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Original Research

# Immune gene expression in head and neck squamous cell carcinoma patients

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**Abstract** ***Background:*** Nivolumab and pembrolizumab targeting programmed cell death protein 1 (PD-1) have recently been approved among patients with recurrent and/or metastatic head and neck squamous cell carcinoma (HNSCC) who failed platinum therapy. We aimed to

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carcinoma;
Immune checkpoints;
Prognostic biomarker;
Gene expression

evaluate the prognostic value of selected immune gene expression in HNSCC.
***Patients and methods:*** We retrospectively assessed the expression of 46 immune-related genes and immune-cell subpopulation genes including immune checkpoints by real-time polymerase chain reaction among 96 patients with HNSCC who underwent primary surgery at Institut Curie between 1990 and 2006. Univariate and multivariate analyses were performed to assess the prognostic value of dysregulated genes.
***Results:*** The Median age of the population was 56 years [range: 35–78]. Primary tumour location was oral cavity (45%), oropharynx (21%), larynx (18%) and hypopharynx (17%). Twelve patients (13%) had an oropharyngeal human papillomavirus–positive tumour. Most significantly overexpressed immune-related genes were *TNFRSF9/4*-1BB (77%), *IDO1* (75%), *TNFSF4/OX40L* (74%) and *TNFRSF18/GITR* (74%), and immune-cell subpopulation gene was *FOXP3* (62%). Eighty-five percent of tumours analysed overexpressed actionable immunity genes, including *PD-1/PD-L1*, *TIGIT*, *OX40/OX40L* and/or *CTLA4*. Among the immune-related genes, high *OX40L* mRNA level ($p = 0.0009$) and low *PD*-1 mRNA level ($p = 0.004$) were associated with the highest risk of recurrence. Among the immune-cell subpopulation genes, patients with high *PDGFRB* mRNA level ($p < 0.0001$) and low *CD3E* ($p = 0.0009$) or *CD8A* mRNA levels ($p = 0.004$) were also at the highest risk of recurrence.
***Conclusions:*** *OX40L and PDGFRB* overexpression was associated with poor outcomes, whereas *PD-1* overexpression was associated with good prognosis in patients with HNSCC treated with primary surgery, suggesting their relevance as potential prognostic biomarkers and major therapeutic targets.


## 1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the seventh cause of cancer with a yearly 40–50% mortality [1]. Classical risk factors for HNSCC include tobacco and alcohol consumption, as well as human papillomavirus (HPV) infection that has been demonstrated to have a prognostic impact [2].

The Cancer Genome Atlas reported the genomic landscape of more than 270 primary HNSCCs [3] with mutations in several oncogenes including *PIK3CA* (21%) and *HRAS* (4%), as well as in tumour suppressor genes including *TP53* (72%), *CDKN2A* (22%), *FBXW7* (5%), *KMT2D* (MLL2) (18%) and *PTEN* (2%) [3,4]. Genomic alterations involving the cell cycle (*TP53*, *CCND1*, *CDKN2A*), as well as *FGFR1* amplifications, and tumour genomic alterations burden were shown to be prognostic and potential therapeutic targets for patients with HNSCC [5]. No relevant biomarkers for tailored therapeutic strategies have been identified in HNSCC to date.

Beside surgery, radiotherapy and chemotherapy, HNSCC treatment includes targeted therapy and immunotherapy. Cetuximab, a monoclonal antibody that targets epidermal growth factor receptor (EGFR), has been the first targeted therapy approved in HNSCC, both in the locally advanced setting combined with radiotherapy [6] and in the first-line recurrent and/or metastatic setting in combination with chemotherapy [7]. Two anti–programmed cell death protein 1 (PD-1) immune checkpoint inhibitors have been approved for the treatment of recurrent and/or metastatic HNSCC refractory to platinum therapy in 2016 [8,9]. These agents are better tolerated than chemotherapy and demonstrated durable responses in a minority of patients [8,9].

At the tumour microenvironment (TME) level, the infiltration of HNSCC by innate and adaptive immune cells has been well documented. Several studies have identified immune cells with a prognostic value, such as CD8+ T cells, Foxp3+ regulatory T cells. The presence of tertiary lymphoid structures was also reported to affect prognosis [10–14]. OX40, PD-1 and CTLA4 were shown to have a significantly higher expression in T-cell subsets isolated from tumours of patients with HNSCC [15]. Few integrative studies reported the prognostic value of immune genes in HNSCC.

We aimed in this study to assess the expression of immune genes and to evaluate their prognostic value in patients with HNSCC who are untreated.

## 2. Patients and methods

### 2.1. Patients

We retrieved samples from patients with HNSCC who underwent upfront surgery at the Institut Curie between 1990 and 2006. We selected 96 patients with complete clinical, histological and biological data and long-term follow-up.

This study was approved by the internal review board of Institut Curie and was conducted in accordance with the ethics principles of the Declaration of Helsinki. In

accordance with the French regulations, all patients were informed that analyses were to be performed on the biological specimens obtained during their treatment, and they did not express their opposition.

### 2.2. Gene selection

Forty-six genes involved in the immune process were selected, including 30 genes defined as immune-related genes and 16 genes that were defined as immune-cell subpopulation genes (Supplementay Table 1). We chose *TBP* (Genbank accession number NM_003194) which encodes the TATA box–binding protein as an RNA control gene.

### 2.3. DNA sequencing and mutation assessment

Targeted DNA sequencing of a selection of 100 genes corresponding to the most frequently altered genes in HNSCC and potential therapeutic targets was performed on Illumina HiSeq2500 sequencer and then annotated in the COSMIC and 1000 genome databases [16], as described in the study by Dubot *et al* [5]. Tumour genomic alteration burden was assessed by summing the number of recurrent deleterious genomic alterations (single nucleotide nariation (SNV) + copy number variantion (CNV)) per sample. Sanger sequencing was also performed to confirm PIK3CA, KRAS, HRAS and NRAS mutations.

### 2.4. Human papillomavirus genotyping

HPV status was assessed at the Pathology Department of the Institut Curie. HPV typing was conducted using total DNA isolated from formalin-fixed tissue blocks. Real-time polymerase chain reaction (RT-PCR) was performed with Sybr® Green and specific primers for HPV16 and HPV18 using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA).

### 2.5. Real-time quantitative polymerase chain reaction

PCR consumables, RNA extraction, ctDNA synthesis and PCR reaction conditions were previously described in detail [17]. Primers are described in Supplementary Table 2. For each investigated gene, mRNA values $\geq 3$ were considered as overexpression and $\leq 0.33$ as underexpression. We previously used the same cut-off value for altered tumour gene expression [17].

### 2.6. Immunohistochemistry/OX40L protein expression

We performed immunohistochemistry (IHC) assay by using the OX40L (rabbit, 59036, 1/100, pH = 9; Cell Signaling Technology) antibody in a series of 20 HNSCCs among the 96 HNSCC patients, corresponding to 10 patients with high *OX40L* mRNA level and 10 with low *OX40L* mRNA level (Supplementary material and methods). We performed immunohistochemical study of the tumour microenvironment by OX40L immunostaining in tumour cells, stromal cancer-associated fibroblasts (CAFs) and mononuclear inflammatory cells (MICs). All quantifications were performed with blinding of 2 expert pathologists to patient status.

