CD8-alpha T-cell infiltration in human papillomavirus-related oropharyngeal carcinoma correlates with improved patient prognosis

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Patients with human papillomavirus (HPV)-related oropharyngeal tumors display improved prognosis. The biological basis of this tumor phenotype is poorly understood. We investigated whether increased lymphocyte infiltrate in HPV-positive oropharyngeal squamous cell carcinomas could account for better prognosis. We previously identified, in an Affymetrix GeneChip analysis of 83 HPV-unrelated and 11 HPV-related squamous cell carcinoma of the oropharynx, several candidate genes, including CD8α and CD3ζ. Their expression was validated in this study by qRT–PCR on an independent clinical series of 144 oropharyngeal tumors. Immunohistochemical staining of tumor specimens was performed to evaluate infiltration of tumor stroma by CD8α+ and CD4+ lymphocytes. The prognostic value of CD8α and CD3ζ expression levels was measured by Kaplan–Meier and Cox regression model analyses. Immune response-related signaling pathways were found to be deregulated in HPV-positive oropharyngeal tumors. Expression of CD8α, CD3ζ, granzyme K, CD28 and integrin αL RNAs was upregulated in HPV-positive lesions when compared with HPV-unrelated tumors (p < 0.05). Stroma of HPV-positive tumors was frequently and strongly infiltrated by CD8α- and CD3ζ-positive T cells. CD8α RNA expression correlated with both improved global (Kaplan–Meier; p = 0.005; Cox regression: p = 0.003) and disease-free (Cox regression: p = 0.04) survival. CD3ζ RNA expression correlated with improved overall survival (Cox regression: p = 0.024). These results suggest that an increased cytotoxic T-cell-based antitumor immune response is involved in improved prognosis of patients with HPV-positive tumors.

The presence of human papillomaviruses (HPVs; mainly HPV type 16) correlates with the onset and development of about 25% of head and neck squamous cell carcinomas (HNSCC).1 HPV-positive cancers of the head and neck arise mainly in the oropharynx and define a subgroup of radio- and chemosensitive tumors with distinct clinical, pathological and molecular features and improved prognosis with respect to their HPV-negative counterparts.2–5 The majority of HPV-related oropharyngeal squamous cell carcinomas (OSCC) express wild-type TP53,6,7 which might be involved in the improved response of tumors to treatment. Despite thorough analysis of gene expression profiles of HPV-positive OSCC by several groups,8–11 no novel prognostic or predictive biomarker has been identified, and the molecular mechanisms that underlie improved prognosis of HPV-related OSCC are poorly understood.

The immune response has an important impact in many HPV-associated tumors and can have either favorable or unfavorable consequences.12 The importance of the immune response in HPV-related OSCC is less well established. An increased T-cell response against HPV E7 epitopes has been detected in the blood of patients with HPV-positive HNSCC.13–15 A more recent study has shown that T cells that proliferate and synthesize inflammatory cytokines upon HPV16 E6 and E7 oncoprotein recognition can be isolated from OSCC biopsies or tumor-draining lymph nodes.16 Lymphocyte infiltration and expression of lymphocyte markers

Key words: human papillomavirus, oropharyngeal cancer, lymphocyte infiltration, CD8 T-cell, prognosis

Additional Supporting Information may be found in the online version of this article.

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have been correlated with improved prognosis of patients with HNSCC. However, the exact role of this immune response at the tumor site has not been established. Important open questions are whether the amount and phenotype of tumor infiltrating lymphocytes (TILs) in the microenvironment of human OSCC differ between HPV-negative and HPV-positive lesions, and whether these differences have an impact on prognosis. We have addressed these issues in this study.

We found in an analysis of the transcriptome of OSCC that HPV-positive lesions display a distinct gene expression profile, including immune response genes, in agreement with data from other groups. We now report a more detailed qRT-PCR-based analysis of the expression of these immune-related genes and have identified markers that are overexpressed in HPV-positive OSCC. Using immunohistochemical staining, we observed abundant infiltration of CD8+ T cells in the stromal microenvironment of HPV-related lesions. High expression of CD8α and CD3ε was found to correlate with improved prognosis.

