

# Does the human papilloma virus affect the prognosis of laryngeal squamous cell carcinoma? An immunohistochemical study

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## Context

The worldwide increase in the prevalence of laryngeal cancer is a major health problem. Human papilloma virus (HPV) infection is a major cause for laryngeal carcinoma, especially in nonsmokers.

## Aim

This study investigated the effect of HPV on laryngeal carcinoma prognosis and whether HPV-associated carcinoma cases carry a better or worse outcome. This was approached by studying the relationship between HPV infection (indicated by p16 immunoreactivity) and various prognostic markers of laryngeal carcinoma such as patients' age, tumor grade, lymph node metastasis, thyroid cartilage infiltration, and immunohistochemical markers of tumor proliferation such as cyclin D1 and p53.

## Materials and methods

This was a preliminary study that included paraffin blocks of 40 cases of laryngeal squamous cell carcinoma, obtained either as laryngoscopic biopsies or laryngectomy samples with or without selective neck dissection. All cases were immunostained using antibodies against p16, cyclin D1, and p53.

## Statistical analysis

The clinical, histopathological, and immunohistochemical data are summarized as percentages and means±SD. The  $\chi^2$ -test was used to assess differences between qualitative variables, whereas the *t*-test and analysis of variance were used for quantitative variables. A *P*-value less than 0.05 was considered to be significant.

## Results

A statistically significant correlation was observed between p16 expression and tumor grade, lymph node metastasis, thyroid cartilage infiltration, and immunoexpressions of cyclin D1 and p53. No other prognostic markers correlated with p16 expression.

## Conclusion

HPV-induced squamous cell carcinoma may carry a worse prognosis in the larynx.

## Keywords:

carcinoma, immunohistochemistry, laryngeal, p16

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## Introduction

Laryngeal carcinoma prevalence is increasing over time worldwide. In the past few decades, the incidence of laryngeal carcinoma has been rising steadily in the USA [1].

A report of the Middle-East Cancer Consortium of the National Cancer Institute in Bethesda, USA, stated that Egypt has one of the highest overall incidence rates of cancer of the oral cavity and larynx among the Middle-East Cancer Consortium countries. In Egypt, the Cancer Pathology Registry of National Cancer Institute of Cairo University declared that during the years 2003/2004 laryngeal cancer was the most frequent among all respiratory cancers (27.84%) and the tenth most frequent among all cancers (1.77%) [2], with the majority of cases being diagnosed at advanced stages [3].

The most common risk factors causing laryngeal carcinoma are tobacco and alcohol use with significant interaction between the two. Other observed risk factors include poor oral hygiene, gastroesophageal reflux, and human papilloma virus (HPV) infection, especially in nonsmokers [4]. Within the Middle East, smoking rates are high, although alcohol consumption is limited. This is especially true for Egypt, where smoking rates are increasing with respect to both cigarettes and water pipe [5].

Infection with various HPV types is related to the development of laryngeal carcinoma independent of

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tobacco and alcohol use [6]. HPV infection plays a role in the molecular pathways through its viral oncoproteins, E6 and E7. These two proteins increase the degradation of p53 and interfere with pRb function, causing upregulation of p16 by losing negative-feedback control. Mutation in the p16-encoding gene leads to p16 overexpression, which has been demonstrated in many cervical and head and neck cancers [7].

The p16 gene is often mutated in squamous cell carcinoma of the larynx, and its overexpression is caused by the HPV E7 protein. Consequently, p16 is assumed to be an indirect marker of HPV-induced laryngeal squamous cell carcinoma [8].

Other chromosomal alterations detected in laryngeal squamous cell carcinoma include cyclin D1, a proto-oncogene that is an important regulator of G1-S phase progression in different cell types, together with its binding partners cyclin-dependant kinases 4 and 6. Cyclin D1 forms active complexes that induce cycle progression by phosphorylating and inactivating the retinoblastoma protein. Thus, overexpression of the cyclin D1 is a common event in cancer [9].

