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Immune checkpoint expression on tumor-infiltrating lymphocytes (TIL) is dependent on HPV status in oropharyngeal carcinoma (OPSCC) – A single-cell RNA sequencing analysis

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ABSTRACT

Introduction: A substantial proportion of head and neck squamous cell carcinoma (HNSCC), particularly oropharyngeal squamous cell carcinoma (OPSCC), is associated with human papillomavirus (HPV), resulting in distinct molecular phenotypes. In this study, we investigated differential immune checkpoint molecule (ICM) expression by HPV status using RNA sequencing data to identify additional ICM targets that may complement anti-PD1 antibodies.

Material and methods: RNA sequencing was performed on 51 OPSCC cases and validated using the TCGA HNSCC dataset. Unsupervised clustering and differential gene expression analyses in R were conducted based on HPV status. Additionally, a published single-cell RNA sequencing (scRNA) dataset of tumor-infiltrating lymphocytes (TIL) and peripheral immune cells (PBMC) (GSE139324) was analyzed with a Seurat pipeline grouped by HPV status.

Results: Our study identified a significant upregulation of all examined ICM in HPV-positive OPSCC through bulk RNA sequencing, validated by the TCGA cohort. Unsupervised clustering revealed a strong association between HPV-positive/-negative and high/low ICM expression cases respectively, indicating overlap between ICM and HPV status. In scRNA analysis, CD27, PD-1, OX-40, and BTLA were significantly more highly expressed on TILs of HPV-positive OPSCC. Conversely, VSIR was increased in PBMC and TILs of HPV-negative OPSCC, while LAG3 expression on PBMC was reduced in HPV-negative OPSCC.

Conclusion: Our study unveils the intricate interplay of ICMs in OPSCC, emphasizing the necessity for personalized therapeutic approaches based on HPV status and immune profiles. The identified ICMs, including PD1, CD27, and CTLA4, are promising candidates for further investigation and may enhance immunotherapeutic interventions in the HPV-dependent treatment strategies for OPSCC.

Background

Head and neck squamous cell carcinoma (HNSCC) is a frequently diagnosed cancer type, with nearly 900,000 new cases reported each year, leading to approximately 450,000 annual fatalities [1].

A quarter of all HNSCC cases and approximately 50–70 % of

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oropharyngeal squamous cell carcinomas (OPSCC) are associated with human papillomavirus (HPV) [2,3]. Chronic infection with a high-risk HPV sub-type can result in the development of OPSCC regardless of classical risk factors such as smoking and alcohol [4]. The different carcinogenesis of HPV^{pos} and HPV^{neg} HNSCC results in distinct molecular phenotypes [5–10]. The majority of HPV^{pos} OPSCC patients exhibit high counts of tumor-infiltrating lymphocytes (TIL) in their tumor tissue as a response to the viral antigen [11–14] in part comprised of HPV-specific immune cells [8,11,15,16].

At the moment, treatment of HNSCC is mainly agnostic of HPV-status and HPV^{pos} OPSCC patients have a much better prognosis than HPV^{neg} patients [17]. The survival benefit of HPV^{pos} OPSCC is most likely attributable to the patient's anti-tumor immune response and independent of individual treatment protocols [18,19]. It appears that a small proportion of patients with HPV^{pos} OPSCC and low TIL status may have disease-related survival rates that are somewhat comparable to those of HPV-negative OPSCC [20,21]. Programmed death 1 (PD1) antibody treatment has been established as a standard of care for platinum-naïve recurrent or metastatic (R/M) HNSCC, either as a monotherapy or in combination with cisplatin and 5-fluorouracil [22], and as a monotherapy for platinum-refractory R/M HNSCC [23,24]. It has already been shown that the inflamed tumor microenvironment in HPV^{pos} HNSCC is associated with improved response to anti-PD1 treatment [14]. The combination of anti-PD1/PD-L1 treatments with conventional treatment in a curative setting is currently under investigation in various clinical trials [25–27]. Besides the co-inhibitory PD1 and PD-L1 molecules further conceivably targetable ICM have been identified. These can be divided into co-stimulatory ICM such as CD137, OX40, glucocorticoid-induced TNFR family-related gene (GITR) and CD27 and co-inhibitory ICM such as cytotoxic T-lymphocyte antigen 4 (CTLA4), B and T lymphocyte attenuator (BTLA), lymphocyte activation gene 3 (LAG3) or T-cell immunoglobulin and mucin-domain containing-3 (TIM3). Publications using immunohistology have already shown that different ICM (e.g. LAG3, TIM3, VISTA) are correlated to increased CD8 + TIL counts and HPVpos OPSCC [28].

As the importance of modulating immune checkpoint molecules (ICM) for HNSCC treatment is increasing, the characterization of ICM expression on a single cell basis differentiated by HPV status could provide evidence for potentially targetable ICM in HPV^{pos} or HPV^{neg} OPSCC.

