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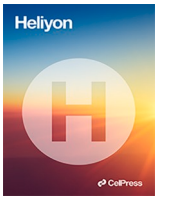

Research article

The causal impact of complement C3d receptor 2 on head and neck cancer microenvironment and its implications for immunotherapy response prediction

Qin Ding [a,b], Wenqian Xu [a,b], Hanxuan Yang [a,b], Wenxi Wu [a,b], Lishui Wu [a,b], Xin Chen [a,b], Hui Liu [a,b,**], Sufang Qiu [a,b,*]

[a] Department of Radiation Oncology, Clinical Oncology School of Fujian Medical University, Fujian Cancer Hospital, Fuzhou, China
[b] Fujian Provincial Key Laboratory of Translational Cancer Medicine, Fuzhou, China

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ABSTRACT

This research dives into the intricate immune landscape of head and neck cancer (HNC), with a keen focus on the roles of specific immune cell subpopulations and their linked genes. We used tumour RNA-seq (in-house cohort: n = 192, TCGA-HNSC: n = 546) and Mendelian randomization to pinpoint key SNPs in immune cells that have a causal connection to HNC. Our discoveries unveil a spectrum of tumour immune phenotypes that either offer protection against or increase the risk of HNC. We underscore the therapeutic promise of Complement C3d Receptor 2 (CR2), a gene closely tied to immune cells, with its increased expression in tumour tissues linked to a more favourable prognosis. This is correlated with heightened immune pathway activity, stronger resistance to radiochemotherapy, and improved immunotherapy responses. Our research emphasises the pivotal role of CR2 in immune regulation and the significance of immune cells in tumour progression, highlighting the potential of CR2-targeted therapeutic interventions.

1. Introduction

Head and neck cancer (HNC) represents a significant global health burden, comprising a diverse group of epithelial malignancies affecting the upper aerodigestive tract, salivary glands, and associated lymph nodes [1]. With an estimated 30 % increase in incidence expected by 2030, understanding the underlying mechanisms of HNC development and progression is crucial for improving patient outcomes [2,3]. The immune system plays a pivotal role in the oncogenesis and progression of HNC, highlighting the importance of exploring the intricate relationship between HNC and immune cell activities [4–6] [4–6] [4–6]. Identifying suitable biomarkers for predicting the immune microenvironment and the efficacy of immunotherapy in HNC patients stands as a critical objective [7]. These biomarkers hold the promise of revolutionizing treatment approaches by enabling personalized therapeutic strategies tailored to the unique immunological landscape of each patient's tumor [8].

However, despite the recognized importance of the immune system in HNC pathophysiology, there remains a significant gap in our

* Corresponding author. Department of Radiation Oncology, Clinical Oncology School of Fujian Medical University, Fujian Cancer Hospital, Fuzhou, China.
** Corresponding author. Department of Radiation Oncology, Clinical Oncology School of Fujian Medical University, Fujian Cancer Hospital, Fuzhou, China.
E-mail addresses: liuhuifj@sina.com (H. Liu), sufangqiu@fjmu.edu.cn (S. Qiu).

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understanding of the causal relationships between specific immune cell activities and HNC. This gap underscores the need for innovative research approaches to unravel these complex interactions. Mendelian randomization (MR) technique, which uses genetic variants as proxies, is applied to rigorously explore the causal associations between immune cell signatures and HNC [9]. This study aims to uncover the complex relationships between HNC and the immune microenvironment, identifying potential predictive biomarkers for treatment responses.

The study employs a multi-step approach (Fig. 1) to explore the genetic basis of HNC in the context of immune cell SNPs. Initial MR analysis identifies SNPs within immune cells causally associated with HNC, utilizing genetic variations as instrumental variables to infer causal links. Subsequently, the study validates mapped genes associated with these SNPs in two datasets: bulk data from Fujian Provincial Cancer Hospital and The Cancer Genome Atlas (TCGA). This validation confirms gene relevance to HNC, reinforcing its role in disease pathogenesis and prediction of therapeutic effects. This comprehensive strategy enhances findings' robustness and generalizability, providing valuable insights into genetic factors influencing immune cell behavior in HNC.