Material and Methods

Patients
All tumor specimens were collected, stored and used with the patients’ informed consent. Patients included in our previous Affymetrix GeneChip analysis were surgically treated for primary head and neck tumors followed by adjuvant radiation therapy. In this present study, the expression levels of genes of interest were evaluated by qRT-PCR on a series of 144 patients. These patients underwent initial surgical resection of their OSCC between 1988 and 2006, followed by postoperative radiotherapy (102 of 144 cases; 71%) or by postoperative chemoradiotherapy (42 of 144 cases; 29%). Fourteen tumors included in this series (3 of 24 HPV-positive tumors: 11 of 140 HPV-negative tumors) were also included in the previous cohort used for the Affymetrix GeneChip Analysis. Treatment was completed in all cases. Hematoxylin-eosin slides of paraffin-embedded tumor specimens were examined by two pathologists. All the tumors were squamous cell carcinomas. The median age of the patients was 53 years (40–82 years). The inclusion criteria were tumor localization (oropharynx), any size (Tx), any lymph node status (Nx) and no clinically evident distant metastases (M0) by conventional clinical and diagnostic radiological examinations (computed tomography). Patients did not have any previous or synchronous neoplasia. The median follow-up period was 53 months (4–236 months).

Tumor samples
Tumor samples were collected at the time of surgery. A fragment was taken near the advancing edge of the primary tumor (avoiding its necrotic center), immediately frozen in liquid nitrogen and stored at –70°C. The rest of the tumor was fixed in 6% buffered formalin and embedded in paraffin for histopathological analysis. Examination of sections adjacent to each tumor fragment showed that the percentage of tumor cells was over 70%. The TNM system of the UICC was used for tumor-node-metastasis staging.

Affymetrix GeneChip data mining—Identification of biological pathways
We used the Affymetrix data generated in our previous study. Four methods were used to compare gene sets and sample groups: GSA 20: R package GSA; globaltest 21: R package globaltest; SAM–GS 22: original R code; and the Tuckey algorithm described in Table 4 of Ref. 23: original R code). Each method yielded a p-value: the lower the p-value, the more the genes in this gene set are differentially expressed between the sample groups. To aggregate the results of the four methods, we first ranked the gene sets (pathways/Gene Ontology (GO) terms/...) for each method (according to p-values), and we then calculated for each gene set the mean rank across the four methods. This mean rank was used as a summarized score. KEGG and Biocarta pathways (and related genes) were obtained, respectively, from ftp://ftp.genome.ad.jp/pub/kegg/paths/hsa and http://www.biocarta.com. GO terms and related proteins were obtained from http://www.geneontology.org. We mapped the biological pathway-related genes or GO term-related proteins to non-redundant HUGO gene symbols. For each GO term, we obtained the list of nonredundant-related proteins identifiers, either directly associated with the GO term or to one of its descendants.

Genomic DNA and RNA extraction and gene expression assays (real-time quantitative reverse transcription-PCR)
Genomic DNA and total RNA were extracted from frozen tissues using DNA/RNA allprep minikits (Qiagen, France), according to the manufacturer’s instructions. The integrity of extracted RNA was verified on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). qRT–PCR were performed as described in Ref. 8. The primer pairs were CD4 (5’-AGGAAGTGAAACCTTGTTGTTG-3’ and 5’-CTCAGCA GACACTGCCACAT-3’), CD8α (5’-AGCTACCCGAGAGT
Tumor Immunology

HPV16 E6/E7 transcripts were analyzed in all the samples by genus, divided into three groups based on their association to cancer: 15 high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82), 3 putative high-risk (26, 53 and 66) and 6 low-risk (6, 11, 42, 43, 44 and 70). The consensus primers BSGP5 and 6 were hybridized to 24 HPV type-specific, 1 tentative scoring. At least 10 fields at high magnification (400×) were assessed per tumor sample. In our scoring system, one unit of positivity was considered to be statistically significant. CD8+ T-cell infiltration in the tumor microenvironment was evaluated by semiquantitative scoring. At least 10 fields at high magnification (400×) were assessed per tumor sample. In our scoring system, one unit of positivity was considered to be statistically significant.