Here, we analyzed bulk RNA sequencing data from a British and a German as well as the TCGA cohort with a focus on differential ICM expression by HPV status. In addition, we studied a published single-cell RNA dataset of primary tumors and related samples of peripheral blood mononuclear cells (PBMC) and TIL.

Methods

Patients

Our own RNA sequencing cohort (main test cohort) consisted of 51 OPSCC primary tumor samples (40 samples obtained from the University Hospital Ulm, Germany, and 11 samples kindly provided by Prof. Ottensmeier from Southampton University, UK). Representative tissue samples were retrieved from the primary tumor site during surgery and snap-frozen in liquid nitrogen. Samples were stored at −80 °C until use. This research was approved by the respective ethics committees (Ulm University: Approval number 222/13; 90/15; UK Medical Research and Ethics Committee: Approval number MREC 09/H0501/90). Patient samples were collected after obtaining written informed consent.

The HPV status was determined via RNA-Seq, with reads aligned to HPV high-risk type genomes using the viGen bioinformatic pipeline [29]. Samples with ≥1000 reads for HPV E6 or E7 RNA or ≥1000 reads for all HPV oncogenes (E1, E2, E4, E5, E6, E7, L1, L2) in summary were considered HPV-positive.

The TCGA RNA-Seq dataset (validation cohort) of HNSCC was downloaded from http://xena.ucsc.edu/ on 5th February 2020. The values used were log2 (TPM + 0.001). Clinical parameters were derived from TCGA annotations. HPV status was based on alignment of RNA to the HPV genome as previously defined (>1000 HPV E6/E7 RNA reads) in 279 patients [30]. For the remaining OPSCC, p16 immunohistochemistry and HPV in-situ hybridization were used for classification as HPV-positive as described previously [31]. Patients without sufficient data to establish their HPV status were excluded. A total of 66 cases of OPSCC were included in the analysis.

RNA sequencing

Cohort 1 (Ulm): Total RNA was extracted using the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) from fresh, snap-frozen tumor samples. RNA sequencing was performed using Illumina's next-generation sequencing methodology [32]. Specifically, total RNA was quantified and quality-checked using an Agilent 2100 Bioanalyzer Instrument (Agilent RNA 6000 Pico). Libraries were prepared from 500 ng of input material using TruSeq Stranded mRNA, following the manufacturer's instructions, and subsequently quantified and quality checked using an Agilent 2100 Bioanalyzer Instrument (DNA 7500 kit). Libraries were pooled and sequenced in a single lane of the HiSeq 2500 System running in 51 cycle/single-end/high output mode. Sequence information was converted to FASTQ format using bcl2fastq (2.20.0.422). High-quality reads were mapped to the human genome (hg38) using STAR (v2.0.9) and, following the removal of multimapping reads, converted to gene-specific read counts for annotated genes using featureCounts (v2.0.0). The total mapping percentage (uniquely mapped reads/total raw reads) was over 75 %.

Cohort 2 (Southampton; Soton): Total RNA from 11 cases of Southampton was extracted from snap-frozen tissue (Maxwell® RSC simplyRNA Tissue Kit (AS1340, Promega, Southampton, UK) and concentration and quality were analyzed using the RNA Nano Kit for the 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA, USA). RNA sequencing was conducted by Edinburgh Genomics (University of Edinburgh, Edinburgh, UK). An automated TruSeq Stranded mRNA-Seq library preparation from total RNA and the NovaSeq sequencing system was used (100 bp paired-end; 1750 M + 1750 M reads, Illumina, San Diego, CA, USA).

scRNA sequencing data from GEO

The raw data of scRNA sequencing were downloaded for 26 samples with paired peripheral blood mononuclear cells (PBMC) and tumor-infiltrating immune cells (TIL) from HNSCC patients (18 HPV^{neg} and 8 HPV^{pos}) (GSE139324; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE139324) [33,34]. The data were bioinformatically processed, concatenated, and analyzed via the Seurat pipeline based on gene count values [35]. The whole dataset of the published TIL and PBMC samples, which were sorted by CD45 and contained all lymphocytes, was included in the analysis.

Data analysis and statistics

RNA sequencing data analysis was performed in R (v4.0.2). The differential expression analysis was performed using DESeq2 (v1.29.16). Clustering in the heatmaps was done using hierarchical clustering within the pheatmap (v1.0.12) package. The scRNA-sequencing data was bioinformatically processed, concatenated, and analyzed via the Seurat pipeline [35,36]. Data were analyzed and graphed using GraphPad Prism (v8.4.2) and R (v4.0.3) with RStudio (v1.2.5033). For ICM-specific group analyses (HPV^{pos} vs. HPV^{neg}) an unpaired, non-parametric Mann-Whitney test was used. When comparing more ICMs a 2-way ANOVA was performed and corrected for multiple comparisons with the Benjamini, Krieger and Yekutieli method. The usage of the different datasets is summarized in a consort diagram (**supplementary**