2. Materials and methods

2.1. MR analysis design

In a two-sample MR analysis, we investigated the causal relationship between 731 immune cell signatures, grouped into seven categories, and HNC. MR employs genetic variants as substitutes for risk factors, necessitating legitimate instrumental variables (IVs) that adhere to three essential conditions [1]: A direct link to the exposure exists [2]; There's an absence of correlation with any intermediate variables that could confound the relationship between exposure and outcome [3]; The outcome is unaffected by these variables through any means other than the exposure itself. All included studies received approval from institutional review boards, with participants providing informed consent.

2.2. Sources of immunity-Wide Genome-wide association study (GWAS) data

Summary statistics for 731 immune traits from GWAS can be found in the GWAS Catalog, with references ranging from GCST0001391 to GCST0002121(10). The dataset includes 118 absolute cell counts, 389 median fluorescence intensities (MFIs) associated with surface antigens, 32 morphological parameters (MPs), and 192 relative cell counts.

Immunophenotypes examined included B cells, CDCs, mature T cells, monocytes, myeloid cells, and TBNK (T cells, B cells, NK cells), including Treg panels. Initial GWAS involved 3757 non-overlapping European participants, imputing approximately 22 million genotyped SNPs with high-density arrays based on a Sardinian reference panel [10]. Analyses adjusted for covariates such as sex, age, and age squared.

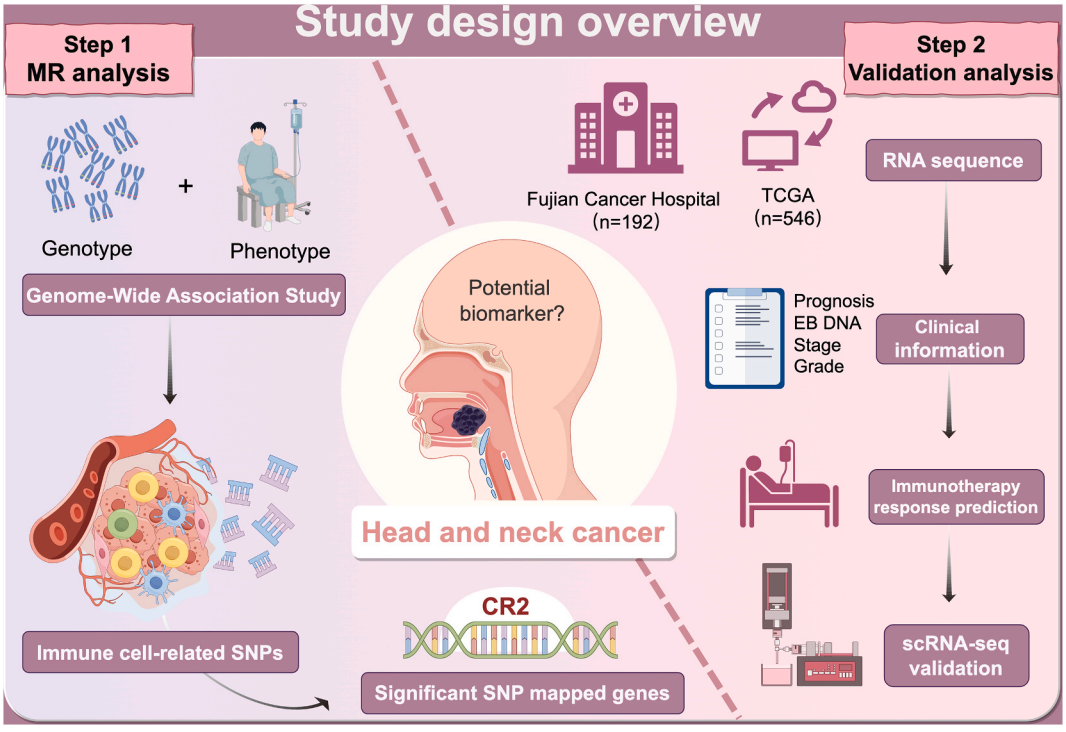

Fig. 1. Flowchart of the study process.

