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# CELL CYCLE, VIRAL AND IMMUNOLOGIC PROFILE OF HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC) AS PREDICTABLE VARIABLES OF TUMOUR PROGRESSION

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**Keywords:** head and neck squamous cell carcinoma; human papillomavirus; proliferation index; p53; macrophage infiltration.

## **Abstract**

*Background.* We wish to determine whether the aberrant expression of cell cycle- or immune response-markers together with **Human Papillomavirus (HPV)** positivity may impact patient survival in different head and neck squamous cell carcinoma (HNSCC) subsets.

*Methods.* 59 HNSCC specimens were analyzed for expression of cell cycle and proliferation markers and macrophage infiltration. HPV status was evaluated by **Polymerase Chain Reaction (PCR)** and DNA sequencing.

*Results.* The HPV presence in oropharynx carcinoma was associated with survival advantage. Low Ki67 expression was associated with favourable outcome in both oropharynx and oral cavity carcinoma. A more favourable outcome was associated with low Cyclin E expression in larynx carcinoma and with low p53 expression in the **squamous cell carcinoma (SCC)** from the oral cavity. Finally, a direct correlation between macrophage infiltration and tumor proliferation index was observed irrespective of the tumor subset.

*Conclusions.* Assessing proliferation, viral and immunologic profile may be crucial to finding more beneficial treatments for the different HNSCC subsets.

## Introduction

Head and neck squamous cell carcinoma of the head and neck district (HNSCC) is the eighth most common malignancy in the world, with approximately 620,000 patients diagnosed with cancer of the oral cavity, nasopharynx and larynx each year.<sup>1</sup> The disease is characterised by local tumour aggressiveness, early recurrence and a high frequency of second primary tumours.<sup>2</sup>

The majority of HNSCC are differentiated squamous cell carcinomas (SCC), which occur in the oral cavity, oropharynx, hypopharynx, and larynx. HNSCC are etiologically heterogeneous, with one subset attributable primarily to human papillomavirus (HPV) infection and another to alcohol and tobacco use. Most HPV-associated HNSCC tends to occur in the oropharynx, with the highest distribution in the tonsils. HPV16 is the predominant genotype in head and neck tumours, with different prevalences among the various head and neck sites. These subsets are clinically and molecularly distinct, and these distinctions extend to patient prognosis. As the role of HPV16 in HNSCC becomes better understood, these HPV16-related cancers are becoming increasingly recognized as a biologically distinct subgroup of HNSCC with a characteristic clinical profile. Indeed, the presence of HPV16 in HNSCC has been correlated with improved survival and may serve as a useful biomarker for prognosis.<sup>3-8</sup>

In contrast, the clinical behaviour of HPV-negative HNSCC is heterogeneous, and the most frequent molecular alteration carried by patients with HPV negative HNSCC is p53 mutation, that has been correlated with a poor response to radiotherapy and chemotherapy.<sup>9</sup> Thus, it is essential to investigate the molecular alterations in advanced HNSCCs that may help to identify patients who are at the greatest risk of progression.

The tumor suppressor protein, p53, is a central processing unit, receiving specific signals, interpreting combinatorial inputs, and determining regulatory output. Signalling to p53 occurs by physical interactions with protein complexes, which covalently modify specific amino acids of p53, mediate subcellular localization of p53, target p53 to specific chromatin sites, and/or dictate p53's cellular concentration.<sup>10</sup> These p53-regulated functions oppose tumor development and progression,

and their dysfunction in a tumor cell is generally caused by direct mutation of p53 or disruption of its signalling interactions.<sup>11</sup> In opposition to p53, which acts as a brake in the cell cycle, is Cyclin E, which is rate limiting for the G1/S-transition in the cell cycle of mammalian cells. Cyclin E binds to and activates CDK2, initiating the processes required for the G1-to-S-phase transition.<sup>12</sup> The ability to enter S-phase requires Cyclin E or cells will arrest at G1-phase, defining the important role of substrates, phosphorylated by Cyclin E/CDK2, in promoting DNA synthesis.<sup>13</sup> Many investigations point to the relevance of Cyclin E alteration in breast cancer. The *Cyclin E* gene is amplified in some breast cancer cell lines and it has been demonstrated that this amplification can result in several-fold overexpression of Cyclin E mRNA that is constitutively expressed across all phases of the cell cycle. Such constitutive overexpression and activation of Cyclin E results in the deregulation of cell cycle progression and chromosomal instability.<sup>11,14</sup> Genetic abnormalities of components of the cell cycle, such as p53 and p16/INK4A, are very common in most tumor types, including HNSCC, and altered expression of cyclins and CDKs has also been identified. However, information on Cyclin E expression in HNSCC has not been provided yet, hampering the possibility to exploit Cyclin E as a marker in association with tumor progression.<sup>15-17</sup>

