Squamous Cell Carcinoma Antigen-encoding Genes *SERPINB3/B4* as Potentially Useful Markers for the Stratification of HNSCC Tumours

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Abstract. *Background/Aim: The squamous cell carcinoma antigen (SCCA), encoded by the genes SERPINB3/B4, is a tumour marker produced by head and neck squamous cell carcinoma (HNSCC). We aimed to examine SERPINB3/B4 mRNA levels and its clinical significance in the therapeutic context. Materials and Methods: We retrieved mRNA expression levels, clinical, pathological and genomic data for 520 HNSCC from The Cancer Genome Atlas (TCGA). Results: HNSCC tumours express high levels of SERPINB3/B4 mRNA. SERPINB3 expression differs depending on Human papillomavirus (HPV) infection status, primary tumour location, grade and differentiation, extension to lymph nodes and extracapsular spread. Interestingly, we observed an association between SERPINB3/B4 and the presence of tumour immune infiltrate as well as the expression of the immune checkpoint regulators PD-L1/PD-L2 that depended on HPV status. Conclusion: Our findings point to potential interest of SERPINB3/B4 for the stratification of HNSCC patients in the therapeutic context.*

Head and neck squamous cell carcinoma (HNSCC) is the 6th most frequent type of solid tumour in the world. Alcohol and

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tobacco use are two important risk factors for these tumours (1, 2). Human papillomavirus (HPV) infection is another important risk factor that is responsible for the recent increase in the incidence of oropharynx tumours in economically developed countries (1, 2). Surgery, radiotherapy, chemotherapy and cetuximab, a monoclonal antibody targeting the epidermal growth factor receptor (EGFR) constitute the mainstay of the treatment of HNSCC (3-5). Despite these therapeutic options, the natural history of HNSCC is marked by a high risk of recurrence and a poor prognosis (1, 2). Recently, targeting the immune checkpoint regulator programmed cell death-1 (PD-1) with monoclonal antibodies, such as pembrolizumab and nivolumab, proved efficacious in the treatment of recurrent/metastatic HNSCC (6-8). Nivolumab is now approved by the FDA for the treatment of patients with recurrent or metastatic HNSCC that fail to respond to platinum-based therapy. While clinical trials are ongoing to define the optimal strategy involving these new molecules, it is clear at this stage that biological markers are needed to adjust the best therapeutic strategy for each individual patient.

The tumour antigen SCCA (Squamous Cell Carcinoma Antigen) was initially isolated from squamous cell carcinoma originating from the cervix (9). Following its discovery, SCCA has been detected in multiple types of primary tumours, and in the serum of cancer patients, making it a protein marker of potential clinical interest in HNSCC (9). In the human genome, SCCA is encoded by two genes (*SERPINB3/SERPINB4*) located on the long arm of chromosome 18 (18q21). The corresponding protein products are two highly homologous protein members of the Serpin family of Ser protease inhibitors that function as suicide substrates for cellular proteases (9). These two proteins are

Table I. *Clinical characteristics of HNSCC patients (n=520).*

Gender	Male (n=382); Female (n=135); N/A (n=3)
Age (median, years)	61
HPV status	HPV-negative (n=72); HPV-positive (n=37); N/A (n=412)
Primary tumour location	Larynx (n=116)
	Oral Cavity (n=313)
	Oropharynx (n=78)
	Hypopharynx (n=10)
	N/A (n=3)
TNM	T0 (n=1), T1 (n=48), T2 (n=134), T3 (n=99), T4/a/b (n=173), TX (n=39), N/A (n=26)
	N0 (n=176), N1 (n=67), N2/a/b/c (n=166), N3 (n=8), NX (n=75), N/A (n=28)
	M0 (n=184), M1 (n=1), MX (n=61), N/A (n=274)

HPV status based on p16 labelling by immunohistochemistry; TNM staging according to the American Joint Committee on Cancer (AJCC).

both localized in the cytosol, but are likely active against distinct intracellular proteases: SCCA1, encoded by *SERPINB3*, is able to inhibit lysosomal cathepsins (isoforms S, L, and K), while SCCA2, encoded by *SERPINB4* inhibits cathepsin G (10). Early studies performed in HNSCC cells have shown that SCCA1 regulates cell death associated with protease activation, for example upon lysosomal leakage (11-13). In agreement with the widely-accepted view that matrix invasion depends on functional proteases, overexpression of *SERPINB3* was also found to reduce cancer cell invasion in matrigel assays (14).

Recent studies performed on multiple types of tumours have unveiled new functional consequences of the neutralization of cellular proteases by *SERPINB3/B4* (15-16). In epithelial cells from various types of adenocarcinomas, the introduction of an activated RAS oncogene was found to induce *SERPINB3/SERPINB4* expression, leading to endoplasmic reticulum stress and a cellular homeostatic response known as the unfolded protein response (UPR) (17, 18). Through its ability to neutralize intracellular proteases and to induce the UPR, *SERPINB3* may contribute to the installation of a pro-inflammatory phenotype characterized by high expression of the cytokine interleukin-6 (IL6) in colorectal and pancreatic adenocarcinomas (15). More recently, mutations of the *SERPINB3/B4* genes were found in melanoma patients receiving the immune checkpoint inhibitor ipilimumab (19). This observation opens up the possibility that *SERPINB3/B4* expression might be a determinant of tumour immunogenicity in melanoma in the therapeutic context (19).