2.3. Data sources for HNC GWAS

The GWAS data utilized in the investigation of HNC were sourced from the UK Biobank (https://www.ukbiobank.ac.uk/). The research conducted a comprehensive GWAS involving 373,122 individuals of European descent (Ncase = 1,106, Ncontrol = 372,016). Following rigorous quality control measures and imputation, 9,655,080 variants were analyzed.

2.4. Selection of IVs

Recent studies determined the significance threshold for IVs linked to each immune trait to be 1×10^{-5} [11]. Using PLINK software (version 1.90), the clumping procedure pruned SNPs with linkage disequilibrium (LD) r^2 threshold <0.1 within a 500 kb distance, with LD r^2 calculations referencing the 1000 Genomes Project panel [12]. For HNC, the significance threshold was set to 5×10^{-8}. The proportion of phenotypic variation explained (PVE) and F-statistics for each IV were used to assess IV strength, thus mitigating the bias from weak instruments. Subsequently, IVs for HNC were retained after excluding those with F statistics below 10.

2.5. Clinical sample collection

Tumor biopsies from nasopharyngeal carcinoma patients at Fujian Cancer Hospital (January 2015–January 2018) were staged using TNM criteria. Detailed clinical characteristics are in Table 1. The study, approved by the Ethics Committee (Reference: K2022-074-01), obtained written informed consent from all participants. External validation utilized data cohorts from TCGA.

2.6. RNA isolation, mRNA library construction, and sequencing

Total RNA was extracted from tumor tissues using a TRIzol reagent kit in accordance with the manufacturer guidelines. Subsequently, mRNA was enriched using oligo(dT)-attached magnetic beads, fragmented, and reverse transcribed to cDNA. Following end repair, a single 'A' nucleotide was added before adapters were ligated to the cDNA. PCR amplification was performed after adapter ligation. BGI Technology Services Co. Ltd performed sequencing on the resulting cDNA library.

2.7. Significant gene identification

The "ggplot2" R package visualized differently expressed genes (DEGs) in Fujian Cancer Hospital's tumor samples. The criteria for identifying DEGs included a fold-change greater than 2 and a *p*-value less than 0.05. The overlap between DEGs and genes mapped to immunophenotype-related SNPs constituted the set of differentially expressed immune-related genes. Key genes predicting survival were identified for in-depth analysis using the "survival" R package.

2.8. Estimation of immune-related factors in multidimensional analysis

To assess immune cell infiltration, we employed various immune scoring methods, including Cibersort and ssGSEA algorithms [13,14]. R package "ESTIMATE" calculated immune, stromal, and EATIMATE scores [15]. Inhibitory immune checkpoints with therapeutic efficacy were obtained from a previous study [16]. Gene Set Variation Analysis (GSVA) used R software (version 4.2.1) to

Table 1
Clinical characteristics of in-house patients.

Gender	Male	Female
n	136	57
Age, mean ± sd	48.8 ± 10.8	47.4 ± 9.8
T, n (%)		
1	24 (12.6 %)	15 (7.9 %)
2	29 (15.3 %)	12 (6.3 %)
3	44 (23.2 %)	18 (9.5 %)
4	37 (19.5 %)	11 (5.8 %)
N, n (%)		
0	13 (6.8 %)	2 (1.1 %)
1	43 (22.6 %)	21 (11.1 %)
2	53 (27.9 %)	25 (13.2 %)
3	25 (13.2 %)	8 (4.2 %)
M, n (%)		
0	126 (66.3 %)	52 (27.4 %)
1	8 (4.2 %)	3 (1.6 %)
x	0 (0 %)	1 (0.5 %)
Grade, n (%)		
1	4 (2.1 %)	0 (0 %)
2	22 (11.6 %)	14 (7.4 %)
3	50 (26.3 %)	23 (12.1 %)
4	58 (30.5 %)	19 (10 %)

