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BRIEF ARTICLE

# Incidence of human papilloma virus in esophageal squamous cell carcinoma in patients from the Lublin region

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## Abstract

**AIM:** To assess the prevalence of human papilloma virus (HPV) in esophageal squamous cell carcinoma (ESCC) in the south-eastern region of Poland.

**METHODS:** The study population consisted of 56 ESCC patients and 35 controls. The controls were patients referred to our department due to other non-esophageal and non-oncological disorders with no gross or microscopic esophageal pathology as confirmed by endoscopy and histopathology. In the ESCC patients, samples were taken from normal mucosa (56 mucosa samples) and from the tumor (56 tumor samples). Tissue samples from the controls were taken from normal mucosa of the middle esophagus (35 control samples). Quantitative determination of DNA was carried out using a spectrophotometric method. Genomic DNA was isolated using the QIAamp DNA Midi Kit. HPV infection was identified following PCR amplification of the *HPV* gene sequence, using primers MY09 and MY11 complementary to the genome sequence of at least 33 types of HPV. The sequencing results were computationally analyzed using the basic local alignment search tool database.

**RESULTS:** In tumor samples, HPV DNA was identified in 28 of 56 patients (50%). High risk HPV phenotypes (16 or/and 18) were found in 5 of 56 patients (8.9%), low risk in 19 of 56 patients (33.9%) and other types of HPV (37, 81, 97, CP6108) in 4 of 56 patients (7.1%). In mucosa samples, HPV DNA was isolated in 21 of 56 patients (37.5%). High risk HPV DNA was confirmed in 3 of 56 patients (5.3%), low risk HPV DNA in 12 of 56 patients (21.4%), and other types of HPV in 6 of 56 patients (10.7%). In control samples, HPV DNA was identified in 4 of 35 patients (11.4%) with no high risk HPV. The occurrence of HPV in ESCC patients was significantly higher than in the controls [28 of 56 (50%) *vs* 4 of 35 (11.4%), $P < 0.001$]. In esophageal cancer patients, both in tumor and mucosa samples, the predominant HPV phenotypes were low risk HPV, isolated 4 times more frequently than high risk phenotypes [19 of 56 (33.9%) *vs* 5 of 56 (8.9%), $P < 0.001$]. A higher prevalence of HPV was identified in female patients (71.4% *vs* 46.9%). Accordingly, the high risk phenotypes were isolated more frequently in female patients and this difference reached statistical significance [3 of 7 (42.9%) *vs* 2 of 49 (4.1%), $P < 0.05$]. Of the pathological characteristics, only an infiltrative pattern of macroscopic tumor type significantly correlated with the presence of HPV DNA in ESCC samples [20 of 27 (74.1%) *vs* 8 of 29 (27.6%) for ulcerative or protruding macroscopic type, $P < 0.05$]. The occurrence of total HPV DNA and both HPV high or low risk phenotypes did not significantly differ with regard to particu-

lar grades of cellular differentiation, phases in depth of tumor infiltration, grades of nodal involvement and stages of tumor progression.

**CONCLUSION:** Low risk HPV phenotypes could be one of the co-activators or/and co-carcinogens in complex, progressive, multifactorial and multistep esophageal carcinogenesis.



**Key words:** Human papilloma virus; Low risk phenotypes; High risk phenotypes; Esophageal cancer; Squamous cell carcinoma; Carcinogenesis

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## INTRODUCTION

Esophageal cancer is the eighth most common cancer and the sixth leading cause of cancer-related death worldwide[1-3]. Overall survival rates range between 5% and 16%[2]. Thus, esophageal cancer poses a considerable medical and public health challenge in many parts of the world. Although there is a marked tendency for increasing incidence rates of esophageal adenocarcinoma in industrialized Western countries, the world leading pathological type of esophageal cancer is still squamous cell carcinoma[2]. Marked geographic, cultural and ethnic variations suggest the importance of environmental influences in the development of this condition. In China, India and Central Asia (the Esophageal Cancer Belt), squamous cell carcinoma still predominates[2]. Significantly high incidence rates have been reported in France, South Africa, Chile, Brazil and the north-eastern Himalayan region[1-3]. The North American and European countries have been found to be low-risk areas[1-3]. The highest incidence and mortality have been reported in Kazakh populations, especially in Xinjiang, China[1-3]. In Europe, the incidence of esophageal cancer is increasing and seems to parallel the decrease in gastric cancer incidence[1-3]. Poland is a low-incidence country with the reported age-standardized annual incidence of 4.5 and 0.7/100.0, for men and women, respectively[4].

