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Original contribution

p16^{INK4A} genetic and epigenetic profiles differ in relation to age and site in head and neck squamous cell carcinomas☆

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Summary Head and neck squamous cell carcinoma (HNSCC) typically affects male smokers older than 55 years. Recently, an increase in the incidence of HNSCC in young adults has been recognized, many of them nonsmokers and females. Functional inactivation of *p16* is known to be a common event in HNSCC, mainly by either deletion or methylation. A previous study by this group has shown that *p16* deletions in HNSCC are significantly associated with age. The primary objective of this study was to evaluate additional molecular alterations of *p16* in HNSCC, specifically in relation to age, site, and human papillomavirus (HPV) status. Patients ranging in age from 22 to 76 years with HNSCC were prospectively identified (n = 24). Methylation-specific polymerase chain reaction and immunohistochemistry were used to evaluate *p16* gene inactivation and p16 protein expression, respectively. HPV 16 status was determined for each case. Overall, *p16* inactivation was a frequent event detected in 46% of cases. Methylation of *p16* was more often detected in females than males ($P = .05$). All cases showing *p16* methylation were from the anterior tongue, and 75% of them were young patients. The results indicate that *p16* methylation is a more common event in those younger than 40 years in contrast to *p16* deletions, which are more common in those older than 40 years. Consequently, it appears that specific modes of inactivation of *p16* in HNSCC are related to specific patient risk profiles. Interestingly, HPV 16 messenger RNA was detected exclusively in HNSCC from the base of tongue lesions and was only found in males. This differs from the patient profile of HNSCC in the young, which affects the anterior tongue and commonly females, thus, making it highly unlikely that this virus is a primary causative agent of HNSCC in these young adults.

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1. Introduction

The typical patient with head and neck squamous cell carcinoma (HNSCC) is older than 55 years, male, and a smoker. However, in recent years, there has been a worldwide increase in the incidence of HNSCC in young adults often with no history of smoking and who are often female [1-7]. Alterations of the *p16* tumor suppressor gene have been consistently reported in HNSCC, with the prevalence of *p16* alterations ranging from 25% to 83% [8-12]. Previous studies have noted that these alterations are predominantly in the form of deletions and methylation-induced epigenetic silencing, and also, that mutation of *p16* is a less common mechanism of inactivation in head and neck cancer [13-15]. Previous work by this group has specifically noted that *p16* deletions in HNSCC occurred in patients older than 40 years [10]. What remained in question, however, was whether *p16* was inactivated by another mode (for example, methylation) in young adults (younger than 40 years old). Immunohistochemistry (IHC) studies alone cannot determine whether p16 has been altered by deletion or methylation. The aim of this study was to correlate age and tumor site with both *p16* gene and p16 protein profiles in HNSCC. Complicating this area is the evidence of a close relationship between p16 protein overexpression and human papillomavirus (HPV) in a subset of head and neck squamous cell cancers [16,17]. Although HPV may be a contributing factor in a subset of head and neck malignancies [18-20], it is clear that its role in HNSCC differs from cervical cancer, where HPV has been established as a primary cause [21]. With the recent increase in HNSCC in young adults, HPV has been considered as a possible risk factor [22-24]. In this study, the relationship between p16 expression and HPV status in all cases was also evaluated.

2. Materials and methods

2.1. Case selection

Ethical approval for this study was acquired from The Dublin Federated Hospitals Ethics Committee. Both fresh snap-frozen tumor tissue and paraffin-embedded tissue were collected from consenting patients at St James Hospital, Dublin. A total of 24 cases of HNSCC were accrued for the approved period. All samples were taken from squamous cell carcinomas (SCCs) occurring in the oral cavity or the oropharynx (*International Classification of Diseases, Ninth Revision*, codes 140, 141, 143-149 inclusive). Medical records of these patients were retrieved. The clinicopathologic features of these tumors are listed in Table 1. The term *young* is used to define patients 40 years or younger at time of diagnosis, whereas *old* is defined as patients older than 40 years at time of diagnosis.

Table 1 Clinicopathologic characteristics of cases

Parameter	n (%)
Age (y)	
≤40	10 (42)
>40	14 (58)
Sex	
Male	17 (71)
Female	7 (29)
Site	
Anterior tongue	10 (42)
Oropharynx	11 (46)
Floor of mouth	1 (4)
Alveolar ridge	2 (8)
Smoking status	
Yes	14 (58)
No	10 (42)
Stage	
I	2 (8)
II	5 (21)
III	8 (33)
IV	9 (38)
Grade of differentiation	
Mild	1 (4)
Moderate	19 (79)
Severe	4 (17)

2.2. Immunohistochemistry

Immunohistochemical staining for p16 protein was performed on 4-μm-thick formalin-fixed paraffin-embedded tissue sections using the DAKO $p16^{INK4A}$ Research Kit (Dako Cytomation, Glostrup, Denmark). This kit uses a monoclonal anti-$p16^{INK4A}$ antibody clone E6H4 (MTM Laboratories AG, Heidelberg, Germany). Controls were included in each assay, comprising positive tissue controls of sections of cervical transformation zone with evidence of high-grade squamous dysplasia (CIN3), negative controls in which the primary antibody was replaced with phosphate-buffered saline, and a control of normal oral mucosal tissue. The immunoreaction cutoff was established by quantifying nuclear and cytoplasmic positivity in normal tissue, which ranged from 0% to 5%. After staining, tissue sections were graded by 2 pathologists (M. E. T., E. M. O.) according to the following scale: 0 (no positive staining), 1 (<10% positive staining), 2 (>10% and <50% positive staining), and 3 (>50% positive staining). The positive samples were then assessed as to whether staining was nuclear or cytoplasmic. Only those showing either nuclear staining only or nuclear and cytoplasmic staining were regarded as positive.

2.3. Methylation

A detailed description of the methylation-specific polymerase chain reaction (MSP) technique used by this group has been previously reported [25]. Briefly, DNA was