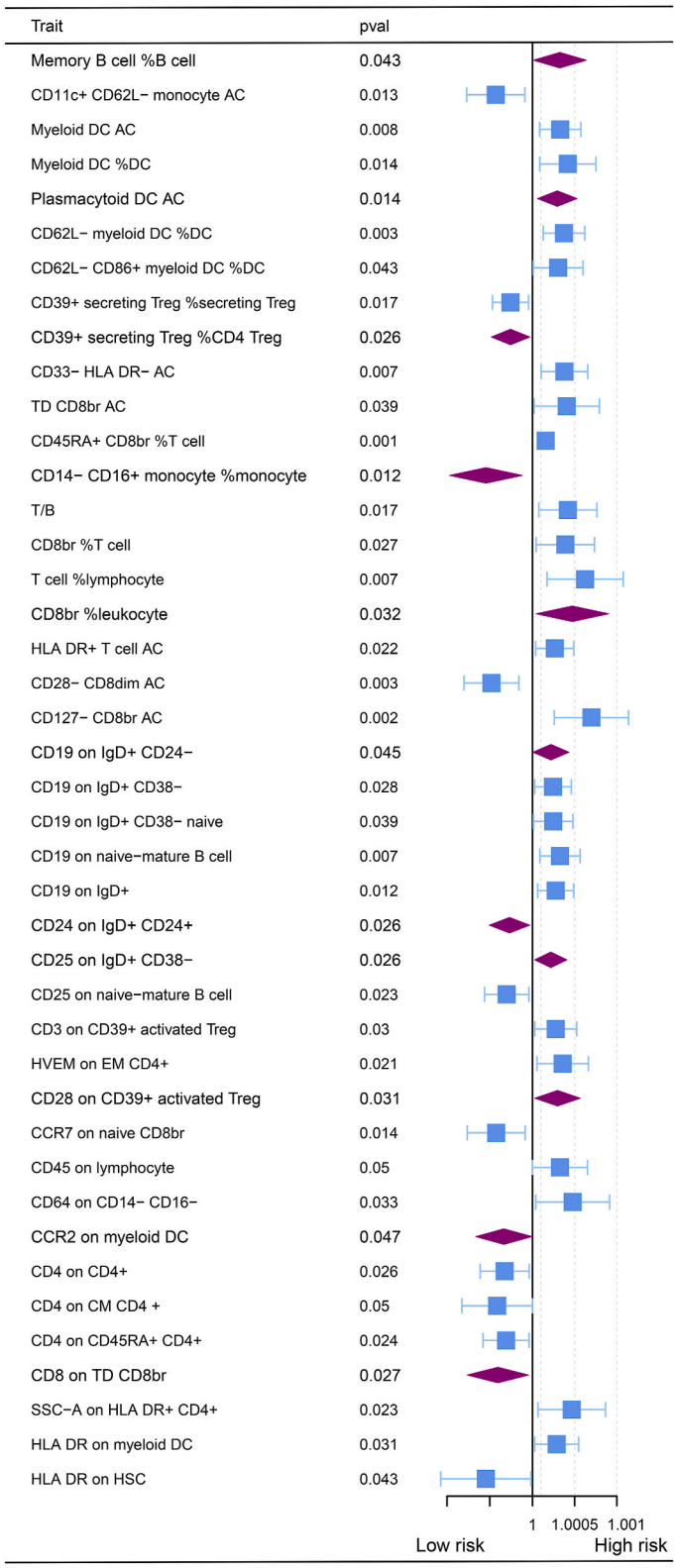

Fig. 2. A forest plot showing the cell subpopulations with potential causal effects on HNC.

calculate pathway scores for each tumor sample from transcriptomic data. This method, employing the "GSVA" package, evaluates the expression levels of genes in predefined gene sets to determine enrichment scores [17,18].

2.9. Chemotherapy and radiotherapy sensitivity assessment

To explore CR2's functional implications in drug sensitivity, the NCI-60 tumor cell line panel was analyzed. Drug sensitivity nuances, measured by half-maximal inhibitory concentration (IC50) values, were extracted from the CellMiner database (https://discover.nci.nih.gov/cellminer/) [19]. A total of 218 FDA-approved drugs and 574 drugs or compounds from clinical trials were chosen for further analysis. The "impute" [20] and "limma" R packages [21] assessed CR2's impact on drug sensitivity. Employing the impute knn function, missing information on certain medications was estimated.