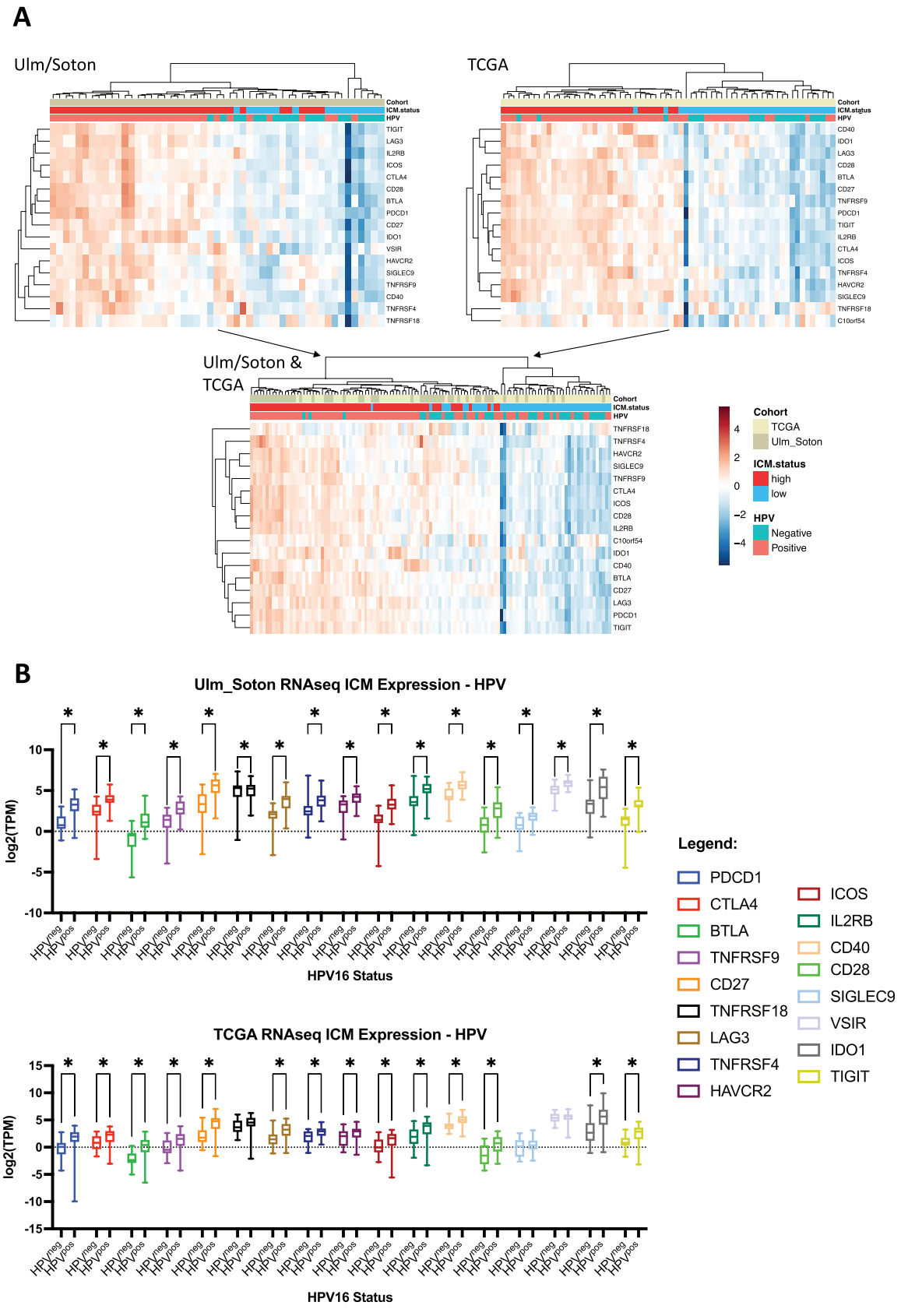

Fig. 1. Unsupervised hierarchical clustering of bulk RNA sequencing data from Ulm/Southampton and TCGA separated and combined (**A**). Box and whiskers plots (min to max, median) showing the different expression levels of ICM for the Ulm/Soton and the TCGA cohort (**B**). *ICM = Immune checkpoint molecule, HPV = human papillomavirus, (Significance level: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).*

