Immune related proteins and tumor infiltrating CD8+ lymphocytes in hypopharyngeal cancer in relation to human papillomavirus (HPV) and clinical outcome

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Abstract

**Background:** Hypopharyngeal cancer (HPSCC) shows a poor clinical outcome, while HPSCC, caused by human papillomavirus (HPV), presents a better outcome. Here, HPCC, immune proteins, and tumor infiltrating CD8+ lymphocytes (CD8+ TILs) were evaluated in relation to HPV and outcome.

**Methods:** Fresh frozen tissue from four HPV-positive HPSCC, 39 HPV-negative HPSCC, and normal samples were analyzed for protein expression by the Proseek immuno-oncology immunoassay. CD8+ TIL numbers evaluated by immunohistochemistry on 144 formalin-fixed biopsies were analyzed in relation to clinical outcome.

**Results:** Proteins differing between HPV-positive and negative HPSCC included CD8A, PD-L1, Fas ligand, and chemokines. High CD8+ TIL numbers were correlated to improve clinical outcome in HPV-negative HPSCC.

**Conclusions:** High expression of immune proteins in HPV-positive HPSCC may explain the better clinical outcome. CD8+ TILs are of relevance for outcome of HPV-negative HPSCC, while tumors with high immune activity but poor patient survival suggest a role for immune therapy.

**KEYWORDS**
CD8-positive lymphocytes, clinical outcome, human papillomavirus, hypopharyngeal cancer, protein expression
1 | INTRODUCTION

Hypopharyngeal cancer, most commonly squamous carcinoma (HPSCC), accounts for approximately 0.4% of all cancers worldwide with around 80,000 cases/year. Most HPSCC is diagnosed at late stage, and shows the poorest outcome among all head and neck cancer sites. The traditional main risk factors for HPSCC are smoking and alcohol. Another risk factor for HNSCC is human papillomavirus (HPV), a major cause of oropharyngeal cancer (OPSCC), especially for tonsillar and base of tongue cancer. Currently, in Stockholm, Sweden, around 70% of tonsillar and base of tongue cancers are caused by HPV, similar to several other countries in the Western world, for example, United States. In HNSCC outside of the oropharynx, the frequency of cancer caused by HPV is much lower. Thus, subsets of oral, laryngeal, nasopharyngeal, and HPSCCs have been reported as HPV positive, although there are large variations in the data presented.

In two earlier studies, 3.6% of 191 HPSCC were found to be caused by HPV, as determined by the presence of HPV DNA combined with the expression of p16INK4A (p16) protein, an established combination to assess for tumors caused by HPV. Notably, patients with HPV-positive HPSCC showed a very good survival, in sharp contrast to those with HPV-negative HPSCC. The correlation between HPV and clinical outcome in HPSCC has also been confirmed in other studies. The well-known fact that patients with HPV-positive OPSCC have a markedly better survival than those with the corresponding HPV-negative tumors has been linked to a more active immune response in these tumors, for example, higher numbers of tumor infiltrating lymphocytes (TILs), especially CD8 positive TILs. In addition, several studies have demonstrated the upregulation of immune related RNA-transcripts in HPV-positive vs HPV-negative OPSCC. The increased immune response toward HPV-positive OPSCC has therefore also been suggested to be due to an adaptive immune response directed against viral antigens.

The involvement of the immune response in HPSCC specifically, has been much less studied, and although some reports have correlated immunological markers to clinical outcome, these have mostly not been associated to tumor HPV status. Thus, Ono et al found in two studies a correlation between CD8+ TILs and clinical outcome in HPSCC, demonstrating a correlation between the adaptive immune response and clinical outcome. Additional studies have investigated the immune response in HNSCC including a subset of HPSCC, for example, References 23 and 26. Because HPSCC usually is in minority in these studies, it is difficult to know to what degree the result is valid for HPSCC alone, and there is thus a need for further studies on HPSCC specifically.

