

**Human papilloma virus in lung cancer tissue**  
**Experience at the Calderón Guardia Hospital,**  
**San José, Costa Rica.**

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## **ABSTRACT**

*Background:* The epidemiology of lung cancer, a major cause of morbidity throughout the world and the leading cause of mortality among adult men and women, has changed substantially in the past few years. Some of these changes can be explained by tobacco consumption, but there is also the possibility of other carcinogenic agents involved in lung cancer etiology, such as Human Papilloma Virus (HPV) and they have described a mechanism by which some of the viral structural proteins interfere with tumor suppressor genes, leading to uncontrolled and fast cellular proliferation.

*Methods:* The objective of this study was to establish the prevalence of HPV in lung cancer (LC) tissue. The study population was a group of 110 patients with histologically confirmed LC. The biopsies of lung tissue were fixed in paraffin and formalin at the Calderon Guardia Hospital, Department of Pathology, from January 2002 to July 2009. The viral DNA was extracted using a system for fixed tissue - QIAmp DNA FFPE-, and the virus detection was done by multiple PCR differentiation on the molecular weight of the E6 gene that differs within genotypes.

*Results:* The prevalence of HPV in LC was 4,5%. Most of the HPV types found were non-oncogenic

*Conclusions:* The prevalence found is very low in comparison with other regions of the world. There was no association found between HPV and LC histology, gender, and tobacco use.

## **Introduction**

Over the last 50 years the incidence of pulmonary neoplasms has increased dramatically world wide, affecting mortality in both men and women <sup>1</sup>. For 2008, LC was the most common cause of cancer death for men and women in USA, 31% and 26% respectively<sup>2</sup>. In Costa Rica LC occupies the 3d place in mortality for men and the 5<sup>th</sup> for women<sup>3</sup>

LC pathogenesis is complex, and there are 2 genetically different types, one tobacco related and the other not tobacco related. The former is associated with KRAS and P53 mutations <sup>4-7</sup> and in non smokers it is associated with alterations of epidermal growth factor receptor (EGFR)<sup>4-6</sup>.

Although genetic variants establish an individual's risk for cancer development, environmental factors could trigger cellular changes with the same capability. Epidemiological studies show that tobacco consumption and its nitrous components are the main, but not the only, risk factors involved in LC development<sup>1, 8, 9</sup>.

The possibility that HPV contributes as a co-carcinogen in LC etiology is of interest, since HPV infection is considered the most common sexually transmitted disease in the world, with more than 50% of the population having been infected with this virus<sup>9,10</sup>. Also, the burden of HPV infections and their consequences is a serious concern worldwide in terms of cost to society and human suffering<sup>11</sup>.

HPV is linked with benign tumors such as laryngeal papillomatosis and with more than 10% of human cancers, affecting the genital tract, skin, esophagus, anus, rectum and larynx<sup>12,13</sup>.

The participation of HPV as an etiologic agent of malignant tumors of the respiratory tract was first mentioned by the German virologist Harald zur Hausen in 1976, and later by K J Syrjänen, a pathologist, who in 1980, suggested the role of the virus in the development of epidermoid carcinoma based on histological findings<sup>14,15</sup>.

HPV is a double helix circular DNA of 8000 bases<sup>16-18</sup>, and about 150 types have been isolated. This virus can be classified as mucosal or cutaneous. The mucosal types are classified according to their malignant potential, as of high risk (16,18, 31,33,35,39,45,51,52, 56, 58, 59 and 68) and low risk (for example 6,11) genotypes<sup>19-21</sup>.

The pharynx and larynx are lined by a stratified non-keratinized epithelium but the lower respiratory tract is mainly covered by simple cylindrical epithelium, producing squamo-columnar junctions. These junctions also occur in smokers since tobacco consumption causes epidermoid metaplasia in multiple sites along the bronchial tree<sup>21</sup>. HPV virions first penetrate basal epithelial layers possibly through micro lacerations or through the squamo-columnar junctions, where they join integrin  $\alpha 6$  receptors<sup>20</sup>. Viral DNA maintains a low replication rate within the cell nucleus until it achieves complete differentiation, and then moves to the epithelial surface where it undergoes an exponential genomic amplification<sup>17,20</sup>.

The genome can be divided in 3 parts: an early region (E), that encodes non structural proteins, a late region (L), that encodes capsid proteins, and a non coding long control region (LCR), which regulates viral replication and gene expression<sup>17,22</sup>.

