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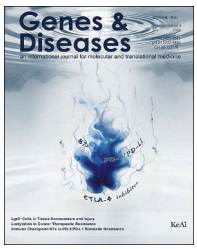

RAPID COMMUNICATION

Immune patterns of the tumor microenvironment in PD-L1$^+$ EGFR$^+$ HNSCC patients received anti-PD-1 and EGFR-based neoadjuvant therapy

Anti-programmed death 1 (PD-1) and epidermal growth factor receptor (EGFR)-based neoadjuvant therapy was confirmed to be effective for the treatment of head and neck squamous cell carcinoma (HNSCC). However, a previous study found that nearly 1/3 of HPV-negative HNSCC experienced no significant reduction or even increase in tumor size.[1] Up till now, few studies have demonstrated the clinicopathological patterns of PD-L1$^+$ EGFR$^+$ HNSCC patients. Therefore, single-cell RNA-sequencing (scRNA-seq) analysis was applied in our retrospective study of 1077 HPV-negative HNSCC patients to characterize the immune patterns of the tumor microenvironment (TME) after anti-PD-1 and EGFR-based neoadjuvant therapy. In this study, we identified for the first time that most PD-L1$^+$ EGFR$^+$ HNSCC patients were in an advanced clinical stage and suffered lymph node metastasis. HPV-negative HNSCC patients experienced a significant reduction of tumor size after receiving 1 course of anti-PD-1 and EGFR-based neoadjuvant therapy. Meanwhile, PD-L1$^+$ EGFR$^+$ HNSCC patients have stronger invasive ability. The CXCL11/CXCR3 pair in PD-L1$^+$ EGFR$^+$ HNSCC cells contributed to the construction of an immunosuppressive TME by recruiting plasmacytoid dendritic cells, regulatory T cells, dendritic cells and CD8$^+$ T cells. CD73, OX40, and TIM-3 are potential targets for immunotherapy sensitization in HPV-negative HNSCC. Our finding provides an immunotherapeutic sensitization strategy for PD-L1$^+$ EGFR$^+$ HNSCC.

Immune checkpoint blockade (ICB) is a successful immunotherapy strategy applying in HNSCC. Currently, combination immunotherapy has become a new strategy for the improvement of efficacy. Anti-PD-1 and EGFR-combined immunotherapy obtained a relatively high objective response rate (ORR) of 45% in HNSCC patients.[1] However, nearly 1/3 of HPV-negative HNSCC experienced no significant reduction or even increase in tumor size. Hence, we first collected 1,077 HNSCC patients between May 2014 and May 2024 and divided the cohort into a PD-L1$^+$ EGFR$^+$ group ($n = 882$) and a non-PD-L1$^+$ EGFR$^+$ group ($n = 195$), which included PD-L1$^+$ EGFR$^-$ ($n = 117$), PD-L1$^-$ EGFR$^+$ ($n = 57$) and PD-L1$^-$ EGFR$^-$ groups ($n = 21$) (Table S1). The number of PD-L1$^+$ EGFR$^+$ HNSCC in the T2 stage ($n = 330$, 37.4%) ($P < 0.0001$), N1-3 stage ($n = 312$, 35.4%) ($P = 0.0324$) and clinical stage III were higher than those in the non-PD-L1$^+$ EGFR$^+$ HNSCC group (Fig. 1A; Table S2). Compared with those combined positive score (CPS) in 1–20, the degree of lymph node metastasis was significantly reduced in PD-L1$^+$ EGFR$^+$ HNSCC patients with CPS scores higher than 20 (Fig. 1B; Table S3). To investigate the relationship between PD-L1 and EGFR levels and the prognosis of HNSCC patients, we collected follow-up information of 1077 HNSCC patients for prognosis analysis. There was no significant difference in recurrence-free survival (RFS) ($P = 0.8711$) or overall survival (OAS) ($P = 0.6620$) between PD-L1$^+$ EGFR$^+$ and non-PD-L1$^+$ EGFR$^+$ HNSCC patients, which was consistent with the prognostic results of 394 HNSCC samples from The Cancer Genome Atlas (TCGA) (Fig. 1C–E). We inferred that PD-L1 or EGFR expression levels play a limited role in predicting the prognosis of HNSCC patients. Twenty-seven HNSCC patients in our cohort received a course of anti-PD-1 (Sintilimab, 200 mg) and EGFR (Nimotuzumab, 200 mg)-based neoadjuvant therapy, 15 of whom were in the PD-L1$^+$ EGFR$^+$ group and 12 in the non-PD-L1$^+$ EGFR$^+$ group. By reviewing the enhanced CT and clinical information (Fig. S1A), we found that HNSCC patients harvested a significant reduction of tumor size after receiving 1 course of neoadjuvant therapy. Although there was no statistically significant difference, PD-L1$^+$ EGFR$^+$ HNSCC patients

