In situ hybridization for human papillomavirus DNA in squamous cell carcinomas of the larynx and inverted papillomas

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There is increasing molecular and epidemiologic evidence that human papillomavirus (HPV) is associated with distinct subset of head and neck lesions. The aim of the present study was to evaluate a possible relationship between the presence of HPV DNA and morphological signs of HPV infections in twenty-six formalin-fixed, paraffin-embedded cases of laryngeal squamous cell carcinomas and seventeen cases of sinonasal inverted papillomas, retrieved from archival material. Ten out of twenty-six cases of cancers and eleven out of seventeen sinonasal inverted papillomas showed morphological signs of HPV infections. All cases have been analyzed using in situ hybridization. The expression of HPV DNA was noted in both examined groups of squamous cell carcinomas and in group of sinonasal inverted papillomas when the morphological signs of HPV infections were present. No significant differences in the frequency of HPV DNA expression were noted in both examined groups of cancers and inverted papillomas (with and without morphological signs of HPV infection). In conclusion, our study confirms the role of HPVs in pathogenesis of inverted papillomas and laryngeal squamous cell carcinomas. However, there is no relevant relationship between the presence of HPV DNA and morphological signs of HPV infections in studied cases.

Key words: human papillomavirus, inverted papilloma, laryngeal cancer.

Introduction

Head and neck cancers are among the most common cancers worldwide and are responsible for 350 000 deaths each year. Nearly 90% of these cancers are squamous cell carcinomas [1, 2]. Head and neck malignancies are characterized by a multiphasic and multifactorial aetiopathogenesis. Smoking and alcohol consumption are the most common risk factors for oral, oropharyngeal, and laryngeal carcinomas. There is increasing molecular and epidemiological evidence that human papillomaviruses (HPVs) may act as a cocarcinogen, along with tobacco, in the causation of these cancers [3, 4].

The role of HPV in the aetiology of anogenital cancers has been firmly established, and infection with this virus has also been shown to have prognostic significance [5, 6]. The similarity of the morphological features of genital and oral HPV-associated lesions was one of the early findings that raised the possibility that HPV might be involved in oral and laryngeal squamous cell carcinomas [7, 8]. Between 15% and 35% of head and neck squamous cell cancers could be associated with high risk HPVs, in particular HPV 16 [9, 10].

Infections with HPV types that have low oncogenic risk, such as HPV 6 and 11, are associated with benign lesions of the anogenital areas known as condylomata acuminata (genital warts), low-grade squamous intraepithelial lesions of the cervix as well as head and neck papillomas [11, 12]. Human papillomavirus 6 and 11 are the most frequently identified HPV subtypes in oral and sinonasal inverted papillomas [13-16]. Inverted papillomas are particularly noteworthy because in contrast to

exophytic papillomas, they are associated with malignancy and have a tendency to recur.

Infections with HPVs are divided basically into three different infection types: those producing specific clinically visible lesions, those remaining subclinical, and those being latent. Histopathological examinations are usually sufficient to reveal characteristic signs of HPV infection including: binucleation, multinucleation, koilocytosis, spindle koilocytes, abnormal mitosis, increased mitotic activity, dyskeratosis and parakeratosis. The most popular viral cytopathic effect of HPV infection is koilocytosis, which is considered a major criterion in HPV infection from the histopathological point of view [17]. Although it is known that molecular methods are the best current method to detect HPVs, the diagnosis is usually established by investigating morphological signs of HPV infection with haematoxylin and eosin stained slides.

The aim of our study was to determine whether an association exists between histopathological signs of HPV infection and presence of HPV DNA in cases of squamous cell carcinomas and inverted papillomas with and without morphological signs of HPV infection.

Material and methods

Patients

Twenty-six formalin-fixed, paraffin-embedded tissue specimens of squamous cell carcinoma of the larynx and 17 of sinonasal inverted papillomas were retrieved from archival material (Chair of Pathomorphology, Medical University of Lodz). The age range for sinonasal inverted papillomas was from 29 to 79 years (mean 53.59) and for squamous cell carcinomas of the larynx was from 47 to 82 years (mean 62.77). Paraffin tissue sections were stained with haematoxylin and eosin and the histological diagnoses were established according to WHO classification [18]. Ten out of 26 cases of squamous cell carcinoma of the larynx and 11 out of 17 sinonasal inverted papillomas showed histological signs of HPV infections (koilocytosis, binucleated squamous cells, increased mitotic activity and abnormal mitoses).

In situ hybridization

Seventeen sinonasal inverted papillomas and 26 squamous cell carcinomas of the larynx were analysed using commercially available HPV DNA probes (Dako, Carpinteria, California, USA). Initially, a wide spectrum biotinylated probe for common HPV subtypes was used, according to the manufacturer's suggested protocol. The wide spectrum probe targets the genomic DNA of HPV types 6, 11, 16, 18, 31, 33, 35, 39, 45, 51 and 52. Further subtyping was carried out in the same way, using specific probes for HPV low risk (HPV 6 and 11) and HPV high risk (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68). Afterwards a catalysed signal amplification system prepared according to the instructions of the manufacturer was used (GenPoint, CSA System for in situ hybridization; Dako).