While these new studies unveil a potential role of *SERPINB3/B4* as a determinant of tumour progression and response to various treatments, no study to date has examined this possibility in HNSCC. In the present study, we aimed to examine the clinical and pathological significance of *SERPINB3/SERPINB4* expression in HNSCC, using data from The Cancer Genome Atlas (TCGA) (20).

Materials and Methods

TCGA datasets. Patient clinical, pathological data and RNA-seq expression values (RNA SeqV2 RSEM), were retrieved from TCGA in September 2017, using Cbioportal at: http://cbioportal.org (21, 22). Altogether, 24 tumour datasets were analysed, representing a total of 10,663 patients and 520 HNSCC (Table I). Gene expression was normalised to TBP (TATA-box binding protein) expression as indicated.

Gene Ontology (GO) analysis. We used the PANTHER classification system (http://pantherdb.org/) to perform a statistical overrepresentation test. The enrichment of Gene Ontology (GO) terms in our gene set was compared to the whole *Homo Sapiens* genome (23), with Bonferroni correction applied.

IMMUNE score analysis. The IMMUNE score was used to evaluate tumour purity, *i.e.* the infiltration levels of immune cells based on gene expression data (24).

Statistical analyses. Pearson correlation, Cox regression, Kruskal-Wallis and Wilcoxon tests were used as indicated and were all performed using R (packages Hmisc and Survival).

Results