Viral proteins E1 and E2 play an important role in the regulation of genomic replication, serving as activators or inhibitors of transcription. Another 3 proteins, known as E5, E6 and E7, have transforming properties and stimulate viral growth, and are encoded by high-risk serotypes for malignancy. Proteins encoded by these genes are multifunctional and interfere with cell cycle regulation<sup>17,20</sup>.

The carcinogenic mechanism starts with the integration of viral genome into host chromosomes. This generates a sustained expression of genes E6 and E7 and the E2

protein loses its inhibitory potential, which constitutes the most relevant genetic change in its transition to a malignant state <sup>17, 19</sup>.

E6 and E7 proteins are pleiotropic regarding oncogenic activity; E6 interacts with tumor suppressors P53 and P16, inducing their rapid degradation and an antiapoptotic effect, chromosomal instability, telomerase activation and interferon blockade amongst others. E7 expression, on the other hand, helps foreign DNA integration into the host cell and inhibits tumor suppressor pRB, provoking increased mutagenesis and interaction with other carcinogens<sup>17, 19-26</sup>.

Some investigators have reported hematogenous transportation of VPH DNA from the original infection site to other organs where eventually neoplasms will appear<sup>25, 27</sup>.

Several studies, have suggested a relationship between HPV and LC, identifying the virus in lung tissue by polymerase chain reaction (PCR) or by in situ hybridization. A meta analysis carried out from the years 1985 to 2000 of 2648 bronchogenic carcinomas reported 536 (21,7%) positive cases of VPH DNA<sup>20, 23</sup>.

Therefore, HPV appears to have a role in human carcinogenesis, and preventive strategies and measures should be taken to reduce the incidence and mortality associated with this virus. Specifically a LC-HPV association has been denied by other authors<sup>28</sup>.

The majority of investigations in this field have come from Europe and some Asian countries. There is only 1 from the USA <sup>26</sup>. At the same time, little information regarding the prevalence of this virus and LC has come from Latin America, with only 2 publications on the subject <sup>24, 29</sup>. The objective of this study is to determine the prevalence of HPV in LC tissue of patients seen at the Calderon Guardia Hospital (HCG) in San Jose, Costa Rica, according to gender, smoking history and viral genotypes

## **Materials and methods**

### ***Patients and sample***

This report is a cross sectional study of patients with biopsy proven primary LC seen at the HCG from January 2002 to July 2009. The size of the sample was calculated using the formula  $N = \frac{4 \ln^2 P}{(1-P)^2} \cdot W^2$  <sup>30</sup> knowing the prevalence of 21.7% established in the world literature <sup>22</sup>. Therefore, to obtain a confidence level of 95% and 80% precision no less than 96 individuals needed to be analyzed. According to the Pathology Department registry for the study period, there were 154 patients with this diagnosis. However, 17 were excluded because there was not enough tissue left for the study or the diagnosis was made by fine needle aspiration. In three patients the diagnosis was made in non-pulmonary tissue, and the paraffin blocks were missing for other 24 cases. Thus, the final sample was made up of 110 patients.

### ***Polymerase chain reaction for viral genome detection (This test was done at the Saenz Renault Laboratory, San Jose, Costa Rica).***

DNA was extracted with the QIAMP DNA FFPE, extraction kit (Cat. #56404; QIAGEN S.A., Courtaboeuf, France) according to the manufacturers instruction. HPV detection was made with the multiplex PCR kit for HPV from Maxim Biotech, Inc. (Cat. # 70215, San Francisco, CA), designed for simultaneous amplified differentiation through molecular weights of HPV gene E6, genotypes 6, 11, 16, 18, 31, 32, 52, and 58. Thirty-five amplification cycles were made with a Perkin-Elmer 2400 thermocycler. Each cycle included denaturation at 94° C for 1 minute and hybridization at 67° C for 2 minutes. Previously 1 cycle of denaturation at 96 ° C for 1 minute and one hybridization at 96 ° C for 4 minutes, with final elongation of 10 minutes at 70° C.

The positive control provided by the MPCR kit manufacturer was used and deionized distilled water was used as a negative control.

The detection of amplified products was made by electrophoresis at 90 volts on 1% (w/v) agarose gels with 0.5 mg/ml of ethidium bromide, and verified by UV light. HPV genotypes were separated by their molecular weights.