Table 1
Clinical, biological and pathological characteristics of the 96 patients with HNSCC in relation with disease-free interval (DFI).

| Characteristics | Patients (%) | Events[a] (%) | DFI[b] |
|---|---|---|---|
| Total | 96 (100) | 45 (47) | |
| Age | | | |
| <56 | 46 (48) | 20 (44) | 0.89 (NS) |
| ≥56 | 50 (52) | 25 (50) | |
| Gender | | | |
| Female | 19 (20) | 8 (42) | 0.71 (NS) |
| Male | 77 (80) | 37 (48) | |
| Alcohol[c] | | | |
| Yes | 50 (70) | 24 (48) | 0.17 (NS) |
| No | 21 (30) | 8 (38) | |
| Tobacco consumption[d] | | | |
| Yes | 58 (73) | 28 (48) | 0.075 (NS) |
| No | 22 (27) | 7 (32) | |
| HPV status | | | |
| Negative | 84 (87) | 42 (50) | 0.032 |
| Positive | 12 (13) | 3 (25) | |
| UICC stage | | | |
| Stage I | 10 (10) | 5 (50) | 0.68 (NS) |
| Stage II | 15 (16) | 6 (40) | |
| Stage III | 12 (13) | 4 (33) | |
| Stage IV | 59 (62) | 30 (51) | |
| Tumour location | | | |
| Oral cavity | 43 (45) | 22 (51) | 0.053 (NS) |
| Oropharynx | 20 (21) | 5 (25) | |
| Larynx | 17 (18) | 8 (47) | |
| Hypopharynx | 16 (17) | 10 (63) | |
| Oncogenes' mutational status[e] | | | |
| Not mutated | 78 (81) | 36 (46) | 0.079 (NS) |
| At least one mutated | 18 (19) | 9 (50) | |
| Number of molecular alterations[f] | | | |
| <3 | 56 (60) | 27 (48) | 0.082 (NS) |
| ≥3 | 37 (40) | 17 (46) | |

DFI: disease-free interval; NS: not significant; HNSCC: head and neck squamous cell carcinoma; UICC: Union for International Cancer Control; HPV: human papillomavirus.
[a] Events: locoregional and/or metastatic recurrence, second cancer.
[b] Log-rank test.
[c] Alcohol use was considered at 10 gr/day or more (i.e. alcohol unit). Information was available for 71 patients.
[d] Tobacco use was considered at 10 pack-years or more. Information was available for 80 patients.
[e] *PIK3CA*, *NRAS*, *HRAS* or *KRAS* oncogenes.
[f] Number of molecular alterations among a selection of 100 genes as previously determined in the study by Dubot *et al*. [5]. Information was available for 93 patients.

### 2.7. Statistical analyses

The clinicopathological features were tested for association with disease-free interval (DFI) by using the log-rank test. DFI was determined from the time of initial diagnosis to the time of the first event among locoregional recurrence, metastatic recurrence or second cancer. The

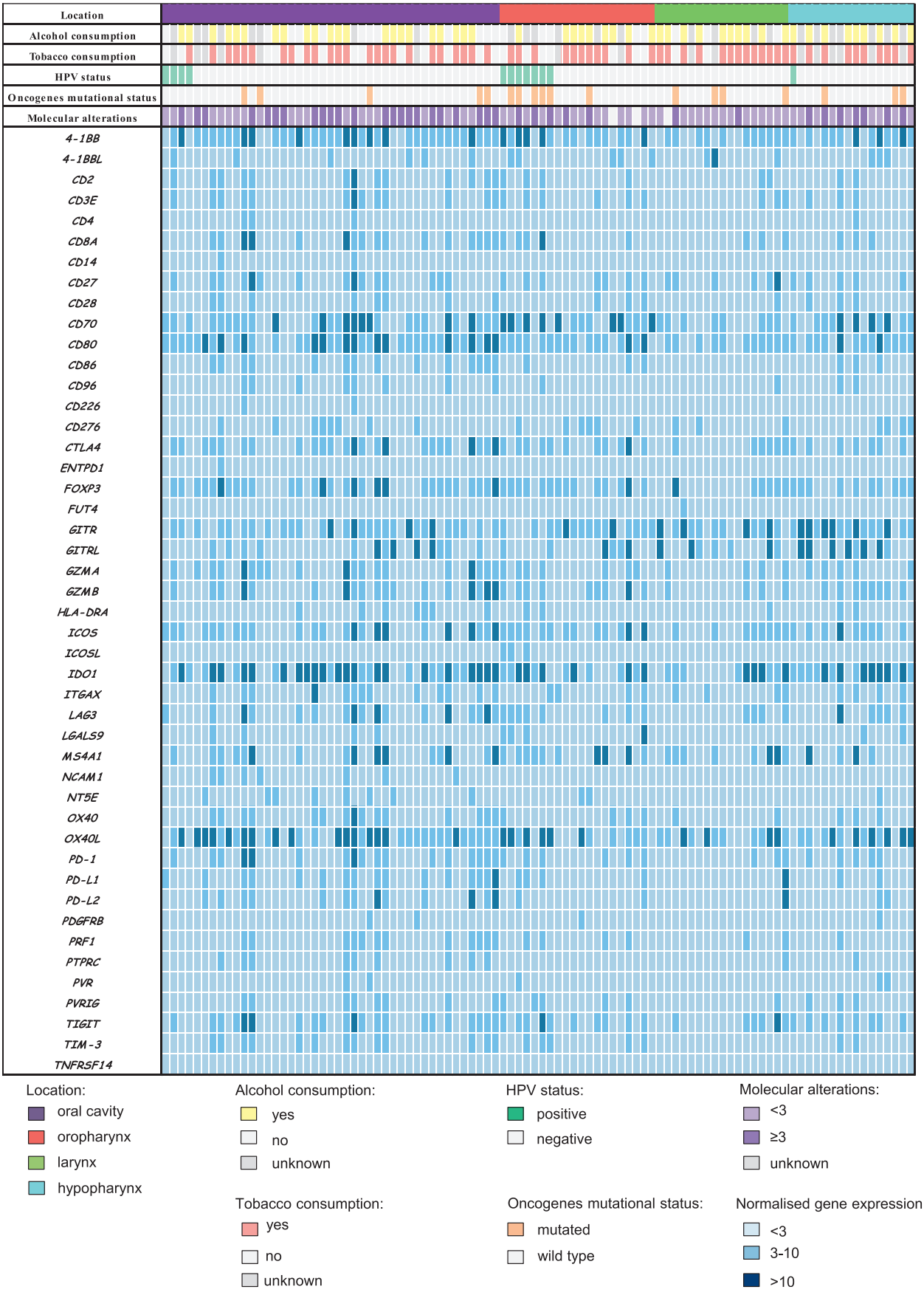



Fig. 1. Gene expression versus molecular alterations and clinical patient characteristics. HPV, human papillomavirus.

Table 2
mRNA expression of 30 immune-related genes relative to normal tissue mRNA level.