Statistical and survival analysis

Statistical and survival analyses were performed using MedCalc statistical software (http://www.medcalc.be/). The relationship of HPV status with other clinical and pathological features was compared using Fisher’s exact test. h-Values <0.05 were considered to be statistically significant. Gene expression levels were compared by drawing Box-and-Whisker plots. Differences between subgroups were evaluated using one-way ANOVA and pairwise comparison with a Student–Newman–Keuls test. Differences were considered to be significant if p < 0.05. Cut-offs for CD8α and CD3ζ were the qRT–PCR values for 90% specificity, that is, 90% of the HPV-negative samples had a qRT–PCR value below the cut-off. Samples were considered positive if the qRT–PCR value was greater than or equal to cut-off. The statistical evaluation of the proportion of CD8α-negative and CD8α-positive, and CD3ζ-negative and CD3ζ-positive samples among HPV-negative and HPV-positive patients was performed with a Fisher’s exact test. CD8+ T-cell infiltration in the tumor microenvironment was evaluated by semiquantitative scoring. At least 10 fields at high magnification (400×) were assessed per tumor sample. In our scoring system, one unit of positivity was considered to be statistically significant.
were considered significant when $p < 0.05$. Statistical comparison of the number of positively stained CD8α-positive cells between CD8α-positive and CD8α-negative (qRT-PCR score; see earlier) was performed with the Mann–Whitney $U$ test, and differences were considered significant when $p < 0.05$.

Disease-free and overall survival comparing negative and positive patients were estimated using Kaplan–Meier curves. Survival time was defined as the interval between the date of surgery and the date of local, locoregional or distant relapse (disease-free survival; uncensored), the date of death for any reason (overall survival; uncensored) or the last date when the patient was known to be disease-free or alive (censored). A log rank test was used to evaluate the differences between subgroups and considered as statistically significant when $p < 0.05$ in two-sided tests. Local and locoregional relapse were defined as persistent or recurrent disease at the primary site or in the cervical lymph nodes, respectively. Distant relapse was defined as disease progression to distant sites (lungs, liver, bones). For overall survival multivariate analysis, the prognostic power of the cut-off while controlling confounders was evaluated using a Cox regression analysis. Hazard ratios with 95% confidence intervals for categorical variables were calculated by entering variables in a stepwise procedure with a $p$ value of 0.1 for removal ($p$-values were derived from a Wald test).

### Results

**Genes involved in cell-based immunity are more highly expressed in HPV-related oropharyngeal tumors**

We have used previously performed Affymetrix GeneChip to analyze gene expression of HNSCC that was HPV-unrelated (83 cases), or HPV-related in which HPV16 E6/E7 transcripts were detected (11 cases). Using Welch $t$-tests, we identified 3,104 probe sets, corresponding to 2,155 unique HUGO gene symbols that were significantly upregulated. Eight hundred and fifty of these genes were highly expressed in HPV-positive when compared with HPV-unrelated tumors.

To gain more insight into the molecular bases of the HPV-dependent tumor phenotype, we searched for signaling pathways and biological processes that are deregulated in HPV-positive OSCC samples. Four methods of pathway enrichment analysis (see “Methods” section) were performed to analyze the GO, KEGG (Kyoto Encyclopedia of Genes and Genomes) and BioCarta databases. Among the pathways that were recovered, most were related to the immune response.

### Table 1. Pathways that are deregulated in HPV-positive as compared to HPV-negative tumors

| Pathway                                                                 | Globaltest $p$-value | GS size | Mean of ranks |
|------------------------------------------------------------------------|----------------------|---------|---------------|
| GO:0000795—synaptonemal complex                                        | 0                    | 21      | 15.2787971    |
| GO:0030217—T-cell differentiation                                       | 3.34E-08             | 125     | 72.9048898    |
| GO:0016538—cyclin-dependent protein kinase regulator activity           | 4.16E-10             | 18      | 73.8513315    |
| GO:0032943—mononuclear cell proliferation                               | 2.53E-08             | 148     | 73.9232793    |
| GO:0046651—lymphocyte proliferation                                     | 2.50E-08             | 146     | 75.221814     |
| GO:0042110—T-cell activation                                            | 1.07E-07             | 266     | 75.392298     |
| GO:0051250—negative regulation of lymphocyte activation                 | 1.17E-09             | 68      | 80.4984472    |
| GO:0045954—positive regulation of natural killer cell mediated cytotoxicity | 3.71E-08             | 16      | 82.0390965    |
| GO:0030097—hemopoiesis                                                 | 4.53E-08             | 379     | 82.1076559    |
| GO:0033077—T-cell differentiation in thymus                             | 3.91E-10             | 52      | 82.8552846    |
| GO:0046649—lymphocyte activation                                        | 2.52E-07             | 239     | 83.3943366    |
| GO:0030098—lymphocyte differentiation                                  | 1.01E-07             | 172     | 84.6458181    |
| GO:0051249—regulation of lymphocyte activation                          | 1.58E-07             | 244     | 86.9611256    |
| GO:0050863—regulation of T-cell activation                              | 1.23E-07             | 190     | 87.784773     |
| GO:0050852—T-cell receptor signaling pathway                            | 1.35E-07             | 82      | 87.8362499    |
| GO:0045321—leukocyte activation                                        | 2.67E-07             | 437     | 88.1441211    |
| GO:0002520—immune system development                                   | 6.70E-08             | 428     | 90.2663013    |
| GO:0030888—regulation of B cell proliferation                          | 2.23E-09             | 45      | 91.1706641    |
| GO:0045580—regulation of T-cell differentiation                         | 4.41E-08             | 68      | 92.8785222    |
| GO:0007140—male meiosis                                                | 1.17E-09             | 20      | 92.9677569    |