Recently, we investigated protein expression in OPSCC in relation to normal tissue, HPV status, and clinical outcome, utilizing Proseek multiplex panels (Olink Bioscience, Uppsala, Sweden) based on proximity extension assay (PEA) technology, and found an increased expression of many immune related proteins in HPV-positive vs HPV-negative tumors. In the present study, we aimed to, with the same method, investigate the expression of immune-related proteins in HPSCC in relation to normal tissue, and tumor HPV-status. Because previous studies demonstrated a relation both to tumor HPV-status and a prognostic role of tumor infiltrating CD8 positive lymphocytes (CD8+ TILs) in OPSCC, we also aimed to investigate the role of CD8+ TILs in formalin-fixed biopsies from HPSCC in relation to tumor HPV-status and clinical outcome.

2 | MATERIALS AND METHODS

2.1 | Patients and tumor biopsies

Patient and tumor characteristics are presented in Table 1. Forty-three pretreatment tumor biopsies of HPSCC, ICD-10 codes C12.9 (pyriform sinus), C13.0 (postericoid region), C13.1 (aryepiglottic fold, hypopharyngeal aspect), C13.2 posterior wall of hypopharynx), C13.8 (overlapping sites of hypopharynx), and C13.9 (hypopharynx, unspecified location) and adjacent normal tissue, from patients treated 2002 to 2013 at the Karolinska University Hospital, were snap frozen and stored at −70°C until cutting of the samples. Analysis of tumor infiltrating CD8+ cells was performed on 149 pretreatment formalin-fixed paraffin-embedded (FFPE) HPSCC pretreatment biopsies, from patients treated 2000 to 2013 at the Karolinska University Hospital. These FFPE samples had been included in two earlier studies. Twenty-two of the fresh frozen tumors were from tumors also included among the FFPE biopsies. The patients were followed up every third month for 2 years and every 6 months thereafter, until 5 years after completed
## Patient and tumor characteristics

|                     | PEA-assay | IHC CD8 TILs |
|---------------------|-----------|--------------|
|                     | All Patients/tumors | Included in analysis | All Patients/tumors | Included in survival analysis |
|                     | n | % | n | % | n | % | n | % |
| Total number        | 43 | 33 | 149 | 128 |
| Gender              |   |    |     |     |
| Male                | 33 | 76.7 | 27 | 81.8 | 111 | 74.5 | 94 | 73.4 |
| Female              | 10 | 23.3 | 6 | 18.2 | 38 | 25.5 | 34 | 26.6 |
| Age                 |   |    |     |     |
| Mean age            | 68.1 | 68.3 | 66.4 | 65.9 |
| Median age          | 70 | 70 | 67 | 66 |
| Age range           | 47-90 | 47-84 | 40-93 | 40-90 |
| ICD-10 code         |   |    |     |     |
| C129, pyriform sinus | 12 | 27.9 | 8 | 24.2 | 77 | 51.7 | 69 | 53.9 |
| C130, postcricoid region | 4 | 9.3 | 2 | 6.1 | 11 | 7.4 | 6 | 4.7 |
| C131, aryepiglottic fold | 0 | 0.0 | 0 | 0.0 | 5 | 3.4 | 3 | 2.3 |
| C132, posterior wall | 7 | 16.3 | 7 | 21.2 | 13 | 8.7 | 13 | 10.2 |
| C138, overlapping sites | 11 | 25.6 | 9 | 27.3 | 24 | 16.1 | 23 | 18.0 |
| C139, unspecified location | 9 | 20.9 | 7 | 21.2 | 19 | 12.8 | 14 | 10.9 |
| TNM classification  |   |    |     |     |
| T1                  | 1 | 2.3 | 1 | 3.0 | 15 | 10.1 | 11 | 8.6 |
| T2                  | 14 | 32.6 | 10 | 30.3 | 49 | 32.9 | 42 | 32.8 |
| T3                  | 17 | 39.5 | 13 | 39.4 | 51 | 34.2 | 45 | 35.2 |
| T4/a/b              | 11 | 25.6 | 9 | 27.3 | 34 | 22.8 | 30 | 23.4 |
| N0                  | 15 | 34.9 | 13 | 39.4 | 47 | 31.5 | 38 | 29.7 |
| N1                  | 2 | 4.7 | 1 | 3.0 | 23 | 15.4 | 21 | 16.4 |
| N2/a/b/c            | 18 | 41.9 | 13 | 39.4 | 71 | 47.7 | 65 | 50.8 |
| N3                  | 5 | 11.6 | 3 | 9.1 | 6 | 4.0 | 3 | 2.3 |
| Nx                  | 3 | 7.0 | 3 | 9.1 | 2 | 1.3 | 1 | 0.8 |
| M0                  | 37 | 86.0 | 27 | 81.8 | 143 | 96.0 | 122 | 95.3 |
| M1                  | 3 | 7.0 | 2 | 6.1 | 3 | 2.0 | 3 | 2.3 |
| Mx                  | 3 | 7.0 | 3 | 9.1 | 3 | 2.0 | 3 | 2.3 |
| Stage               |   |    |     |     |
| 1                   | 0 | 0.0 | 0 | 0.0 | 6 | 4.0 | 5 | 3.9 |
| 2                   | 4 | 9.3 | 4 | 12.1 | 22 | 14.8 | 19 | 14.8 |
| 3                   | 7 | 16.3 | 4 | 12.1 | 26 | 17.4 | 20 | 15.6 |
| 4a/b/c              | 28 | 65.1 | 21 | 63.6 | 91 | 61.1 | 81 | 63.3 |
| Stage unknown\*     | 4 | 9.3 | 4 | 12.1 | 4 | 2.7 | 3 | 2.3 |
| HPV/p16-status      |   |    |     |     |
| Positive            | 4 | 9.3 | 4 | 12.1 | 7 | 4.7 | 0 | 0.0 |
| Negative            | 39 | 90.7 | 29 | 87.9 | 142 | 95.3 | 128 | 100.0 |
| Treatment           |   |    |     |     |
| RT                  | 13 | 30.2 | 9 | 27.3 | 60 | 40.3 | 58 | 45.3 |
| Reduced RT (50-54)  | 3 | 7.0 | 3 | 9.1 | 3 | 2.0 | 0 | 0.0 |
For the survival analysis, in this study the clinical outcome for the first 3 years was utilized. The study was performed according to permissions 02-009 and 2009/1278-31/4 from the Regional Ethics Committee, Karolinska Institutet. Informed consent was obtained from all the patients.