Whether or not HPV-induced laryngeal carcinoma cases carry a better prognosis than other cause-related cases has been our study question. This was investigated by studying the relationship between HPV infection, through its indirect marker p16, and various prognostic markers of laryngeal carcinoma such as patients' age, tumor grade, lymph node metastasis, lymphovascular invasion (LVI), thyroid cartilage infiltration, and immunohistochemical markers of tumor proliferation such as cyclin D1 and p53.

## Materials and methods

This was a preliminary study that used paraffin blocks of 40 cases of laryngeal squamous cell carcinoma. The specimens were obtained either as laryngoscopic biopsies or as laryngectomy samples with or without selective neck dissection. Each paraffin block was recut by a rotatory microtome into 5- $\mu$ m-thick samples, mounted on glass slides to be stained by hematoxylin and eosin for routine histopathological examination, and then mounted on charged slides for p16, cyclin D1, and p53 immunostaining. Immunostaining was performed for all cases by BenchMark IHC/ISH staining module (Ventana, Roche Corporation, Basel, Switzerland).

### Immunohistochemical staining and evaluation

#### P16

The antibody used was mouse antihuman monoclonal antibody, clone sc-1661, code F-12, and dilution 1 : 50

(Santa Cruz Biotechnology Inc., Santa Cruz, California, USA). Cervical squamous cell carcinoma samples were used as the positive control. Only cells showing nuclear staining were considered positive. P16 was graded depending on the intensity as weak, moderate, and marked staining [10].

#### Cyclin D1

The antibody used was rabbit antihuman monoclonal antibody, clone GR-005, code 61-0090-2, and dilution 1 : 50 (Genemed, San Francisco, California, USA). Mantle cell lymphoma samples were used as the positive control. Positive staining was considered only in the nuclei of tumor cells. The intensity of nuclear staining was graded from 0 to 3. The distribution of positive cells in tumors was also graded as follows: grade 1—staining in lesser than 10% of tumor cells; grade 2—staining in 10–50% of tumor cells; and grade 3—staining in more than 50% of tumor cells. Finally, cases were divided into expressors and nonexpressors. Cyclin D1 expressors were cases showing moderate or intense nuclear staining in greater than and equal to 10% of tumor cells [9].

#### P53

The antibody used was mouse antihuman monoclonal antibody, clone sc-98, code pab 1801, and dilution 1 : 50 (Santa Cruz Biotechnology Inc.). Colonic carcinoma samples were used as the positive control. When greater than 10% of nuclear-stained tumor cells were detected in the section, the tumor was considered to be p53 positive [11].

### Statistical analysis

The previously mentioned clinical, histopathological, and immunohistochemical data were summarized as percentages and means $\pm$ SD. They were coded, entered, and analyzed using the statistical package (SPSS, version 15; SPSS Inc., Chicago, USA). The descriptive statistics were computed to summarize the mean, the SD for quantitative variables, and the percentage for qualitative variables. The  $\chi^2$ -test was used to assess differences between qualitative variables, whereas the *t*-test and analysis of variance were used for quantitative variables. A *P*-value less than 0.05 was considered to be significant.

## Results

This study included 40 cases of laryngeal squamous cell carcinoma. The age of patients ranged between 40 and 82 years, with a mean age of 61.6 $\pm$ 9.96 years. The studied cases showed male predominance, with a male-to-female ratio of 9 : 1. Different grades of differentiation were