| Gene | Protein | Alias | Head and neck normal tissue[a] | Head and neck squamous cell carcinomas | p-value[b] | % under expression | % normal expression | % over expression |
|---|---|---|---|---|---|---|---|---|
| | | | n = 27 | n = 96 | | | | |
| HLA-DRA | Ligand | | 1.0 (0.01–5.87) | 0.74 (0.01–9.95) | 0.91 (NS) | 41% | 47% | 13% |
| LAG3 | Receptor | | 1.0 (0.29–4.96) | 2.01 (0.2–16.7) | 0.001 | 6% | 58% | 35% |
| PVR | Ligand | CD155 | 1.0 (0.66–3.93) | 1.22 (0.47–4.78) | 0.28 (NS) | 0% | 95% | 5% |
| PVRIG | Receptor | CD112R | 1.0 (0.23–7.45) | 1.45 (0.17–8.13) | 0.24 (NS) | 5% | 77% | 18% |
| TIGIT | Receptor | | 1.0 (0.04–9.66) | 2.77 (0.09–23.4) | <0.0001 | 2% | 51% | 47% |
| CD96 | Receptor | | 1.0 (0.14–4.74) | 1.12 (0.12–7.28) | 0.59 (NS) | 8% | 79% | 13% |
| CD226 | Receptor | | 1.0 (0.49–4.16) | 0.85 (0.08–4.3) | 0.05 (NS) | 10% | 88% | 2% |
| TNFSF9 | Ligand | CD137L, 4-1BBL | 1.0 (0.17–5.4) | 1.54 (0.14–10.1) | 0.011 | 4% | 78% | 18% |
| TNFRSF9 | Receptor | CD137, 4-1BB | 1.0 (0.23–8.39) | 5.05 (0.61–29.9) | <0.0001 | 0% | 23% | 77% |
| TNFSF18 | Ligand | GITRL | 1.0 (0–36.1) | 1.81 (0.05–58.4) | 0.063 (NS) | 9% | 55% | 35% |
| TNFRSF18 | Receptor | GITR | 1.0 (0.02–4.21) | 4.13 (0.57–17.4) | <0.0001 | 0% | 26% | 74% |
| ICOSLG | Ligand | ICOSL, B7H2 | 1.0 (0.46–2.52) | 0.64 (0.1–7.98) | 0.0003 | 8% | 89% | 3% |
| ICOS | Receptor | | 1.0 (0.11–6.99) | 3.45 (0.27–24.6) | <0.0001 | 1% | 44% | 55% |
| OX40L | Ligand | TNFSF4, CD134L, CD252 | 1.0 (0–9.94) | 6.52 (0.24–48.0) | <0.0001 | 1% | 25% | 74% |
| OX40 | Receptor | TNFRSF4, CD134 | 1.0 (0.29–2.73) | 2.12 (0.17–13.0) | <0.0001 | 1% | 74% | 25% |
| CD70 | Ligand | TNFSF7 | 1.0 (0.25–4.32) | 4.21 (0.22–92.0) | <0.0001 | 1% | 34% | 65% |
| CD27 | Receptor | TNFRSF7 | 1.0 (0.05–18.6) | 1.92 (0.04–14.3) | 0.11 (NS) | 7% | 58% | 34% |
| TIM-3 | Ligand | HAVCR2 | 1.0 (0.35–2.03) | 1.57 (0.24–8.3) | 0.0006 | 1% | 78% | 21% |
| LGALS9 | Receptor | GALECTINE-9 | 1.0 (0.34–3.02) | 1.21 (0.1–10.2) | 0.31 (NS) | 5% | 84% | 10% |
| PD-L1 | Ligand | CD274 | 1.0 (0.31–2.79) | 1.26 (0.16–38.7) | 0.056 (NS) | 6% | 70% | 24% |
| PD-L2 | Ligand | PDCD1LG2 | 1.0 (0.29–2.75) | 1.45 (0.27–13.6) | 0.035 | 4% | 77% | 19% |
| PD-1 | Receptor | PDCD1, CD279 | 1.0 (0.22–8.09) | 1.97 (0.11–14.2) | 0.019 | 6% | 62% | 32% |
| ENTPD1 | Ligand | CD39 | 1.0 (0.42–2.48) | 1.02 (0.2–3.26) | 0.93 (NS) | 2% | 97% | 1% |
| IDO1 | Ligand | | 1.0 (0.09–8.68) | 6.41 (0.14–184.4) | <0.0001 | 2% | 23% | 75% |
| NT5E | Ligand | CD73 | 1.0 (0.32–2.35) | 1.03 (0.06–6.35) | 0.79 (NS) | 6% | 82% | 12% |
| TNFRSF14 | Receptor | HVEM | 1.0 (0.42–1.6) | 0.61 (0.07–1.75) | 0.0006 | 18% | 82% | 0% |
| CD276 | Receptor | B7H3 | 1.0 (0.34–1.79) | 2.05 (0.26–7.61) | <0.0001 | 1% | 77% | 22% |
| CD80 | Ligand | B7-1 | 1.0 (0.16–5.5) | 5.35 (0.67–23.0) | <0.0001 | 0% | 20% | 80% |
| CD86 | Ligand | B7-2 | 1.0 (0.37–2.61) | 1.81 (0.27–5.98) | <0.0001 | 1% | 78% | 21% |
| CD28 | Receptor | | 1.0 (0.31–6.49) | 1.32 (0.1–8.16) | 0.36 (NS) | 5% | 79% | 16% |

[a] Median (range) of gene mRNA levels; the mRNA values of the samples were normalised such that the median of the 27 head and neck normal tissues mRNA values was equal to 1.
[b] Kruskal-Wallis H test.

clinicopathological and biological characteristics were tested for association with transcript-level expression by using chi-square tests for categorical variables. The association between clinical variables and RNA levels was tested using Kruskal-Wallis H tests. Cox proportional hazard regression was used to estimate hazard ratio (HR) and their 95% confidence intervals (95% CI) for covariates associated with DFI, showing significance at $p < 0.1$ on univariate analysis. Differences between two populations were judged significant at confidence levels greater than 95% ($p < 0.05$) [18]. Unsupervised hierarchical cluster analyses were performed using Morpheus algorithm to identify homogenous genes and tumour groups regarding molecular data.

## 3. Results

### 3.1. Patient characteristics

The characteristics of the 96 patients with untreated HNSCC are listed in Table 1. The median age was 56 years [range: 35–78]. Most patients were male with tobacco and alcohol consumption. Twelve patients (13%) were HPV-positive, with a majority with oropharyngeal cancer. Pathological staging showed a high proportion of stage IV. The main tumour location was the oral cavity (45%), followed by the oropharynx (21%), larynx (18%) and hypopharynx (17%). Most patients had less than three tumour genomic alterations as previously determined [5]. HPV infection was the only characteristic that significantly impacted DFI ($p = 0.032$, log-rank test), with a higher DFI reported for HPV-positive patients.

### 3.2. mRNA expression of immune genes

Fig. 1 illustrates mRNA expression of the 46 immune genes according to clinical and molecular characteristics of the 96 patients with HNSCC.

#### 3.2.1. mRNA expression of the immune-related genes

Among the 30 immune-related genes analysed, 18 genes were significantly deregulated in HNSCC tumours as

Table 3
mRNA expression of 30 immune-related genes in HNSCC according to HPV status and the number of molecular alterations.