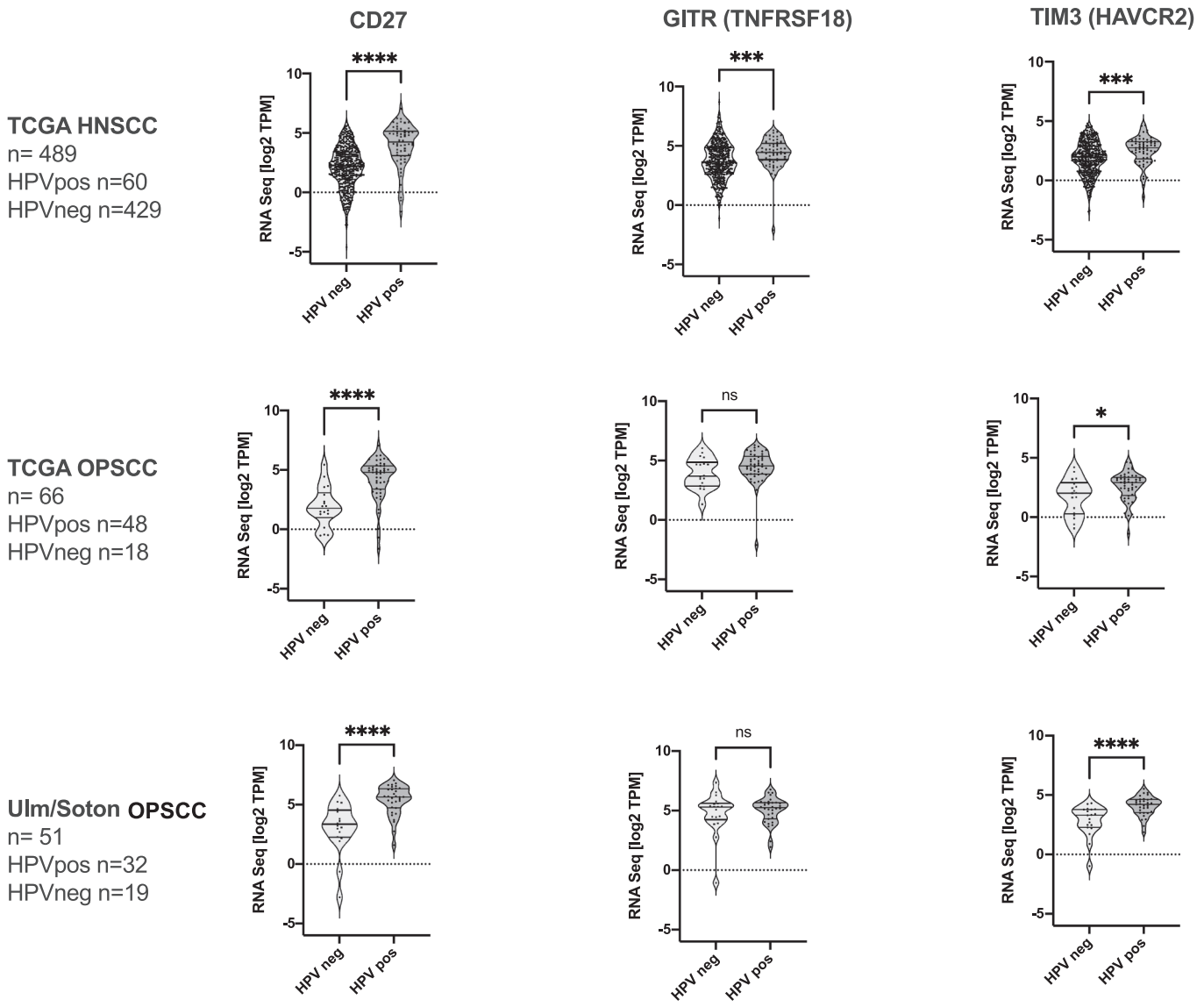

Fig. 2. Immune-Checkpoint Molecule (ICM) RNA expression data of HNSCC TCGA, Ulm/Soton cohort. CD27, GITR, and TIM3 RNA expression data (log2 TPM) is shown for the TCGA whole HNSCC cohort, the TCGA OPSCC cohort, and the Ulm/Soton cohort. Significant differences between the HPV groups are marked with asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Fig. 1). The survival analysis (Kaplan-Meier) was performed by a curve comparison with the Log-rank (Mantel-Cox) test. The source of the survival data was clinical records from Ulm, Germany, and Southampton, UK, as well as publicly available clinical annotations for the OPSCC cases of the TCGA HNSCC dataset (TCGA-HNSC). The cumulative ICM-expression score was calculated using the median of the expression values of all analyzed ICM: PDCD1, CTLA4, BTLA, TNFRSF9, CD27, TNFRSF18, LAG3, TNFRSF4, HAVCR2, ICOS, IL2RB, CD40, CD28, SIGLEC9, IDO1, TIGIT, HLA-G, HLA-E, and VISTA.

Results

Immune checkpoint molecule (ICM) expression differs by HPV status

An unsupervised clustering analysis using the respective ICM revealed a strong separation of ICM-low and ICM-high cases, indicating a clear overlap between the ICM status and the HPV status in both the test cohort and the TCGA OPSCC validation cohort. After combining both datasets, the clustering became even more pronounced (Fig. 1**A**).

