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Immune gene expression in head and neck squamous cell carcinoma patients

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Abstract

Background: Nivolumab and pembrolizumab targeting PD-1 have recently been approved among recurrent and/or metastatic head and neck squamous cell carcinoma (HNSCC) patients who failed platinum therapy. We aimed to 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 using RT-PCR among 96 HNSCC patients 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: Median age of the population was 56 years [range: 35–78]. Primary tumor location was oral cavity (45%), oropharynx (21%), larynx (18%), and hypopharynx (17%). Twelve patients (13%) had an oropharyngeal HPV-positive tumor. 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 tumors analyzed 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 outcome whereas *PD-1* overexpression was associated to good prognostic in HNSCC patients 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 HNSCC [3] with mutations in several oncogenes including *PIK3CA* (21%) and *HRAS* (4%), as well as in tumor 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 tumor 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 EGFR (epidermal growth factor receptor), 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-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 tumor 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 were also reported to affect prognosis [10–14]. OX40, PD-1 and CTLA-4

were shown to have a significantly higher expression in T-cell subsets isolated from tumors of HNSCC patients [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 untreated HNSCC patients.

2. Patients and Methods

2.1. Patients

We retrieved samples from HNSCC patients 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 analyzes 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 (Supplementary 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 & 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 Dubot *et al.*, 2018. Tumor genomic alterations burden was assessed by summing the number of recurrent deleterious genomic alterations (SNV+CNV) per sample. Sanger sequencing was also performed to confirm PIK3CA, KRAS, HRAS and NRAS mutations.

2.4. HPV 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 PCR was performed with Sybr®Green and specific primers for HPV16 and 18 using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA).

2.5. Real time quantitative RT-PCR

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 ≥ 3 were considered as overexpressions and ≤ 0.33 as underexpressions. We previously used the same cut-off value for altered tumor gene expression [17].

2.6. Immunohistochemistry/OX40L protein expression

We performed IHC assay by using OX40L (Cell Signaling Technology, rabbit, 59036, 1/100, pH9) antibody in a series of 20 HNSCC 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 tumor microenvironment by using OX40L immunostaining in tumor cells, stromal cancer associated fibroblasts (CAFs) and mononuclear inflammatory cells (MICs). All quantifications were performed with blinding to patient status by 2 expert pathologists.

2.7. Statistical analyses

The clinicopathological features were tested for association with disease-free interval (DFI) by using 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 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 hazards 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 tumor groups regarding molecular data.

3. Results

3.1. Patient characteristics

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

3.2. mRNA expression of immune genes

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

3.2.1 mRNA expression of the immune-related genes

Among the 30 immune-related genes analyzed, 18 genes were significantly deregulated in HNSCC tumors as compared to 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 tumors (*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 tumors. Expression of *PVRIG* was significantly higher in HPV-positive tumors, whereas *GITR*, *NT5E* and *CD276* expressions were significantly lower in HPV-positive tumors (Table 3). All immune-related genes except *ICOSLG* showed no modification in the expression profile in HNSCC who harbored oncogenes mutations (*NRAS*, *HRAS*, *KRAS* or *PIK3CA*) as

compared to not mutated. Regarding tumor genomic alterations burden, 20 out of the 30 immune-related genes (67%) were significantly overexpressed in tumors with less than three tumor 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 to genes coding a ligand protein (Supplementary Figure 1).

3.2.2 mRNA expression of the immune-cell subpopulation genes

Among the 16 immune-cell subpopulation genes analyzed, 7 genes (44%) were significantly deregulated in HNSCC tumors as compared to normal head and neck tissue ($p < 0.05$). *FOXP3* was overexpressed, while only *NCAM1* was underexpressed in more than 50% of the tumors as compared to 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 tumor genomic alterations.

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

3.3. OX40L protein expression using immunochemistry

Using IHC, OX40L protein was expressed in epithelial cancer cells for most tumor samples (H Score: 1-2.5) with a predominantly cytoplasmic location in various populations of the TME, including mononuclear inflammatory cells (MICs), fibroblasts and muscle (Supplementary Figure 3).

3.4. Prognostic value of immune gene expression

Twelve out 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 (Figure 2).

Eight out 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 (Figure 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 tumors analyzed, 82 tumors (85%) overexpressed at least one of these six genes. Thirty three tumors (34%) overexpressed simultaneously *TIGIT*, *CTLA4*, *PD-1/PD-L1* and *OX40/OX40L*. No tumor exclusively overexpressed *PD-1* or *OX40*. *PD-L1* was exclusively overexpressed in only one sample (1%). *TIGIT* was exclusively overexpressed in only two samples (2%) and *CTLA4* in three samples (3%) whereas *OX40L* was exclusively overexpressed in 29 samples (30%) (Figure 4A).