| Gene | Protein | Alias | HPV- versus HPV+ | | | Not mutated versus mutated oncogenes | | | Number of molecular alterations <3 versus ≥3 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | HPV−[a] | HPV+ | p-value[b] | Not mutated | Mutated | p-value | <3 | ≥3 | p-value |
| | | | n = 84 | n = 12 | | n = 78 | n = 18 | | n = 56 | n = 37 | |
| HLA-DRA | Ligand | | 0.75 (0.01−9.95) | 0.15 (0.02−6.14) | 0.69 (NS) | 0.73 (0.01−6.63) | 1.11 (0.03−9.95) | 0.59 (NS) | 0.78 (0.02−9.95) | 0.65 (0.01−4.69) | 0.19 (NS) |
| LAG3 | Receptor | | 1.88 (0.2−16.7) | 3.04 (0.71−9.02) | 0.23 (NS) | 2.03 (0.20−15.6) | 1.78 (0.77−16.7) | 0.48 (NS) | 2.79 (0.20−15.6) | 1.20 (0.24−16.7) | <0.0001 |
| PVR | Ligand | CD155 | 1.27 (0.53−4.78) | 1.02 (0.47−3.51) | 0.41 (NS) | 1.20 (0.51−4.78) | 1.25 (0.47−4.33) | 0.38 (NS) | 1.32 (0.47−4.33) | 1.07 (0.53−4.78) | 0.29 (NS) |
| PVRIG | Receptor | CD112R | 1.6 (0.17−8.13) | 2.47 (0.47−6.39) | 0.047 | 1.31 (0.17−8.13) | 1.80 (0.49−6.27) | 0.14 (NS) | 1.93 (0.38−8.13) | 0.91 (0.17−5.39) | <0.0001 |
| TIGIT | Receptor | | 2.71 (0.09−23.4) | 4.07 (1.09−10.7) | 0.23 (NS) | 2.59 (0.09−23.4) | 4.01 (0.67−13.1) | 0.13 (NS) | 3.95 (0.67−23.4) | 1.83 (0.09−13.1) | <0.0001 |
| CD96 | Receptor | | 1.06 (0.12−7.28) | 1.79 (0.30−3.85) | 0.27 (NS) | 1.04 (0.12−7.09) | 1.28 (0.35−7.28) | 0.27 (NS) | 1.64 (0.30−7.09) | 0.79 (0.12−7.28) | <0.0001 |
| CD226 | Receptor | | 0.83 (0.08−4.30) | 1.03 (0.32−2.61) | 0.43 (NS) | 0.76 (0.08−3.95) | 0.88 (0.37−4.30) | 0.20 (NS) | 1.24 (0.16−3.95) | 0.49 (0.08−4.30) | <0.0001 |
| TNFSF9 | Ligand | CD137L | 1.54 (0.14−10.1) | 1.35 (0.69−3.78) | 0.71 (NS) | 1.60 (0.14−6.27) | 1.40 (0.40−10.1) | 0.63 (NS) | 1.56 (0.20−5.13) | 1.52 (0.14−10.1) | 0.80 (NS) |
| TNFRSF9 | Receptor | CD137 | 5.05 (0.61−25.2) | 6.08 (2.32−29.9) | 0.26 (NS) | 4.83 (0.61−29.9) | 7.21 (1.33−19.3) | 0.15 (NS) | 6.43 (1.33−29.9) | 4.39 (0.61−18.8) | 0.005 |
| TNFSF18 | Ligand | GITRL | 2.030.05−58.4) | 1.33 (0.33−6.46) | 0.36 (NS) | 1.85 (0.16−58.4) | 1.49 (0.05−6.34) | 0.51 (NS) | 1.73 (0.05−58.4) | 2.44 (0.18−57.8) | 0.74 (NS) |
| TNFRSF18 | Receptor | GITR | 4.26 (0.57−17.4) | 2.99 (0.89−9.66) | 0.0097 | 4.23 (0.57−17.4) | 3.74 (0.72−11.0) | 0.16 (NS) | 3.70 (0.57−17.4) | 6.80 (0.58−14.8) | 0.002 |
| ICOSLG | Ligand | ICOSL | 0.61 (0.10−2.01) | 0.82 (0.33−7.98) | 0.10 (NS) | 0.57 (0.10−7.98) | 0.88 (0.26−7.15) | 0.014 | 0.73 (0.3−7.98) | 0.54 (0.10−1.46) | 0.079 (NS) |
| ICOS | Receptor | | 3.13 (0.27−24.6) | 4.45 (2.05−7.77) | 0.27 (NS) | 3.40 (0.27−24.6) | 4.03 (0.37−9.28) | 0.45 (NS) | 4.49 (0.37−24.6) | 2.13 (0.27−9.28) | <0.0001 |
| OX40L | Ligand | TNFSF4 | 6.52 (0.24−48.0) | 9.35 (0.43−18.1) | 0.91 (NS) | 5.85 (0.24−39.5) | 7.39 (1.58−48.0) | 0.40 (NS) | 7.94 (0.39−40.0) | 5.45 (0.24−48.0) | 0.32 (NS) |
| OX40 | Receptor | TNFRSF4 | 2.12 (0.17−13.0) | 2.09 (0.94−4.67) | 0.54 (NS) | 2.12 (0.17−13.0) | 2.27 (0.94−4.40) | 0.99 (NS) | 2.23 (0.57−13.0) | 1.70 (0.17−4.65) | 0.011 |
| CD70 | Ligand | TNFSF7 | 4.10 (0.22−88.2) | 5.85 (0.87−92.0) | 0.34 (NS) | 4.41 (0.22−88.2) | 3.93 (0.66−91.4) | 0.45 (NS) | 4.31 (0.66−92.0) | 4.07 (0.22−68.4) | 0.23 (NS) |
| CD27 | Receptor | TNFRSF7 | 1.9 (0.04−14.3) | 2.79 (0.49−5.75) | 0.45 (NS) | 1.82 (0.04−14.3) | 2.64 (0.13−5.75) | 0.46 (NS) | 2.79 (0.13−14.3) | 0.93 (0.04−7.54) | <0.0001 |
| TIM-3 | Ligand | HAVCR2 | 1.56 (0.24−8.30) | 1.87 (0.71−3.49) | 0.89 (NS) | 1.52 (0.24−5.63) | 2.02 (0.89−8.30) | 0.13 (NS) | 2.01 (0.70−5.63) | 1.16 (0.24−8.30) | <0.0001 |
| LGALS9 | Receptor | GALECTINE-9 | 1.19 (0.10−10.20) | 1.86 (0.26−5.38) | 0.15 (NS) | 1.22 (0.10−10.2) | 1.19 (0.59−5.04) | 0.16 (NS) | 1.38 (0.26−6.85) | 0.89 (0.10−5.04) | 0.003 |
| PD-L1 | Ligand | CD274 | 1.17 (0.16−38.7) | 1.77 (0.47−8.83) | 0.21 (NS) | 1.17 (0.16−10.9) | 1.72 (0.47−38.7) | 0.14 (NS) | 1.72 (0.23−38.7) | 0.74 (0.16−9.02) | <0.0001 |
| PD-L2 | Ligand | PDCD1LG2 | 1.42 (0.27−13.6) | 1.51 (0.29−6.27) | 0.94 (NS) | 1.41 (0.27−13.6) | 1.84 (0.29−10.2) | 0.41 (NS) | 1.79 (0.29−13.6) | 1.17 (0.27−4.81) | 0.003 |
| PD-1 | Receptor | PDCD1 | 1.92 (−0.11−14.2) | 2.60 (0.74−6.34) | 0.23 (NS) | 1.92 (0.11−12.2) | 2.18 (0.85−14.2) | 0.17 (NS) | 2.67 (0.27−12.2) | 0.97 (0.11−14.2) | <0.0001 |
| ENTPD1 | Ligand | CD39 | 1.02 (0.20−3.26) | 0.76 (0.41−1.83) | 0.091 (NS) | 1.02 (0.20−3.26) | 1.03 (0.45−1.93) | 0.77 (NS) | 1.17 (0.41−3.26) | 0.76 (0.20−2.13) | 0.001 |
| IDO1 | Ligand | | 6.41 (0.14−184.4) | 6.21 (1.70−128.3) | 0.84 (NS) | 6.27 (0.14−138.9) | 7.83 (1.34−184.4) | 0.33 (NS) | 8.35 (0.14−128.3) | 4.45 (0.15−184.4) | 0.035 |
| NT5E | Ligand | CD73 | 1.15 (0.18−6.35) | 0.44 (0.06−2.87) | 0.008 | 1.15 (0.18−6.35) | 0.89 (0.06−4.95) | 0.34 (NS) | 1.16 (0.06−6.35) | 0.99 (0.18−5.10) | 0.96 (NS) |
| TNFRSF14 | Receptor | HVEM | 0.61 (0.07−1.75) | 0.66 (0.21−1.27) | 0.68 (NS) | 0.59 (0.07−1.53) | 0.70 (0.31−1.75) | 0.12 (NS) | 0.71 (0.21−1.53) | 0.45 (0.07−1.75) | <0.0001 |
| CD276 | Receptor | B7H3 | 2.15 (0.26−7.61) | 1.25 (0.59−4.81) | 0.005 | 2.07 (0.26−7.61) | 1.91 (0.59−4.81) | 0.72 (NS) | 1.99 (0.59−4.81) | 2.13 (0.26−7.61) | 0.22 (NS) |
| CD80 | Ligand | B7-1 | 5.47 (0.67−23.0) | 4.68 (1.99−9.38) | 0.59 (NS) | 5.23 (0.67−23.0) | 7.06 (1.28−16.1) | 0.55 (NS) | 6.20 (1.28−23.0) | 3.66 (0.67−16.0) | 0.002 |
| CD86 | Ligand | B7-2 | 1.76 (0.27−5.98) | 2.05 (0.77−3.09) | 0.98 (NS) | 1.71 (0.27−5.98) | 2.26 (0.77−5.09) | 0.46 (NS) | 2.29 (0.68−5.98) | 1.36 (0.27−4.56) | 0.001 |
| CD28 | Receptor | | 1.23 (0.10−8.16) | 1.72 (0.59−2.92) | 0.47 (NS) | 1.17 (0.10−8.16) | 1.54 (0.58−2.92) | 0.84 (NS) | 1.85 (0.48−8.16) | 0.90 (0.10−5.18) | <0.0001 |

[a] Median (range) of gene mRNA levels; the mRNA values of the samples were normalised such that the median of the 27 head and neck normal tissues mRNA values was equal to 1.
[b] Kruskal-Wallis H Test.

compared with normal head and neck tissue ($p < 0.05$), all being overexpressed except *ICOSLG* and *TNFRSF14* (Table 2). Seven genes were overexpressed in more than 50% of tumours (*4*-1BB, *GITR*, *ICOS*, *OX40L*, *CD70*, *IDO1* and *CD80*). *OX40L* had the highest mRNA level with a median of 6.52-fold and was overexpressed in 74% of tumours. Expression of *PVRIG* was significantly higher in HPV-positive tumours, whereas *GITR*, *NT5E* and *CD276* expressions were significantly lower in HPV-positive tumours (Table 3). All immune-related genes except *ICOSLG* showed no modification in the expression profile in patients with HNSCC who harboured oncogene mutations (*NRAS*, *HRAS*, *KRAS or PIK3CA*) as compared with those with no mutation. Regarding tumour genomic alteration burden, 20 of the 30 immune-related genes (67%) were significantly overexpressed in tumours with less than three tumour genomic alterations except for *GITR*, which showed a lower mRNA level (Table 3). Unsupervised hierarchical clustering analyses of 96 HNSCC samples with the 30 immune-related genes showed that the majority of genes coding a receptor protein clustered together as compared with genes coding a ligand protein (Supplementary Fig. 1).