### 2.2 Analysis of HPV DNA positivity and p16INK4A expression

HPV-positive status was defined as being both HPVDNA and p16INK4A (p16) positive. The 149 FFPE pretreatment biopsies had previously been analyzed for HPVDNA and p16 overexpression as presented in two earlier studies. As described earlier, presence of HPV was analyzed by a multiplex bead-based assay evaluated for the presence of 24 or 27 HPV types on a MagPix instrument (Luminex Corp., Austin, Texas), after HPV-specific PCR amplification. For the fresh frozen tumors, the corresponding FFPE biopsies were, when not done previously, analyzed for HPVDNA and p16 expression, as described above. For cases where FFPE material was lacking, cuts were made from the fresh frozen biopsies and analyzed for the presence of HPVDNA and p16 expression. In those cases, DNA was extracted with the Qiagen Blood and tissue kit (Qiagen, Hilden, Germany), before the analysis for the 27 HPV types in the multiplex HPV assay.

### 2.3 Sample preparation for Proseek analysis

Samples were prepared for protein analysis essentially as described in Ramqvist et al. Briefly, six cuts were made from each biopsy (1 × 5 μm, 4 × 20 μm, and 1 × 5 μm), frozen, and embedded in optimal cutting temperature compound. The first and last slides were used for evaluation of tumor content by an experienced pathologist and only tumors with ≥40% tumor cells were included in the protein evaluation and 28/33 samples included in the analysis had ≥70% tumor cells. All normal samples were checked to be free from tumor tissue. The four 20 μm cuts from each sample were pooled, dissolved in RIPA buffer, and frozen at −70°C until analysis on the Proseek panel as described in Ramqvist et al.