Figure 4B describes the clinical and molecular characteristics of the 82 HNSCC patients with at least one overexpressed immune gene among the six actionable genes of interest.

4. Discussion

We assessed the prognostic value of selected immune genes expression in a retrospective analysis of 96 HNSCC patients 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 tumors analyzed 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 tumor cells as compared to normal head and neck tissue. Tumors 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 tumors whereas natural killer cells specific genes (i.e. *NCAM1*) were underexpressed. Furthermore we observed a high *FOXP3* mRNA level not correlated to recurrence. This suggests a high numbers of T regulatory cells that was also reported in HNSCC patients [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 HNSCC patients, OX40L was expressed in the microenvironment of the tumor 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 characterized by an immunosuppressive environment in triple negative breast cancer patients [21]. No clear cell location of 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 tumor cells following anti-PD1 treatment was strongly associated with an objective response in HNSCC patients [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 had also a poor prognosis although less significant than *PD-1* which is consistent to KEYNOTE-048 trial interim findings who 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 renal cell carcinoma [27] and pancreatic adenocarcinoma [28] patients.

HNSCC patients 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 early breast cancer patients [29]. *CD8* overexpression correlated with prolonged overall survival and recurrence-free survival in bladder cancer patients [19].

Eighty-five percent of HNSCC tumors 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 tumors and had the worst prognostic value, suggesting that OX40L is a relevant therapeutic target for HNSCC patients.

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 1 trial evaluating an anti-OX40 agonistic monoclonal antibody (9B12) in 30 patients with refractory metastatic solid malignancies showed a favorable safety profile and tumor shrinkage in 12 patients [30].

In addition in our series, thirty four percent of HNSCC tumors co-expressed *PD-1/PD-L1*, *TIGIT*, *OX40/OX40L*, and *CTLA4* together suggesting that a combination of immune checkpoint inhibitors may be a relevant therapeutic strategy in HNSCC patients. 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 HNSCC patients treated with primary surgery. On the contrary *PD-1* overexpression was associated to good prognostic. 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 HNSCC patients 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

Pr. Le Tourneau has participated in advisory boards of MSD, BMS, Merck Serono, Astra Zeneca, Novartis, Roche and Nanobiotix.

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Tables

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

	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)	
Tumor 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)	

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 Dubot et al., 2018. Information was available for 93 patients.

DFI: disease-free interval; NS: not significant; HNSCC: head and neck squamous cell carcinoma; UICC: Union for International Cancer Control

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

Gene	Protein	Alias	Head and neck normal tissue ^a (n=27)	Head and neck squamous cell carcinomas (n=96)	p-value ^b	% under expression	% normal expression	% over expression
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 normalized such that the median of the 27 head and neck normal tissues mRNA values was equal to 1
b Kruskal Wallis's H Test

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 (n=84)	HPV+ (n=12)	p-value ^b	Not mutated (n=78)	Mutated (n=18)	p-value	<3 (n=56)	≥3 (n=37)	p-value
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 normalized such that the median of the 27 head and neck normal tissues mRNA values was equal to 1
^b Kruskal Wallis's H Test

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 (n=27)	Head and neck squamous cell carcinoma (n=96)	p-value ^b	% under expression	% normal expression	% over expression
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	hematopoietic 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%

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

LT: T cell; LB: B cell; NK: Natural killer, LTc: Cytotoxic T lymphocyte

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- versus 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	hematopoietic 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)

^a Median (range) of gene mRNA levels; the mRNA values of the samples were normalized such that the median of the 27 head and neck normal tissues mRNA values was equal to 1
^b Kruskal Wallis's H Test

LT: T cell; LB: B cell; NK: Natural killer, LTc: Cytotoxic T lymphocyte

Figures legends

Figure 1. Gene expression versus molecular alterations and clinical patient characteristics

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

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

Figure 4. Clinical characteristics and molecular alterations of the main targeted actionable genes in immunotherapy

Supplementary material

Supplementary Figures

Supplementary Figure 1. Heat map of the 30 immune related genes mRNA levels in our series of 96 HNSCC

Supplementary Figure 2. Heat map of the 16 immune-cell subpopulation genes mRNA levels in our series of 96 HNSCC