To examine the influence of CR2 on the sensitivity to radiotherapy, we evaluated the radiotherapy tolerance of our internal cohort samples employing the GSVA technique [17]. The score for each sample was calculated through the ssGSEA approach in R(13).

2.10. Prediction of the immunotherapy response

To assess CR2's predictive performance for immunotherapy response, we collected immunotherapy cohorts from the GEO database and the TIGER website (http://tiger.canceromics.org/#/). This included our in-house cohort and melanoma-GSE91061(22), melanoma-GSE93157 [22]. We visually compared response proportions in high and low CR2-expressed groups. The predictive power of CR2 expression was evaluated using receiver operating characteristic (ROC) analysis in these cohorts with the R package "ROC".

2.11. Single-cell RNA-seq analysis

This study processed single-cell RNA sequencing data through quality control, downscaling, and clustering with the Seurat package (version 4.0.4) [23]. To ensure data integrity, genes in fewer than three cells and cells with detections of fewer than 250 genes were removed. Additionally, cells with mitochondrial gene content exceeding 35 % were filtered out [24]. The logNormalize method normalized the data. Uniform Manifold Approximation and Projection (UMAP) facilitated unsupervised classification and unbiased cell population visualization in two-dimensional maps [25]. Cell type annotations were obtained through TISCH (http://tisch.compgenomics.org/) [24]. The "FindAllMarkers" function identified marker genes within clusters with a threshold of an absolute $\log_2$FC of at least 0.3 and a minimum cell cluster fraction of 0.25.

2.12. Cell–cell chat analysis

CellChat software (version 1.1.3) analyzed cell-cell communication by inferring ligand-receptor interactions [26]. Cell groups with fewer than 10 cells were excluded to refine the analysis. Statistical significance of communication was determined through pairwise tests on probability values, enabling a detailed examination of molecular dialogues.

2.13. Statistical analysis

All MR analyses were performed in R software (http://www.Rproject.org).

To investigate the causal relationship between 731 immunophenotypes and HNC, diverse statistical methods were applied using the "TwoSampleMR" package (version 0.4.3) [27]. These methods encompassed MR-Egger, Weighted median, Inverse variance weighted (IVW), Simple mode, and Weighted mode [28]. Cochran's Q statistic and p-values were utilized to assess consistency among instrumental variables (IVs), testing for heterogeneity [29]. To mitigate potential bias from horizontal pleiotropy, the MR-Egger method was employed, detecting horizontal pleiotropy through a statistically significant intercept term [30]. The MR-PRESSO method identified and excluded horizontal pleiotropic outliers, minimizing their impact on causal estimation [31].

Two-tailed unpaired Student's t-test or Wilcoxon test was used for two-group comparisons, and the Kruskal–Wallis test for multiple groups, considering data distribution and variance characteristics. This approach tailors statistical analysis appropriately based on data nature and assumptions.

3. Result

3.1. Exploration of the causal effect of immunophenotypes on HNC

In our findings, 28 immune phenotypes were identified with a risk-promoting effect on HNC, while 14 immune phenotypes exhibited a protective effect (Fig. 2, Table S1). Using the IVW method, we found positive Beta values for certain cell subtypes (B cell, cDC, Maturation stages of T cell, Monocyte, Myeloid cell, TBNK, Treg panel) consistently indicating an increased risk of HNC. This trend was consistent across three other methods. Conversely, negative Beta values for specific cell subtypes (B cell, cDC, Maturation stages of T cell, Monocyte, Myeloid cell, Treg panel) consistently suggested a decreased risk of HNC, confirmed by three other methods. In addition, MR-Egger's intercept and MR-PRESSO's global test ruled out the possibility of cross-sectional pleiotropy in these four associations (Table S2). The detailed information obtained from Cochran's Q statistic and corresponding p values confirms no heterogeneity of the observed causal relationships (Table S3).