A hypergeometric test was performed on GeneChip data. Pathways (GO terms), globaltest $p$-values, gene set (GS) size and mean of ranks are shown. Pathways are ranked according to the mean of ranks. Only pathways with GS size > 10 were retained. Signaling pathways that related to the immune response are shown in bold.
Information Table 1) encode factors known to be (1) inflammatory chemokines (CXCL9; CXCL10; CXCL11) and their receptor (CXCR3) that are involved in T-cell recruitment and migration to the site of inflammation, (2) required for priming the interaction between antigen-presenting cells (APCs) and naive lymphocytes [CD2, integrin subunit αL (ITGAL) and integrin subunit β2 (ITGB2)], (3) members of the major histocompatibility complex I (HLA-C), (4) subunits of the T-cell receptor (TCR/CD3 complex (CD3δ, CD3ε, CD3ζ), (5) involved in TCR/CD3 costimulation (CD28, CD86), (6) involved downstream of the TCR/CD3 activation pathway [ZAP70, LCK, LCP2, granzyme K (GZMK), T-cell-restricted intracellular antigen-1 (TIA1)] and (7) CD4+ and CD8+ T cell-specific markers (CD4 and CD8α, respectively).

To validate our data, we assessed the expression of genes that encode factors involved in APC/T-cell interactions (ITGAL; ITGB2; CD28; CD86), members of the MHC I (HLA-C), markers of CD4+ and CD8+ T cells (CD4 and CD8α, respectively), members of the TCR (CD3ζ) and products synthesized by functional CD8+ T cells (GZMK). These analyses were performed on RNA samples from a consecutive series of 144 OSCC. The clinical and pathological features of these patients are presented in Table 2. Only HPV DNA- and RNA-positive samples were considered to be HPV-positive, as in our previously published work. Consistently with what is known in the literature, a Kaplan–Meier analysis of overall survival at 5 years showed that HPV is a marker for improved prognosis (p = 0.012; Supporting Information Fig. 2).

To determine whether immunity-related genes were altered in HPV-positive patients, we performed one-way ANOVA statistical analysis of variance. The expression of ITGAL (p = 0.003), CD28 (p = 0.016), CD3ζ (p = 0.001), CD8α (p < 0.001), GZMK (p < 0.001) and CD4 (p = 0.032) was significantly upregulated in HPV-positive samples (Fig. 1).

The microenvironment of HPV-related OSCC tumors displays increased CD8+ T-cell infiltration

To detect T cells in the tumor stroma, we immunostained formalin-fixed paraffin-embedded tumor specimens from 10 HPV-related and 7 HPV-negative patients of our cohort, with antibodies raised against CD8α, CD3ζ, CD4 and CD3. Sections were counterstained with hematoxylin and eosin to visualize tumors and stroma. Tissue samples with high levels of infiltrating lymphocytes were selected for analysis. Representative samples are shown in Figure 2a. In HPV-negative samples (top panels), CD8α and CD3ζ stainings were observed at the level of the plasma membrane of some TILs in the stroma surrounding carcinoma cells. However, both the intensity of staining and the number of positive cells were strongly increased in HPV-related samples. CD4-positive and, to a lesser extent, CD3-positive staining, were observed in the stroma of HPV-positive lesions (Fig. 2a; bottom panels). This staining was weaker in HPV-negative tissue sections. Using semiquantitative assessments of CD8α staining, we found significantly higher CD8α scores in the stroma of HPV-positive than in HPV-negative OSCC (Fig. 2b).