Supplementary Figure 3. OX40L protein expression in two HNSCC patients

Supplementary Tables

Supplementary Table 1. Description of immune-related genes and immune-cell

Supplementary Table 2. Oligonucleotide primer sequences used for qRT-PCR genes subpopulation genes

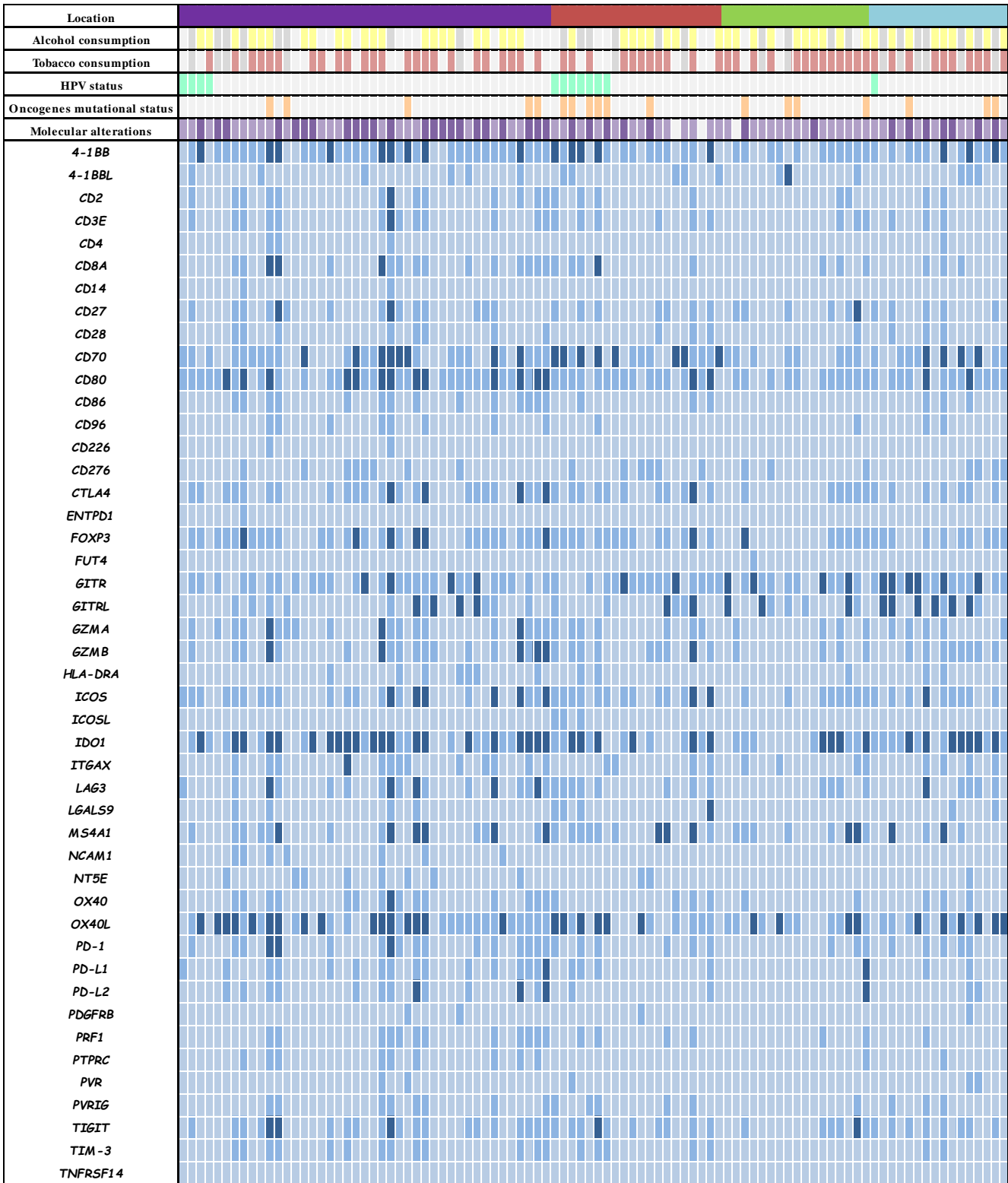
Supplementary Table 3. Relationship between mRNA expression of immune-related genes and disease-free interval (DFI) in the 96 HNSCC

Supplementary Table 4. Relationship between mRNA expression of immune-cell subpopulation genes and disease-free interval (DFI) in the 96 HNSCC

Supplementary Table 5. Multivariate COX analysis of disease-free interval (DFI) in our series of 96 HNSCC

Supplementary Patients and Methods

Figure 1.