In Western countries, esophageal carcinomas with squamous cell differentiation typically arise after many years of tobacco and alcohol abuse[5]. Other causes include chronic mucosal injury due to hot beverages and malnutrition[5]. An etiologic role of different microorganisms such as fungi, bacteria and viruses has also been proposed for the pathogenesis of esophageal squamous cell cancer (ESCC)[5,6]. Of these microorganisms, human papilloma virus (HPV), cytomegalovirus (CMV) and Epstein-Barr virus (EBV) have been investigated[5,6]. The role of HPV in the etiology of ESCC has been debated for the past 30 years[7]. The prevalence of HPV in esophageal cancer seems to be higher in areas with a high incidence of ESCC, such as China and South Africa. In 1982, Syrjanen *et al*[7] found that HPV infection caused pathological lesions in esophageal cancer specimens. The prevalence of HPV in ESCC in subsequent studies has shown high variability ranging from 0% to 67%[8-12]. The issue of HPV incidence in ESCC patients in the Polish population has not yet been addressed. In this study we evaluated the prevalence of HPV DNA in patients treated in a regional specialized center for esophageal cancer surgery in Poland, where squamous cell type differentiation of esophageal cancer still predominates.

## MATERIALS AND METHODS

### *Study population*

HPV identification was carried out on 147 esophageal tissue samples taken from 91 patients treated between 2007 and 2010 in the Second Department of General and Gastrointestinal Surgery and Oncological Surgery of the Alimentary Tract, Medical University of Lublin, Poland. The study population consisted of 56 patients with ESCC (49 males aged 56.22 ± 7.34 years; 7 females aged 58.85 ± 7.77 years) and 35 controls with normal esophageal mucosa (30 males aged 33.73 ± 9.62 years; 5 females aged 45.91 ± 5.72 years). The detailed pathological characteristics of the ESCC patients are shown in Table 1. The controls were patients referred to our department due to other non-esophageal and non-oncological disorders with no gross and microscopic esophageal pathology as confirmed by endoscopy and histopathology. All investigated patients and controls were smokers.

The study was approved by the Ethics Committee of the Medical University of Lublin (0254/212/2007) and carried out in accordance with the principles of the Helsinki Declaration. Written informed consent was obtained from all the subjects.

Tissue samples in ESCC patients were taken during surgery from the specimen or during preoperative esophagoscopy. The first set of samples was taken from normal esophageal mucosa 10 cm proximal to the edge of the tumor (56 mucosa samples). A second set of samples was then taken from the tumor (56 tumor samples). Tissue samples in the controls were taken during esophagogastro-duodenoscopy from normal mucosa of the middle esophagus (35 control samples). Tissue samples were divided into two parts: one part was sent

**Table 1 Clinico-pathological characteristics of esophageal squamous cell carcinoma patients investigated from Lublin region**

| Characteristic | *n* (%) |
|---|---|
| Gender | |
| Male | 49 (87.5) |
| Female | 7 (12.5) |
| Age (yr) | |
| > 50 | 46 (82.1) |
| < 50 | 10 (17.9) |
| Macroscopic type | |
| Ulcerative | 10 (17.9) |
| Infiltrative | 6 (10.7) |
| Ulcerative/infiltrative | 21 (37.5) |
| Protruding | 19 (33.9) |
| Grading | |
| G1 | 4 (7.1) |
| G2 | 43 (76.8) |
| G3 | 9 (16.1) |
| Staging | |
| Ⅱ | 30 (53.6) |
| Ⅲ | 19 (33.9) |
| Ⅳ | 7 (12.5) |
| Tumor | |
| T2 | 19 (33.9) |
| T3 | 29 (51.8) |
| T4 | 8 (14.3) |
| Nodes | |
| N0 | 25 (44.6) |
| N1 | 31 (55.3) |

for histopathological assessment and the other was stored in a freezer at -70 ℃ for molecular testing. Before molecular testing for HPV, histopathological assessment of all samples was carried out by two independent experienced pathologists. ESCC was confirmed in all tumor samples and no microscopic pathology was identified in the mucosa and control samples.