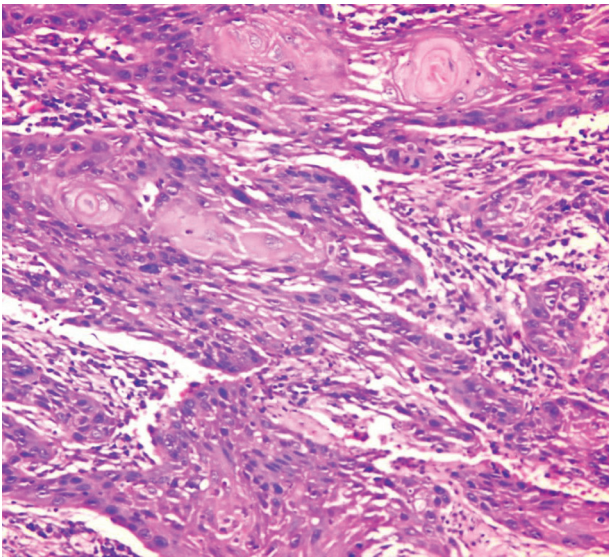
encountered, and most of the cases were grade 2 (50%) as shown in Fig. 1, followed equally by grades 1 and 3 (Fig. 2) (22.5% each). The least encountered was carcinoma *in situ* (5%). Regarding keratinization, 33 (87%) cases were keratinized squamous cell carcinoma. Selective neck dissection was received in 34 cases only, of which 5 (15%) cases showed lymph node metastases as shown in Fig. 3. Infiltration of the thyroid cartilage was found in only 13 out of 30 (43%) cases received with underlying cartilage.

### Immunohistochemical expression

#### P16

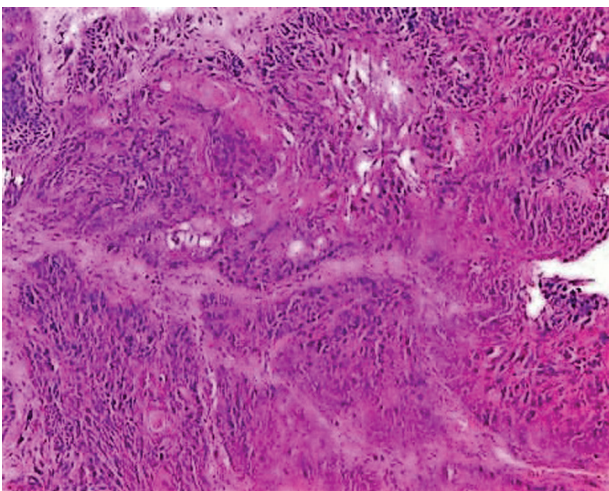
Positive p16 expression was observed in 36 (90%) cases, in contrast to only four (10%) cases that were negative for

Figure 1



A case of keratinizing squamous cell carcinoma of the larynx, grade 2 (hematoxylin and eosin,  $\times 200$ ).

Figure 2



A case of laryngeal squamous cell carcinoma, grade 3 (hematoxylin and eosin,  $\times 100$ ).

p16. The positively stained cases showed weak expression in 13 (36%) cases as seen in Fig. 4, moderate expression in 16 (44.5%) cases as seen in Fig. 5, and only seven (19.5%) cases showed marked expression as seen in Fig. 6.

#### Cyclin D1

Regarding cyclin D1 expression, cases were divided into two groups: cyclin D1 expressors (Fig. 7), which included 20 (50%) cases, and cyclin D1 nonexpressors, which also included 20 (50%) cases.

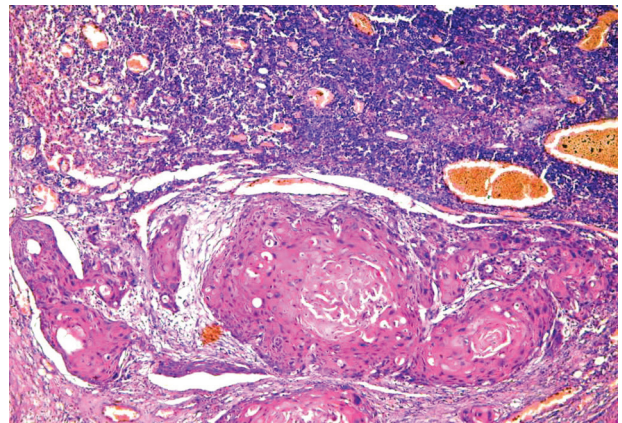
#### P53

With respect to p53, 30 (75%) cases were p53 positive, as shown in Fig. 8, whereas 10 (25%) cases showed negative reaction.