Location:
 ■ oral cavity
 ■ oropharynx
 ■ larynx
 ■ hypopharynx

Alcohol consumption:
 ■ yes
 ■ no
 ■ unknown

HPV status:
 ■ positive
 ■ negative

Molecular alterations:
 ■ <3
 ■ ≥3
 ■ unknown

Tobacco consumption:
 ■ yes
 ■ no
 ■ unknown

Oncogenes mutational status:
 ■ mutated
 ■ wild type

Normalized gene expression:
 ■ <3
 ■ 3-10
 ■ >10

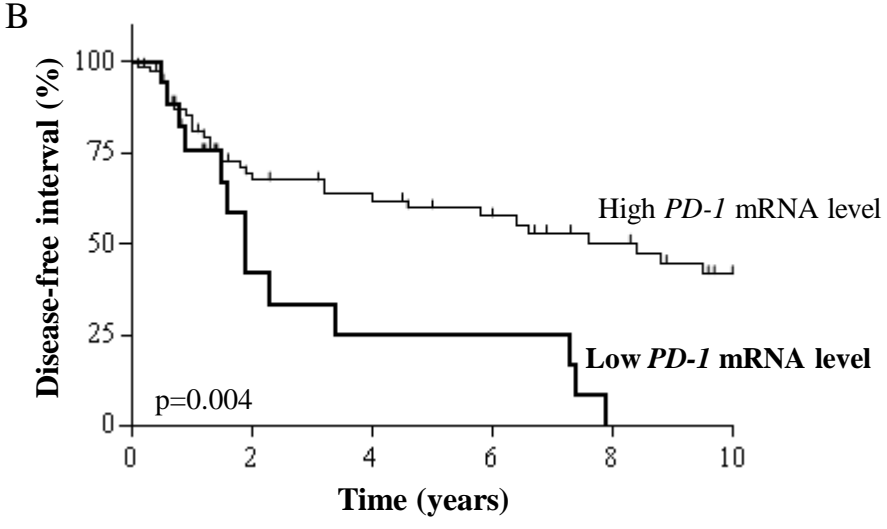
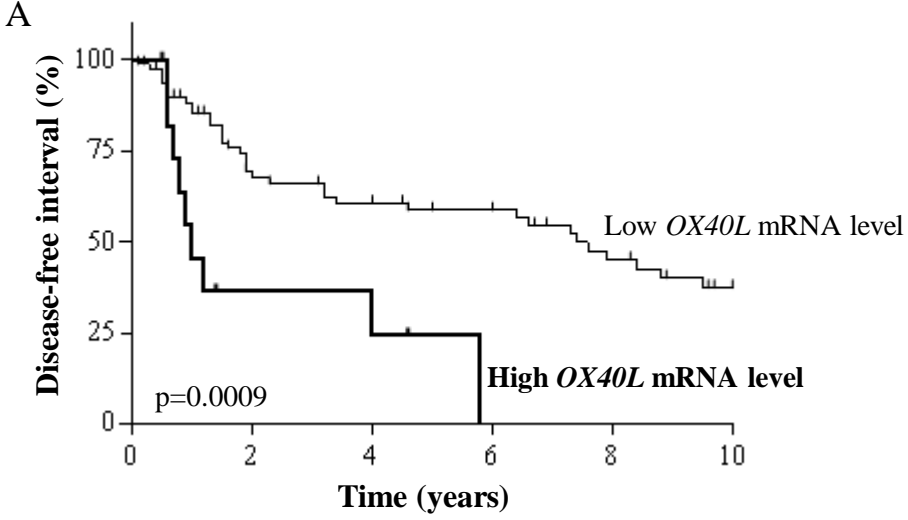


Figure 3.