### DNA isolation

Genomic DNA was isolated from esophageal cancer tissue and healthy esophageal mucosa by proteinase digestion and phenol extraction or using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions.

The esophageal tissue samples were homogenized following the addition of 1 mL buffer with the following composition: 0.01 mol Tris-HCl pH 7.5; 0.01 mol ethyl enediaminetetraacetic acid; 0.6% odium dodecyl sulfate. The homogenate was incubated for 30 min at room temperature. Then, K proteinase was added reaching a final concentration of 50 mg/L and incubated for 24 h at 37 ℃. Following incubation, half the volume of phenol: chloroform: isoamyl alcohol (in a ratio of 25:24:1) mixture was added to the solution; it was shaken for 15 min at room temperature and centrifuged for 15 min at 3000 *r*/min. The remaining volume of phenol, chloroform, isoamyl alcohol mixture was added to the water phase obtained, shaken energetically and then centrifuged. The above procedures were repeated until complete purification of DNA manifested as a lack of interphase was achieved. Then, half the volume of isopropylene alcohol and 0.1 volume of 3 mol acetate at pH 7.0 were added to the water phase obtained.

The DNA samples obtained in this manner were then rinsed in 80% ethanol and dissolved in distilled water after drying. The samples with dissolved DNA were stored at 20 ℃. Quantitative determination of the DNA obtained was carried out by spectrophotometry using an automatic spectrophotometer manufactured by Pharmacia Co. In order to determine the amount of DNA in a given sample, 1 μL of the sample was dissolved in 69 μL of re-distilled water and after calibration of the spectrophotometer, the sample was placed in its measuring chamber. After automatic processing of the data measured, the result was read in mg/L.

**Table 2 Human papilloma virus identifying primers for polymerase chain reaction study**

| Primers | Region of amplification | Sequence 5′-3′ | Product size (bp) |
|---|---|---|---|
| MY09 | L1 | CGTCCMARRGGAWACTGATC | 450 |
| MY11 | | GCMCAAGGWCATAAYAATGG M = A + C, R = A + G, W = A + T, Y = C + T | |
| HPV16/L1A | L1 | GCCTGTGTAGGTGTTGAGGT | 264 |
| HPV16/L1B | | TGGATTTACTCCAACATTGG | |
| ME12 | HPV18/E6 | CACGGCGACCCTACAAGC-TACCTG | 404 |
| ME50 | | TGCAGCACGAATTGGCACTG-GCCTC | |

HPV: Human papilloma virus.

### HPV identification

Genomic DNA was isolated from study tissues using the QIAamp DNA Midi Kit (QIAGEN) according to the manufacturer's protocol. HPV infection was identified following polymerase chain reaction (PCR) amplification of the *HPV* gene sequence, using primers MY09 and MY11 (Table 2) complementary to the genome sequence of at least 33 types of HPV viruses as described previously[13]. The reaction mixture contained 15 mg/L DNA and the following reagents: 0.5 U Taq DNA polymerase (Fermentas), PCR buffer and magnesium chloride at a final concentration of 1.5 mmol (Fermentas), primers at a final concentration of 0.25 mmol each and dNTPs (Promega) at a final concentration of 0.2 mmol. PCR products were run in 1.2% agarose gel electrophoresis with ethidium bromide and visualized in ultraviolet light. The product length was assessed according to the MassRuler marker (Fermentas). PCR products were randomly eluted from agarose gel using QIAGEN and sequenced in order to confirm the presence of the expected product. The sequencing results were computationally analyzed using the basic local alignment search tool database. These were sequenced in the Sequencing Laboratory Adam Mickiewicz University Poznań.