### Correlation between p16 expression and different prognostic factors of laryngeal squamous cell carcinoma

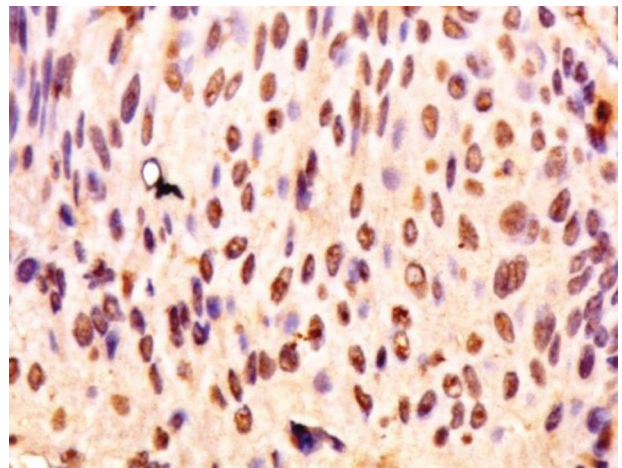
Regarding age of patients, 8/16 (50%) cases less than 60 years showed moderate or intense p16 expression,

Figure 3



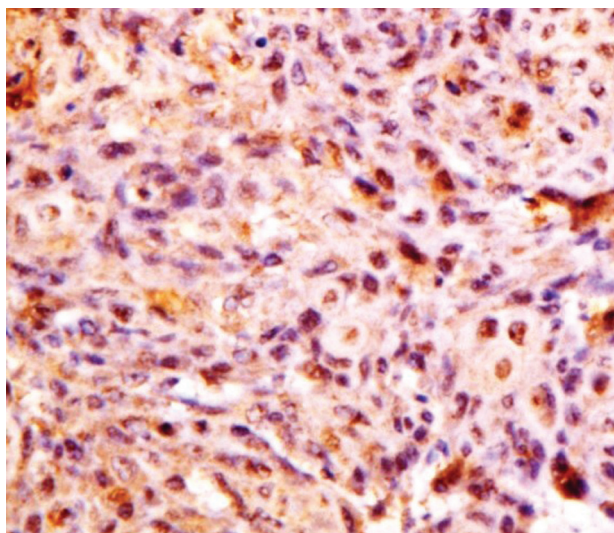
A case of lymph node metastasis in squamous cell carcinoma (hematoxylin and eosin,  $\times 200$ ).

Figure 4



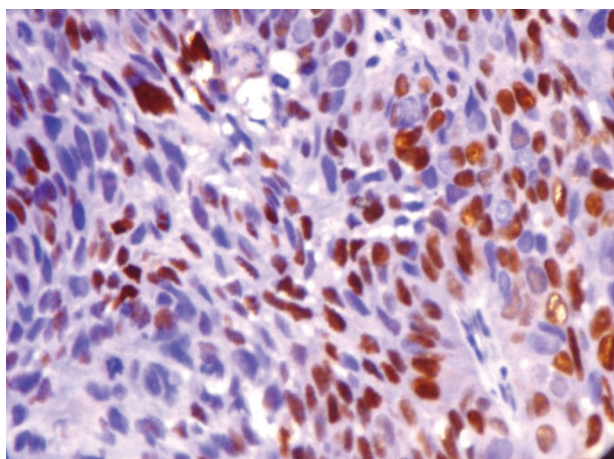
A case of laryngeal squamous cell carcinoma showing positive p16 expression of weak intensity (p16,  $\times 400$ ).