We selected 17 targetable ICM for our analysis. Inhibitory ICM, namely PD1, CTLA4, BTLA, LAG3, HAVCR2 (TIM3), SIGLEC9, VSIR (C10orf54), IDO1, TIGIT and stimulatory ICM, namely TNFRSF9 (CD137), CD27, TNFRSF18 (GITR), TNFRSF4(OX40), ICOS (CD278), IL2RB, CD40, CD28 were selected. In a multiple comparisons analysis, we found a statistically significant higher expression of all ICM in HPV^{pos} OPSCC in our test cohort (Ulm and Soton) with higher p-values for GITR and VSIR. The same result was observed in the TCGA validation cohort except for a lack of statistically significant differences between HPV^{pos} and HPV^{neg} cases for TNFRSF18 (GITR) and SIGLEC9 as well as VSIR (Fig. 1**B**).

CD27, GITR, and TIM3 RNA expression in HNSCC primary tumors differ by HPV status

We used the HNSCC RNA sequencing dataset from TCGA (n = 489) and our own HNSCC RNA sequencing cohort (n = 51) for a DeSeq2 analysis with a comparison of HPV^{pos} with HPV^{neg} HNSCC cases. Across all TCGA HNSCC cases (n = 489) we discovered statistically significantly higher ICM expression values in HPV^{pos} HNSCC cases for the ICM CD27, GITR, and TIM3 (Fig. 2). To validate these results, we repeated the

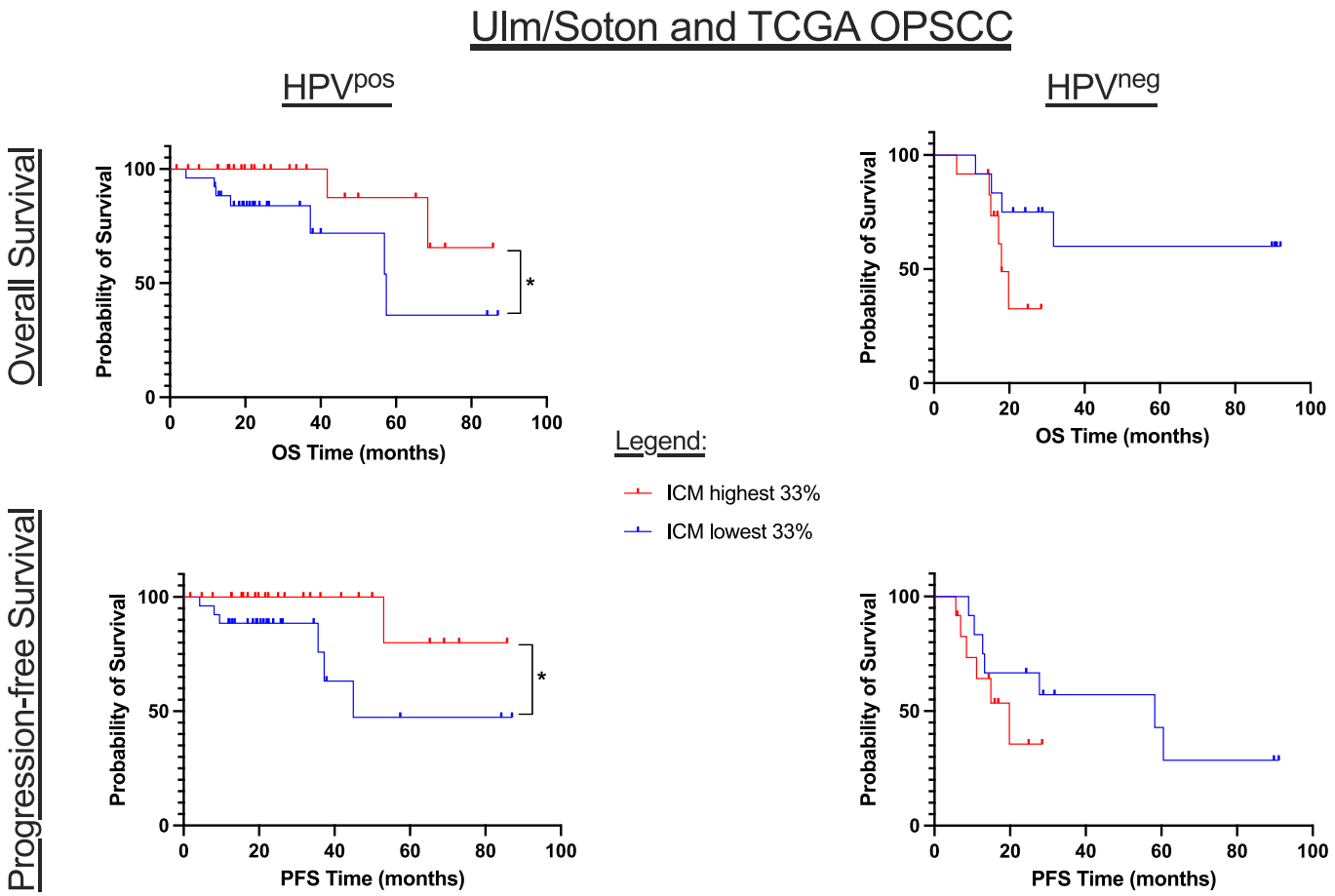

Fig. 3. Kaplan-Meier curves show the overall survival and the progression-free survival of the HPV^{pos} and the HPV^{neg} OPSCC patients for their cumulative ICM expression value. *(Significance level: * $p < 0.05$).*