High expression of SERPINB3/SERPINB4 in HNSCC. In order to examine the expression levels of *SERPINB3* and *SERPINB4* in various types of tumours, we performed an analysis of their mRNA expression levels, normalized to TBP (*TATA box-Binding protein*) mRNA expression (Figure 1). *SERPINB3* was found to be expressed at significant levels in a broad variety of tumours, with the highest expression levels in cervical, head and neck and lung squamous tumours (Figure 1A). The same pattern of expression was detected with *SERPINB4*, even though the expression levels of *SERPINB4* were on average 4x lower than those of SERPINB3 (Figure 1B). In HNSCC tumours, the expression levels of *SERPINB3* were highly correlated with those of *SERPINB4* ($R^2=0.92$, $p<0.001$) (Figure 1C).
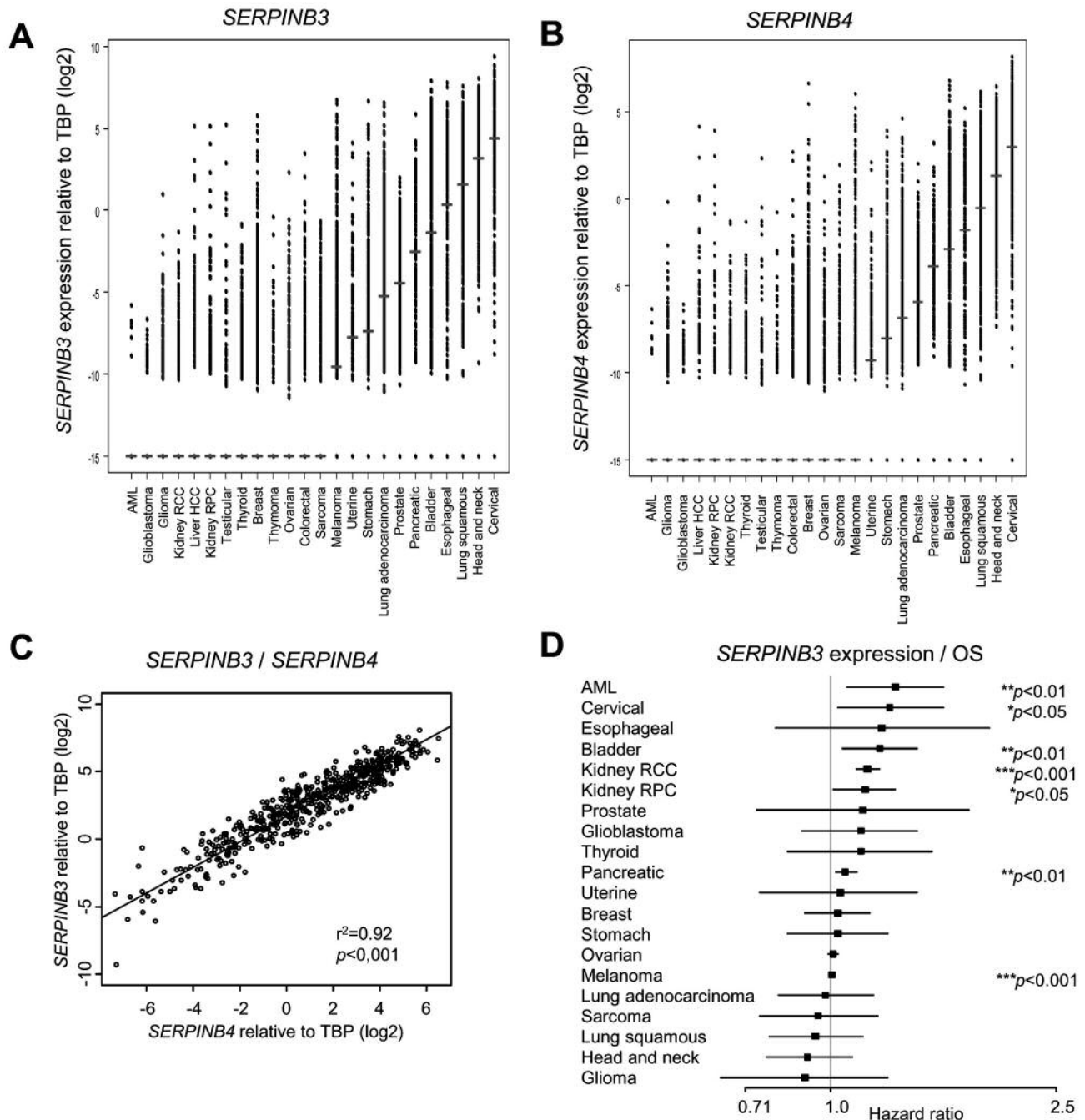
Figure 1. *Expression of SERPINB3 and SERPINB4 in different human tumours. A, B. Each data point represents the expression levels of SERPINB3 (A) or SERPINB4 (B) as log2(RSEM values normalized to TBP) in one tumour. Horizontal lines indicate the median expression values. Samples with zero expression are plotted on the bottom of the y axis. C. Pearson correlation between SERPINB3 and SERPINB4 expression (log2(RSEM values normalized to TBP)) in HNSCC (Pearson r²=0.92, p<0.001) D. A forest plot showing the hazard ratios and 95% confidence intervals for overall survival for each cancer type, with respect to SERPINB3 expression (Cox proportional hazards regression model). *p<0.05, **p<0.01, ***p<0.001.*