### Table 2. Histopathological, Clinical and Biological Features of a Cohort of 144 Patients with a Squamous Cell Carcinoma of the Oropharynx

| Age | HPV-negative (N = 120) | HPV-positive (N = 24) |
|-----|-----------------------|----------------------|
| <53 | 39 (32.5%) | 7 (29.2%) | p = 0.936 |
| ≥53 | 81 (67.5%) | 17 (70.8%) |
| Gender | | | |
| Male | 108 (90.0%) | 19 (79.2%) | p = 0.248 |
| Female | 12 (10.0%) | 5 (20.8%) |
| Tobacco consumption | | | |
| <10 pack/year | 3 (2.5%) | 10 (41.7%) | p < 0.0001 |
| ≥10 pack/year | 114 (95.0%) | 14 (58.3%) |
| Nonavailable | 3 (2.5%) | / |
| Alcohol consumption | | | |
| None | 13 (10.8%) | 18 (75.0%) | p < 0.0001 |
| Moderate | 44 (36.7%) | 5 (20.8%) |
| Important | 51 (42.5%) | 1 (4.2%) |
| Nonavailable | 12 (10.0%) | / |
| Histology | | | |
| Well differentiated | 21 (17.5%) | 1 (4.2%) | p = 0.002 |
| Moderately differentiated | 74 (61.7%) | 10 (41.6%) |
| Poorly/ nondifferentiated | 25 (20.8%) | 13 (54.2%) |
| Histological size (pT) | | | |
| pT1 | 4 (3.3%) | 3 (12.5%) | p = 0.251 |
| pT2 | 45 (37.5%) | 7 (29.2%) |
| pT3 | 55 (45.8%) | 13 (54.2%) |
| pT4 | 16 (13.4%) | 1 (4.2%) |
| Pathological lymph node status (pN) | | | |
| pN0 | 24 (20.0%) | 3 (12.5%) | p = 0.252 |
| pN1 | 28 (23.3%) | 3 (12.5%) |
| pN2 | 58 (48.3%) | 17 (70.8%) |
| pN3 | 10 (8.4%) | 1 (8.2%) |
CD8\(\alpha\) and CD3\(\zeta\) overexpressions correlate with improved prognosis

To determine whether there is a correlation between CD8\(\alpha\) + T cells levels in the stroma of OSCC and prognosis, we analyzed the levels of CD8\(\alpha\) and CD3\(\zeta\) expression according to 5-year overall survival and 2-year disease-free survival. qRT–PCR analysis using CD8\(\alpha\)-specific primers was interpretable in 143 of 144 cases. We selected a cut-off value for CD8\(\alpha\) that corresponded to 90% specificity (i.e., 90% of the HPV-negative samples had CD8\(\alpha\) qRT–PCR levels below this cut-off). According to this criterion, 12 of 119 HPV-negative OSCC (10%) and 10 of 24 HPV-positive OSCC (42%) were CD8\(\alpha\)-positive, which is a statistically significant difference (Fisher’s exact test; \(p = 0.006\)). In addition, we found a good correlation (Fig. 2c; \(p < 0.05\); Mann–Whitney U test) between the CD8\(\alpha\) mRNA score as determined with this stratification system and the amount of CD8\(\alpha\)-positively labeled cells that were observed in paraffin-embedded specimens (see above). Using Kaplan–Meier analysis, we found that patients with high-CD8\(\alpha\) expression levels (CD8\(\alpha\)-positive) had improved 5-year overall survival (Fisher’s exact test; \(p = 0.005\); Fig. 3a). When survival data were corrected for potential confounding factors such as age, history of tobacco smoking, tumor histology and stage (pT and pN) in Cox regression analysis, the expression of CD8\(\alpha\) retained its predictive value (\(p = 0.003\); Table 3). Using Kaplan–Meier analysis, patients with high-CD8\(\alpha\) levels (CD8\(\alpha\)-positive) had a nonsignificant trend for improved 2-year disease-free survival (\(p = 0.074\); Fig. 3b). Using multivariate Cox regression analysis, CD8\(\alpha\) expression correlated with improved disease-free survival at 2 years (\(p = 0.040\); Table 3). Using a similar approach, we calculated a cut-off value for CD3\(\zeta\) expression. Twelve of 120 HPV-negative samples (10%) and 11 of 24 HPV-related tumors (46%) were found to be CD3\(\zeta\)-positive, which is a significant difference (Fisher’s exact test; \(p = 0.001\)). Kaplan–Meier analysis showed that CD3\(\zeta\)-positive patients displayed a nonsignificant trend for better overall survival at 5 years (\(p = 0.107\)). We did not observe any significant impact of CD3\(\zeta\) expression levels on 2-year disease-free survival (Fig. 3d and Table 3). The Cox multivariate analysis suggested that CD3\(\zeta\) expression predicts a favorable 5-year overall outcome (\(p = 0.024\); Table 3).