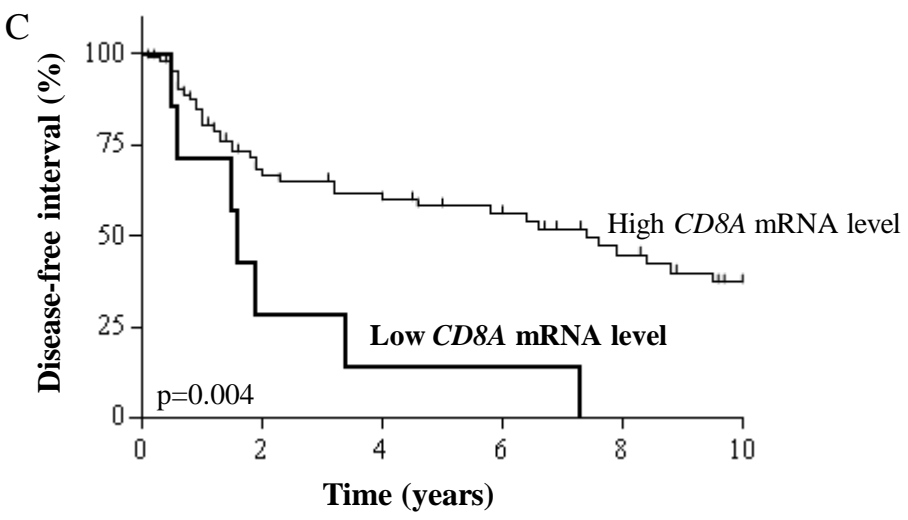
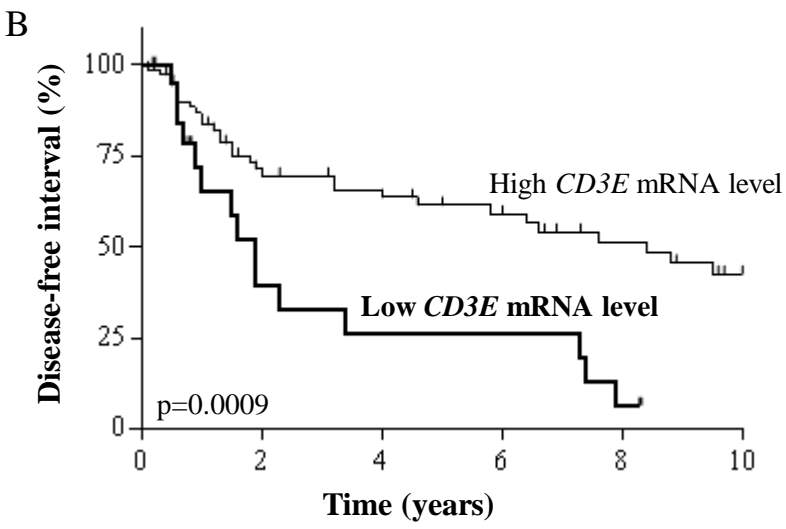
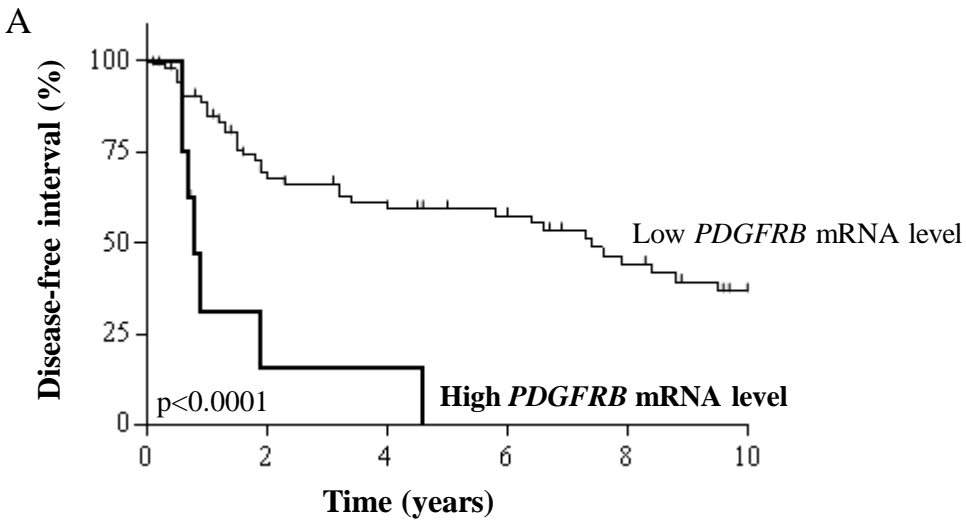
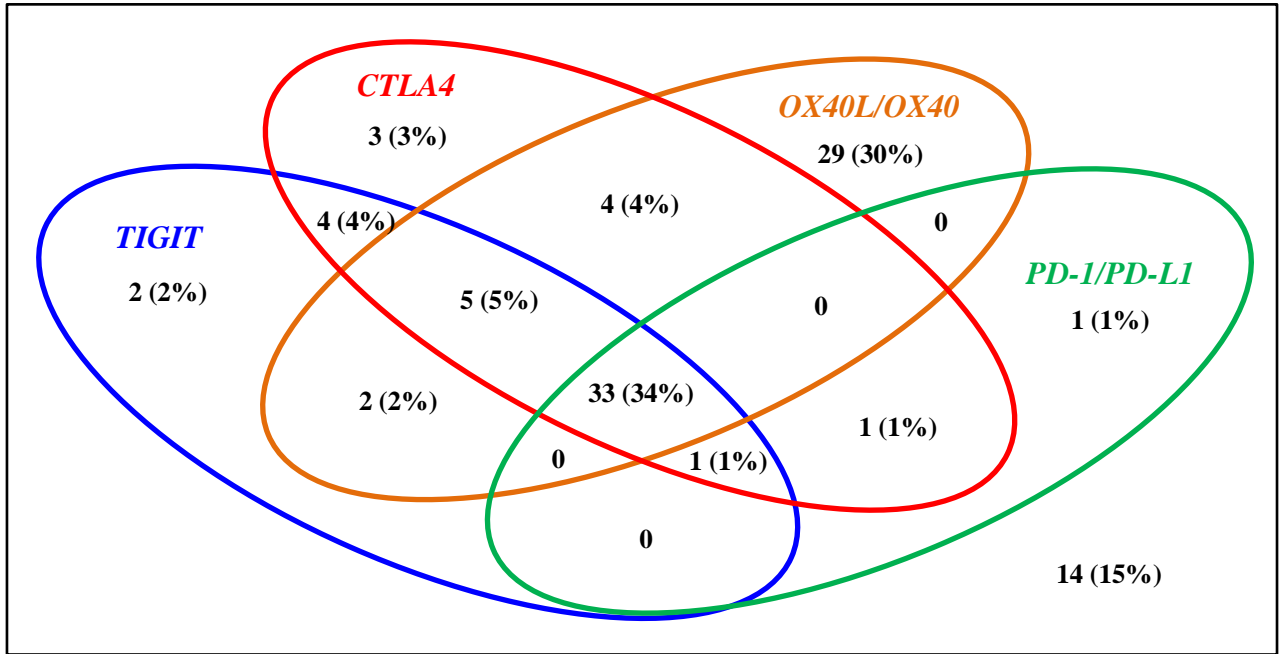
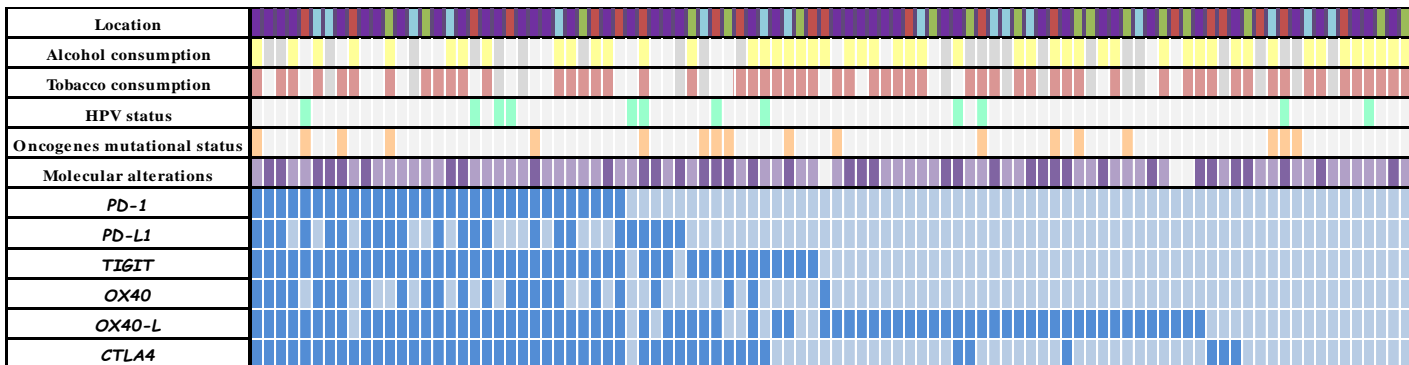


Figure 4.

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



Location:

- oral cavity
- oropharynx
- larynx
- hypopharynx

Alcohol consumption:

- yes
- no
- unknown

Tobacco consumption:

- yes
- no
- unknown

HPV status:

- positive
- negative

Oncogenes mutational status:

- mutated
- wild type

Molecular alterations:

- <3
- ≥3
- unknown

Normalized gene expression:

- <3
- ≥3