### 2.4 Analysis on the Proseek immuno-oncology panel and evaluation of data

Sample aliquots were analyzed for the presence of 92 proteins with the Immuno-Oncology Proseek multiplex immunoassay (Olink Bioscience, Uppsala, Sweden), at the Clinical Biomarkers facility, Science for Life Laboratory, Uppsala University. Concentrations of each protein were reported as normalized protein concentration (NPX) in a 2-log scale, and limit of detection (LOD) was defined as 3SD above background. The assay included two internal controls and a detection control. Quality control and data preprocessing (including normalization) of PEA data were made according to the recommendations of the manufacturer.

All evaluations were performed on log-transformed NPX data. For presentation of protein ratios in Tables 2 and 3, values and log ratios were converted to linear. Log transformed was analyzed using Qlucore Omics Explorer 3.5 (Qlucore, Lund, Sweden), including heatmaps, principal component analysis (PCA)-plots, and analysis of significance. Differences in protein expression levels between categories of samples (tumor vs normal, HPV positive vs negative) were evaluated by t test on log-
transformed data. False discovery rate was evaluated according to the method by Benjamin and Hochberg.  

### 2.5 Immunohistochemistry for p16 and CD8 positive TILs

Evaluation of CD8+ TILs was performed essentially as in Nasman et al.  

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**TABLE 2** Proteins with the most prominent differences between hypopharyngeal cancer and normal samples

| Protein   | Ratio tumor/normal | Adjusted P value |
|-----------|--------------------|-------------------|
| Higher in tumors |                     |                   |
| IL6       | 81.4               | 9.97E-25          |
| CCL3      | 80.4               | 4.58E-23          |
| MMP12     | 63.7               | 1.00E-23          |
| CAIX/CA9  | 61.1               | 4.33E-21          |
| IL8       | 40.9               | 4.96E-17          |
| CXCL5     | 30.9               | 6.21E-15          |
| CCL20     | 27.5               | 4.56E-14          |
| CCL4      | 24.6               | 4.03E-19          |
| CXCL1     | 20.5               | 1.19E-13          |
| MCP-3/CCL7| 20.3               | 1.42E-21          |
| CXCL13    | 19.2               | 6.18E-11          |
| MCP-2/CCL8| 15.8               | 2.71E-15          |
| TNFRSF9   | 14.9               | 6.11E-15          |
| CXCL10    | 14.9               | 9.64E-11          |
| MCP-1/CCL2| 12.8               | 4.46E-17          |
| CXCL11    | 11.6               | 4.80E-10          |

**Lower in tumors**

| Protein | Ratio tumor/normal | Adjusted P value |
|---------|--------------------|-------------------|
| Gal-1   | 0.60               | 8.66E-05          |
| DCN     | 0.57               | 3.77E-05          |
| PTN     | 0.41               | 3.85E-05          |
| CCL23   | 0.40               | 3.90E-05          |
| IL33    | 0.32               | 7.12E-05          |

Notes:

- **Higher in tumors**
  - 2-log ratios transformed into linear ratios.
  - Adjusted for false discovery rate.

- **Lower in tumors**
  - 2-log ratios transformed into linear ratios.

**TABLE 3** Protein with significant differences in expression between human papilloma virus positive and negative hypopharyngeal cancer

| Protein | Ratio HPV positive/negative tumors | P value |
|---------|-----------------------------------|---------|
| Higher in HPV positive | | |
| CD8A | 6.74 | .04 |
| CXCL11 | 5.79 | .04 |
| FASLG | 5.66 | .002 |
| CXCL10 | 5.44 | .03 |
| IL12 | 4.51 | .009 |
| CXCL9 | 3.82 | .02 |
| PD-1 | 3.60 | .02 |
| KLRD1 | 3.42 | .008 |
| MCP-4 | 3.18 | .02 |
| PD-L1 | 2.96 | .04 |
| CD244 | 2.87 | .02 |
| CCL17 | 2.86 | .04 |
| CCL4 | 2.83 | .04 |
| GZMH | 2.77 | .047 |
| CRTAM | 2.63 | .003 |
| IL13 | 2.26 | .03 |

**Lower in HPV positive**

| Protein | Ratio HPV positive/negative tumors | P value |
|---------|-----------------------------------|---------|
| HO-1 | 0.78 | .04 |
| MIC-A/B | 0.34 | .02 |

**Notes:**

- **Higher in HPV positive**
  - 2-log ratios transformed into linear ratios.
  - Not adjusted for false discovery rate.