#### 3.2.2. mRNA expression of the immune-cell subpopulation genes

Among the 16 immune-cell subpopulation genes analysed, 7 genes (44%) were significantly deregulated in HNSCC tumours as compared with the normal head and neck tissue ($p < 0.05$). *FOXP3* was overexpressed, while only *NCAM1* was underexpressed in more than 50% of the tumours as compared with the normal head and neck tissue (Table 4).

Expressions of *PDGFRB* and *NCAM1* were significantly different according to HPV status (Table 5). All immune-cell subpopulation genes except *PDGFRB* and *FUT4* were significantly underexpressed in HNSCC with more than three tumour genomic alterations.

A similar unsupervised hierarchical clustering analysis of 96 HNSCC samples with the 16 immune-cell subpopulation genes showed that the lymphocyte-specific genes also clustered together (Supplementary Fig. 2).

### 3.3. OX40L protein expression by immunochemistry

By IHC, the OX40L protein was expressed in epithelial cancer cells for most tumour samples (H Score: 1–2.5) with a predominantly cytoplasmic location in various populations of the TME, including MICs, fibroblasts and muscle (Supplementary Fig. 3).

### 3.4. Prognostic value of immune gene expression

Twelve of the 30 immune-related genes (40%) were associated with a short DFI in univariate analysis, including four genes with a high mRNA level and eight genes with a low mRNA level (Supplementary Table 3). High *OX40L* mRNA level ($p = 0.0009$) and low *PD*-1 mRNA levels ($p = 0.004$) were associated with the highest risk of recurrence (Fig. 2).

Table 4
mRNA expression of 16 immune-cell subpopulation genes relative to normal tissue mRNA level.

| Gene | Cellular specificity | Alias | Head and neck normal tissue[a] | Head and neck squamous cell carcinoma | p-value[b] | % under expression | % normal expression | % over expression |
|---|---|---|---|---|---|---|---|---|
| | | | n = 27 | n = 96 | | | | |
| ITGAX | Dendritic cells | | 1.0 (0.22–3.23) | 2.04 (0.52–13.0) | <0.0001 | 0% | 74% | 26% |
| PDGFRB | Fibroblast | | 1.0 (0.22–5.89) | 1.1 (0.17–5.58) | 0.87 (NS) | 3% | 93% | 4% |
| PTPRC | Haematopoietic cells | CD45 | 1.0 (0.39–6.41) | 1.24 (0–6.1) | 0.63 (NS) | 5% | 83% | 12% |
| MS4A1 | LB | CD20 | 1.0 (0.06–161.9) | 1.57 (0.02–51.2) | 0.14 (NS) | 22% | 39% | 40% |
| CTLA4 | LT | CD152 | 1.0 (0.03–5.05) | 3 (0.21–15.1) | <0.0001 | 1% | 49% | 50% |
| PRF1 | LT | | 1.0 (0.43–3.34) | 1.35 (0.12–8.62) | 0.13 (NS) | 8% | 72% | 20% |
| CD3E | LT | | 1.0 (0.26–7.59) | 1.58 (0.13–11.2) | 0.18 (NS) | 6% | 68% | 26% |
| CD2 | LT | | 1.0 (0.19–6.73) | 1.62 (0.14–13.2) | 0.20 (NS) | 8% | 70% | 22% |
| FOXP3 | LTc | | 1.0 (0–5.16) | 3.98 (0.33–18.2) | <0.0001 | 0% | 39% | 62% |
| CD8A | LTc | | 1.0 (0.21–6.54) | 1.46 (0.08–17.4) | 0.65 (NS) | 10% | 62% | 28% |
| CD4 | LT helper | | 1.0 (0.56–2.71) | 1.04 (0.11–5.33) | 0.62 (NS) | 6% | 90% | 4% |
| GZMA | LT/NK | | 1.0 (0.35–6.15) | 2.16 (0.11–18.9) | 0.011 | 8% | 55% | 37% |
| GZMB | LT/NK | | 1.0 (0.2–18.4) | 2.16 (0.09–22.2) | 0.0002 | 2% | 57% | 41% |
| NCAM1 | NK | CD56 | 1.0 (0.1–7.6) | 0.15 (0.01–7.56) | <0.0001 | 66% | 27% | 7% |
| CD14 | Macrophages/monocytes | | 1.0 (0.39–3.54) | 1.17 (0.17–3.41) | 0.19 (NS) | 3% | 95% | 2% |
| FUT4 | Neutrophils | CD15 | 1.0 (0.35–4.18) | 0.7 (0.13–3.37) | 0.002 | 12% | 88% | 1% |

LT: T cell; LB: B cell; NK: natural killer, LTc: cytotoxic T lymphocyte.
[a] Median (range) of gene mRNA levels; the mRNA values of the samples were normalised such that the median of the 27 head and neck normal tissues mRNA values was equal to 1.
[b] Kruskal-Wallis H Test.

Table 5
mRNA expression of immune-cell subpopulation genes in HNSCC according to HPV status and the number of molecular alterations.