### Statistical analysis

From the statistical analysis of the results obtained from

**Table 3 Human papilloma virus status in esophageal squamous cell carcinoma patients investigated from Lublin region**

| HPV risk | *n* (%) | $\chi^2$ |
|---|---|---|
| Tumor samples (*n* = 56) | | |
| High risk | 5 (8.9) | |
| Low risk | 19 (33.9)[b] | 24.487 |
| Others | 4 (7.1) | |
| Total | 28 (50.0)[d] | 21.185 |
| Mucosa samples (*n* = 56) | | |
| High risk | 3 (5.3) | |
| Low risk | 12 (21.4)[b] | 23.451 |
| Others | 6 (10.7) | |
| Total | 21 (37.5)[d] | 27.521 |
| Control samples (*n* = 35) | | |
| High risk | 0 (0) | |
| Low risk | 2 (5.7) | |
| Others | 2 (5.7) | |
| Total | 4 (11.4) | |

[b]$P < 0.01$ *vs* human papilloma virus (HPV) high risk, Fisher test; [d]$P < 0.01$ *vs* control, Fisher test.

HPV (+) and HPV (-) patients, correlation tables containing structure indices were compiled. A method of statistical interference, verification of hypotheses based on homogeneity and independence test $\chi^2$, was used. The differences between groups of ESCC samples according to analyzed clinico-pathological parameters were compared using the Mann-Whitney *U* test or the Kruskal-Wallis test depending on the distribution of the grouping variable. Statistical significance was found at $P < 0.05$. The statistical analyses were performed on an IBM PC, using SPSS 8.0 PL for Windows 95 and Statistica 5.0.

## RESULTS

The results of molecular testing which evaluated the occurrence of HPV in 147 esophageal tissue samples from patients with ESCC and controls are shown in Table 3. In tumor samples, HPV DNA was identified in 28 of 56 samples (50%). High risk HPV DNA (16 or/and 18) was found in 5 of 56 samples (8.9%), low risk in 19 of 56 samples (33.9%) and other types of HPV (37, 81, 97, CP6108) in 4 of 56 samples (7.1%). In mucosa samples, HPV DNA was isolated in 21 of 56 samples (37.5%). High risk HPV DNA was confirmed in 3 of 56 samples (5.3%), low risk HPV DNA in 12 of 56 samples (21.4%), and other types of HPV in 6 of 56 samples (10.7%). In control samples, HPV DNA was identified in 4 of 35 samples (11.4%). In control samples, no high risk HPV DNA was isolated. Two samples contained low risk HPV DNA (5.7%) and other types of HPV were found in 2 of 56 cases (5.7%).

The occurrence of HPV DNA in ESCC tissue and unaffected esophageal mucosa taken from patients with esophageal cancer was significantly higher than in control samples taken from healthy esophageal mucosa [28 of 56 (50%) *vs* 4 of 35 (11.4%), $P < 0.001$]. In esophageal cancer patients, both in tumor and mucosa samples, the predominant HPV phenotype was low risk HPV DNA, and was isolated approximately 4 times more frequently than high risk phenotypes. This difference reached statistical significance [19 of 56 (33.9%) *vs* 5 of 56 (8.9%), $P < 0.001$].

**Table 4 Clinico-pathological characteristics of esophageal squamous cell carcinoma patients investigated from Lublin region**