DeSeq2 analysis using only OPSCC cases of TCGA and our own Ulm/Soton cohort separately. The differences observed for CD27 in the entire TCGA cohort were also statistically significant in the OPSCC subgroup of the TCGA cases and in our own Ulm/Soton cohort (Fig. 2). The differences for TIM3 and GITR were observed not only in the TCGA OPSCC cohort but also in the Ulm/Soton cohort. However, it was not significant for GITR in the Ulm/Soton cohort and for TIM3 in the TCGA OPSCC cohort, most likely due to low case numbers in the HPV^{neg} group. Combining the TCGA and the Ulm/Soton cohort revealed a highly significant difference for CD27 ($p < 0.0001$), and a significant difference for TIM3 ($p = 0.0248$) while GITR did not reach significance (not shown).

Survival benefit of ICM expression only in HPV^{pos} cases

We used the ICM status to group the HPV^{pos} and HPV^{neg} cases into the lowest and highest third of ICM expression. In HPV^{pos} OPSCC, we observed a statistically significant improvement in both overall survival (OS) and progression-free survival (PFS) with high ICM expression levels ($p < 0.05$). In contrast, for HPV^{neg} OPSCC cases, no statistically significant difference in OS or PFS was observed between high and low ICM expression groups. Interestingly, a trend was noted in which higher ICM expression was associated with slightly worse outcomes, although this effect did not reach statistical significance. This suggests that the survival benefit of increased ICM expression may be unique to HPV^{pos} cases and not applicable to HPV^{neg} OPSCC (Fig. 3).

scRNA sequencing

To validate the findings of the bulk RNA sequencing we performed a scRNA sequencing analysis of the published dataset GSE139324. After dividing of the data into HPV^{pos} and HPV^{neg}, the analysis confirmed the higher expression of CD27 of the tumor-infiltrating lymphocytes (TIL) in HPV^{pos} OPSCC. Also, PD-1, OX-40, and BTLA were statistically higher expressed in the TILs of HPV^{pos} OPSCC. Interestingly, VSIR was increased in PBMC and TILs of HPV^{neg} OPSCC, and LAG3 expression on PBMC was reduced in HPV^{neg} OPSCC. The summarized differences in the TILs were: CD27, CD28, TIGIT, PD-1, BTLA, TNFRSF9 (CD137), and CD40 were elevated in HPV^{pos} OPSCC, while ICOS, TNFRSF4 (OX40), TNFRSF18 (GITR), and ENTPD1 (CD39) were increasingly expressed in HPV^{neg} OPSCC (selection of ICM shown Fig. 4, all ICM shown in **supplementary** Fig. 2). The ratio calculation comparing the expression of CTLA4 between TILs and PBMCs revealed a 140-fold higher expression in HPV-negative cases, whereas the ratio was only 13-fold higher in HPV-positive cases. For LAG3 we also observed a higher expression ratio for HPV^{neg} (17 *vs.* 7), respectively.

Discussion

In this study, we present a comprehensive analysis of the expression of immune checkpoint molecules (ICM) in HNSCC, with a particular focus on the impact of HPV status. Our findings reveal substantial differences in ICM expression profiles, suggesting distinct immune landscapes in HPV-positive (HPV^{pos}) and HPV-negative (HPV^{neg}) OPSCC.

Our investigation highlights significant variations in ICM expression between HPV^{pos} and HPV^{neg} OPSCC cases in the bulk RNA sequencing