Figure 5



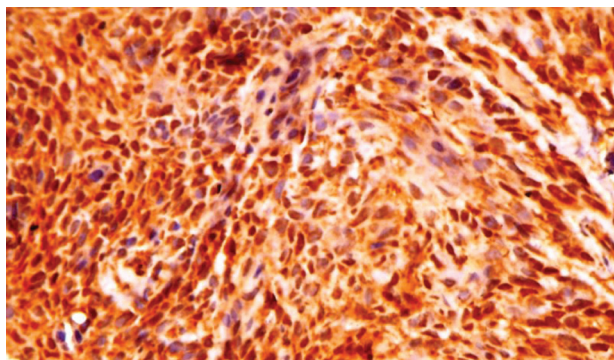
A case of laryngeal squamous cell carcinoma showing positive p16 expression of moderate intensity (p16,  $\times 400$ ).

Figure 6



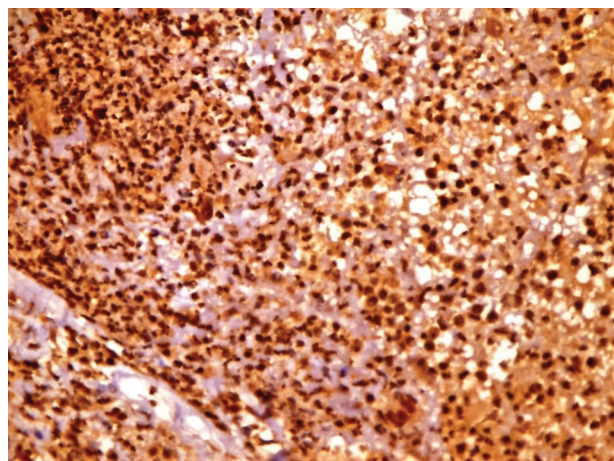
A case of laryngeal squamous cell carcinoma showing positive p16 expression of marked intensity (p16,  $\times 400$ ).

Figure 7



A case of laryngeal squamous cell carcinoma that is considered as a Cyclin D1 expressor (cyclin D1,  $\times 400$ ).

Figure 8



A case of laryngeal squamous cell carcinoma showing positive p53 expression (p53,  $\times 200$ ).

whereas 15/24 (63%) cases greater than and equal to 60 years showed moderate or intense p16 intensity. These results denote a statistically insignificant correlation between age of patients and p16 expression ( $P=0.37$ ). Another statistically insignificant correlation was found between p16 expression and keratinization of the tumor, as 18/33 (54%) keratinized cases showed moderate or strong expressions, whereas all the five nonkeratinized cases showed moderate or strong expressions ( $P=0.2$ ).

Marked p16 staining was noticed in only one (11.1%) case of grade 1, three (15%) cases of grade 2, and three (33.3%) cases of grade 3 carcinomas but in none of the in-situ carcinoma cases. A significant relationship between degree of tumor differentiation and p16 intensity ( $P=0.03$ ) was observed. Lymph node metastasis, which is a very important prognostic factor, showed a statistically significant correlation with p16 expression, as moderate or marked p16 expression was noticed in all the five (100%) cases of positive lymph node metastasis but in only 16/29 (55%) cases of negative lymph node metastasis ( $P=0.003$ ). Thyroid cartilage infiltration also showed a statistically significant correlation with p16 expression, as all the 13 (100%) cases with thyroid cartilage infiltration showed moderate or marked p16 expressions ( $P=0.005$ ), comparable with lymph node metastasis. The correlations between p16 expression and cyclin D1 on the one hand and p53 on the other hand are demonstrated in Tables 1 and 2, respectively.

### Discussion

The worldwide increase in laryngeal carcinoma prevalence is a major health problem. A number of

**Table 1 Correlation between p16 expression and cyclin D1**

Cyclin D1	P16 expression [n (%)]				Total [n (%)]
	Negative	Weak	Moderate	Strong	
Positive	2 (10)	0 (0)	11 (55)	7 (35)	20 (50)
Negative	2 (10)	13 (65)	5 (25)	0 (0)	20 (50)
Total	4 (10%)	13 (32.5%)	16 (40%)	7 (17.5%)	40 (100)

$P < 0.0001$ .

