV detection rates in SCCs as well as in AC have been reported when compared to one another, so that this question does not seem to be answered yet [22-25].

To our knowledge there are only two published case-control-studies on the current subject that only include SCCs. A Chinese study published in 2012 compared 45 cases of primary SCC with 16 controls of patients without malignant disease. In 19 of the 45 lung cancers HPV DNA was detected by PCR while in none of the controls HPV DNA was detected [26]. In contrast a study from Italy published in 2012 detected no statistically significant difference between the detected HPV prevalence in 50 cases of SCC and 23 controls, 4% and 4.3% respectively [27]. In our own meta-analysis the published HPV prevalence in SCCs was significantly higher than in ACs, 17.9% and 9.6% respectively [14]. This was mainly due to a much higher HPV prevalence in SCC compared to ACs in lung cancer patients in Asia. Furthermore the difference between HPV positive cases and controls was statistically significant all histological subtypes included, 31.3% *vs.* 5.4% ($p<0.01$) [14]. This highlights the considerable divergence between data around the globe and suggests that geographic location and ethnicity may cause more interference in HPV lung infection and possible carcinogenesis than might be expected.

Within the published literature a huge variety of HPV detection methods are used. The most commonly used methods are PCR and ISH. In a study from China 141 NSCLC tumor specimens were analyzed with both nested PCR and in situ hybridization [28]. Testing

was done for HPV 16 and HPV 18 separately and in both cases the HPV detection rate by nested PCR was higher. The concordance between HPV 16 and HPV 18 detection by PCR and ISH were 73% and 85.5% respectively.

A different study conducted in Canada showed a concordance rate of 100% between ISH and PCR while the overall detected HPV prevalence was only 1.5% [25]. In our study PCR was chosen as detection method because of its reported high sensitivity and specificity [29].

A possible loss of specific parts of the HPV DNA originating by the tissue processing method has been discussed. The tissue analyzed in our study was formalin-fixed paraffin-embedded which is the most common tissue processing method because it allows to store tissue for long periods of time. In a study from the USA on a cohort of 399 primary SCCs and ACs HPV detection was done on both formalin-fixed and ethanol-fixed tissue specimens [30]. HPV DNA was not detected in any of them. In other studies, the authors report that HPV detection was done on tissue processed in more than one way, but unfortunately the results were not reported separately [28,31-34].

In our study all PCR analysis were done on resected lung tissue. Some of the studies report on bronchoscopic biopsies which provide smaller amounts of tissue and therefore only a limited amount of testing can be done. Still, we did not detect any HPV DNA.

There is a large methodical heterogeneity between the published studies conducted on a possible association between HPV infection and lung cancer. We minimized methodological bias by including SCC as only histological subtype. We only analyzed a small group of tumor tissue which is associated with a large statistical bias. We also only used PCR as sole detection method and the analyzed tissue was formalin-fixed and paraffin-embedded which might result in lower detection rates.

## Conclusion

Even though huge progress has been made in the therapy of lung cancer, if diagnosed late it still has a poor prognosis. Identifying modifiable risk factors therefore are of vital importance. Many studies have detected HPV in lung cancer, but no causal association has been proven yet. In our own study using nested PCR we were not able to detect HPV in primary SCC. To further investigate these conflicting results more studies most of all on larger cohorts will be necessary to prove a possible role of HPV infection in German patients diagnosed with primary lung cancer.

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