CD8\(\alpha\) identifies subgroups of HPV-related tumors with distinct prognosis

Interestingly, our Kaplan–Meier analysis of 5-year overall survival demonstrated that the statistical power of CD8\(\alpha\) expression levels (\(p = 0.005\); Fig. 3a) is higher than the statistical power of HPV (\(p = 0.012\); Supporting Information Fig. 2). CD8\(\alpha\) expression retains its prognostic value for both 5-year overall survival (\(p = 0.011\); Table 3) and 2-year disease-free survival (\(p = 0.040\); Table 3) when HPV is included in a Cox regression together with other potential confounders [age; tobacco use; tumor size (pT); histology; lymph node involvement (pN)]. Interestingly, HPV was not found to have prognostic value in a Kaplan–Meier analysis of the 5-year overall survival rate of CD8\(\alpha\)-negative patients (\(p = 0.258\); Supporting Information Fig. 3). CD8\(\alpha\)-positive patients with HPV-negative tumors displayed a nonsignificant trend for improved global survival (\(p = 0.138\); Fig. 3e) with respect to their CD8\(\alpha\)-negative counterparts. However, the stratification of HPV-related patients according to CD8\(\alpha\) expression levels showed that HPV-positive CD8\(\alpha\)-positive patients have an improved 5-year overall survival when compared with HPV-positive CD8\(\alpha\)-negative patients (\(p = 0.040\); Fig. 3f). Interestingly, when HPV-positive patients were stratified according to both the presence of HPV and the CD8\(\alpha\) score, GZMK gene expression was found to be significantly more expressed in HPV-positive CD8\(\alpha\)-positive than in HPV-negative CD8\(\alpha\)-positive patients (\(p = 0.006\); one-way ANOVA; Fig. 2d). In addition, the GZMK gene expression had a tendency to be higher in HPV-positive CD8\(\alpha\)-positive patients than in HPV-positive CD8\(\alpha\)-negative (\(p = 0.056\); one-way ANOVA).

Discussion

In this study, we have found that HPV-related OSCCs express higher levels of immune response-related genes, that they are infiltrated to a greater extent with cytotoxic CD8+ T cells, and that infiltration correlates with improved prognosis. One possible interpretation of these data is that an increased CD8+ T-cell-based immune response could be involved in the better response of HPV-positive oropharyngeal carcinoma to therapy.

Previous studies indicate that lymphocyte infiltrate has an impact on HNSCC progression. Cytotoxic CD8+ T cells...
have been shown to be the effectors of antitumor immunity.\textsuperscript{28} A weak lymphocytic response at the tumor/host interface strongly correlates with local recurrence and death.\textsuperscript{17} Similarly, patients with lymph node mononuclear cells and PBMCs that display unresponsive CD3 receptors are at increased risk for lymph-node invasion.\textsuperscript{29} CD3\(\alpha\) expression together with increased numbers of intratumoral dendritic cells has been reported to be a good prognostic marker of overall survival for patients with oral carcinoma.\textsuperscript{18}

There is evidence for a specific immune response to HPV-related HNSCC. Mouse models suggest that the ability to mount a robust T-cell-based immune response plays an important role in HPV-related tumor growth and response to therapy. HPV-related xenografts grow more quickly in \textit{Rag1} immune-compromised than in wild-type mice.\textsuperscript{30} Cisplatin-based therapy results in tumor clearance in wild type but not in \textit{Rag2} mice.\textsuperscript{31} Two studies have shown that the proportion of T cells that are specific for HPV16 E7 peptides among CD8\(\alpha\) T cell is significantly increased (three to fourfold) in PBMC of patients with HPV-positive oropharyngeal cancer when compared with HPV-negative patients.\textsuperscript{13,32} More recently, a flow cytometry analysis of peripheral blood samples of patients with OSCC before treatment showed that HPV-positive patients have an increased number of