- **Lower in HPV positive**
  - 2-log ratios transformed into linear ratios.
  - Not adjusted for false discovery rate.

### 2.6 Evaluation of patient survival

For analysis of survival in relation number of CD8+ TILs by IHC, survival was measured in days from diagnosis until an event occurred, or until 3 years after diagnosis when patients were censored. For calculation of progression-free survival (PFS), disease recurrence, or death of any cause was considered as events. For evaluation of disease-specific survival (DSS), death with documented relapse was considered as event while patients that had deceased before 3 years from other causes were censored at time of death. For overall survival (OS), death of any cause was considered as an event. Kaplan-Meier estimator was used for evaluation of PFS, DSS, and OS. Differences in the survival of patients in relation to CD8+ TILs were evaluated using the log-rank test. The Cox proportional hazards regression analysis was used for the calculation of hazard ratios in the univariate or multivariate...
analysis. Calculations and analyses were performed using IBM SPSS Statistics, version 26.0.

3 | RESULTS

Forty-three HPSCC (Table 1) and their corresponding normal samples were analyzed on the Proseek Immuno-Oncology panel covering 92 proteins. Three proteins (IL-5, IL-21, and IFN-gamma) were excluded from the analysis due to values below the LOD (as defined by Olink) in >70% samples. Nine tumor samples with tumor content ≤30%, and one sample that failed in the assay were excluded from further analysis. The final analysis included 33 samples, 29 HPV-negative, and 4 HPV-positive cases (Table 1). Furthermore, one sample with some tumor cells in the normal sample was excluded from the comparison between normal and tumor samples.

3.1 | Comparison between tumor and normal samples

As demonstrated on the heatmap in Figure 1A based on all included samples, tumor and normal samples in general presented a clear separation. This separation was further demonstrated in a PCA-plot (Figure 1B). Totally 66/89 (74%) evaluated proteins differed significantly in expression between tumor and normal samples, with 65/89 (73%) after adjusting for false discovery rate. Of these, 53 were significantly higher in the tumors. In Table 2, the 16 proteins with >10 times higher average expression in tumors are presented. Besides the hypoxia related CAIX (CA9), matrix metalloproteinase-12 (MMP12), and the TNF-receptor TNFRSF19, all were chemokines or cytokines. Few proteins (eg, IL-33, PTN, CCL23, and DCN) presented with a significantly lower expression in the tumors compared to the normal samples, and the differences were smaller than for the reverse comparison. The five of these with the largest differences are also presented in Table 2.

3.2 | Comparison between HPV positive and negative tumors

In general, the difference in expression levels between the HPV-positive and HPV-negative tumors was less prominent than between tumors vs normal tissues. In Tables 3, 18 proteins with significant differences in HPV-
positive vs HPV-negative tumors are presented. The 16 proteins with higher expression in the HPV-positive cases included proteins related to tumor infiltrating immune cells (eg, CD8, FASLG, PD-1, and KLRD1), thereby indicating a strong immune infiltration. In addition, several cytokines, especially CXCL9, 10, 11, and IL12, presented with a strong difference. Only two proteins, MICA/B and HO-1, had a significantly higher expression in HPV-negative tumors. None of the 18 proteins showed a significant difference after adjusting for false discovery rate.

When a heatmap was generated based on these 18 proteins, the tumors samples separated in two distinct clusters, differing in immune infiltration (Figure 1C). One cluster including only HPV-negative samples and the other cluster, with high expression of for example, FAS ligand, PD-1 (PDCD1), PD-L1, CXCL9, 10, and 11, included all HPV-positive tumors, as well as 6 HPV-negative tumors, indicating a strong immune activity also in these tumors. The clustering of HPV-positive samples together with some of the HPV-negative samples can also be observed in the unselected PCA-plot in Figure 1B, including both tumor and normal samples. In total, 23/33 tumor samples, 19 HPV negative, and 4 HPV positive, came from patients receiving curative treatment (Table 1) and of these only 6 patients, 3 with HPV-negative and 3 with HPV-positive tumors, survived relapse free for >3 years. For this reason, protein expression was not evaluated in relation to survival.