Genetic and cell biology studies indicate that tumour growth is not just determined by accumulation of genetic alterations by malignant cancer cells, but also by tumour stroma. Numerous host cells, such as inflammatory cells, endothelial cells and fibroblasts, are recruited to and activated in the microenvironment of a developing tumour. Subsequent reciprocal interactions between these stromal cells, their mediators, and genetically altered “initiated” cells are indispensable for carcinogenesis. Infiltration of leukocytes into the neoplastic microenvironment is a common feature of many epithelial malignancies. The attracted monocytes differentiate into tumour-associated macrophages (TAMs) at the tumour site and are thought to produce various cytokines that promote tumour progression.<sup>18,19</sup> Consistent with this hypothesis, we have recently observed that the number of infiltrating macrophages was significantly associated with progression to malignancy.<sup>20</sup>

This study was undertaken to investigate the expression pattern of cell cycle regulators, such as p53 and Cyclin E, the proliferation activity by Ki-67 immunostaining, and HPV status in 59 advanced HNSCC in an effort to identify factors with potential clinical implications. The number of tumor-associated macrophages present in the tumors was also semi-quantitatively determined.

## **Materials and methods**

### ***Patients and samples collection***

Fifty-nine cases of primary squamous cell carcinomas (SCC) of the head and neck region diagnosed and treated at the Department of Otolaryngology and the Department of Clinical Oncology, San Giovanni Battista Hospital, Turin, between 1995 and 2004 were included in the study. The clinical and pathological staging and identification of anatomical sites of the lesions were based on the International Union Against Cancer TNM classification of malignant tumours (2003). All patients underwent surgery as a first approach. As shown in Table 1, primary tumours sites were: 22 SCC in the oropharynx, 25 in the oral cavity and 12 in the larynx. Six tumours were histologically well-differentiated, 27 were moderately differentiated, and 26 were poorly differentiated. Samples were grouped by TNM **classification** as follows: 10 in T1, 17 in T2, 14 in T3, 18 in T4; 14 in N0, 12 in N1, 24 in N2, 6 in N3, 3 in Nx and 57 in M0 and 2 in M1. The mean age of the patients was 59 years (range, 37-75), the male to female ratio was 48 to 11, and the follow-up interval ranged from 5 to 167 months.

### ***Immunohistochemistry***

Serial sections, 2- $\mu$ m thick, were cut from each selected block and de-waxed. Antigen unmasking was performed by microwaving in a conventional pressure cooker, where the slides were placed for 30 min in a 10 mM citrate buffer at pH 6.0. To abolish endogenous peroxidase activity, sections were immersed for 10 min in 3% hydrogen peroxide solution, buffered in PBS 1X at pH 7.3, and then incubated sequentially with Protein Blocking Agent (UltraTech HRP Streptavidin-Biotin Universal Detection System, Immunotech, Marseille, France) to reduce non-specific binding. Afterwards, the slides were incubated with the primary monoclonal antibodies (mAb) (mouse) for 1.5 hours at room temperature in a humid chamber. The biotinylated secondary antibody was applied followed by incubation with streptavidin-horseradish peroxidase complex (Immunotech, Marseille, France). As a negative control, a separate set of slides was incubated with PBS 1X instead of primary mouse mAbs. The immunologic reactions were developed at room

temperature with 3, 3'-diamino-benzidine tetrahydrochloride (**DAB**) solution (Roche, Mannheim, Germany), counterstained with Mayer's Haematoxylin, dehydrated and finally mounted with EUKITT (Bioptica, Milan, Italy). The following anti-sera were used: (1) Ki67 (clone MIB-1, working dilution 1:100), (2) CD68 (clone PG-M1, 1:50 dilution) provided by DAKO Cytomation, Denmark; (3) p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; clone DO-1; working dilution 1:5000); (4) Cyclin E (Santa Cruz Biotechnology, Santa Cruz, CA, USA; clone HE12, working dilution 1:200).