3.2. Exploring the expression pattern of the candidate biomarker CR2

We identified 69 genes linked to SNPs across significant immune cell subpopulations (Table S4). Intersecting these genes with DEGs from Fujian Cancer Hospital's internal dataset revealed five immune-related differentially expressed genes: NAT8, SPATA48, FCGR3A,

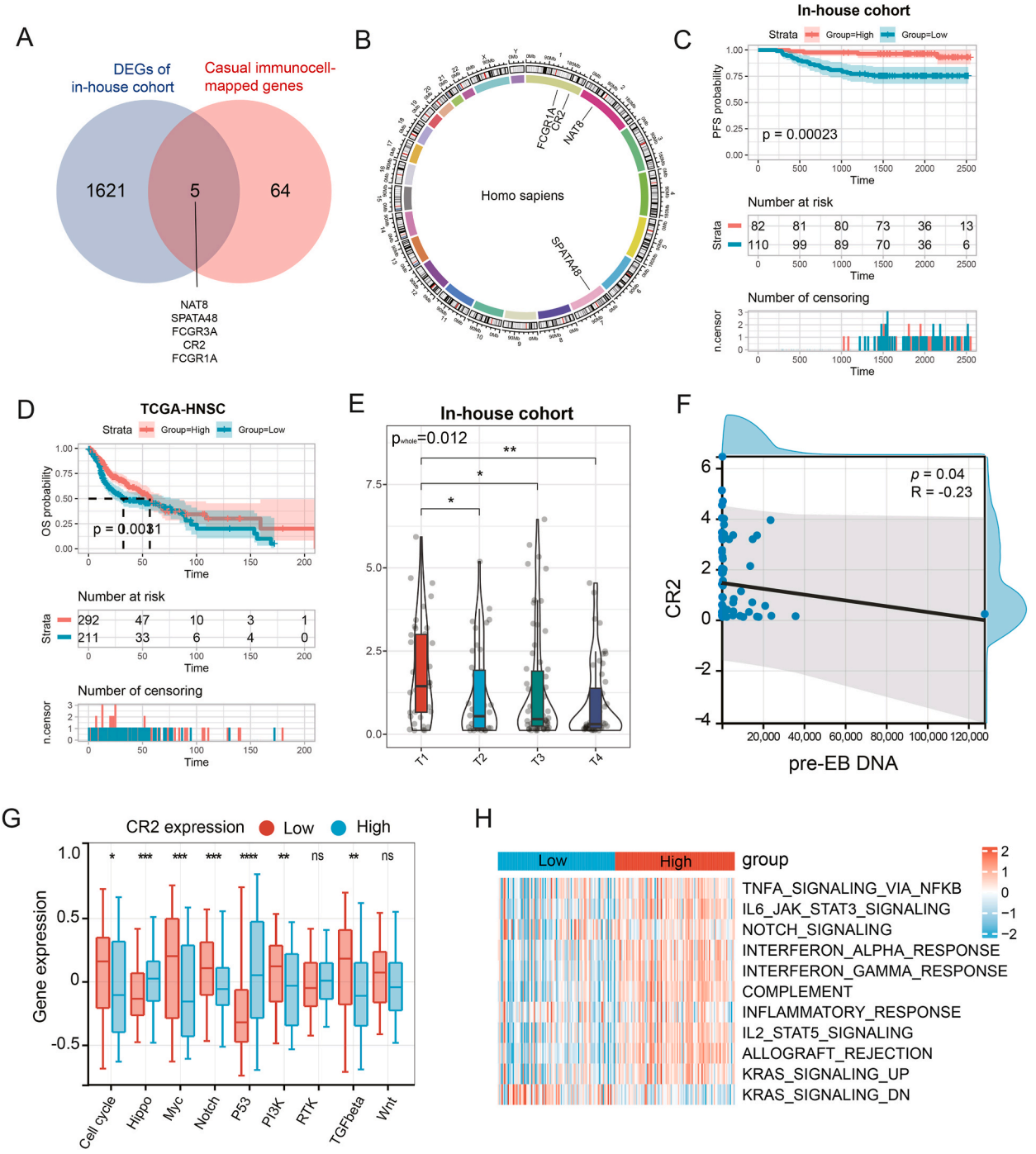

Fig. 3. CR2 expression pattern. **A.** Venn diagram showing the intersection of DEGs in the in-house cohort and causal immunocell SNP mapped genes. **B.** Chromosomal localization of the genes. **C.** Survival analysis of CR2 expression in the in-house cohort. **D.** Survival analysis of CR2 expression in the TCGA-HNSC cohort. **E.** Comparison of CR2 expression levels across different T stages. **F.** Correlation between CR2 expression and EB DNA copies in patients of the in-house cohort. **G.** Evaluation of the difference in the scores of the top ten carcinogenic pathways between patients with high and low expression of CR2. **H.** Assessment of ALLMARKER scores in patients with high and low CR2 expression.