3.3 | Tumor infiltrating CD8+ lymphocytes in relation to tumor HPV status

Totally 149 FFPE HPSCC samples were analyzed for numbers of tumor infiltrating CD8+ lymphocytes (CD8+ TILs). Seven of these (4.7%) were from HPV-positive HPSCC. On average, HPV-positive HPSCC had more CD8+ TILs than HPV negative (27.1 vs 14.7), similar to the results obtained in the immuno-oncology assay (Figure 2). However, this difference did not reach significance (0.085), likely due to the low number of HPV positive samples.

3.4 | Tumor infiltrating CD8+ lymphocytes in relation to clinical outcome

For evaluation of CD8+ TILs in relation to survival, only HPV-negative HPSCC samples were included. As presented earlier, patients with HPV-positive HPSCC have a much better clinical outcome compared to those with HPV-negative HPSCC, and notably all six patients with HPV-positive HPSCC and receiving curative treatment survived.10,11 As these tumors also have a higher number of CD8+ TILs they were excluded from further analysis in order to investigate the relation between CD8+ TILs and clinical outcome in patients with HPV-negative HPSCC. The final analysis included 128 HPV-negative tumor samples from patients receiving curative treatment (Table 1). Samples were
separated into four quartiles based on the number of CD8+ TILs. After initial analysis, the three lowest quartiles were combined and compared to those in the highest quartile. As presented in Figure 3, patients with tumors in the highest quartile had a significantly improved PFS, DSS, and OS (\(P = .033\), \(P = .011\), and \(P = .037\)).

Parameters potentially related to survival, such as sex, age, T and N classification, and number of CD8+ TILs, were evaluated using the Cox proportional hazards model. Notably, high number of CD8+ TILs showed significant correlation to improve PFS, DSS, and OS, both in univariate and multivariate analysis, while other parameters showed significance for some but not all evaluations, Table 4.

### 4. DISCUSSION

In this study, cancer and immune-related proteins were analyzed in fresh frozen HPSCC biopsies and corresponding normal samples, and differences in protein expression between tumor and normal tissue, as well as between HPV-positive and HPV-negative HPSCC were identified. In addition, a correlation between high numbers of CD8+ TILs and survival in HPV-negative HPSCC was demonstrated in a larger set of FFPE HPSCC biopsies.

#### TABLE 4  Univariate and multivariate Cox regression analysis for 3-year survival in patients with human papilloma negative hypopharyngeal cancer

| Factor                  | Univariate HR 95% CI P value | Multivariate HR 95% CI P value |
|-------------------------|------------------------------|--------------------------------|
| 3-year progression-free survival (PFS) |                             |                                |
| Sex\(^a\)               | 0.456 (0.244-0.854) .01      | 0.528 (0.274-0.965) .047       |
| Age\(^b\)               | 1.019 (0.994-1.045) .13      | 1.019 (0.992-1.046) .17        |
| Tumor size\(^c\)        | 0.507 (0.301-0.853) .01      | 0.52 (0.305-0.888) .02         |
| Nodal status\(^d\)      | 0.45 (0.240-0.843) .01       | 0.477 (0.251-0.907) .02        |
| CD8+ TILs\(^e\)         | 2.17 (1.135-4.149) .02       | 2.542 (1.317-4.907) .005       |