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**Figure 2.** (a) Immunohistochemical analysis of HPV-negative (top panels) and HPV 16-positive (bottom panels) tumor specimens. Hematoxylin and eosin staining is shown (left panels; H&E; magnification \(\times 200\)). Highlights of the inset in the H&E micrographs are presented in the other panels and show the CD8\(\alpha\), CD3\(\zeta\), CD4 and CD3 markers (magnification \(\times 400\)). (b) Semiquantitative and statistical analysis of the CD8\(\alpha\) staining in 10 HPV-related and 7 HPV-negative patients **\(p < 0.001\) (Mann–Whitney U test). Differences were considered significant when \(p < 0.05\). (c) Evaluation and statistical analysis of the number of CD8\(\alpha\)-positive-stained cells in tumor samples stratified according to the CD8\(\alpha\) score (CD8\(\alpha\)-negative vs. CD8\(\alpha\)-positive) determined by qRT-PCR. *\(p < 0.05\) (Mann–Whitney U test). Differences were considered significant when \(p < 0.05\). (d) \textit{GZMK} gene expression analysis in HPV-positive and -negative patients stratified according to the CD8\(\alpha\) score (CD8\(\alpha\)-negative vs. CD8\(\alpha\)-positive). A one-way ANOVA analysis of the data was performed. Differences were considered significant when \(p < 0.05\).
Figure 3. Univariate Kaplan–Meier analyses of the prognostic values of the expression levels of CD8α (a, b, c, d, e, f) and CD3ε (c, d, e, f). Five-year overall survival (a, c, e, f) and 2-year disease-free survival (b, d) were used as end points. Analyses were performed on the whole cohort (a–d; N = 144), on HPV-negative patients (e; N = 119) and on HPV-positive patients (f; N = 24). Differences were considered significant when p < 0.05.
Table 3. Multivariate analyses of the prognostic value of CD8α and CD3ζ expression levels by a Cox regression model that include the patients’ age, tobacco consumption, tumor histology, size (pT) and lymph node involvement (pN), HPV status.