| Gene | Cellular specificity | Alias | HPV- verus HPV+ (84 versus 12) | | | Not mutated versus mutated oncogenes (78 versus 18) | | | Total number of alterations <3 versus ≥3 (56 vs 37) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | HPV-[a] | HPV+ | p-value[b] | Not mutated | Mutated | p-value | <3 | ≥3 | p-value |
| ITGAX | Dendritic cells | | 2.14 (0.52−13.0) | 1.77 (0.55−6.03) | 0.22 (NS) | 1.98 (0.52−13.0) | 2.20 (0.55−6.64) | 0.33 (NS) | 2.35 (0.55−13.0) | 1.61 (0.52−6.25) | 0.002 |
| PDGFRB | Fibroblast | | 1.19 (0.21−5.58) | 0.64 (0.17−1.73) | 0.007 | 1.13 (0.21−3.55) | 1.07 (0.17−5.58) | 0.62 (NS) | 1.19 (0.17−5.58) | 0.99 (0.21−3.31) | 0.41 (NS) |
| PTPRC | Haematopoietic cells | CD45 | 1.15 (0.00−6.10) | 1.49 (0.59−2.91) | 0.53 (NS) | 1.14 (0.00−6.10) | 1.44 (0.50−3.94) | 0.21 (NS) | 1.58 (0.00−6.10) | 0.74 (0.11−4.96) | <0.0001 |
| MS4A1 | LB | CD20 | 1.50 (0.02−51.2) | 2.81 (0.10−9.06) | 0.80 (NS) | 1.43 (0.02−51.2) | 2.05 (0.07−8.55) | 0.99 (NS) | 3.18 (0.04−42.1) | 0.48 (0.02−51.2) | 0.0002 |
| CTLA4 | LT | CD152 | 2.85 (0.21−15.1) | 3.83 (1.79−5.15) | 0.48 (NS) | 3.00 (0.21−15.1) | 3.20 (0.48−9.05) | 0.68 (NS) | 3.86 (0.48−15.1) | 1.97 (0.21−7.91) | 0.0002 |
| PRF1 | LT | | 1.35 (0.12−8.62) | 1.31 (0.58−4.87) | 0.98 (NS) | 1.32 (0.12−7.67) | 1.57 (0.40−8.62) | 0.14 (NS) | 1.57 (0.24−8.46) | 1.01 (0.12−8.62) | 0.002 |
| CD3E | LT | | 1.35 (0.13−11.2) | 2.33 (0.62−6.84) | 0.092 (NS) | 1.35 (0.13−11.2) | 2.07 (0.29−7.10) | 0.24 (NS) | 2.30 (0.29−11.2) | 0.90 (0.13−7.10) | <0.0001 |
| CD2 | LT | | 1.44 (0.14−13.2) | 2.21 (0.61−6.78) | 0.11 (NS) | 1.44 (0.14−13.2) | 1.87 (0.27−8.55) | 0.26 (NS) | 2.34 (0.27−13.2) | 0.86 (0.14−8.55) | <0.0001 |
| FOXP3 | LTc | | 3.74 (0.33−18.2) | 5.42 (1.83−8.76) | 0.26 (NS) | 3.97 (0.33−18.2) | 4.53 (1.08−10.5) | 0.63 (NS) | 5.13 (1.08−18.2) | 2.73 (0.33−10.5) | <0.0001 |
| CD8A | LTc | | 1.38 (0.08−17.4) | 1.98 (0.29−10.5) | 0.21 (NS) | 1.33 (0.08−11.8) | 1.98 (0.38−17.4) | 0.062 (NS) | 2.34 (0.16−11.8) | 0.72 (0.08−17.4) | <0.0001 |
| CD4 | LT helper | | 1.04 (0.11−5.33) | 1.25 (0.50−2.46) | 0.30 (NS) | 1.04 (0.11−5.33) | 1.05 (0.48−4.16) | 0.50 (NS) | 1.23 (0.37−5.33) | 0.66 (0.11−4.16) | <0.0001 |
| GZMA | LT/NK | | 2.16 (0.11−19.0) | 2.16 (0.50−7.85) | 0.60 (NS) | 2.11 (0.11−18.9) | 2.16 (0.32−16.7) | 0.44 (NS) | 2.55 (0.29−18.9) | 1.40 (0.11−16.7) | 0.003 |
| GZMB | LT/NK | | 2.16 (0.09−22.2) | 2.22 (0.57−8.70) | 0.65 (NS) | 2.16 (0.09−22.2) | 2.73 (0.38−14.0) | 0.52 (NS) | 2.38 (0.09−22.2) | 1.65 (0.24−14.0) | 0.016 |
| NCAM1 | NK | CD56 | 0.19 (0.01−7.56) | 0.05 (0.03−1.13) | 0.033 | 0.16 (0.01−7.56) | 0.12 (0.03−3.12) | 0.75 (NS) | 0.19 (0.01−7.56) | 0.12 (0.01−3.01) | 0.048 |
| CD14 | Macrophages/monocytes | | 1.18 (0.17−3.41) | 1.10 (0.46−1.70) | 0.23 (NS) | 1.12 (0.17−3.41) | 1.29 (0.46−2.65) | 0.34 (NS) | 1.33 (0.40−3.41) | 0.86 (0.17−2.06) | 0.002 |
| FUT4 | Neutrophils | CD15 | 0.71 (0.13−3.37) | 0.57 (0.29−2.66) | 0.34 (NS) | 0.70 (0.13−3.37) | 0.85 (0.37−2.66) | 0.16 (NS) | 0.76 (0.24−2.66) | 0.64 (0.13−3.37) | 0.098 (NS) |

LT: T cell; LB: B cell; NK: natural killer, LTc: cytotoxic T lymphocyte.

[a] Median (range) of gene mRNA levels; the mRNA values of the samples were normalised such that the median of the 27 head and neck normal tissues mRNA values was equal to 1.

[b] Kruskal-Wallis H Test.

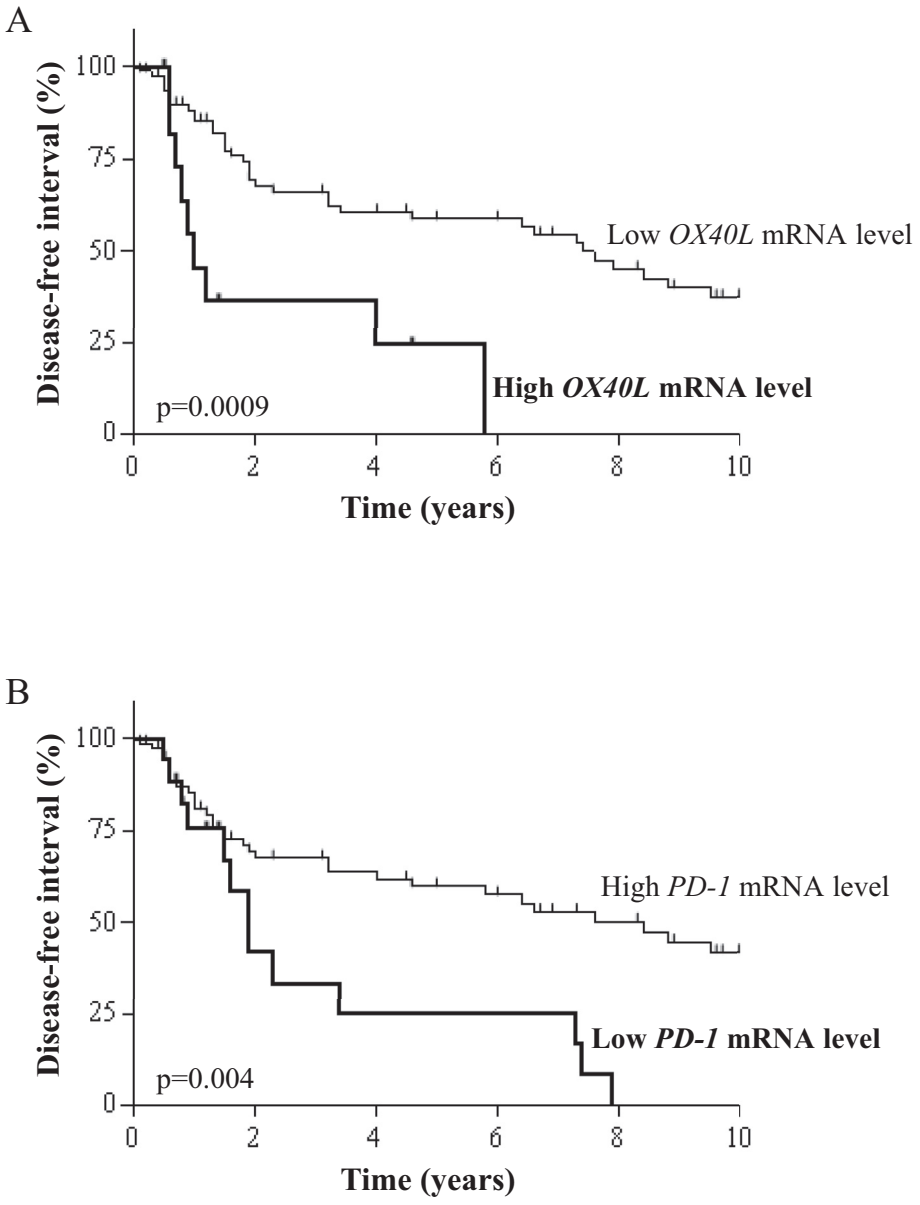



Fig. 2. Relationship between disease-free interval and (A) *OX40L* and (B) *PD*-1 mRNA level.

Eight of the 16 immune-cell subpopulation genes (50%) were associated with a short DFI, including two genes with a high mRNA level and six genes with a low mRNA level (Supplementary Table 4). High *PDGFRB* mRNA level ($p < 0.0001$) and low *CD3E* or *CD8A* mRNA levels ($p = 0.0009$ and $p = 0.004$, respectively) were associated with the highest risk of recurrence (Fig. 3).