We therefore limited our subsequent analyses to the regulation of *SERPINB3*. To address the possibility that the expression of SCCA might have a prognostic value, we calculated the hazard ratio for overall survival across the different types of primary tumours included in this analysis (Figure 1D). In cervical tumours, expression of *SERPINB3* was associated with a hazard ratio>1, representing a significantly reduced OS and an unfavorable outcome
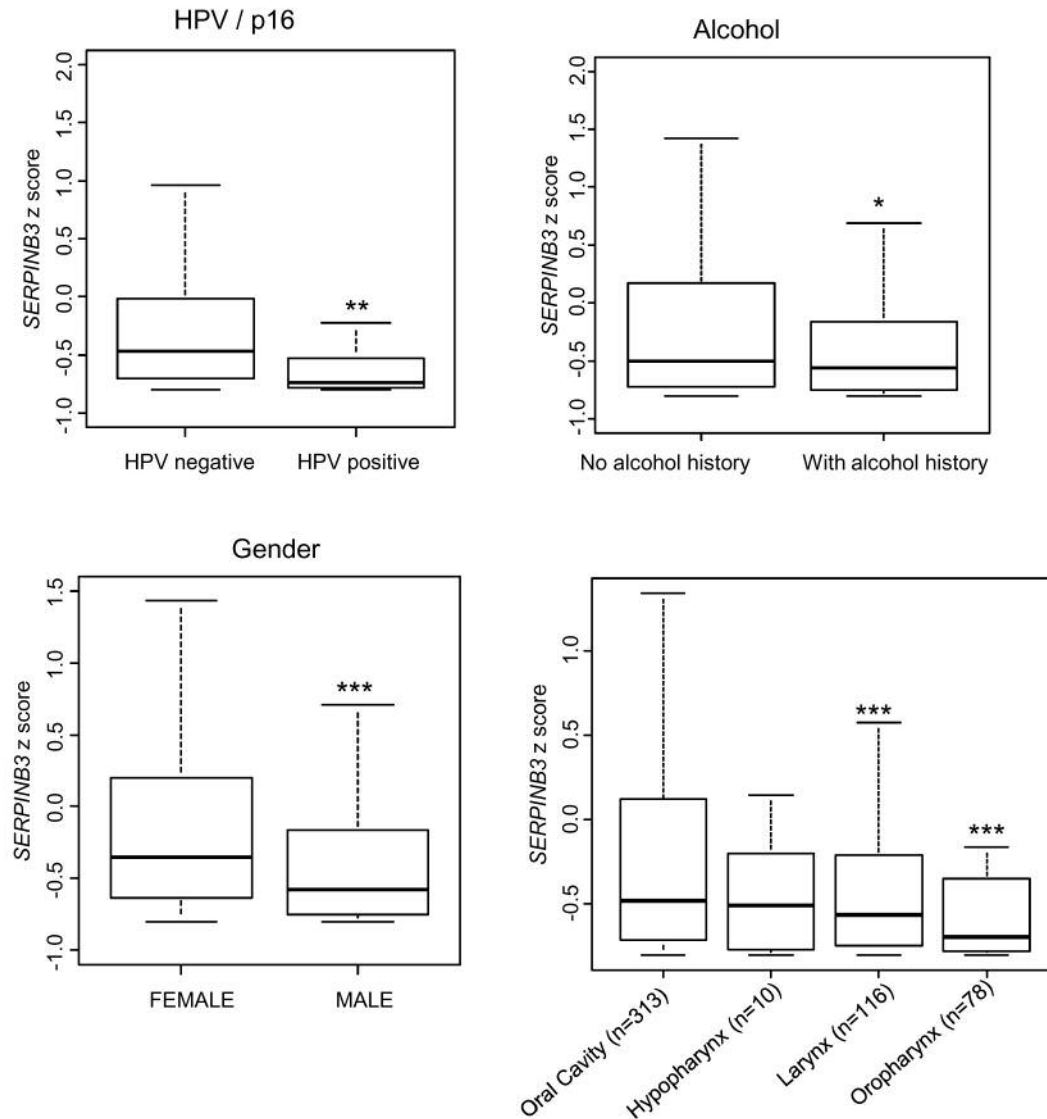
Figure 2. *Clinical correlates of SERPINB3 in HNSCC. Boxplots showing SERPINB3 expression (z scores) in HNSCC patients based on HPV status (p16) (HPV+ n=72, HPV- n=37), history of alcohol use (with alcohol history n=346, no alcohol history n=160) and gender (male n=382, female n=135). The lower panel is an analysis of SERPINB3 expression depending on the location of the primary tumour. The groups were compared to the highest SERPINB3 expressor "Oral cavity". Wilcoxon test was used for statistical analyses. *p<0.05, **p<0.01, ***p<0.001.*