Statistical methods

The differences between groups were tested using non-parametric χ^2 test. Results were considered statistically significant if $p < 0.05$.

Results

Of the 11 cases of sinonasal inverted papillomas with morphological signs of HPV infection, 1 was positive for HPV DNA using the wide spectrum probe and for HPV DNA subtypes 6 and 11 (Fig. 3). In addition, 1 case was positive for the HPV high risk probe and 1 for the wide spectrum HPV probe only. None of the 6 cases of sinonasal inverted papillomas without morphological signs of HPV infection was positive for any HPV DNA probes.

One of the 10 cases of squamous cell carcinoma with morphological signs of HPV infection was positive for both HPV DNA probes: the wide spectrum probe and the HPV high risk probe. In addition, positive reactions for HPV DNA using the high risk probe were noted in 3 examined cases of squamous cell carcinomas of the larynx where the morphological signs of HPV infection were present (Fig. 1). Four of the 16 cases of squamous cell carcinoma without morphological signs of HPV infection were positive for both HPV DNA probes – the wide spectrum probe and the high risk probe (Fig. 2) – and 1 case was positive for the high risk probe only.

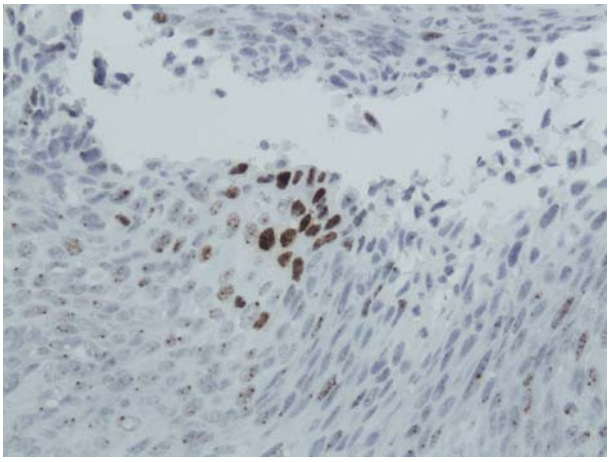

Fig. 1. Positive DNA expression for high risk HPV probe in squamous cell carcinoma of the larynx with morphological signs of HPV infection. In situ hybridization. Magnification 200×

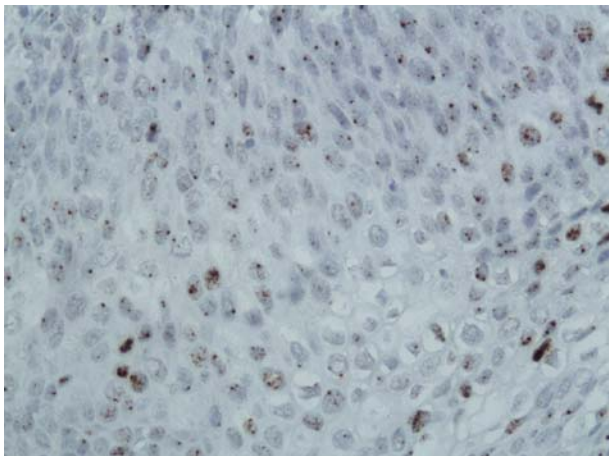

Fig. 2. Positive DNA expression for high risk HPV probe in squamous cell carcinoma of the larynx without morphological signs of HPV infection. *In situ* hybridization. Magnification 200×

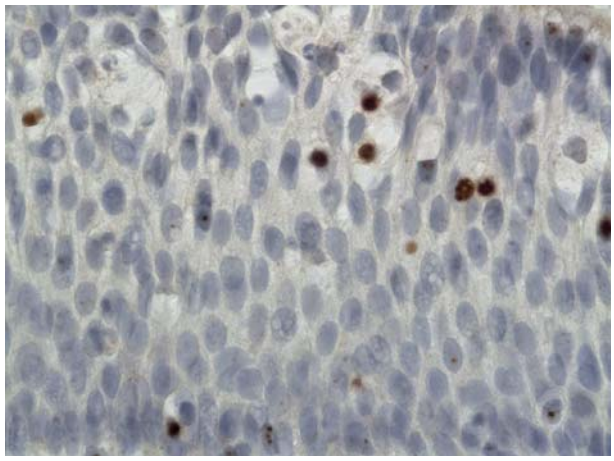

Fig. 3. Positive DNA expression for low risk HPV probe in sinonasal inverted papillomas. *In situ* hybridization. Magnification 400×

Table I. Summary of results of *in situ* hybridization for human papillomavirus (HPV) DNA in squamous cell carcinomas of larynx (SCC) and inverted papillomas (IP)

	Number of cases positive for HPV/total number of cases
SCC with morphological signs of HPV infection	4/10
SCC without morphological signs of HPV infection	5/16
IP with morphological signs of HPV infection	3/11
IP without morphological signs of HPV infection	0/6

SCC – squamous cell carcinoma
IP – inverted papilloma

No significant differences in the frequency of HPV infection were noted between the two examined groups (with and without morphological signs of HPV infection) of sinonasal inverted papillomas ($p = 0.15$) or between the two groups of squamous cell carcinomas of the larynx ($p = 0.64$).