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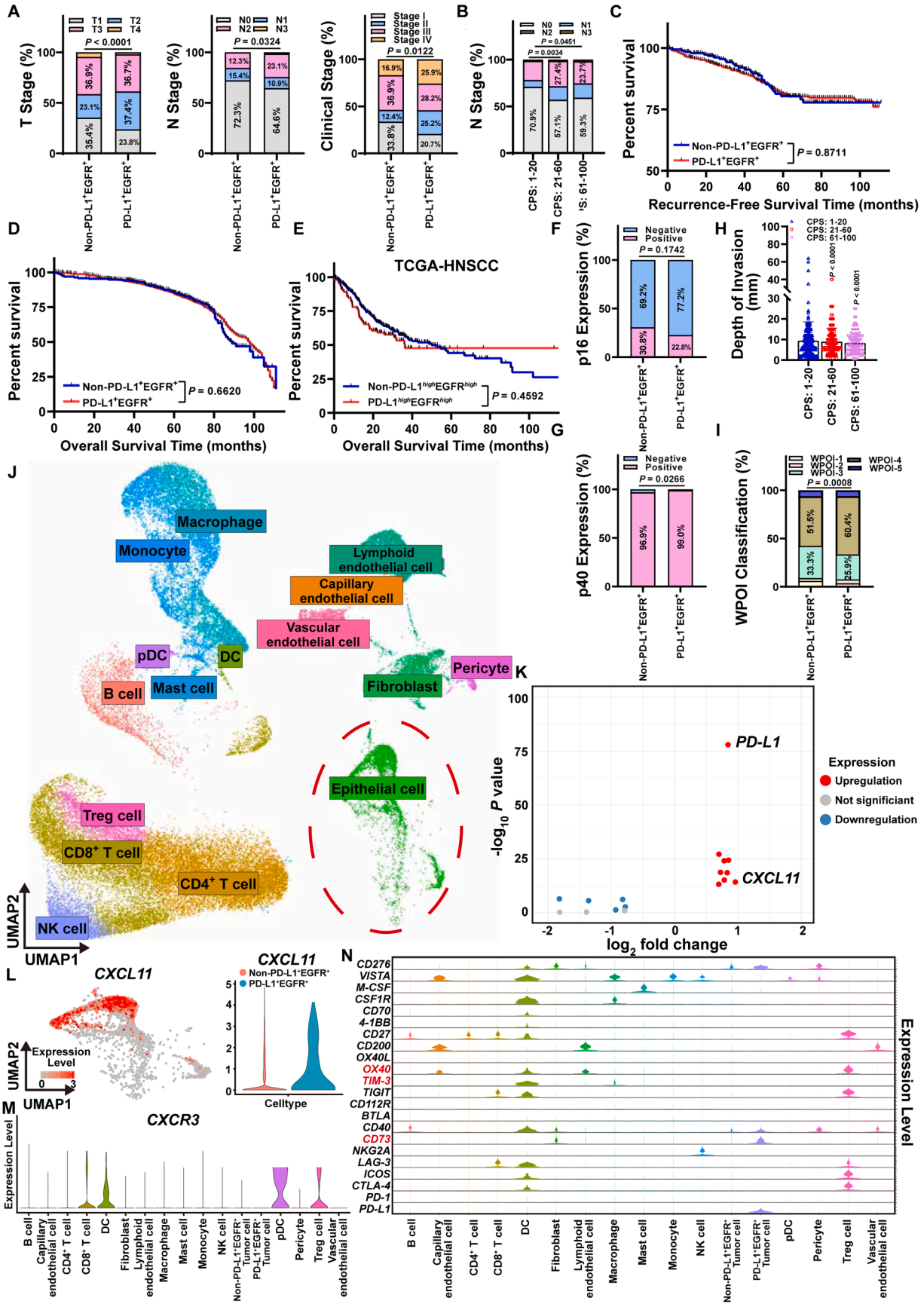

Figure 1 Immune patterns of the TME in PD-L1^{+} EGFR^{+} HNSCC. **(A)** Proportion of T stage (left), N stage (middle) and clinical stage (right) between PD-L1^{+} EGFR^{+} and non-PD-L1^{+} EGFR^{+} HNSCC patients. **(B)** Proportion of N stage among the three subgroups of

exhibited better outcomes after receiving anti-PD-1 and EGFR-based combination therapy (Fig. S1B).