*OX40L*, *PD-1*, *PDGFRB*, *CD3E* and *CD8A* immune genes were significantly associated with a short DFI (*OX40L*: $p = 0.005$, *PD-1*: $p = 0.019$, *PDGFRB*: $p = 0.0004$, *CD3E*: $p = 0.011$, and *CD8A*: $p = 0.016$) (Supplementary Table 5) in a multivariate analysis, taking into account all clinical parameters associated with a short DFI with a p-value <0.1 (Table 1).

### 3.5. Expression of actionable immune genes

*PD-1*/*PD-L1*, *TIGIT*, *OX40*/*OX40L* and *CTLA4* are currently major actionable genes in the context of immunotherapy strategies. Among the 96 tumours analysed, 82 tumours (85%) overexpressed at least one of these six genes. Thirty-three tumours (34%) simultaneously overexpressed *TIGIT*, *CTLA4*, *PD-1*/*PD-L1* and *OX40*/*OX40L*. No tumour exclusively overexpressed *PD-1* or *OX40*. *PD-L1* was exclusively overexpressed in only one sample (1%). *TIGIT* was

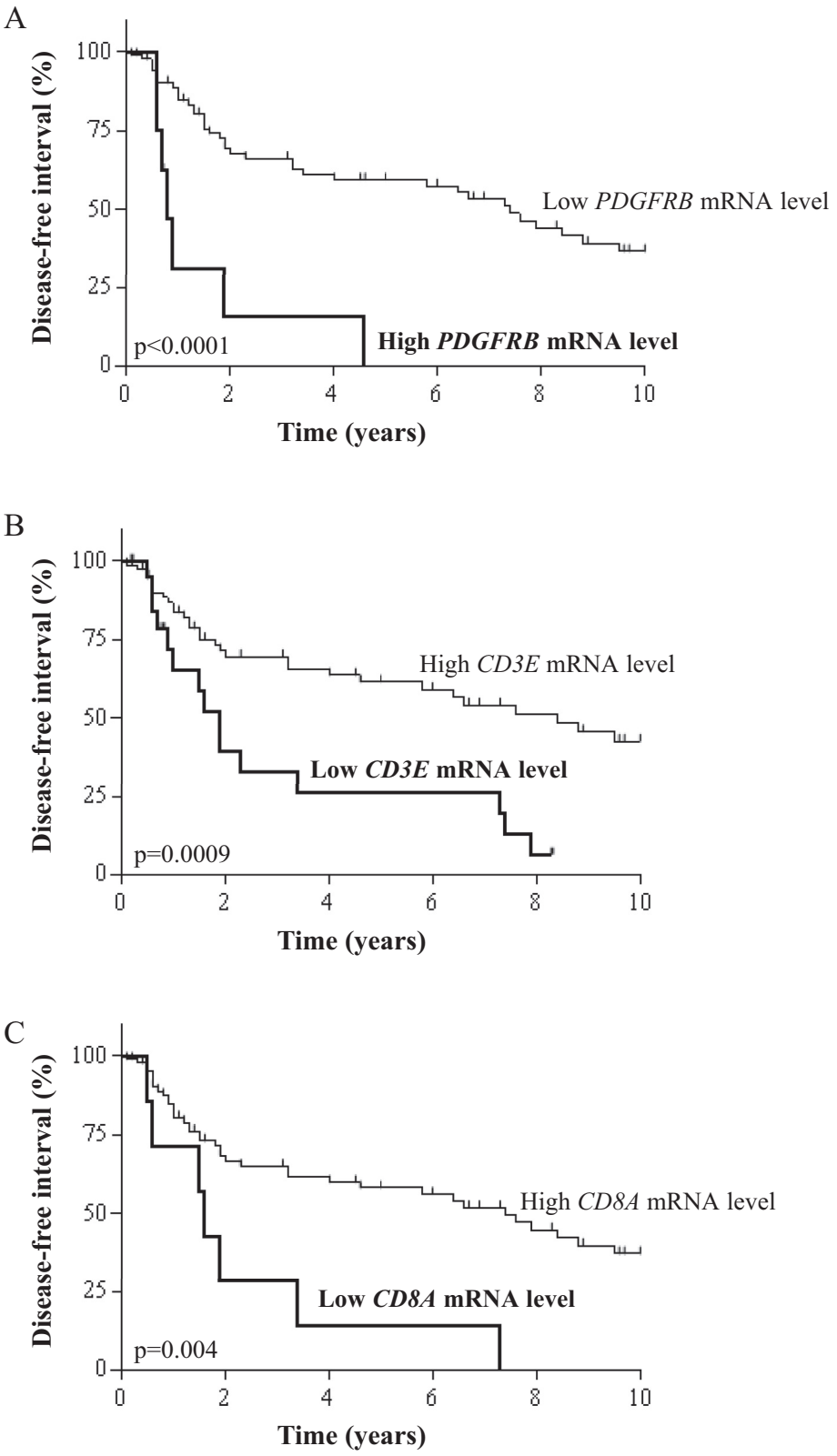
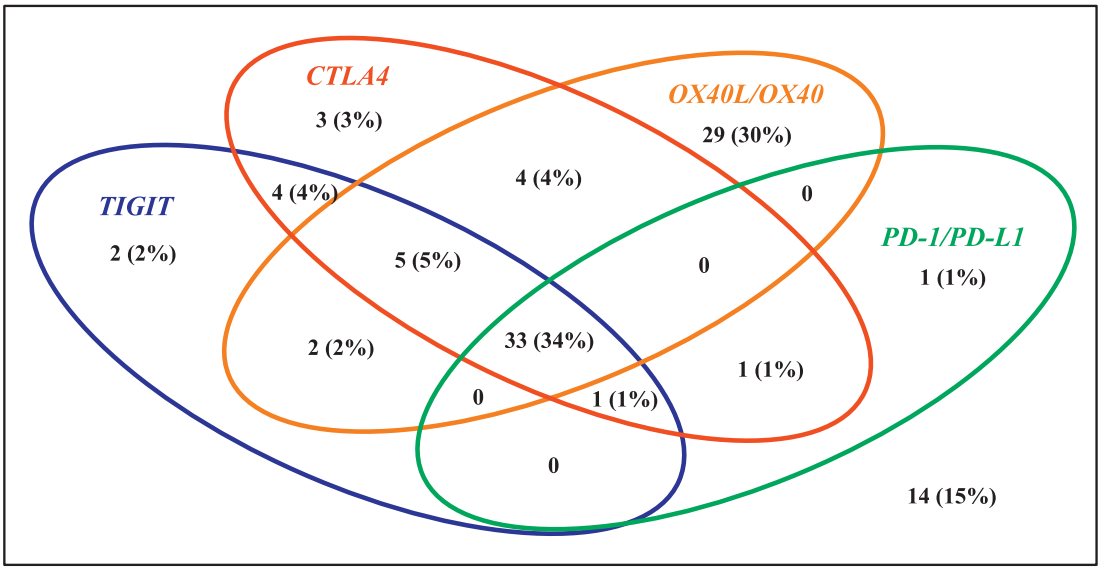



Fig. 3. Relationship between disease-free interval and (A) *PDGFRB*, (B) *CD3E* and (C) *CD8A* mRNA level.