### ***Statistical analysis***

Variables recorded, obtained from the clinical records, were: gender, age at the time of diagnosis, residence, tobacco use, histological type of LC, presence or absence of HPV in the paraffin block, and genotype. The data was analyzed with SPSS 11.0 (SPSS Inc. Chicago, IL) and with Epidat version 3.1.

Chi square was used for descriptive analysis of qualitative variables and Student's t-test for quantitative variables in 2 groups. Also, in order to compare multiple quantitative variables, the ANOVA test was used complemented with the Bonferroni test.

The Hospital Bioethics Committee approved this study (CLOBI number: HCG-028-05-2009). No specific informed consent was obtained from the participating patients, since the individual study of their pathological specimen involved no risk for them and the local Bioethics Committee (CLOBI) agreed with this request.

### **Results**

Biopsy material for the study was obtained for 110 patients. There were 70.9% males, age range 30 to 91 years with a mean of 64.65 (sd  $\pm 12,3$ ). For the women the age range was 40 to 88 years, with a mean of 63,19 years (s.d.  $\pm 11,6$ ). There was no significant difference in age according to gender ( $p=0.57$ ), Table 1

Patients with adenocarcinoma were diagnosed younger (mean age 60,81 years SD 11,7) than those with epidermoid carcinoma (mean age 69,97 years SD 10,3) ( $p=0.009$ ).

Only 89 charts of the 110 had information regarding smoking habits. Smoking prevalence in the whole sample was 73%, (C.I.: 95% 63,2 – 82,1). It was significantly higher in men (87,1%, C.I.: 95% 77,9 – 96,2) than in women (40,7%, C.I.: 95% 20,3 – 61,12) ( $p = 0,000$ ). On the other hand, the prevalence ratio for gender was 2.14 (C.I.: 95% 1,4 -3-4,  $p=0,00001$ ), showing the association between males and smoking.

There is a statistically significant association of male gender and some tumors ( $p=0,008$ ). In these cases, smoking related types were adenocarcinoma ( $p=0,0016$ ) and epidermoid carcinoma ( $p= 0,0047$ ).

There was no statistical difference in LC histologic type between smokers and non smokers ( $p= 0.07$ ). Men consumed an average of 45.16 pack/years, whereas women smoked 4.78 pack/years ( $p=0.00001$ ).

HPV was found in 5 of the 110 LC tissue samples studied, for a 4.54% prevalence (C.I.: 95% 1,5-10,3). Three were from men and 2 from women ( $p=0,627$ ).

HPV genotype 11 was present in 3 cases and HPV 6 in 1 case; all of them were of low-grade malignancy. The other case was positive for HPV 52, an oncogenic HPV type.

Three out of the 5 cases were adenocarcinomas ( $p=0,7$ ), 2 in non-smoking females and 1 in a male smoker. The other 2 cases (both males) had epidermoid carcinoma; one a known smoker and the smoking status of the other was not known.

## **Comment**

This study showed some important facts regarding LC behavior in patients at the CGH. Males predominate in a 2:1 ratio, contrary to the global trend, in which LC is increasing in women, and it is expected that the gender LC incidence will be equal in the next decade <sup>2, 31</sup>.

When comparing gender and histological type, males have higher percentage of lung neoplasm regardless of histological type. All the patients with small cell carcinoma and adenosquamous carcinoma were males. However, there was no statistical



association. Epidermoid and adenocarcinoma, were shown to be linked to male gender (p 0.008)

The literature shows that small cell carcinoma and epidermoid carcinoma predominate in males, but in the last decade the latter has decreased 40% and there is a reduction in mortality for both tumors in men<sup>31</sup>.

In this study adenocarcinoma was the most prevalent, as it is in other parts of the world. From 1970 on adenocarcinoma has become more frequent than epidermoid and small cell carcinoma, and currently is the most common pulmonary malignancy in females<sup>9, 31</sup>. This could be explained by lower tobacco consumption, and also suggests that other carcinogens could be implicated in the pathogenesis of LC, such as HPV<sup>32, 33</sup>. Since pulmonary adenocarcinoma is most common in women, and represents 81% of all LC in them, it has been suggested, but not demonstrated, that estrogens may have some role in the histological and molecular characteristics of this neoplasm. This could explain some gender differences<sup>9, 34</sup>.