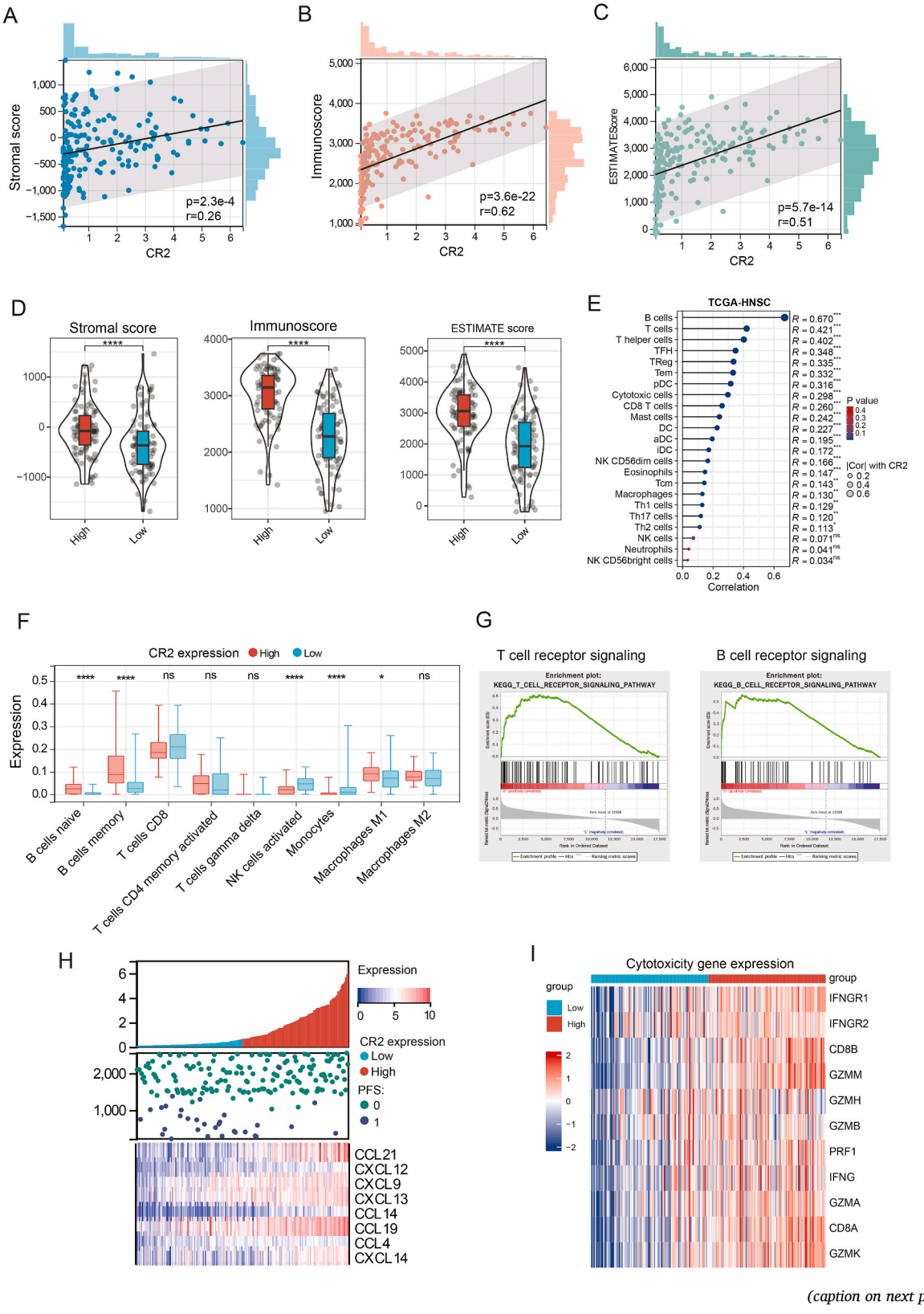

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Fig. 4. The relationship between CR2 and immune tumor microenvironment.**A–C.** Correlation assessment between CR2 and stromal score **(A)**, immunoscore **(B)**, and ESTIMATE score **(C)** within the in-house cohort. **D.** Difference in stromal score, immunoscore, and ESTIMATE score between high and low CR2 expression groups within the in-house cohort. **E.** ssGSEA method used to assess the correlation between CR2 and different types of immune cells. **F.** CIBERSORT method used to evaluate the difference in the infiltration level of different types of immune cells between high and low CR2 expression groups. **G.** High CR2 expression group enriched in T and B cell receptor pathways. **H.** Risk plot showing that as CR2 expression increases, the probability of disease progression decreases, and cytokine expression increases. **I.** Difference in cytotoxicity gene expression between high and low CR2 expression groups.

CR2, and FCGR1A (Fig. 3A).

In Fig. 3B, chromosomal localization maps for the five genes are shown. Survival analysis was conducted, excluding SPATA48 and FCGR3A due to predominantly zero expressions, impeding analysis. Among NAT8, CR2, and FCGR1A, only CR2 showed significant survival prediction capability (Fig. 3C–D, Figs. S3a–c). In internal cohort analysis, high CR2 expression correlated with improved PFS (Fig. 3C) and OS (Fig. S3c) compared to low expression counterparts.

Taking into account the clinical data of the patients, CR2 expression was inversely related to the progression of T-stage, N-stage, and M-stage (Fig. 3E, Fig. S3d). Additionally, there was a negative correlation between CR2 expression and EB DNA copies (Fig. 3G). Furthermore, patient samples from high- and low-CR2 expression groups exhibited significant disparities in scores across ten carcinogenic pathways (Fig. 3G). Furthermore, elevated CR2 expression correlates with high enrichment of cytokine-related HALLMARK in our cohort, such as complement signal, inflammatory response signal (Fig. 3H).