### ***Interpretation of immunohistochemical staining***

The intensity of immunohistochemical staining was evaluated in five areas of the slide sections. Positive cells were stained dark brown. Ki67, Cyclin E, and p53 expression was evaluated by determining the percentage of squamous cancer cells showing nuclear immunoreactivity, with cut-off values that differed depending on the type of antibody used. Inflammatory cells positive for CD68 immunostaining showed a cytoplasmatic reactivity within the tumour mass. Immunoreactivity was referred to as low or high. CD68 staining was detected as a cytoplasmatic immunoreactivity in inflammatory cells within the tumour mass.

### ***HPV testing***

The presence and typing of HPV DNA was assayed by highly sensitive polymerase chain reaction (PCR) and sequencing. DNA was extracted from 15 µm paraffin-embedded tissue sections using a commercial extraction kit (NucleoSpin Tissue, Machery-Nagel, Germany), according to the manufacturer's specified protocol. DNA integrity was confirmed by amplification of the *β-globin* gene. To increase the sensitivity of HPV detection, nested PCR assays were performed using MY09/MY11 as the outer, and GP5+/GP6+ as the inner primers.<sup>21</sup> A 50 µL reaction mixture consisted of 1 µM of each primer, 300 ng of the extracted sample, 1X Taq Buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1 mM MgCl<sub>2</sub> and 0.1% gelatine), 200 µM of each of the four dNTPs and 1 unit of Taq DNA Polymerase (Sigma, St. Louis, MO, USA). The PCR products were verified by direct sequencing with the GP5+/GP6+ primers on a DNA sequencer (PRIMM, Milan, Italy). HPV

type was determined on the basis of >90% homology with HPV sequences deposited in GenBank **using the BLAST network service at NCBI (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>).**

The negative controls were samples with water replacing target DNA in the reaction mixture. The positive control was DNA from cervical cancer cells positive for high-risk HPV (Casky). Standard precautions concerning spatial separation of pre- and post-PCR steps, aliquoting of reagents, and single use of scalpels for processing tissue specimens were strictly followed.

### ***Statistical analysis***

A one-way ANOVA with Bonferroni post-test was used for determining the statistical significance of differences between the disease groups for Ki67, p53, Cyclin E, CD 68 expression. Correlations were obtained using the Pearson test when it could be assumed that data were sampled from Gaussian populations, and the Spearman test for non-parametric correlations. All tests were two-tailed and a probability value  $p < 0.05$  was considered statistically significant.

We estimated survival curves using the Kaplan-Meier method, and compared them using a two-sided log-rank test. Analyses were carried out with GraphPad Prism 4.03 software (**GraphPad software, San Diego, CA, USA; [www.graphpad.com](http://www.graphpad.com)**).

## Results

### *HPV DNA detection*

**The overall frequency of HPV infection was 50% in the oropharynx SCC, 36% in the oral cavity SCC, and 58.3% in the larynx SCC. These percentages, although slightly higher, are still in the range reported by other investigators<sup>4,22,23</sup>. HPV type was identified as HPV16 in all tumors, with the sole exception of two laryngeal carcinomas that were positive for HPV6. HPV infection did not correlate with tumour differentiation, node status or with the other clinical features evaluated (Table 1, Table 2).**

### *Immunohistochemical studies*

The expression of Ki67, Cyclin E, and p53 as proliferation and cell cycle markers, and of CD68 as macrophage infiltration marker, were examined by immunohistochemistry in a series of clinical samples of HNSCC. The results of the immunohistochemical assays are summarized in Table 2 and illustrated in Figure 1. In normal epithelia, Ki67-positive cells were mostly located in the basal cell layer, whereas in the tumour mass they were distributed in all levels of the epithelium. Remarkably, in low-grade squamous cell carcinomas, positive Ki67 staining was detected only in cells at the periphery of the tumor cell nests (Figure 1, panel A), such that the central keratinizing areas and adjacent tumor cells were negative. Conversely, the staining was more diffuse in high-grade carcinomas (Figure 1, panel B). Overall, the proliferative activity, as defined by Ki67 immunostaining, was significantly related to tumor differentiation and histological diagnosis. Three of 22 (13.7%) oropharyngeal SCC, 1 of 25 (4%) oral SCC, and 3 of 12 (25%) laryngeal SCC were considered negative. For Ki67 immunostaining, a significant association with a particular anatomical site was not observed ( $p=0.1716$ ) (Table 2).