(hazard ratio=1.27, *p*<0.05). No significant effect of the *SERPINB3* status was found for HNSCC patients.

SERPINB3 expression is associated with clinical and pathological characteristics in HNSCC. The levels of *SERPINB3* were significantly higher in the population of patients with a HPV-negative status (as determined from the expression of the viral oncoprotein p16) (Figure 2). A modest, yet significant link to alcohol exposure was found: HNSCC tumours arising in a context of alcohol intake expressed slightly lower levels of *SERPINB3* (Figure 2). No link was found to smoking (data not shown). Male patients expressed

lower levels of *SERPINB3* mRNA compared to female patients (Figure 2). Expression levels of *SERPINB3* in HNSCC differed according to the localization of the primary tumour (Figure 2). Tumours arising in the oral cavity had significantly higher *SERPINB3* expression levels compared to other locations, such as the larynx and oropharynx (Figure 2).

We examined the association of *SERPINB3* expression levels with HNSCC staging, as defined by the American Joint Committee on Cancer Code (AJCC). We found no significant difference in *SERPINB3* expression levels according to tumour volume (Figure 3A). Extension to lymph nodes and
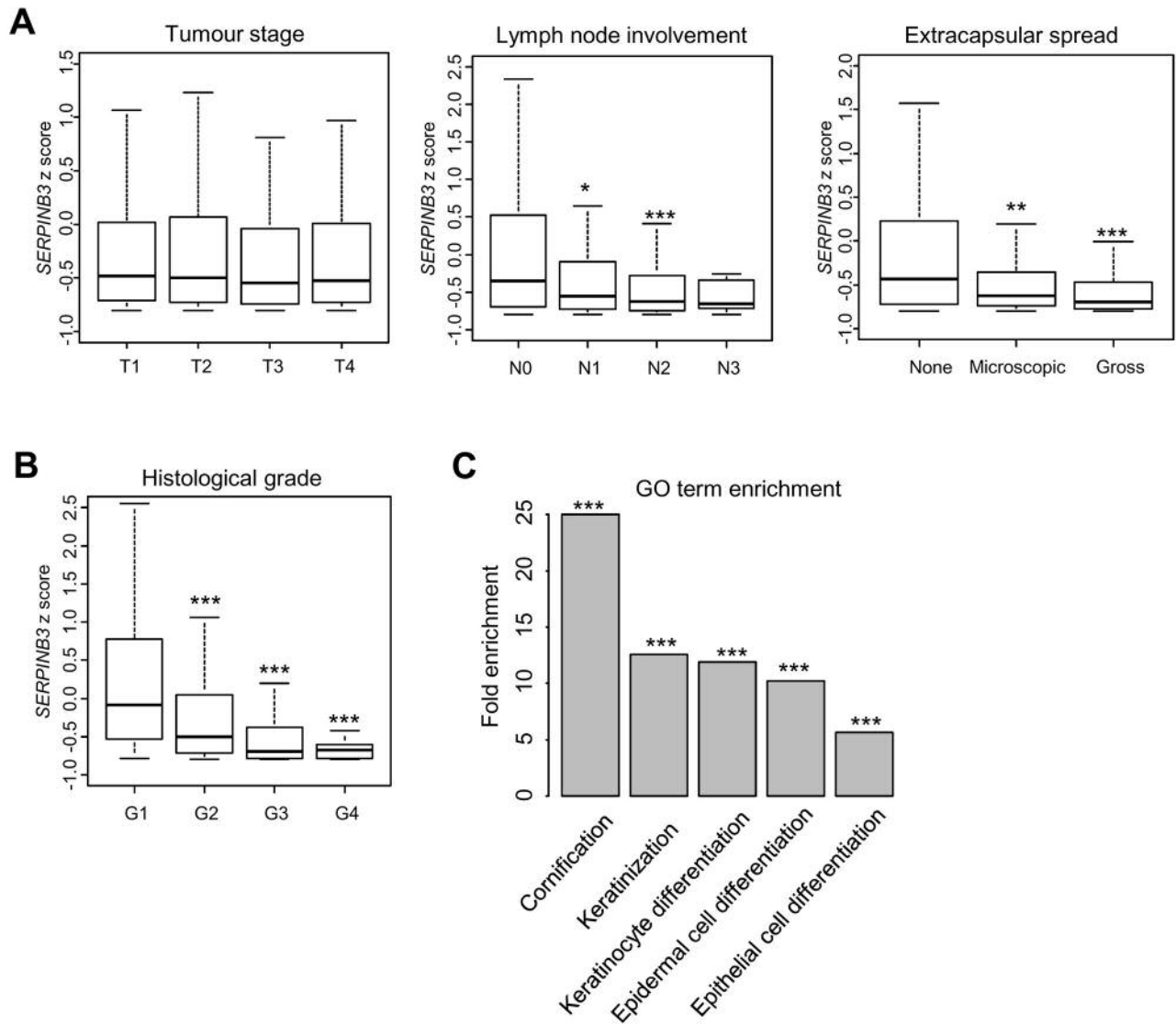
Figure 3. *Pathological correlates of SERPINB3 in HNSCC. A. Boxplots representing SERPINB3 expression (z score) in HNSCC tumours of different stage (n=520). Tumour stage and lymph node involvement are given according to the American Joint Committee on Cancer. T: T1 (n=48), T2 (n=134), T3 (n=99), T4 (n=173). N: N0 (n=176), N1 (n=67), N2 (n=166), N3 (n=8). Extracapsular spread was estimated as absent (n=243), microscopic (n=74) or gross (n=36). B. Boxplots of SERPINB3 expression (z scores) in HNSCC tumours according to histological grade (G1 to G4, with G1 representing well differentiated tumors and G3/4 poorly differentiated tumors): G1 (n=61), G2 (n=302), G3 (n=125), G4 (n=7). Wilcoxon test was used for statistical analyses compared to control. *p<0.05, **p<0.01, ***p<0.001. C. A Gene ontology (GO) analysis was performed on the panel of genes that correlate with the expression of SERPINB3 in HNSCC and cervical tumours (n=115). For each indicated GO term, we present a calculation of the fold-enrichment. ***p<0.001.*

extracapsular spread were however associated with lower expression levels of *SERPINB3* (Figure 3A). HNSCC tumours of high grade (G2, G3 and G4) had significantly lower expression levels of *SERPINB3* compared to those that were well-differentiated (G1) (Figure 3B). To support this observation suggesting that *SERPINB3* is a marker of epidermal differentiation, we performed a gene ontology