| Characteristic | HPV positive | | HPV high risk | | HPV low risk | |
|---|---|---|---|---|---|---|
| | *n* (%) | *P* value | *n* (%) | *P* value | *n* (%) | *P* value |
| Gender | | | | | | |
| Male | 23 (46.9) | 0.4435[1] | 2 (4.1) | 0.0294[1] | 17 (34.7) | 0.1254[1] |
| Female | 5 (71.4) | | 3 (42.9) | | 2 (28.6) | |
| Age (yr) | | | | | | |
| > 50 | 25 (54.4) | 0.7274[1] | 3 (6.5) | 0.2332[1] | 18 (39.1) | 0.2217[1] |
| < 50 | 3 (30.0) | | 2 (20.0) | | 1 (10.0) | |
| Macroscopic type | | | | | | |
| ULC | 2 (20.0) | 0.0293[2] | 0 (0.0) | 0.1125[2] | 2 (20.0) | 0.1448[2] |
| INF | 5 (83.3) | | 1 (16.7) | | 4 (66.7) | |
| ULC/INF | 15 (71.4) | | 3 (14.3) | | 9 (42.9) | |
| PROT | 6 (31.6) | | 1 (5.3) | | 4 (21.1) | |
| Grading | | | | | | |
| G1 | 3 (75.0) | 0.3234[2] | 1 (25.0) | 0.2638[2] | 2 (50.0) | 0.2911[2] |
| G2 | 19 (44.2) | | 3 (7.0) | | 15 (34.9) | |
| G3 | 6 (66.7) | | 1 (11.1) | | 2 (22.2) | |
| Staging | | | | | | |
| Ⅱ | 11 (36.7) | 0.2720[2] | 3 (10.0) | 0.6106[2] | 6 (20.0) | 0.3537[2] |
| Ⅲ | 14 (73.7) | | 2 (10.5) | | 10 (52.6) | |
| Ⅳ | 3 (42.9) | | 0 (0.0) | | 3 (42.9) | |
| Tumor | | | | | | |
| T2 | 11 (57.9) | 0.3458[2] | 3 (15.8) | 0.1013[2] | 7 (36.8) | 0.3125[2] |
| T3 | 15 (51.7) | | 1 (3.5) | | 11 (37.9) | |
| T4 | 2 (25.0) | | 1 (12.5) | | 1 (12.5) | |
| Nodes | | | | | | |
| N0 | 12 (48.0) | 0.7604[1] | 3 (12.0) | 0.2484[1] | 7 (28.0) | 0.4626[1] |
| N1 | 16 (51.6) | | 2 (6.5) | | 12 (38.7) | |

[1]Mann-Whithey *U* test; [2]Kruskal-Wallis test. HPV: Human papilloma virus; ULC: Ulcerative; INF: Infiltrative; PROT: Protruding.

A higher prevalence of HPV DNA was identified in female patients (71.4% *vs* 46.9%). Accordingly, high risk HPV phenotypes were isolated more frequently in female patients and this difference reached statistical significance [3 of 7 (42.9%) *vs* 2 of 49 (4.1%), $P < 0.05$] (Table 4). Of the pathological characteristics, only an infiltrative pattern of macroscopic tumor type significantly correlated with the presence of HPV DNA in ESCC samples [20 of 27 (74.1%) *vs* 8 of 29 (27.6%), $P < 0.05$] (Table 4). The occurrence of total HPV DNA and both HPV high or low risk phenotypes did not significantly differ with regard to particular grades of cellular differentiation, phases in depth of tumor infiltration, grades of nodal involvement and stages of tumor progression. The detailed distribution of total HPV DNA, high and low risk HPV phenotypes according to particular clinico-pathological characteristics are shown in Table 4.

## DISCUSSION

The role of HPV in the etiology of ESCC is not clear. To date, over 70 studies have tested for HPV in esophageal cancer tissue[13] and the reported HPV detection rate ranged from 0% to 67%[8-12]. The data from China[14] pro-

vided the most convincing evidence to date that HPV is not directly involved in ESCC carcinogenesis, at least in China. HPV DNA contamination cannot be ruled out as an explanation for high HPV occurrence in ESCC tissue studies with less stringent tissue procurement and processing protocols. A few possible reasons for this variation between studies include small study sites, different HPV detection assays, interlaboratory variability and suboptimal sample collection and handling leading to contamination[1,15].

The incidence of HPV infection in ESCC patients was significantly higher in comparison to controls with normal esophageal mucosa. In our study we identified HPV DNA in 50% of ESCC patients, but contrary to the Chinese data[1], the detected HPV types had low oncogenic potential. A high risk 16 or/and 18 HPV DNA type was only detected in 5 of 56 cases (8.92%). HPV types with low oncogenic potential were found 4 times more frequently than high risk HPV types both in tumor tissue and distant mucosa in patients with ESCC. These differences reached statistical significance.

HPV type 16 was detected in 16% of ESCC patients in Uppsala, Sweden[16] and this was higher than in our population. Xu *et al*[17] reported that the detection rate of infection with all HPV types (16, 18 and 58) in ESCC tissue in Chinese patients was 50%. This number corresponded well with data observed in our study. When investigated by nested polymerase chain reaction, HPV DNA was identified in all ESCC tumor tissue in German patients[18]. Concomitantly, another potentially oncogenic virus considered to be involved in ESCC malignant transformation was EBV DNA which was detected in 35% of these patients.