A different immunophenotype pattern was observed with the other markers employed. Cyclin E immunostaining was considered positive when Cyclin E expression was equal or greater than 20% in all three anatomical sites. According to this cut-off value, 14 of 22 samples (63.6%) in the oropharynx, 17 of 25 (68%) in the oral cavity, and six of 12 in the larynx (50%) were above the

cut-off of 20% and turned out to be positive. Thus, no significant correlation between Cyclin E expression and a particular anatomical site was found ( $p=0.5666$ ) (Table 2).

p53 immunostaining was considered positive when its expression was 15% or more in all anatomical sites (Figure 1, panel E and F). Based on this cut-off value, 14 of 22 (63.6%) oropharyngeal SCC and 16 of 25 (64%) oral SCC samples were positive. In contrast, 10 of 12 (83.3%) larynx SCC samples were positive for p53 immunostaining. No significant correlation between p53 expression and a particular anatomical site was found ( $p=0.4347$ ) (Table 2).

Primary tumor macrophage content has been demonstrated to be a strong predictor of tumor aggressiveness in HNSCC. To assess if macrophage infiltration could affect clinical outcome, we examined the expression of the macrophage marker CD68. All sample tissues studied contained cells positive for the macrophage marker CD68. Tumour Associated Macrophages (TAMs) were predominantly located in the tumour stroma, although they were also observed between tumour cells. TAMs were distributed heterogeneously in the tumour tissues and were often numerous in advanced stage carcinomas. Stained macrophages were also observed in normal tissues surrounding the tumour mass, only in the stroma, and their number was lower. The CD68 immunophenotype results were classified as high in 13 out of 22 (59.1%) and in 16 out of 25 (64%) tumors derived from the oropharynx and oral cavity, respectively. In contrast, only four out of 12 (33.3%) larynx SCC were classified as high, indicating an inverse correlation between macrophage infiltration and this particular anatomical site. However, this association was not significant ( $p=0.1983$ ) (Table 2).

Next, potential associations between the presence/absence of HPV and expression of cell cycle markers were examined. Among the 59 tumors, there was a strong association between HPV presence and reduced Ki67 expression ( $p=0.0026$ ) (Table 3). All tumors that were HPV negative were more likely to have up-regulated Ki67 (32 out of 32, 100%), while of the 27 HPV positive tumors, 20 tumors had up-regulated Ki67 (74%) and seven had down regulated Ki67 (26%) expression. In contrast, there was no association between the presence/absence of HPV and Cyclin E and p53 expression, away from the tumor site.

### *Statistical Analysis*

Patients with oropharyngeal carcinomas testing HPV16-positive had a better clinical outcome than those testing HPV-negative. The Kaplan-Meier analysis showed that patients with HPV16-positive tumors had significantly improved overall survival compared to those with HPV-negative tumors ( $p=0.0211$ ) (**Figure 2**). In contrast, in carcinomas of the oral cavity and larynx, HPV-positivity seemed to be a negative prognostic factor, although the significance of the Kaplan-Meier analysis was low ( $p=0.2802$  and  $p=0.5439$ , respectively) (**data not shown**).

Ki67 expression in carcinomas derived from the oropharynx tended to be inversely correlated with prognosis, i.e. patients with cancer expressing Ki67 at a level  $<25\%$  were found to have a significantly better clinical outcome than those with Ki67 at expression levels  $\geq 25\%$  ( $p=0.0188$ ) (Figure 3, panel A). A very favourable outcome was observed in the HPV-positive oropharynx subset in association with a Ki67 immunostaining score below the cut-off value of 25%. The survival benefit observed in these patients, amounting to a 2.48 relative mortality reduction at five years, occurred irrespective of tumor stage. In contrast, all HPV-negative patients displayed an immunostaining score above 25% with a survival curve that did not overcome five years. Similarly, an immunostaining score below 25% in SCC from oral cavity showed a strong correlation with a better follow-up ( $p=0.0240$ ) (Figure 3, panel B). In contrast, no association between Ki67-positivity below 25% and a survival benefit in patients with carcinoma arising in the larynx was found ( $p=0.2467$ ) (**data not shown**).

