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# Langerhans cell number is a strong and independent prognostic factor for head and neck squamous cell carcinomas

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ABSTRACT

*Objectives:* Head and neck squamous cell carcinomas (HNSCCs) exhibit great biological heterogeneity and relatively poor prognosis. Tobacco and alcohol consumption is involved in the cause of the majority of these cancers, but over the last several years, Human Papilloma Virus (HPV) infection has increased specifically in oropharyngeal cancers and become an additional risk factor. Here, we evaluated the number of Langerhans cells (LCs) in HNSCC and reporting its prognostic power in comparison to other risk factors.

*Materials and methods:* Our clinical series was composed of 25 tumor-free peritumoral epithelium, 64 low-grade dysplasia, 54 high-grade dysplasia and 125 carcinoma samples. HPV was detected by E6/E7 qPCR and p16 immunohistochemistry. CD1a-positive LCs were counted in intra-tumoral and stromal compartments as well as lymph nodes. MIP-3α was assessed in carcinomas using immunohistochemistry.

*Results:* Univariate Cox regression analyses demonstrated that high LC number is associated with longer recurrence-free survival in both intra-tumoral and stromal compartments and longer overall survival in stromal compartment. Tobacco and alcohol habits, but not HPV status, are also correlated with poor prognoses in terms of recurrence. Multivariate analyses reported stromal LC number as a strong prognostic factor independent of tobacco, alcohol and HPV status. Moreover, LC number is higher in tumors and invaded lymph nodes than dysplastic lesions but it decreases in HPV-positive cancer patients. Further, LC number correlates with MIP-3α expression.

*Conclusion:* These findings suggest that LC number is a significant and independent prognostic factor for HNSCC. LC infiltration is increased in cancer lesions but decrease with HPV infection.



## Introduction

The incidence of head and neck squamous cell carcinoma (HNSCC) exhibits significant variation around the world and is predominantly prevalent in Asia and Northern Europe. Approximately 550,000 new cases are diagnosed each year in the world, and thus, these cancers rank 6th in incidence [1]. The classical risk factors for HNSCC remain tobacco and alcohol use. However, since Gillison's study in 2000, human papilloma virus (HPV) infection has become recognized as a new risk factor for these cancers [2]. In this context, over the past several years, some countries have seen an increase in oropharyngeal cancers associated with HPV-16 in the young, non-smoker and non-drinker population [3].

In contrast, the question of the HPV-related prognosis in HNSCC remains controversial. Indeed, several studies have demonstrated that HNSCC patients with HPV infection, particularly younger, non-smoking and non-drinking patients with oropharyngeal cancer, exhibit better prognoses than do patients without this infection [4–7]. However, our group and others have observed the opposite findings [8–10]. One of the reasons of this discrepancy may be the heterogeneity of HNSCC regarding tobacco and alcohol

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consumption in addition to HPV infection (transcriptionally active or not). In this regard, in a group of HPV-positive patients, Ward et al. demonstrated that heavy-smokers have shorter survival times than do non- and light-smokers [11]. In Western countries, we are faced with two HPV-positive populations: one is younger, non-smoking and non-drinking and exhibits a better prognosis and a lower incidence [12], and the other smokes and drinks and exhibits a poorer prognosis and higher incidence [13,14]. Moreover, a recent international study that included 3680 patients demonstrated that oropharyngeal, oral cavity and laryngeal cancers are attributable to HPV in 18.5%, 3% and 1.5% of the cases, respectively, based on HPV-DNA, HPV E6 mRNA and $p16^{INK4a}$ immunostaining [15]. These findings clearly indicate that tobacco and alcohol consumptions remain major risk factors for HNSCC.

Langerhans cells (LCs) belong to the dendritic cell family and are antigen-presenting cells for T lymphocytes localized in the epithelium [16]. Several studies have reported that the LC number is increased in the leukoplakia lesions of patients with smoking habits and in oral squamous cell carcinomas [17,18]. Similarly, HPV infection modulates the tumor immune environment as demonstrated by Nguyen et al., who reported that the number of LCs (CD1a-positive cells) is reduced in the stroma of younger oropharyngeal cancer patients with HPV infection [19].

Given this context, we examined the number of LCs in a large clinical series composed of 25 cases with tumor-free peritumoral epithelium, 64 cases with low-grade dysplasia, 54 cases with high-grade dysplasia and 125 cases with carcinomas including 82 HPV-negative and 43 HPV-positive HNSCCs. We evaluated whether the intra-tumoral and stromal LC infiltrates might be useful as prognostic factor in HNSCC patients and we compared the prognostic power of LC number to other risk factors including tobacco and alcohol consumption as well as HPV status.