A search of the most frequently used databases (Medline, PubMed) using the terms esophageal squamous cell carcinoma, HPV and Poland, revealed a lack of studies investigating the occurrence of HPV DNA in ESCC in the Polish population. To our knowledge this is the first report from Poland elaborating this issue.

Analysis of the prevalence of HPV DNA in ESCC samples according to different clinico-pathological parameters revealed that HPV was more frequently isolated in female patients and in tumors showing an infiltrative growth pattern (Table 4). In the majority of studies, investigators did not confirm statistically significant differences between HPV-positive and HPV-negative cases with regard to clinical and pathologic findings[19-26]. Nevertheless, Antonsson *et al*[20] reported that patients with HPV-positive ESCC tumors were younger than those with HPV-negative tumors and had higher body mass index. In turn, Harrera *et al*[21] in a Mexican population found that male patients were most commonly affected and the male:female ratio in HPV-positive ESCC increased two-fold in comparison with HPV-negative cases. Dreilich *et al*[16] found a significantly higher rate of the HPV 16 phenotype in advanced compared with localized cancer but this did not translate into prognostic significance. Although isolated data may suggest some association between HPV and aggressiveness and prognosis of ESSC, generalized and coherent conclusions concerning the influence of HPV and its particular phenotypes on clinico-pathological characteristics of ESCC cannot be drawn.

In summary, we identified low risk HPV DNA types in ESCC patients significantly more frequently than other types (37, 81, 97, CP6108). This may suggest that low risk HPV types could be one of the co-activators or/and co-carcinogens in complex, progressive, multifactorial and multistep esophageal carcinogenesis. HPV DNA occurrence does not preclude development of the squamous cell phenotype of esophageal carcinoma.

## COMMENTS

### Background

Esophageal cancer is the eighth most common cancer and the sixth leading cause of cancer-related death worldwide. Overall survival rates range between 5% and 16%. Although there is a marked tendency for increasing incidence rates of esophageal adenocarcinoma in industrialized Western countries, the world leading pathological type of esophageal cancer is still squamous cell carcinoma. Marked geographic, cultural and ethnic variations suggest the importance of environmental influences in the development of this condition. In Europe, the incidence of esophageal cancer is increasing and seems to parallel the decrease in gastric cancer incidence. Poland is a low-incidence country with the reported age-standardized annual incidence of 4.5 and 0.7/100.0, for men and women, respectively.

### Research frontiers

Esophageal carcinoma is multifactorial in origin and typically arises after many years of tobacco and alcohol abuse. Other causes include chronic mucosal injury due to hot beverages and malnutrition. An etiologic role of different microorganisms such as fungi, bacteria and viruses has also been proposed for the pathogenesis of esophageal squamous cell cancer (ESCC). Of these microorganisms, human papilloma virus (HPV), cytomegalovirus and Epstein-Barr virus have been investigated. The role of HPV in the etiology of ESCC has been debated for the past 30 years.

### Innovations and breakthroughs

In this study, the authors found HPV DNA in 50% of ESCCs. High risk HPV phenotypes (16 or/and 18) were found in 8.9%, low risk in 33.9% and other types of HPV (37, 81, 97, CP6108) in 7.1% of the patients. Low risk HPV DNA types were identified significantly more frequently than other types (37, 81, 97, CP6108). This may suggest that low risk HPV types could be one of the co-activators or/and co-carcinogens in complex, progressive, multifactorial and multistep esophageal carcinogenesis.

### Applications

The data on the prevalence of different types of HPV may contribute to the design of future strategies for the prevention of HPV-related malignancies, including the development of effective vaccines.

### Terminology

HPVs are DNA viruses that infect basal skin and mucosal cells. HPVs are categorized according to their cervical oncogenicity-based risk, with high, probably high, low and undetermined risks.

### Peer review

In this manuscript, the authors investigated the prevalence of HPV in esophageal squamous cell carcinoma in Poland. Overall positive rate of HPV was similar to the previous reports. However HPV DNA type was different from other countries. Since this is the first report to investigate about relationship between HPV DNA type and esophageal squamous cell carcinoma, this study is certainly valuable in this research field.

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