A similar investigation of Cyclin E expression yielded different results. HPV-positive SCC turned out to have a similar level of Cyclin E expression when compared to HPV-negative SCC ( $p=0.4182$ ) (Table 3), irrespective of tumor site. Low Cyclin E expression ( $<20\%$ ) was not associated with increased overall patient survival in either the oropharynx or the oral cavity ( $p=0.5308$  and  $p=0.5594$ , respectively) (**data not shown**). In contrast, Cyclin E expression below 20% in SCC from the larynx showed a strong association with a more favourable outcome (five-year survival,  $p=0.0046$ ) (**Figure 3, panel C**).

p53 nuclear staining was rare in normal epithelium, where its expression was generally restricted to isolated basal and parabasal epithelial cells. In testing the prognostic role of p53 status, significant results were obtained when SCC from oral cavity were investigated. Kaplan-Meier analysis showed a median survival time of 57.5 months for p53 expression below the cut-off point of 15%, versus a median survival time of 16 months for p53 above 15%. The difference was statistically significant ( $p=0.0236$ , **Figure 3, panel D**). No such correlation of p53 expression was demonstrated with oropharynx- and larynx-derived tumours ( $p=0.2600$  and  $p=0.8673$ , respectively) **(data not shown)**.

Finally, when a comparative analysis of the expression rate for the above cell proliferation markers was attempted, an inverse correlation between p53 and cyclin E expression was found ( $p=0.0421$ ,  $r=-0.2655$ ) (**Figure 4, panel A**), independent of the tumor site. Moreover, in accord with previous results, when a correlation between macrophage infiltration as measured by CD68 staining and tumor proliferation index as measured by Ki67 staining was evaluated, a direct association between the two markers' expression was observed. The median macrophage counts were significantly higher in those tumors expressing Ki67 above the cut-off point ( $>25\%$ ) independently of the anatomical site, suggesting that the number of infiltrating macrophages was significantly associated with progression to malignancy (**Figure 4, panel B**).

## Discussion

HNSCC, including tumors derived from oropharynx, larynx, and oral cavity are etiologically heterogeneous with one subset derived from the oropharynx attributable primarily to HPV infection and another to alcohol and tobacco abuse. All subsets are clinically and molecularly distinct, and these distinctions extend to patient prognosis, making it difficult to assess their malignancy and predict the outcome of treatment. Molecular markers defining certain genotypes and phenotypes, and representing tumor subgroups with more homogenous behaviour must thus be found for the different HNSCC types.

In this study, we have attempted to identify different molecular markers, including HPV DNA, to be used as prognostic factors for each tumor subgroup. Consistent with results reported by other investigators, our studies demonstrate that HPV-positive oropharyngeal cancers comprise a distinct molecular and pathologic disease entity that is causally associated with HPV infection and has a markedly improved prognosis. We examined the prevalence of HPV in SCC of the oropharynx, larynx and oral cavity. In addition, the clinical outcome of the patients was reviewed and correlated with the presence of HPV, the proliferation index of tumor cells (Ki67 and Cyclin E), and p53 expression rate.

The Kaplan-Meier analysis showed that patients with HPV-positive oropharyngeal carcinomas had a better overall survival than those with HPV-negative carcinomas. Such an association has not been found in patients harbouring SCC from both the oral cavity and larynx. The mechanisms underlying the better clinical outcome of HPV-positive oropharynx SCC remain unexplained. A few studies have concluded that the reason for a better prognosis could be explained by an enhanced radiosensitivity of these HPV-positive SCC in comparison to HPV-negative tumors.<sup>1,24,25</sup> It was suggested that the interaction between E6 and p53 does not result in its full functional abrogation as compared with mutated TP53 showing p53 overexpression. This would justify an increased radiosensitivity sustained by a functioning p53 protein.<sup>26</sup> Whatever the explanation, HPV-positive oropharyngeal SCC may comprise a distinct molecular and pathologic

disease entity that is causally associated with HPV infection, and has a markedly improved prognosis.