(GO) analysis of the genes that correlated with *SERPINB3* expression in SCC (Figure 3C). We identified 115 genes that correlated with *SERPINB3* mRNA in HNSCC and cervical cancer (Pearson R^2>0.30). The GO term "cornification" (accession: GO:0070268) was the most significantly enriched biological process (25-fold enrichment, *p*<0.001), followed by "keratinization" (GO:0031424, 12.5-fold enrichment,
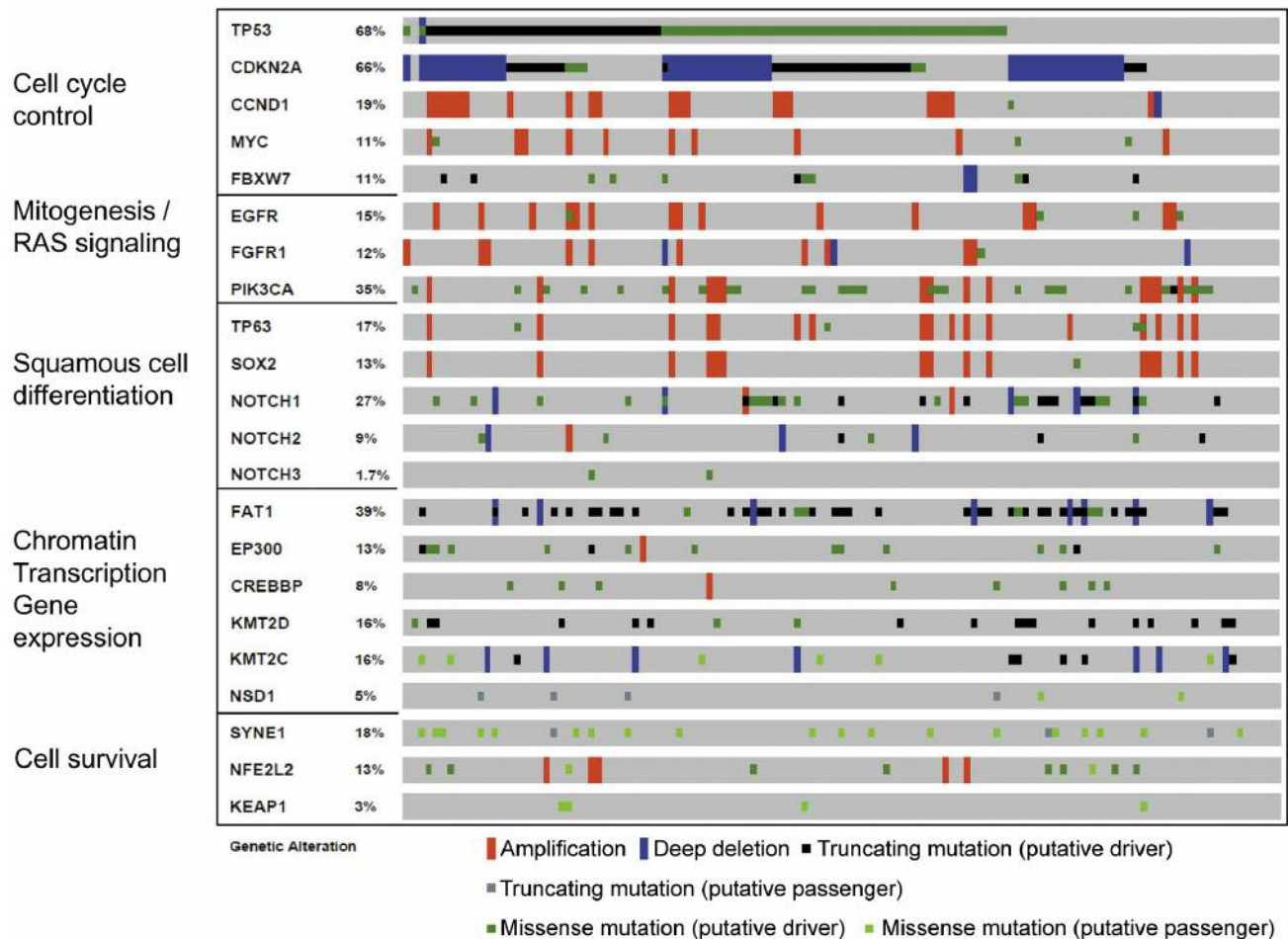
Figure 4. *Genetic profile of SERPINB3 expressing HNSCC. An ONCOPRINT representation of gene alterations most commonly found in HNSCC tumours with high expression levels of SERPINB3 (z >0, n=119). For each gene, we present a summary of the main types of alterations (amplification, deletion, missense or truncating mutations, putative drivers or passenger mutations). The genes are classified according to their main cellular function, as previously reported by Dotto et al. (25).*

$p<0.001$), "keratinocyte differentiation" (GO:0030216, 11.9-fold enrichment, $p<0.001$), "epidermal cell differentiation" (GO:009913, 10.2-fold enrichment, $p<0.001$) and "epithelial cell differentiation" (GO:0030855, 5.7-fold enrichment, $p<0.001$) (Figure 3C). We concluded that *SERPINB3* mRNA is a marker of epithelial differentiation in HNSCC.

SERPINB3 as a potential marker of tumour immunogenicity in HNSCC. We examined the possibility that *SERPINB3* expression might be related to specific genomic events in HNSCC tumours (20, 25). An Oncoprint representation, analysing the genomic events in HNSCC tumours with a positive z score for *SERPINB3* mRNA was constructed (Figure 4). This analysis revealed the lack of association between *SERPINB3* overexpression and the presence of gene alterations in the pathways identified as being essential in

SCC (25): cell cycle control (*TP53, CDKN2, CCND1, MYC, FBXW7*), mitogenesis and RAS signaling (*EGFR, FGFR1, PIK3CA*), squamous cell differentiation (*TP63, SOX2, NOTCH1/2/3*), chromatin and transcription/gene regulation (*FAT1, EP300, CREBBP, KMT2D, KMT2C, NSD1*), cell survival (*SYNE1, NF2L2, KEPA1*) (Figure 4). We also explored the possibility that SERPINB3 status may be associated with tumour mutational burden, by comparing SERPINB3 mRNA expression with the number of somatic mutations in HNSCC tumours. No association was seen between these two parameters (data not shown).