#### 3.5  Comparison of tumor infiltrating CD8+ cells and CD8A expression

For 22 tumors, both CD8A values from the PEA analysis and data on CD8+ TILs were available. These values were compared and found to be correlated, although with a rather low correlation coefficient (\(r = 0.46\), \(P = .029\)) (Figure 4). The main discrepancies were some tumors with high CD8+ TILs counts, but with very low PEA values for CD8A. These discrepancies were possibly due to that the fresh frozen and FFPE biopsies were taken from different parts of the tumor.
The vast majority, 73%, of the proteins included in the Immuno-Oncology panel showed a significant upregulation in tumors vs normal tissue. This reflects both the increased activity of the immune defense in the tumors, as well as that the proteins included in the Immuno-Oncology panel were selected for being related to immune activity. The proteins with the strongest upregulation in the tumor tissue were mainly chemokines and cytokines, for example, CCL2-4, 7-8 and 20, CXCL9-11 and 13, and IL6 and 8. This expression pattern is very similar to the pattern earlier obtained for tonsillar and base of tongue cancer analyzed with the same Proseek panel.27 Also in a study on breast cancer, a similar expression pattern with upregulation of CCL2-4 and 7-8, CXCL 9-11, and IL-6 in cancer vs benign lesions was observed.30,31 Notably, upregulation of CCL2-4, 8, and CXCL9-11 and 13 are all part of a 12-chemokine gene expression signature associated with lymph node-like structures in melanoma, colorectal cancer, breast cancer and melanoma, and proposed to indicate a potential for immune therapy.32-34 High expression levels of CXCL9 and 10, as well as CCL20 were also observed, when cytokines and chemokines from supernatants in cell suspensions from HNSCC samples were analyzed.35

All HPV-positive tumors included in the Immuno-Oncology panel demonstrated a high immune activity in comparison to the average HPV-negative tumor (Figure 1C). This included both immune related surface proteins (CD8A, Fas ligand, PD-1, and PD-L1), and some chemokines and chemokines (eg, IL-12 and CXCL9-11). The high expression of CD8A, Fas ligand, and PD-1 indicates a high immune infiltration in the HPV-positive HSPCC, and is similar to results obtained earlier for HPV-positive vs HPV-negative OPSCC.27 IL-12 is involved in the priming and activation of T-cells, while CXCL9 and 10 are engaged in the trafficking of T-cells to tumors.36

The higher infiltration by CD8+ TILs in HPV-positive vs HPV-negative HSPCC, evaluated by IHC in the present study, further demonstrates a more active immune response in the latter tumors. This is in line with earlier studies showing a more active immune response in HPV-positive OPSCC, especially an increased number of CD8 + TILs, and this has been considered to be a major reason for the increased survival in patients with HPV-positive OPSCC.15-17,37-39 Two earlier studies on the HPS CC FFPE samples included in the present study have demonstrated a markedly favorable clinical outcome in patients with HPV-positive HSPCC, as compared to patients with HPV-negative HSPCC.10,11 Notably, all seven patients with HPV-positive HSPCC included in the present study survived without recurrence for more than 3 years.

The analysis of CD8+ TILs in HPV-negative HSPCC demonstrated that patients with HSPCC having the highest quartile of number of CD8+ TILs had significantly better PFS and OS independently of sex, age, and T or N classification. This indicates that the immune defense is of major importance for clinical outcome also for patients harboring HPV-negative HSPCC. Some earlier studies have evaluated CD8+ TILs or expression of PD-1 and PD-L1 in HSPCC, both in relation to survival, and in some cases also tumor HPV-status.20,21,23,24,40 Ono et al found a positive correlation between a high number of CD8+ TILs and survival in two studies.20,21 In addition, they found an increased survival for the combination low PD-L1 expression with high numbers of CD8+ TILs.21 Birtalan et al found a high PD-L1 expression on immune cells to be linked to an improved survival for patients with HPV-negative tumors. In contrast, de Ruither et al did not find a correlation between number of CD8+ TILs or tumor PD-L1 expression and survival.40 Also Schneider et al could not show a correlation between the expression of PD-L1 on the tumor cells and survival, although the numbers of PD-1 positive TILs did correlate to increased survival.24 They also did not find any difference in PD-1 expression on TILs and PD-L1 expression on tumor cells between HPV-positive and HPV-negative tumors. However, because they did not detect PD-1 on the tumor cells in their study, this indicated a difference to the higher PD-1 and PD-L1 expression in HPV-positive HSPCC found in the present study. A study indirectly confirming the relation between CD8+ TILs and survival in HPS CC is a study on the relation between the expression of B7-H3, CD8+ TILs, and
clinical outcome in HPSCC, by Katayama et al.\textsuperscript{25} High expression of B7-H3, an accessory co-inhibitor of T-cell responses, was shown to be correlated to worse prognosis and negatively correlated to high CD8+ TILs.