A different pattern in association with the prognostic markers employed emerged when HNSCC from different anatomical sites were analyzed. First, the presence of HPV16 in oral cavity and larynx SCC did not correlate with a better clinical outcome. Second, low p53 expression in the oral cavity was found to be a predictive factor for good prognosis. At 96 months, 62% of patients with p53 expression <15% were still alive, whereas none of the patients with p53 expression >15% were alive at 24 months (p=0.0236).

A high proliferation rate has been correlated with aggressive behaviour of tumors from various anatomical sites. In HNSCC, several data suggest that cell proliferation indices are reliable and reproducible indicators of tumor aggressiveness.<sup>27</sup> Consistent with previous findings, we observed that SCC from the oropharynx and oral cavity with lower proliferation index displayed a better overall survival (p=0.0188 and p=0.0240 respectively). This correlation was not found for larynx SCC (p=0.2467).

Although elevated cell proliferation activity is one of the most remarkable features of HNSCC, abnormalities in cell cycle regulation have yet to be thoroughly investigated in this type of tumor. HNSCC tissue microarray analysis demonstrated multiple alterations at various checkpoints of cell cycle progression with a median overall survival and time-to-progression longer in patients with cyclin A-expressing tumors. Moreover, positive expression of cyclin E in tumors was also associated with an increased median time-to-progression.<sup>15</sup> However, no correlation of these expression markers with a particular tumor location was observed. In the present study, no significant correlation between cyclin E expression and a particular anatomical site was found irrespectively from the presence of HPV. However, low cyclin E expression levels (<20%) in SCC from the larynx showed a strong association with a more favourable outcome (five-year survival, p=0.0046), suggesting that cyclin E is a good predictor of patient response in this particular anatomical site. The *cyclin E* gene has been found to be amplified and the cyclin E protein

constitutively expressed in several breast cancer cell lines.<sup>28,29</sup> Consistent with our conclusion, elevated levels of cell cycle protein Cyclin E assessed by immunohistochemistry analysis have been associated with a poor prognosis following breast cancer and malignant ovarian germ cell tumors.<sup>30,31</sup> Moreover, high levels of Cyclin E as measured by western blotting were the strongest independent factor in predicting survival following breast cancer.<sup>32</sup>

Infiltration of leukocytes into the neoplastic microenvironment is a common feature of many epithelial malignancies. Extensive analysis of human tumor samples has revealed that abundance of innate immune cells, in particular macrophages, correlates with angiogenesis and poor prognosis. In line with these findings, when a correlation between macrophage infiltration as measured by CD68 staining and tumor proliferation index as measured by Ki67 staining was evaluated, a direct association between the two markers' expression was observed, suggesting that the number of infiltrating macrophages in HNSCC was significantly associated with progression to malignancy.

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Table 1. Characteristics of the **head and neck squamous cell carcinoma (HNSCC)** patients included in the study

<b>CHARACTERISTICS</b>	<b>OROPHARYNX</b>	<b>ORAL CAVITY</b>	<b>LARYNX</b>
	<b>No. (%)<sup>†</sup></b>	<b>No. (%)<sup>†</sup></b>	<b>No. (%)<sup>†</sup></b>
<b>Number of patients</b>	22	25	12
<b>Median age</b>	59 years	59 years	58 years
<b>Male/female</b>	20/2	16/9	12/0
<b>T classification</b>			
T1	6 (27.2)	3 (12)	1 (8.3)
T2	10 (45.5)	4 (16)	3 (25)
T3	2 (9.1)	7 (28)	5 (41.7)
T4	4 (18.2)	11 (44)	3 (25)
<b>N classification</b>			
N0	5 (22.7)	6 (24)	3 (25)
N1	6 (27.3)	3 (12)	3 (25)
N2	7 (31.8)	12 (48)	5 (41.7)
N3	2 (9.1)	3 (12)	1 (8.3)
Nx	2 (9.1)	1 (4)	0
<b>M classification</b>			
M0	22 (100)	25 (100)	10 (83.3)
M1	0	0	2 (16.7)
<b>Histological grade</b>			
well differentiated	1 (4.5)	4 (16)	1 (8.3)
moderately differentiated	8 (36.4)	12 (48)	7 (58.3)
poorly differentiated	13 (59.1)	9 (36)	4 (33.3)

<sup>†</sup> Number of patients (percentage of patients) from each anatomic site

Table 2. Evaluation of the biological parameters referring to tumour anatomic sites