Furthermore, we determined to explore the molecular reasons for the unsatisfactory efficacy of anti-PD-1 and EGFR-based neoadjuvant therapy in PD-L1$^+$ EGFR$^+$ HNSCC patients. The majority of PD-L1$^+$ EGFR$^+$ HNSCC patients were negative for p16 (77.8%) and positive for p40 (99.0%) (Fig. 1F and G). And 87.2% of HNSCC patients in our cohort were positive for EGFR expression. There was no significant difference in the DOI between the PD-L1$^+$ EGFR$^+$ group and the non-PD-L1$^+$ EGFR$^+$ group ($P = 0.9221$) (Fig. S2F). Additionally, the DOI of PD-L1$^+$ EGFR$^+$ HNSCC decreased as the CPS score increased (Fig. 1H). The worst pattern of invasion (WPOI) is a histopathology-based assessment of the invasive characteristics at the leading edge of solid tumors. PD-L1$^+$ EGFR$^+$ HNSCC had a higher WPOI-4 subtype (60.4%), whereas non-PD-L1$^+$ EGFR$^+$ HNSCC was predominantly the WPOI-3 subtype (33.3%), suggesting that PD-L1$^+$ EGFR$^+$ HNSCC has a stronger invasive ability (Fig. 1I; Fig. S2H).

To further explore the TME landscape of PD-L1$^+$ EGFR$^+$ HNSCC, we analyzed a public scRNA-seq data of HNSCC (GSE164690). Epithelial cells were extracted and reannotated into PD-L1$^+$ EGFR$^+$ tumor cells and non-PD-L1$^+$ EGFR$^+$ tumor cells based on the levels of PD-L1 and EGFR expression (Fig. 1J; Fig. S3A and S3B). By performing differentially expressed gene (DEG) analysis between the two types of tumor cells, we identified 9 up-regulated DEGs and 4 down-regulated DEGs (Table S4), with *CXCL11* being the most significantly up-regulated DEG (Fig. 1K). CXCL11, predominantly expressed in PD-L1$^+$ EGFR$^+$ tumor cells, was significantly higher than that in non-PD-L1$^+$ EGFR$^+$ tumor cells (Fig. 1L; Fig. S3D—S3F). The chemokine C-X-C motif receptor 3 (CXCR3) is known as a receptor of CXCL11 and plays a vital role in regulating the migration and activation of immune cells in the TME by responding to the recruitment of CXCL11. We found that *CXCR3* was mainly expressed in the plasmacytoid dendritic cells (pDCs), regulatory T (Treg) cells, dendritic cells (DCs) and CD8$^+$ T cells in HNSCC, the former two of which are key immunosuppressive cells constituting the TME (Fig. 1M). Meanwhile, *PD-1* was highly expressed in CD8$^+$ T cells and Treg cells, but was expressed at a lower level in pDCs sand DCs (Fig. S3C). In addition, the up-regulated guanylate binding protein (GBP1) is associated with the IFN-γ-induced immune activation.[2] The overexpression of tryptophanyl-tRNA synthetase (*WARS*) can decrease the level of PD-1 on the surface of CD8+ T cells.[3] *CTSC*, a well-known apoptosis-associated gene, may act as a driver of apoptosis in tumor cells.[4] These DEGs discussed above can be regarded as the potential response to anti-tumor immunity in PD-L1$^+$ EGFR$^+$ tumor cells. However, there are still several significant DEGs (i.e., *F3*, *TGFBI*) associated with resistance to immunotherapy.[5] In summary, PD-L1$^+$ EGFR$^+$ tumor cells recruit immunosuppressive cells such as pDCs and Treg cells through the *CXCL11/CXCR3* ligand—receptor pair to constitute an immunosuppressive microenvironment of HNSCC. Besides, CD8$^+$ T cells and DCs are fundamental for sensitizing PD-L1$^+$ EGFR$^+$ HNSCC to anti-PD-1 and EGFR-based combination immunotherapy.

According to studies of ICIs in solid tumors, we summarized all immune checkpoints for which ICIs have been developed (Fig. S4). In addition to *PD-L1*, *CD73* was specifically expressed on PD-L1$^+$ EGFR$^+$ tumor cells, which are potential targets for immunotherapy, such as Oleclumab, JAB-BX102, and Mupadolimab, to inhibit immune evasion in HPV-negative HNSCC. The immunostimulatory molecule, *OX40*, was specifically up-regulated on Treg cells. Tumor immunotherapy sensitization can be achieved by using monoclonal antibodies to specifically block the immunostimulatory molecule *OX40* (e.g., Rocatinlimab) to inhibit Treg cell activation. Since TIM-3 is specifically up-regulated in HNSCC, Sabatolimab, a monoclonal antibody targeting TIM-3, is more likely to restrain DC-mediated anti-tumor immunity by blocking TIM-3 (Fig. 1I).