Nevertheless, it is of note that in the present study the immuno-oncology assay did not discriminate between protein expression on tumor and immune cells. Notably, in the studies by Birtalan et al and Schneider et al, HPSCC were in a minority, whereas the studies by Ono et al included only HPSCC. Also for OPSCC, an increased PD-1 and PD-L1 expression has also been demonstrated for HPV-positive vs HPV-negative tumors.\textsuperscript{35,41}

Although the increased survival for HPSCC patients with high numbers of CD8+ TILs confirms the results obtained by Ono et al, there is an important difference, as Ono et al did not evaluate tumor HPV-status, whereas here only HPV-negative tumors were included in the survival analysis.\textsuperscript{20,21} Despite the prevalence of HPV-positive HPSCC is low, as such tumors generally have both a higher number of CD8+ TILs and better survival, the inclusion of HPV-positive HPSCC in an analysis may have affected the interpretation, because it cannot be established if the increased survival was due to the possible presence of HPV-positive tumors. It was therefore important to evaluate this correlation separately for HPV-negative HPSCC, and confirm that the relation between high numbers of CD8+ TILs and survival was also the case for these tumors.

Also for head and neck cancers from other subsites, high numbers of CD8+ TILs have been found to be correlated to improve clinical outcome. For OPSCC, this has been demonstrated both for HPV-positive and HPV-negative tumors.\textsuperscript{15,16} In a meta-analysis on oral cancer, Huang et al showed that high numbers of CD8+ TILs, as well as CD45RO+ TILs and CD57+ TILs, were correlated to improve OS for oral squamous cell carcinoma.\textsuperscript{42} Also, for laryngeal squamous cell carcinoma, increased numbers of CD8+ TILs have been shown to be related to improve survival.\textsuperscript{43} However, it should be noted that in these latter studies tumors were not separated based on HPV status.

Intriguingly, when the immuno-oncology assay was used to evaluate expression of proteins significantly correlated to HPV-status in HPSCC (Figure 1C), a subgroup of six HPV-negative tumors showed a similar expression of proteins related to immune activity as the four included HPV-positive tumors. Although the patients with HPV-negative tumors in this small group did not survive, and three did not even obtain complete curative treatment, the data still indicate a much more active immune response in these tumors, possibly making them more responsive to different types of immune therapy, for example, with checkpoint inhibitors. This is further demonstrated in the evaluation of DSS. Although HPSCC patients with tumors with a high infiltration of CD8+ TILs have an increased survival, as shown for DSS in Figure 3B, around 30% in this group, excluding those dying from other causes, still succumb to the disease within 3 years and may benefit from treatment directed at improving the immune response. On the other hand, patients with low number of CD8+ TILs and a low probability of survival may benefit from additional treatment, for example, local surgery, which only a minority received in this cohort. Evaluation of the number of CD8+ TILs may thus serve as an aid for choice of treatment. However, further studies where clinical outcome is evaluated both in relation to treatment and number of CD8+ TILs are needed before this can be implemented in a clinical setting.

There are several limitations in the present study. The main limitation is the low number of HPV-positive tumors, both in the immuno-oncology assay and the evaluation of CD8+ TILs by IHC. Thus, the result presented here must be interpreted with caution. That few of the patients with HPV-negative tumors included in the immuno-oncology assay survived, precluded the possibility to evaluate this result in relation to survival. Another important limitation is that patients receiving different treatments have been evaluated together in the survival analysis, as the patient group was not large enough for a subgroup analysis based on different treatments. Thus, there may be differences in the relation between CD8 TILs and treatment outcome depending on the treatment received.

In conclusion, when comparing protein expression in HPSCC with normal tissue, the most prominent differences were found for chemo- and cytokines. HPV-positive HPSCC showed a clearly higher immune activity than corresponding HPV-negative tumors, similar to what has been observed earlier for HPV-positive OPSCC. In addition, high numbers of CD8+ TILs correlated to better clinical outcome for patients with HPV-negative HPSCC. Finally, some HPV-negative HPSCC had a high expression of immune-related proteins, but poor clinical outcome, and could be especially suitable for immune therapy, for example, with checkpoint inhibitors.

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