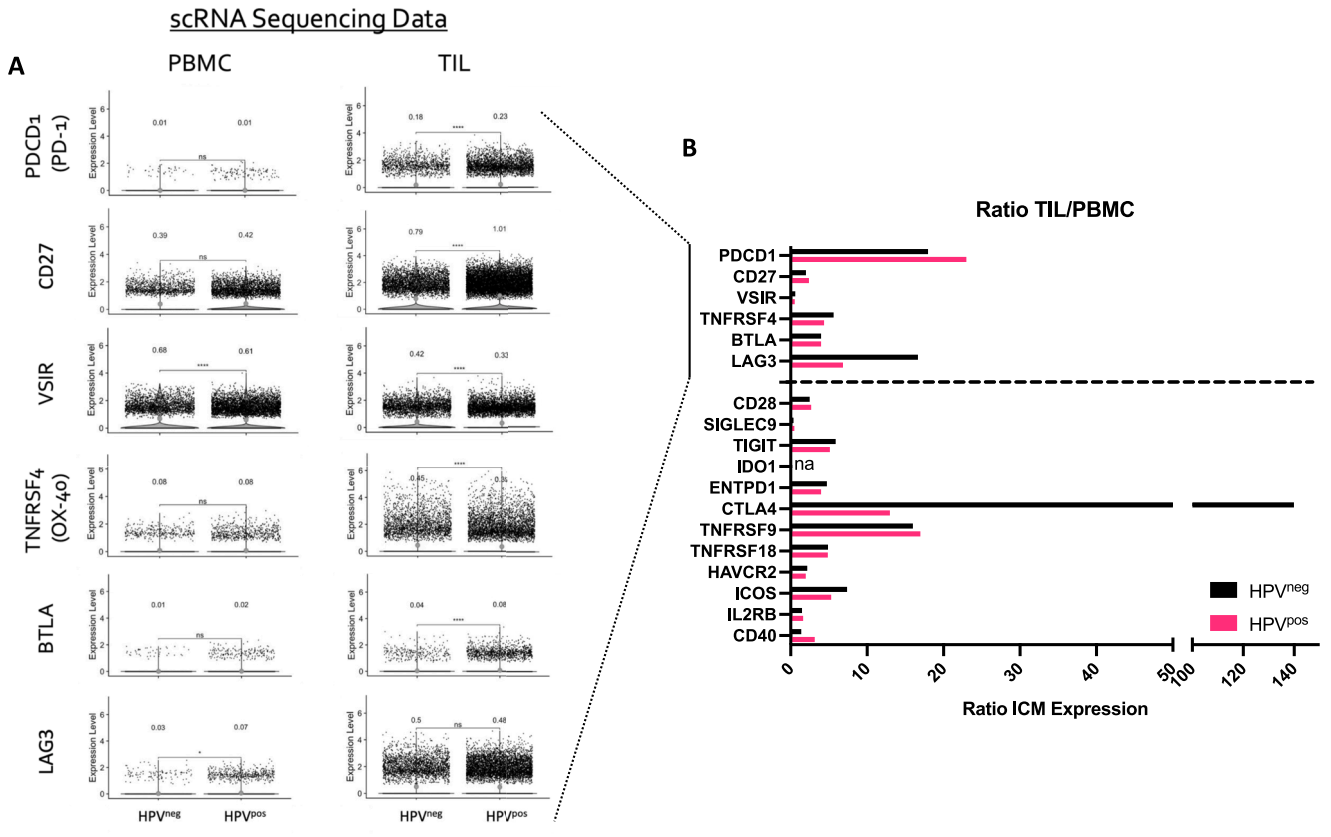

Fig. 4. Gene expression of selected ICM in peripheral immune blood cells and the tumor-infiltrating immune cells using the scRNA sequencing dataset (**A**). Bar graph showing the ICM expression ratio in TILs in comparison to the PBMC expression (**B**). ("Expression level" = Seurat pipeline output of normalized sc RNA gene counts, *Significance level: * $p < 0.05$, **** $p < 0.0001$).*

cohort. The higher expression of ICMs in HPV^{pos} samples prompts intriguing questions regarding the involvement of immune cells in the tumor microenvironment (TME). To minimize bias due to the higher immune infiltration of HPV^{pos} we included a scRNA sequencing analysis. Our findings of higher ICM expression on TILs in the HPV^{pos} OPSCC might be indicative of an ongoing immune response against the tumor [37]. This may also explain why HPV^{pos} OPSCC patients respond better to immune checkpoint blockade [14] and standard treatment due to HPV-specific T cells [20,38]. The bulk RNA sequencing ICM expression data were combined and used to investigate the influence on survival. We found a survival benefit for overall and progression-free survival only for the HPV^{pos} OPSCC group if a higher ICM expression was present. This correlation between ICM expression and improved survival rates underlines the immune-related mechanism and the crucial role of ICM in modulating the anti-tumor immune response.

The observed alterations in stimulatory and inhibitory ICMs might also change over time and treatment, indicating a dynamic interplay within the immune landscape [39]. These changes emphasize the need for personalized therapeutic strategies that consider the current immune state of the OPSCC in a specific patient.

In this study, we observed a statistically higher expression of the T cell activation marker CD137 on TILs in HPV^{pos} OPSCC, which highlights the immune activation in the TME due to the viral antigen and the higher chance of a positive response to immune checkpoint therapy. A published immunohistology study highlighted the expression of immune checkpoint proteins LAG-3, TIM-3, and VISTA in OPSCC, correlating their levels with CD8 + T-cell inflammation and HPV status [28]. In our analysis we also observed increased expression of PD-1 in TILs in HPV^{pos} cases. Our scRNA sequencing analysis also revealed higher LAG3 expression ratios between TILs and PBMCs in HPV^{neg} cases compared to the more modest increases in HPV^{pos} cases, underscoring the distinctive immune checkpoint patterns tied to HPV status in OPSCC.