According to the literature patients with small cell or epidermoid carcinoma are heavier smokers than those with adenocarcinoma<sup>31, 35</sup>.

The prevalence of 4.5% of HPV in LC tissue found in the present study is very similar to the one reported by Bouchet et al in France of 7%<sup>36</sup>. Although much lower than the 21.7% reported previously by Syrjänen<sup>22</sup>.

Even though these numbers are small, the mere presence of viral material in these tissues, is an intriguing fact. The same findings have been reported in tissue with squamous metaplasia as shown by Syrjänen<sup>22</sup> and Yousem in Pittsburgh, PA, USA.<sup>37</sup>

In general the prevalence of nuclear viral material in LC tissue varies from 0 to 79%, being the highest in Okinawa, Japan (58% to 79%), followed by Taiwan (55%), Iran (25%), Kentucky USA (22%) and Italy (21%)<sup>20-26</sup>. There are only 2 Latin American studies, one from Chile, where the prevalence was 22% and the other from 3 countries: México, Colombia and Peru, reporting a prevalence of 28%<sup>20,24, 29</sup>.

The variations in prevalence found amongst the different investigations, are felt to be due to the interaction of geographic, racial, demographic, environmental and genetic factors, and even of sexual practices from region to region<sup>1, 8, 38</sup>. Another factor influencing these results, is the heterogeneity of methodologies employed in the studies, for example the type of lung sample used (fresh, frozen, formalin fixed, or in paraffin blocks), low viral load, and the method used to detect the HPV genome<sup>1,9</sup>. PCR in agarose gel electrophoresis, the method here utilized, has greater sensitivity than *in situ* hybridization<sup>12, 20, 29</sup>.

Viral genotypes found in this investigation are classified as of low risk (HPV6 and HPV 11) and 1 is of intermediate risk (HPV 52). Both HPV 6 and HPV 11 have been associated with LC<sup>20</sup>. Migration from laryngeal papillomatosis to the lungs has been demonstrated. HPV 11 has been found both in lung primaries and metastatic lesions<sup>25, 39 40</sup>.

The role of low risk HPV in the etiology of LC is still unclear. DNA integration to epithelial cells over expresses oncoproteins E6 and E7 stimulating cell proliferation. This increases the presence of epidermal growth and its ligands. It is believed that the role of HPV 6 in carcinogenesis is to produce a mitogenic stimulus originating mutations in growth control genes<sup>26</sup>.

No association was found in HPV positive patients and gender, histological type nor tobacco usage, even though some studies have linked epidermoid carcinoma and tumor differentiation, although other studies associate it with adenocarcinoma<sup>20, 33, 41</sup>. Several publications report greater prevalence of the virus in women and even more if they are not smokers<sup>38, 42</sup>.

The limitations of this study are the smallness of the lung samples taken endoscopically, since tissue containing viral DNA could be left out of the specimen. In fact all the positive cases were from surgically removed material. The other limitation could be the use of archival tissue held in the pathology department for several years<sup>39</sup>.

Prospective studies need to be conducted to provide the investigators with larger and fresher samples to improve the sensitivity.

Another interesting issue could be the follow up of patients with HPV infections or uterine cervix neoplasm, measuring HPV DNA in peripheral blood to see if they develop malignant tumors in other organs or not. Finally would be interesting to observe how LC behaves now that preventive measures against HPV can be taken.

In conclusion HPV prevalence in LC of patients from the CGH in Costa Rica is low in comparison with other regions of the world. This can be due to geographic or epidemiologic characteristics or to the different methods utilized for viral detection by other authors. We found no relationship of HPV with gender, histological type of LC nor smoking habits.

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**Table 1:** Characteristics of Lung Cancer Patients at the Calderon Guardia Hospital from 2002 to 2009

	Male	Female	Total
Number of Patients (n)	78	32	110
Mean Age (years)	64.65	63.19	
Smoking History			
Smokers (n)	54	11	65
Non-smokers (n)	8	16	24
Unknown	-	-	21
Number (packs/year)	45.1	4.78	
Histological Type (n):			
Adenocarcinoma	36	26	62
Epidermoid	27	2	29
Mixed	2	0	2
Large Cell	4	2	6
Small Cell	6	0	6
Carcinoid	3	2	5
HPV (n) :			
Positive	3	2	5
Negative	75	30	105