		PRIMARY TUMOUR SITES			
		Oropharynx	Oral cavity	Larynx	
		No. (%) <sup>†</sup>	No. (%) <sup>†</sup>	No. (%) <sup>†</sup>	
<b>Number of patients</b>		22	25	12	
<b>HPV infection</b>		11 (50)	9 (36)	7 (58.3)	
<b>Ki67</b>	Low (%)	3 (13.7)	1 (4)	3 (25)	p= 0.1716
Cut-off 25%	High (%)	19 (86.3)	24 (96)	9 (75)	
<b>Cyclin E</b>	Low (%)	8 (36.4)	8 (32)	6 (50)	p=0.5666
Cut-off 20%	High (%)	14 (63.6)	17 (68)	6 (50)	
<b>p53</b>	Low (%)	8 (36.4)	9 (36)	2 (16.7)	P=0.4347
Cut-off 15%	High (%)	14 (63.6)	16 (64)	10 (83.3)	
<b>CD68</b>	Low (%)	9 (40.9)	9 (36)	8 (66.7)	p=0.1983
	High (%)	13 (59.1)	16 (64)	4 (33.3)	

<sup>†</sup> Number of patients (percentage of patients) from each anatomic site

Table 3. Histological markers in **head and neck squamous cell carcinoma (HNSCC) patients** grouped by HPV status

	<b>HPV - No. (%)<sup>†</sup></b>	<b>HPV + No. (%)<sup>†</sup></b>	<b>Fisher's exact test</b>
<b>Ki67</b>			
<25%	0	7 (11.9)	
≥25%	32 (54.2)	20 (33.9)	p=0.0026
<b>Cyclin E</b>			
<20%	10 (17)	12 (20.3)	
≥20%	22 (37.3)	15 (25.4)	p=0.4182
<b>p53</b>			
<15%	11 (18.6)	7 (11.9)	
≥15%	21 (35.6)	20 (33.9)	p=0.5758

<sup>†</sup> Number of patients (percentage of patients)

## Legends to figures

**Figure 1.** Immunohistochemical expression of Ki67, Cyclin E, p53 and CD68 in low expressing (left panels) or high expressing (right panels) **head and neck squamous cell carcinoma (HNSCC)**. Immunohistochemical staining shows positive cells in brown, counterstained with haematoxylin (**magnification 200X**).

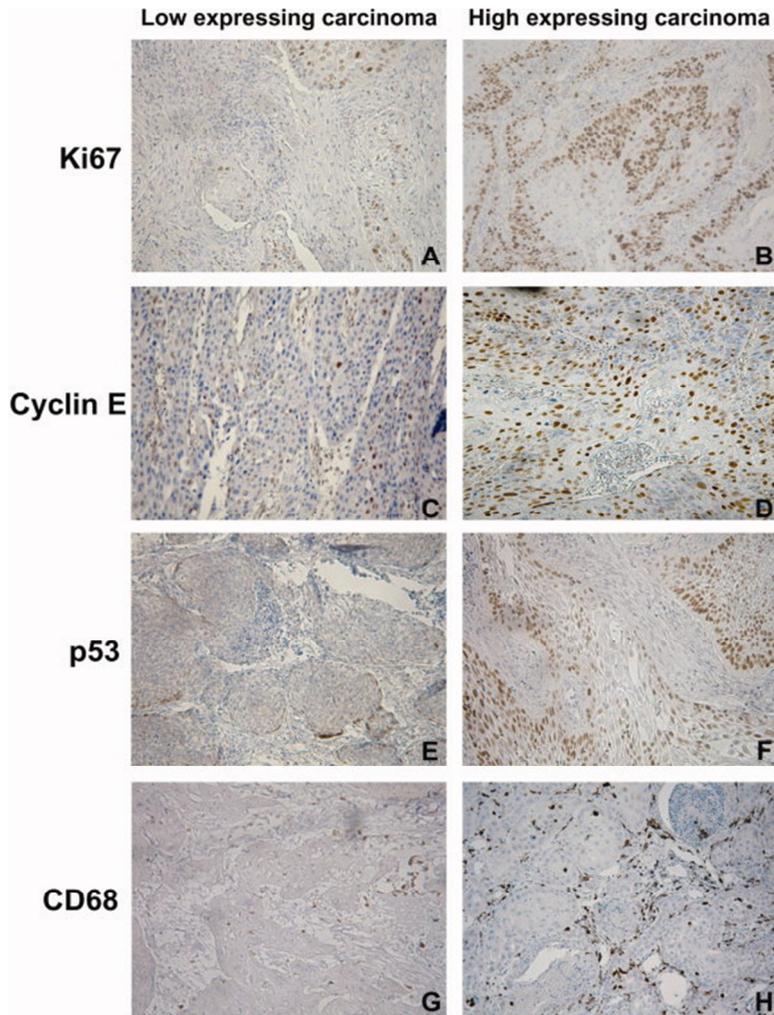
**Figure 2.** Cumulative prognostic value of **human papillomavirus (HPV)** positivity for patients with squamous cell carcinoma (**SCC**) of the oropharynx (**A**), oral cavity (**B**) and larynx (**C**), expressed as probability of overall survival.