The results of *in situ* hybridization are summarized in Table I.

Discussion

Human papillomavirus infection of human tissues is reflected in a spectrum of histological changes. According to many authors, koilocytosis is a pathognomonic sign of HPV infection serving as a foundation for molecular biology studies. Although molecular methods are available for detecting HPV infections, the presence of morphological features of HPV infection in tissues is still fundamental for histopathological diagnosis. It is a routinely used method particularly in centres in which sophisticated molecular methods are unavailable.

Inverted papillomas have been studied extensively for their association with HPV 6 and HPV 11 [13-16]. Positive reaction for a low risk HPV probe, found in our study in 1 case with morphological signs of HPV infections, may suggest the possible involvement of HPV 6 and HPV 11 in aetiology of sinonasal inverted papillomas. It is well known that inverted papillomas are lesions that have a tendency for transformation into squamous cell carcinoma. Many authors suggest that high risk HPV, particularly HPV 16, may be involved in malignant transformation of inverted papillomas [19-21]. The positive reaction for the high risk HPV probe observed in our study may support the hypothesis that HPV infection may play a role in the progression of inverted papillomas to carcinomas. Lack of the expression of HPV DNA in the remaining cases of inverted papillomas with and without morphological features of HPV infection could be caused by the presence of HPV subtypes other than those investigated in our study. Guan [22] observed that HPV 57 may be related to the pathogenesis of nasal inverted papillomas. It is also possible that the negative results in both tested groups of inverted papillomas could reflect the aetiology of these lesions a different than viral. The role of HPV in the pathogenesis of inverted papillomas is still questionable and some analysis suggests that incidental expression of HPV may occur as well. For instance, Syrjänen [2] demonstrated HPV in approximately 11% of cases of normal oral mucosa.

Our results suggest involvement of HPV in pathogenesis of squamous cell carcinoma of the larynx. The 40% positivity rate of high risk HPV

subtypes in tissue samples from patients with squamous cell carcinoma with morphological signs of HPV infection observed in our study is similar to that seen in other studies [8, 13, 23, 24]. It is worth noting that even though morphological signs of HPV infections were present in all tested samples, HPV DNA was detected only in some of the examined samples. The literature data suggest that HPV 16 and HPV 18 are the main HPV subtypes aetiologically involved in the development of squamous cell carcinomas of the larynx [2, 13, 25]. In contrast to other findings, we observed 3 positive results with the high risk HPV probe only. It is possible that subtypes of HPV 56, 58, 59, 68, which are not present in the wide spectrum probe, may play a role in pathogenesis of squamous cell carcinoma in these cases. It is well known that the aetiology of squamous cell carcinomas of the larynx is complex, also demographic and individual variables exists. In our opinion, this is one of the reasons for the highly variable detection of HPV DNA.

In the group of cancers without morphological signs of HPV infections, we obtained unexpected findings. Four of the 16 cases of squamous cell carcinoma were positive for both the wide spectrum HPV probe and the high risk HPV probe. Additionally, 1 case was positive for the high risk probe only. Our results support the presumption of HPV involvement in the pathogenesis of squamous cell carcinomas of the larynx in cases without morphological signs of HPV infections as well. To our knowledge, this is the first study evaluating the frequency of HPV DNA in cancers without signs of HPV infection. It must be taken into consideration that tissues for HPV studies are usually qualified with special emphasis on the presence of morphological signs suggesting HPV infection. Absence of morphological signs of HPV infection in cases with positive HPV DNA expression provides interesting insights into the histopathological value of diagnosis based on routinely stained samples. On the other hand, our results may point, on the one hand, to underestimation of HPV infection in cases without morphological signs of HPV infection but positive for HPV DNA, and to overestimation in cases with morphological signs of HPV infection but negative for HPV DNA. In our opinion the histopathological analyses of haematoxylin and eosin stained tissues are not sufficient for accurate evaluation of HPV infection and should be a screening method of HPV infection only.

In summary, our study confirms the role of HPVs in the pathogenesis of sinonasal inverted papillomas and laryngeal squamous cell carcinoma. However, we also demonstrated the existence of relevant discrepancies between the presence of HPV DNA and morphological signs of HPV infection, because only in some of the examined cases morphological signs of HPV infection were associated with the presence of HPV DNA.

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