**Table 2 Correlation between p16 expression and p53**

P53	P16 expression [n (%)]				Total [n (%)]
	Negative	Weak	Moderate	Strong	
Positive	1 (3.3)	10 (33.3)	13 (43.4)	6 (20)	30 (75)
Negative	3 (30)	3 (30)	3 (30)	1 (10)	10 (25)
Total	4 (10%)	13 (32.5%)	16 (40%)	7 (17.5%)	40 (100)

$P < 0.0001$ .

molecular epidemiological studies have detected the association of HPV infection with laryngeal carcinoma. Other causes have been implicated in the etiology of laryngeal carcinoma. This study was concerned with the effect of HPV on laryngeal carcinoma prognosis and whether HPV-associated laryngeal carcinoma carries a better or worse outcome. P16 immunoe expression, a surrogate marker for HPV infection, often parallels HPV infection status in laryngeal squamous cell carcinoma [12]. Thus, by comparing p16 immunoreactivity with different prognostic markers of laryngeal carcinoma, we can predict the prognostic effect of HPV on laryngeal carcinoma.

This study was conducted on 40 Egyptian cases diagnosed with laryngeal squamous cell carcinoma. Immunohistochemical expressions of cyclin D1 and p53 were studied as prognostic markers of tumor proliferation in laryngeal carcinoma. P16 immunohistochemical study was performed as a marker for HPV infection and compared with different prognostic markers.

This study did not reveal a statistically significant correlation between p16 expression and patients' age or tumor keratinization. These results are in agreement with those observed by Yuen *et al.* [13] and Hwang *et al.* [14], who also found that p16 did not correlate with patients' age. A surprise finding in this study was the insignificant correlation between p16 expression and tumor keratinization, because it is well known that HPV infection usually causes nonkeratinizing squamous cell carcinoma [15]. This could be attributed to our overall small sample size or to the small number of nonkeratinized squamous cell carcinoma cases included in our study (7/40). Besides, most of the studies relating HPV infection to

nonkeratinized squamous cell carcinoma were more concerned with head and neck carcinomas, paying more attention to oropharyngeal carcinomas rather than laryngeal carcinomas, such as the study by Shinohara *et al.* [16], who found a significant correlation between p16 expression and nonkeratinization of tumor in oropharyngeal squamous cell carcinoma.

A statistically significant correlation was observed in this study between p16 expression and tumor grade, but this was not the case in the study by Yuen *et al.* [13] and the study by Şimşek *et al.* [17].

P16 was statistically correlated with lymph node status in our study and also in the study by Young *et al.* [18]. On the contrary, the study by Yuen *et al.* [13] showed no correlation between p16 expression and lymph node status. Similar insignificant correlations between p16 expression and lymph node status were observed in the study by Hwang *et al.* [14]. Regarding LVI, our study revealed no significant correlation with p16 expression unlike Şimşek *et al.* [17], who found a statistically significant correlation between the increased expression of p16 and LVI in all his studied cases. With regard to thyroid cartilage infiltration, our study showed that it significantly correlated with p16 expression, but we were unable to compare our results with those of others, because to our knowledge no previous studies have compared thyroid cartilage infiltration with p16 immunoe expression.

In this study, there was a significant correlation between p16 expression on the one hand and cyclin D1 and p53 on the other hand. Nevertheless, the study carried out by Krecicki *et al.* [19] observed no correlation between p16 and cyclin D1 expressions. As for p53, to our knowledge, no correlation has been observed earlier between p16 and p53 expressions in laryngeal squamous cell carcinoma. However, unlike our results, Shinohara *et al.* [16] found an inverse significant correlation between p16 and p53 expressions in oropharyngeal carcinoma.

## Conclusion

We conclude from this study that HPV-induced laryngeal squamous cell carcinoma cases may be prognostically worse than HPV-negative cases.

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Nil.

## Conflicts of interest

There are no conflicts of interest.

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