The elevated CD27 expression in both peripheral and tumor samples of HPV^{pos} cases aligns with its known role as a co-stimulatory molecule. The positive correlation with TIL-high tumors also suggests a potential avenue for enhancing anti-tumor immune responses. Dysregulation of the interplay between CD27 and its receptor CD70 is known to be associated with tumor progression and immune suppression [40]. If CD27 is activated it acts as a stimulus for antigen-specific T cell expansion [41] and is shown to be a marker of an inflamed TME [14]. This highlights a possible treatment strategy, especially for HPV^{pos} OPSCC, activating CD27 on TILs to enhance anti-tumor immune response [42]. However, first clinical approaches in combination with Nivolumab did not show any superior anti-tumor responses [43].

Regarding the CTLA4 scRNA expression between blood and tumor compartments in HPV^{neg} cases, it unveils intricate interactions between the peripheral immune cells and TILs in the TME. We observed a considerably higher expression of CTLA4 on the TILs in comparison to PMBCs in HPV^{neg} (140 times higher), this might explain the lower response rates to anti-PD1 treatment in HPV^{neg} cases in comparison to HPV^{pos}. This can also potentially influence intra-tumoral T_{REG} activity. The higher expression of OX40 in HPV^{neg} OPSCC may encourage its targeting to enhance anti-tumor immunity, as the OX40 – OX40 ligand interaction can lead to a reduction of regulatory T cell activity [44].

Our study revealed a higher expression of CD39 on TILs in HPV^{neg} OPSCC, which may indicate the increased release of immunosuppressive adenosine in the TME [45]. Therefore, ongoing research targeting CD39 is ongoing and shows promising first results in a reduction of adenosine as well as the reduction of T_{REG} activity [46]. On the other hand, some studies have indicated that CD39 expression, especially in combination

with CD103 expression, identifies antigen-experienced TIL, so-called tissue-resident memory cells, in the TME [47–49].

The distinct ICM expression patterns in HPV^{pos} and HPV^{neg} cases underscore the necessity for tailored therapeutic strategies dependent on the patient's HPV status. Underlying functional analyses and further investigations are required to explore the immunological interaction and the feasibility and efficacy of such interventions.

While our study provides valuable insights into ICM expression in HPV^{pos} OPSCC, it is essential to acknowledge certain limitations. Although our findings confirm previous research highlighting differences in ICM expression between HPV^{pos} and HPV^{neg} OPSCC [50,51], we offer new perspectives through the integration of bulk tumor sequencing data with single-cell RNA sequencing and by comparing the ICM expression between tumor-infiltrating lymphocytes and peripheral immune cells.

Additionally, the scRNA sequencing should be performed in future after only sorting for target cell populations like $CD8^+$ or $CD4^+$ T cells separately for a more in-depth analysis.

Overall, despite being partially sconfirmatory in nature, our comprehensive analysis enriches the existing literature and underscores the potential of various ICMs, such as PD1, CD27, and CTLA4, as targets for immune modulation therapy in HPV^{pos} OPSCC. This work emphasizes the need for personalized therapeutic approaches based on HPV status and immune profiles, paving the way for more effective immunotherapeutic interventions for OPSCC patients and future investigations in this area.

CRediT authorship contribution statement

Adrian von Witzleben: Writing – original draft, Visualization, Software, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ayla Grages:** Formal analysis, Data curation. **Jaya Thomas:** Writing – review & editing, Validation, Software, Methodology, Formal analysis, Data curation. **Jasmin Ezić:** Software, Formal analysis, Data curation. **Cornelia Brunner:** Writing – review & editing, Supervision. **Patrick J. Schuler:** Writing – review & editing. **Johann M. Kraus:** Writing – review & editing, Software. **Hans A. Kestler:** Writing – review & editing, Software. **Julius M. Vahl:** Writing – review & editing. **Johannes Doescher:** Writing – review & editing. **Emma V. King:** Writing – review & editing. **Christian H. Ottensmeier:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Thomas K. Hoffmann:** Writing – review & editing, Supervision. **Simon Laban:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Simon Laban: Advisory Boards: Merck Sharp & Dohme (MSD), Bristol Myers Squibb (BMS), Astra Zeneca (AZ). Honoraria: MSD, BMS, AZ, Merck Serono. Johannes Döscher: Advisory Boards: Merck Serono. Honoraria: Merck Serono. Thomas K. Hoffmann: Advisory Boards: MSD, BMS. Honoraria: MSD, BMS, Merck Serono. Patrick Schuler: Advisory Boards: BMS. All other authors did not declare a conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.oraloncology.2024.107107.

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