To address the possibility that *SERPINB3/B4* could be a determinant of immune response during HNSCC progression and therapeutic response, we turned our attention to three biological parameters: i) the level of tumour immune infiltration; ii) the expression of the immune checkpoint
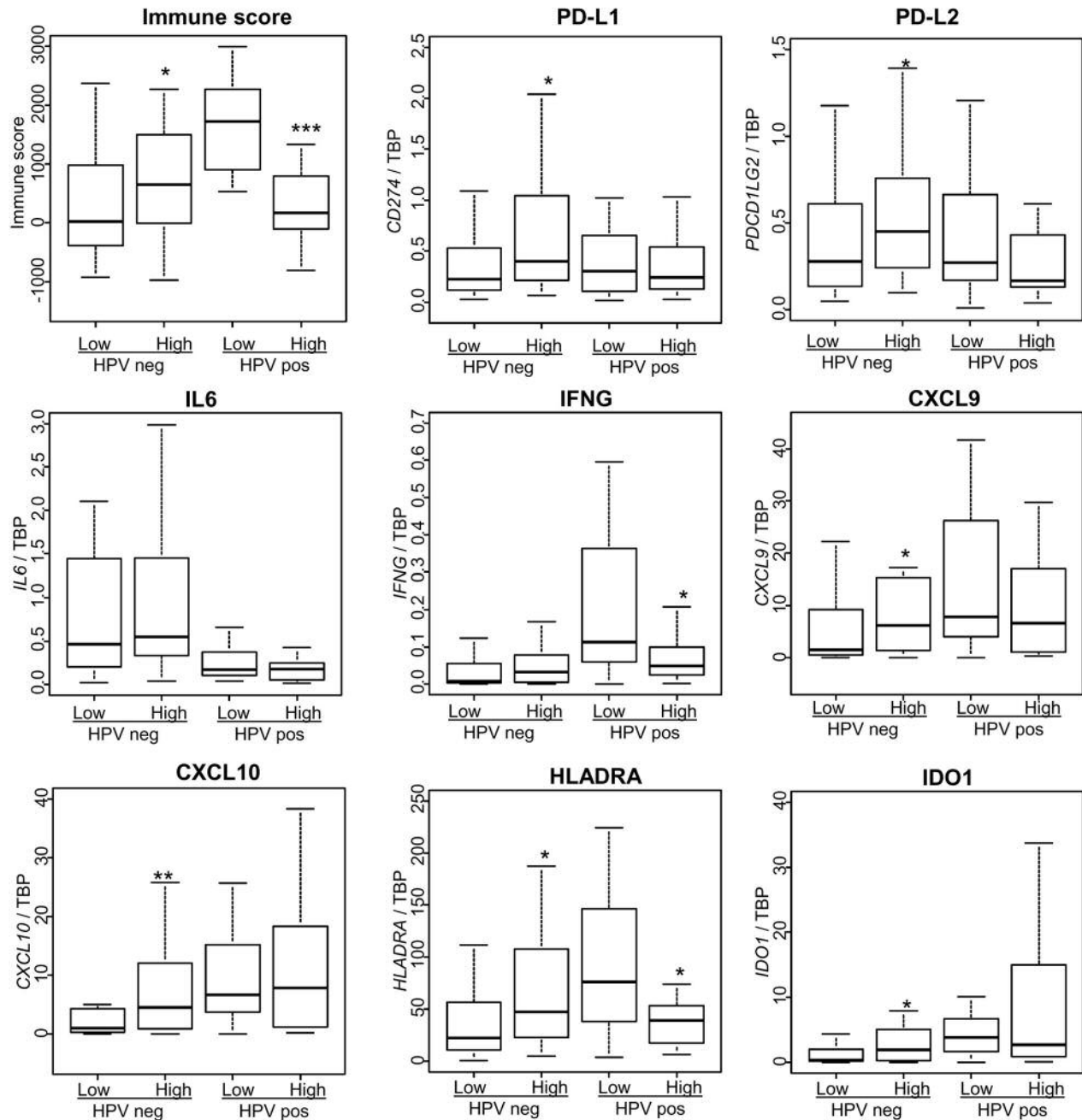
Figure 5. *SERPINB3 status as a potential determinant of tumour immunogenicity. The IMMUNE score, representing tumour immune cell infiltration, was calculated using the ESTIMATE algorithm comparing HNSCC tumors with high SERPINB3 expression (above the median) to tumours with low SERPINB3 expression (below the median) in patients stratified by HPV status (p16) (HPV+ n=72, HPV- n=37). The four groups were tested for their expression of the genes encoding the immune checkpoint modulators PD-L1 (CD274) and PD-L2 (PDCD1LG2) and those associated with the inflammatory and immune responses (IL6, IFNG, CXCL9, CXCL10, HLADRA and IDO1). Wilcoxon test was used to compare the high- and low-expression groups. $*p<0.05$, $**p<0.01$, $***p<0.001$.*