|                      | 5-Year overall survival | 2-Year disease-free survival |
|----------------------|-------------------------|-----------------------------|
|                      | OR  95% CI  p           | OR  95% CI  p               |
| **Age**              |                         |                             |
| ≥53 versus <53       | 3.27 1.71–6.25 0.0004    | 3.09 1.45–6.58 0.004        |
| **Tobacco**          |                         |                             |
| ≥10 pack/year versus <10 pack/year | / / NS  / / NS | / / NS / / NS |
| **Histology**        |                         |                             |
| Poorly/nondiff. versus well/mod. diff. | / / NS  / / NS | / / NS / / NS |
| **pT**               |                         |                             |
| pT3/pT4 versus pT1/pT2 | / / NS  / / NS | / / NS / / NS |
| **pN**               |                         |                             |
| pN1+ versus pN0      | 2.63 1.20–5.73 0.016    | 4.29 1.34–3.75 0.015        |
| **CD8α**             |                         |                             |
| CD8α-pos versus CD8α-neg | 0.22 0.08–0.59 0.003 | 0.34 0.12–0.94 0.040        |
| **Age**              |                         |                             |
| ≥53 versus <53       | 3.30 1.73–6.33 0.0003    | 2.97 1.40–6.32 0.005        |
| **Tobacco**          |                         |                             |
| ≥10 pack/year versus <10 pack/year | / / NS  / / NS | / / NS / / NS |
| **Histology**        |                         |                             |
| Poorly/nondiff. versus well/mod. diff. | / / NS  / / NS | / / NS / / NS |
| **pT**               |                         |                             |
| pT3/pT4 versus pT1/pT2 | / / NS  / / NS | / / NS / / NS |
| **pN**               |                         |                             |
| pN1+ versus pN0      | 2.77 1.27–6.06 0.011    | 3.13 1.13–8.68 0.029        |
| **CD3ζ**             |                         |                             |
| CD3ζ-pos versus CD3ζ-neg | 0.40 0.18–0.88 0.024 | / / NS / / NS |
| **Age**              |                         |                             |
| ≥53 versus <53       | 3.49 1.82–6.69 0.0002    | 3.09 1.45–6.58 0.004        |
| **Tobacco**          |                         |                             |
| ≥10 pack/year versus <10 pack/year | / / NS  / / NS | / / NS / / NS |
| **Histology**        |                         |                             |
| Poorly/nondiff. versus well/mod. diff. | / / NS  / / NS | / / NS / / NS |
| **pT**               |                         |                             |
| pT3/pT4 versus pT1/pT2 | / / NS  / / NS | / / NS / / NS |
| **pN**               |                         |                             |
| pN1+ versus pN0      | 2.71 1.24–5.92 0.013    | 4.29 1.34–3.75 0.015        |
| **HPV**              |                         |                             |
| HPV-pos versus HPV-neg | 0.36 0.14–0.91 0.031 | / / NS / / NS |
| **CD8α**             |                         |                             |
| CD8α-pos versus CD8α-neg | 0.27 0.10–0.73 0.011 | 0.34 0.12–0.95 0.040        |
circulating CD8+ T cells and a lower CD4+/CD8+ lymphocyte ratio. Interestingly, these features correlate with complete and partial tumor response to induction chemotherapy. However, increased infiltration of the stroma of HPV-related oropharyngeal tumors with CD8+ TILs remains controversial. On the basis of a tissue-microarray analysis, Wansom et al. found no significant difference in TIL infiltrates among HPV-positive and HPV-negative patients. In contrast, a recent flow cytometry analysis of cells extracted from HPV-positive oropharyngeal tumors by Dr. Guy Bronner. We are grateful to Dr. Jean-Luc Pr...s of human papillomavirus (HPV) infection in the microenvironment of oropharyngeal tumors. Our semiquantitative analysis of immunostaining data was performed on a limited number of samples (10 HPV-positive vs. 7 HPV-negative), and results should therefore be interpreted cautiously. Nevertheless, we observe that tumor-infiltrating T cells are found more frequently and more abundantly in HPV-related HNSCC. Enrichment of TILs in HPV-related OSCC is consistent with the higher expression of proinflammatory chemokines (CXCL 9, 10 and 11) and chemokines receptors (CXCR3), known to be required for the recruitment of CD8 T cells. Improved tumor stroma infiltration with TILs is also consistent with the higher expression of ITGAL, CD28, CD3ζ, CD8α, GZMK and CD4 genes in HPV-related lesions. In addition, a good correlation was observed between CD8α mRNA expression levels and the number of CD8α+ cells detected in the tumor stroma. Finally, CD8α and CD3ζ expression, which are both hallmarks of an activated CD8+ T-cell-based immune response, correlate with improved disease-free and overall survival. Despite the modest size of our HPV-positive cohort (N = 24), CD8α expression was found to be a prognostic factor that is independent of HPV and to correlate with improved prognosis of HPV-related patients. Similarly, a study by Thurlow et al. showed that HPV-related oropharyngeal tumors preferentially express adaptive immune response genes and that this signature outcompetes HPV markers as a favorable prognostic indicator. Interestingly, we found that, among HPV-positive patients, the CD8α score defines two clinical subpopulations with distinct prognosis: HPV-related tumors with high-CD8α expression correlate with prolonged survival when compared with CD8α-negative lesions. HPV–CD8α-positive tumors also show a tendency for higher GZMK gene expression. These observations suggest that, despite the increased recruitment of TILs to HPV-related OSCC, initiation of an efficient immune response requires some additional regulatory mechanisms, which fail to be switched on in a subset of lesions. Therefore, our data suggest that the CD8+ T-cell infiltrate and immune response activation are involved in the improved prognosis of HPV-positive patients. The antigens that trigger this host response remain to be strictly identified. However, Heusinkveld et al. recently found that HPV16-specific TILs could be extracted from a majority of HPV-positive tumor cultures (six of eight HPV-related tumors). It is therefore reasonable to speculate that the T-cell infiltrate we observe is raised against viral antigens.

Our findings, together with the current knowledge of the biology and physiopathology of HPV-related OSSC, are a good rationale for considering preclinical evaluation of immunotherapy for the treatment of HPV-positive HNSCC. Immune-based therapy of cervical cancer patients using HPV16 E6 and E7 long peptides efficiently triggers CD4+ and CD8+ T-cell immunity. A similar approach could be used to modulate existing treatment protocols for OSCC, in order to boost the immune response, with the aim of sparing patients the toxicity of chemoradiation therapy. In addition, it would be of interest to further evaluate whether the infiltrate of HPV-positive OSCC can be used as a biomarker to predict tumor response to therapy.

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