A) Venn diagram of the mRNA surexpression of *TIGIT*, *CTLA4*, *OX40L/OX40* and *PD-1/PD-L1*




B) Clinical characteristics and molecular alterations of 82 HNSCC patients for whom a potential actionable immune target is available

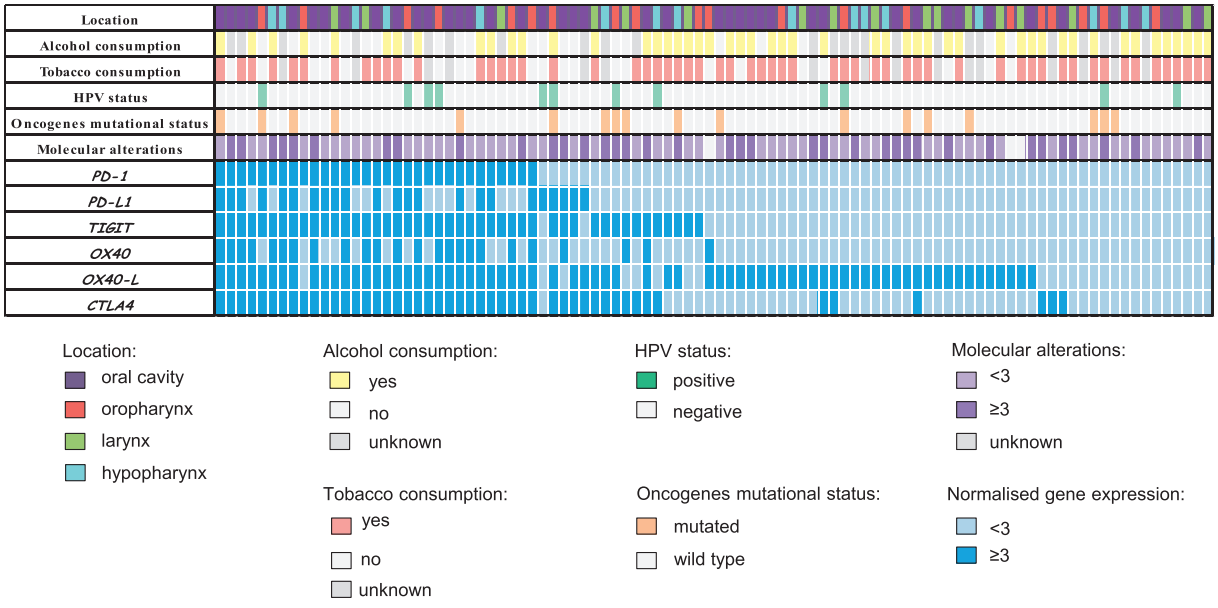



Fig. 4. Clinical characteristics and molecular alterations of the main targeted actionable genes in immunotherapy. HPV, human papillomavirus.

exclusively overexpressed in only two samples (2%) and *CLTA4* in three samples (3%), whereas *OX40L* was exclusively overexpressed in 29 samples (30%) (Fig. 4A). Fig. 4B describes the clinical and molecular characteristics of the 82 patients with HNSCC with at least one overexpressed immune gene among the six actionable genes of interest.

## 4. Discussion

We assessed the prognostic value of selected immune gene expression in a retrospective analysis of 96 patients with HNSCC who underwent primary surgery at Institut Curie. Our results show that most significantly overexpressed genes were *4*-1BB (77%), *IDO1* (75%),

*OX40L* (74%) and *GITR* (74%) immune-related genes and *FOXP3* (62%) immune-cell subpopulation genes. Eighty-five percent of tumours analysed overexpressed actionable immunity genes, including *PD-1*/*PD-L1*, *TIGIT*, *OX40*/*OX40L* and/or *CTLA4*. High *OX40L* mRNA level ($p = 0.0009$) and low *PD*-1 mRNA level ($p = 0.004$) were associated with the highest risk of recurrence. Patients with high *PDGFRB* mRNA levels and low *CD3E* or *CD8A* mRNA levels also were at the highest risk of recurrence. Overall, around half of immune genes had a deregulated mRNA level in tumour cells as compared with the normal head and neck tissue. Tumours with a low number of genomic alterations had higher mRNA levels of immune genes.

More specifically, our results show that among immune-cell subpopulation genes, antigen-presenting cells, dendritic cells and B and T cells had a higher mRNA level in tumours, whereas natural killer cell–-specific genes (i.e. *NCAM1*) were underexpressed. Furthermore, we observed a high *FOXP3* mRNA level not correlated to recurrence. This suggests the high number of T regulatory cells that was also reported in patients with HNSCC [10]. Positive associations were observed between *OX40L* and *FOXP3* mRNA levels and between *OX40L* and *CD4* mRNA levels using the Spearman rank correlation test ($r = +0.27$, $p = 0.0083$ and $r = +0.46$, $p < 0.0001$, respectively).

*OX40L*, *PD-1*, *PDGFRB*, *CD3E* and *CD8A* were associated with a poor prognosis in our study, especially a high mRNA level of *OX40L*. The prognostic value of *OX40L* expression is controversial in the literature and depends on the type of cancer [19,20]. The overexpression of *OX40L* was significantly associated with a higher risk of recurrence in bladder cancer [19], whereas it was associated with prolonged progression-free survival in glioblastoma [20].

In our patients with HNSCC, OX40L was expressed in the microenvironment of the tumour cell notably in fibroblasts at the protein level. In this regard, we observed a marked positive association between *OX40L* and *PDGFRB* mRNA levels using the Spearman rank correlation test ($r = +0.47$ and $p < 0.0001$, data not shown). Similarly, the expression of *OX40L* was detected in a subset of carcinoma-associated fibroblasts characterised by an immunosuppressive environment in patients with triple-negative breast cancer [21]. No clear cell location of the OX40L protein was reported in the literature for HNSCC.

Low mRNA level of *PD-1* correlated with a poor prognosis in our series. The absence of *PD-L1* mRNA overexpression in circulating tumour cells after anti–PD-1 treatment was strongly associated with an objective response in patients with HNSCC [22]. The prognostic value of *PD*-1 mRNA level was also reported in high-grade serous ovarian carcinoma [23] and in non-small cell lung cancer [24]. Low mRNA level of PD-L1 also had a poor prognosis although less significant than *PD-1* which is consistent to KEYNOTE-048 trial (NCT02358031) interim findings that reported an improved overall survival and duration of response versus standard therapy in patients with PD-L1–positive recurrent or metastatic HNSCC [25].

Our results also demonstrated that high mRNA level of *PDGFRB*, a major fibroblast-specific gene, was associated with a poor prognosis. Similar results were reported in prostate cancer [26]. A high PDGFRB protein expression was also reported to correlate with a short survival in patients with renal cell carcinoma [27] and pancreatic adenocarcinoma [28].

Patients with HNSCC with low mRNA levels of *CD3E* or *CD8A* had a poor prognosis. Similarly, high *CD3* and *CD8* mRNA expression was associated with a decreased risk of relapse in patients with early breast cancer [29]. *CD8* overexpression correlated with prolonged overall survival and recurrence-free survival in patients with bladder cancer [19].

Eighty-five percent of HNSCC tumours had at least an actionable immune gene overexpression (*PD-1*/*PD-L1*, *TIGIT*, *OX40L*/*OX40*, and *CTLA4*) in our series. *OX40L* mRNA level was overexpressed in 74% of tumours and had the worst prognostic value, suggesting that OX40L is a relevant therapeutic target for patients with HNSCC.

Several clinical trials evaluate OX40L agonists either as single agent or in combination with anti–PD-1/PD-L1 agents (NCT02315066, NCT02923349, NCT02410512, NCT02221960, NCT02705482). A phase I trial evaluating an anti-OX40 agonistic monoclonal antibody (9B12) in 30 patients with refractory metastatic solid malignancies showed a favourable safety profile and tumour shrinkage in 12 patients [30].

In addition in our series, 34% of HNSCC tumours co-expressed *PD-1*/*PD-L1*, *TIGIT*, *OX40*/*OX40L* and *CTLA4*, suggesting that a combination of immune checkpoint inhibitors may be a relevant therapeutic strategy in patients with HNSCC. Several clinical trials are ongoing with other immunotherapy drugs such as GITR (e.g. NCT01239134 or NCT02697591) or 4-1BB (e.g. NCT03364348) generally administered in combination in advanced solid malignancies.

## 5. Conclusions

*OX40L* and *PDGFRB* overexpression was associated with poor outcome in patients with HNSCC treated with primary surgery. On the contrary, *PD-1* overexpression was associated with good prognosis. These results suggest their relevance as potential prognostic biomarkers to be validated in an independent cohort. Immunotherapy was recently demonstrated to improve overall survival in the recurrent and/or metastatic setting of patients with HNSCC with pembrolizumab [9] and nivolumab [8] that target PD-1. However, only a

minority of patients benefit from these agents [8,9], highlighting the urgent need to identify other relevant immune targets.

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### Conflict of interest statement

C.L.T. has participated in advisory boards of MSD, BMS, Merck Serono, Astra Zeneca, Novartis, Roche and Nanobiotix. All other authors report no conflict of interest.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2019.08.028.

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