modulators PD-L1/PD-L2; iii) the local production of immune cytokines (26). HNSCC patients were stratified according to their HPV status, and we used the IMMUNE score, reported by Yoshihara *et al*. (24) to analyse the levels of tumour immune infiltration (Figure 5). We observed that HPV-negative tumours with high *SERPINB3* (above median) had a significantly higher IMMUNE score compared to HPV-negative tumours with low *SERPINB3* (below median). An

opposite effect was found with HPV-positive tumours that were characterized by a higher IMMUNE score compared to those that were HPV-negative: in HPV-positive tumours, overexpression of *SERPINB3* was associated with a significantly reduced lMMUNE score (Figure 5). Interestingly, HPV-negative tumours with high levels of *SERPINB3* were also found to express PD-L1 (*CD274*) and PD-L2 (*PDCD1LG2*) mRNA at higher levels than those with low levels of *SERPINB3*, an effect that was not seen in HPV-positive tumours (Figure 5). Finally, we examined the mRNA expression levels of the cytokines interleukin-6 (*IL6*) and interferon-γ (*IFNG*), as well as the Interferon-γ gene signature recently reported by Chow *et al*. in HNSCC (27). High levels of *SERPINB3* were not significantly associated with higher levels of *IL6* or *IFNG* mRNA, but there was a positive association with the *IFNG* signature (*CXCL9, CXCL10, HLADRA, IDO1*) in HPV-negative tumours (Figure 5).

Discussion

Analysis of genetic alterations and gene expression profiles holds potential for the stratification of HNSCC in the therapeutic context (28). In the present study, we used data retrieved from TCGA in order to explore the pathological and clinical significance of the tumour marker SCCA in HNSCC. Our analyses confirmed the broad spectrum of expression of *SERPINB3/B4* across tumour types, and the high expression levels of *SERPINB3/B4* in HNSCC compared to other tumours. We found that *SERPINB3* mRNA levels differed significantly depending on tumour etiology (alcohol, HPV), the location of the primary tumour, tumour grade, extension to lymph nodes and extracapsular spread. Expression levels of *SERPINB3* were not related to any of the genetic alterations reported to play a role in HNSCC progression (25). Interestingly, tumour *SERPINB3* mRNA levels were found to be associated with potential biological determinants of tumour immunogenicity: in HPV-negative HNSCC, high levels of *SERPINB3* expression were associated with a high tumour immune infiltrate score and high expression levels of the immune checkpoint modulators PD-L1 and PD-L2.

Measuring serum concentrations of SCCA constitutes a simple method that has previously been proposed to be of potential use for the diagnosis and follow-up of HNSCC patients (29-32). While the information gained from serum analysis *vs*. mRNA levels in tumours are likely different, it is interesting to consider the regulation of *SERPINB3/B4* mRNA levels in HNSCC and its potential implications for the use of SCCA. Firstly, we found that high expression of *SERPINB3/B4* mRNA is present in tumours other than HNSCC. Secondly, we observed that high mRNA levels of *SERPINB3/B4* are characteristic of less aggressive tumours in HNSCC. High *SERPINB3* expression was negatively

associated with tumour extension to lymph nodes and extracapsular spread, two essential pathological features that are used clinically to identify high risk HNSCC tumours that require adjuvant radiochemotherapy (3, 4). Both facts suggest that SCCA might not be suited for diagnostic use (33). On the other hand, the *SERPINB3/B4* status of HNSCC tumours could be helpful at the time of initial assesment of tumour agressiveness. Based on our study, we propose that analyses centered on the *SERPINB3* status may help physicians to adapt the follow-up of individual HNSCC patients, and help in the detection of tumour recurrence at an early stage.

The finding that HNSCC tumours with a HPV-negative status and high expression of *SERPINB3* mRNA have significantly higher immune score values and higher expression levels of the genes encoding PD-L1 and PD-L2 is an important finding of the present study. Indeed, recent studies have established the clinical efficacy of checkpoint inhibitors directed against programmed cell death-1 (PD-1) and PD-L1 for the treatment of recurrent/metastatic HNSCC (6-8). Because of the cost of these treatments and their toxicity, identifying biological parameters that predict their efficacy in individual patients is required (26, 34). Out of the five most important parameters that define the «tumour immunogram» (26), we found that at least three are associated with the *SERPINB3* status: i) the presence of an immune infiltrate; ii) high expression of the immune checkpoint modulator PD-L1, a marker associated with a better response to immunotherapy in HNSCC (7, 8, 35); iii) tumour sensitivity to immune effectors, such as Ifn-γ, as shown here with the *IFNG* signature reported by Chow *et al*. (27). Interestingly, similarly to our observations with *SERPINB3*, high expression levels of another tumour marker, Alpha-foetoprotein (AFP), were recently found in hepatocellular carcinoma cells with increased expression of PD-L1 (36). Based on our results and those of other recent studies on *SERPINB3/B4* (15, 19), we propose that further clinical studies are warranted to examine whether SCCA would be useful for predicting the efficacy of immune checkpoint inhibitors in HNSCC patients.

Acknowledgements

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