## Materials and methods

### *Study population and clinical data*

Formalin-fixed paraffin-embedded HNSCC specimens were obtained from 125 patients who underwent curative surgery at CHU Sart-Tilman (Liège, Belgium), Saint-Pierre Hospital (Brussels, Belgium) and EpiCURA Baudour Hospital (Baudour, Belgium) during the years 2001–2012. Among these patients, 82 (72%) were not infected, 21 (18%) were infected with a transcriptionally non-active HPV (HPV+/p16-) and 11 (10%) were infected with a transcriptionally active HPV (HPV+/p16+). Based on the patients' tobacco use and/or alcohol consumption, they were classified as smokers and non-smokers and as drinkers and non-drinkers at the time of the HNSCC diagnosis (Supplementary Data 1). This retrospective study was approved by the Institutional Review Board.

### *DNA extraction*

The formalin-fixed paraffin-embedded tissue samples were sectioned ($10 \times 5$ µm), de-paraffinized and digested with proteinase K via overnight incubation at 56 °C. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Benelux, Belgium) as previously described [20].

### *Detection of HPV by polymerase chain reaction (PCR) amplification*

HPV DNA detection was performed using PCR with GP5+/GP6+ primers (synthesized by Eurogentec, Liege, Belgium) that amplify a consensus region located within the L1 region of the HPV genome as previously described [20].

### *Real-time PCR amplification of the HPV type-specific DNA*

All of the DNA extracts were tested for the presence of 18 different HPV genotypes using TaqMan-based real-time PCR that targeted type-specific sequences of the viral genes as previously described [20].

### *Immunohistochemistry*

#### *p16 immunostaining*

All HPV-positive samples were immunohistochemically evaluated for p16 expression using the recommended mouse monoclonal antibody (CINtec p16, Ventana, Tucson, USA) and an automated immunostainer (Bond-Max, Leica Microsystems, Wetzlar, Germany). All tumor samples were fixed in 4% buffered formaldehyde for 24 h, dehydrated and embedded in paraffin. Immunohistochemistry was performed on 5-µm thick sections mounted on silane-coated glass slides. The tissue sections were placed in a Leica Bond-Max immunostainer, deparaffinized, subjected to epitope retrieval solution (pH 6) for 10 min and incubated with p16 antibody for 30 min (Ventana, Tucson, USA). Then, polymer detection was performed using Bond Polymer Refine Detection according to the manufacturer's protocol (Leica, Wetzlar, Germany). Finally, the slides were counterstained with hematoxylin and luxol fast blue. Tissue sections from cervical lesions were used as positive sample controls. For negative staining controls, the incubation step with the primary antibody was omitted from the protocol. The expression of p16 was defined as positive only when both the nucleus and cytoplasm were stained and over 70% of the tumor cells were stained.

#### *CD1a and MIP-3α immunostaining*

The Langerhans cells and the chemokine MIP-3α were detected by immunohistochemistry using a CD1a-mouse monoclonal antibody (Dako, Glostrup, Denmark) at a dilution of 1:20 and a MIP-3α-mouse polyclonal antibody (Tebu-Bio, Boechout, Belgium) at a dilution of 1:100. First, the tumor samples were deparaffinized in two xylene baths and then rehydrated in four ethanol baths of decreasing concentrations (100% to 70%). Next, the samples were immersed in a 4.5% $H_2O_2$/methanol bath and finally in distilled water. Then, epitope retrieval was performed by immersing the samples in EDTA buffer (Dako, Glostrup, Denmark), followed by heating in a pressure cooker, incubation in a protein block without serum (Dako, Glostrup, Denmark) and subsequent incubation with the primary antibody overnight. Finally, the samples were incubated with a PowerVision Poly-HRP-anti-mouse IgG (Klinipath, Duiven, Holland), and the antigens were visualized via the addition of a solution of 3–3′ diaminobenzidine-hydrogen peroxide-EDTA buffer (Liquid DAB, San Ramon, USA) before coloring with Mayer's hemalum (Klinipath, Duiven, Holland) and mounting with a synthetic balm (Thermo Scientific, Pittsburg, USA).

The LCs were counted in 5 fields in each area (i.e., the NE, LGD, HGD, CA and nodes) with an AxioCam MRC5 optical microscope (Zeiss, Hallbergmoos, Germany) at 400× magnification.

MIP-3α staining was evaluated under the same microscope used for the LC counts but at 200× magnification. Ten fields on each slide were randomly selected and the percentage of positivity (labeling index, LI) was assessed.

### *Statistical analyses*

The medians of the independent data groups were compared using the nonparametric Mann-Whitney test (2 groups) or the Kruskal-Wallis test (more than 2 groups). When the latter test was significant, the Dunn post hoc test was used to compare each pair of groups (to avoid multiple comparison effects).