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 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/SERPXB4) 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...
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/SERPINO4 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/SERPINO4 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) mRNA 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/SERPINO4 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 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).
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 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 $r^2=0.92$, $p<0.001$) 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). *$p<0.05$, **$p<0.01$, ***$p<0.001$. 

Saidak et al: SERPINB3/B4 in HNSCC
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
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 R²>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 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.
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, CDKN2A, 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, KEAP1) (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...
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 IMMUNE 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 assessment of tumour aggressiveness. 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-fetoprotein (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.

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References