Our study, as the first study to investigate the clinical features, pathological characteristics and TME landscapes of PD-L1$^+$ EGFR$^+$ HNSCC, identified the clinical characteristics of most PD-L1$^+$ EGFR$^+$ HNSCC patients with advanced clinical stage and lymph node metastasis status for the first time. Meanwhile, PD-L1$^+$ EGFR$^+$ HNSCC possesses a more aggressive invasive ability. Based on the scRNA-seq analysis of HPV-negative HNSCC, the CXCL11/CXCR3 ligand-receptor pair was further recognized as a key factor in the construction of an immunosuppressive TME recruited by PD-L1$^+$ EGFR$^+$ HNSCC cells. In addition, CD73, OX40, and TIM-3 may become essential targets for immunotherapy sensitization in HPV-negative HNSCC. Our finding provides a fundamental mechanism for immunotherapeutic failure in HPV-negative HNSCC and an immunotherapeutic sensitization strategy for PD-L1$^+$ EGFR$^+$ HNSCC. Although the above finding holds

PD-L1$^+$ EGFR$^+$ HNSCC patients based on the CPS. **(C)** Kaplan—Meier curves exhibiting the recurrence-free survival of PD-L1$^+$ EGFR$^+$ (red) and non-PD-L1$^+$ EGFR$^+$ (blue) HNSCC patients in our cohort. **(D)** Kaplan—Meier curves exhibiting the overall survival of PD-L1$^+$ EGFR$^+$ (red) and non-PD-L1$^+$ EGFR$^+$ (blue) HNSCC patients in our cohort. **(E)** Kaplan—Meier curves exhibiting the overall survival of PD-L1$^+$ EGFR$^+$ (red) and non-PD-L1$^+$ EGFR$^+$ (blue) HNSCC patients in HNSCC samples from TCGA database. **(F)** Proportion of p16 expression between PD-L1$^+$ EGFR$^+$ and non-PD-L1$^+$ EGFR$^+$ HNSCC patients. **(G)** Proportion of p40 expression between PD-L1$^+$ EGFR$^+$ and non-PD-L1$^+$ EGFR$^+$ HNSCC patients. **(H)** Levels of DOI among the three subgroups of PD-L1$^+$ EGFR$^+$ HNSCC patients based on the CPS. DOI: depth of invasion. **(I)** Proportion of WPOI classification between PD-L1$^+$ EGFR$^+$ and non-PD-L1$^+$ EGFR$^+$ HNSCC patients. WPOI: worst pattern of invasion. **(J)** Unsupervised clustering analysis (UMAP) and cell type annotation exhibiting 16 types of cells in the public scRNA-seq data (GSE164690) of HPV-negative HNSCC. Epithelial cells in the dotted circle (green) refer to tumor cells. **(K)** Volcano plot showing the DEGs between PD-L1$^+$ EGFR$^+$ and non-PD-L1$^+$ EGFR$^+$ tumor cells. Blue: down-regulated DEGs, red: up-regulated DEGs, grey: DEGs without statistical significance. **(L)** Feature plot (left) revealing the expression level of *CXCL11* in HPV-negative HNSCC. Violin plot (right) for the expression level of *CXCL11* between PD-L1$^+$ EGFR$^+$ and non-PD-L1$^+$ EGFR$^+$ tumor cells. Blue: PD-L1$^+$ EGFR$^+$ tumor cells, red: non-PD-L1$^+$ EGFR$^+$ tumor cells. **(M)** Violin plot exhibiting the expression level of *CXCR3* in HNSCC cells. **(N)** Violin plots exhibiting the expression levels of 22 immune checkpoints among cells in the TME of HPV-negative HNSCC. OX40, TIM-3 and CD73 (red) are considered potential targets for the sensitization of immune therapy in HNSCC.

promise for tumor immunotherapy, future studies based on anti-PD-1 and EGFR-based combination immunotherapy should still be conducted in real-world clinical trials to evaluate its efficacy in PD-L1^{+} EGFR^{+} HNSCC.

CRediT authorship contribution statement

Yuan Zhi: Writing — original draft, Methodology, Investigation, Data curation. **Wenhao Ren:** Validation, Project administration, Investigation, Funding acquisition. **Shaoming Li:** Resources, Methodology, Formal analysis, Data curation. **Jingjing Zheng:** Project administration, Investigation, Data curation. **Jianzhong Song:** Supervision, Investigation, Data curation. **Ling Gao:** Writing — review & editing, Project administration, Conceptualization. **Keqian Zhi:** Writing — review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Ethics declaration

This study was approved by the Ethical Committee of the Affiliated Hospital of Qingdao University (No. AHQU-MAL20210604) to the Department of Oral and Maxillofacial Reconstruction, the Affiliated Hospital of Qingdao University, Shandong, China.

Conflict of interests

The authors declare that there are no competing interests in this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2025.101902.

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