**Figure 3.** Cumulative prognostic value of the indicated markers for patients affected by squamous cell carcinoma (**SCC**) of the oropharynx (**A**), oral cavity (**B, D**) and larynx (**C**), expressed as probability of overall survival. Only significant survival curves are shown, as other biomarkers had no statistically-detectable impact on survival.

**Figure 4.** **A)** Correlation between p53 and Cyclin E expression in **head and neck squamous cell carcinoma (HNSCC)** ( $p=0.0421$ ,  $r=-0.2655$ ). The line represents the calculated regression line. **B)** Correlation of immunohistochemical expression of CD68 with Ki67. The levels of CD68 expression showed positive correlation with Ki67 immunostaining ( $p=0.0128$ ).

# Figures

## Figure 1



## Figure 2

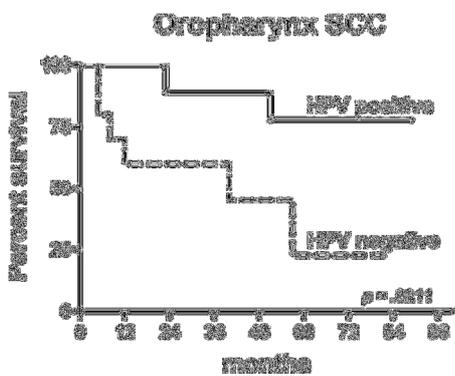


Figure 3

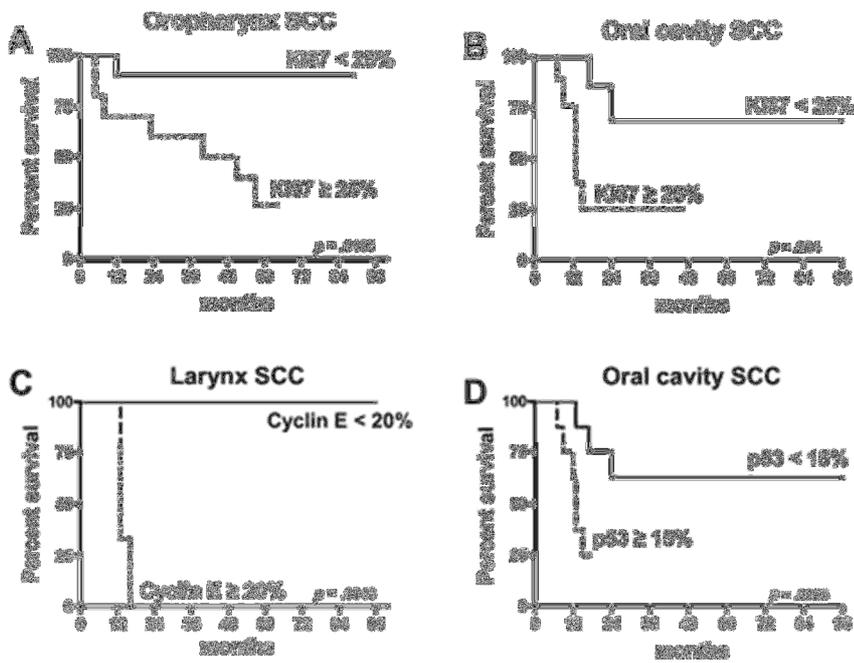


Figure 4