3.3. Assessment of the tumor immune microenvironment

We evaluated the immune microenvironment variations in patients with high and low CR2 expression, emphasizing immunity scores and immune cell infiltration. CR2 expression positively correlated with immunity, stromal, and ESTIMATE scores, showing significant differences between high and low CR2 expression groups in our cohort (Fig. 4A–D) and TCGA-HNSC cohort (Fig. 3e). Additionally, a significant positive correlation was identified between CR2 expression and 23 immune cell types, based on TCGA-HNSC data (Fig. 4E). Utilizing CIBERSORT, the subgroup characterized by high CR2 expression exhibited greater immune cell infiltration, significantly differing from the low CR2 expression subgroup (Fig. 4F).

Furthermore, high CR2 expression was associated with an enrichment in the T and B cell receptor signaling pathway (Fig. 4G). Additionally, elevated CR2 expression was linked to a reduced rate of disease progression among patients, accompanied by higher cytokine expression (Fig. 4H). Cytotoxicity gene expression was also found to be elevated.

3.4. Predictive power of the efficacy of conventional treatment and immunotherapy

Chemotherapeutic agents used in head and neck squamous carcinoma treatment, face resistance in patients with high CR2 expression, including drugs like METHOTREXATE, Paclitaxel, and Oxaliplatin (Fig. 5A). High CR2 expression correlates with increased radioresistance scores (Fig. 5B), suggesting potential radiotherapy insensitivity. Given this insensitivity to standard treatment, focus shifted to immunotherapy response in patients with high CR2 expression. A positive correlation between CR2 expression and immune checkpoint expression was found, validated in TCGA-HNSC cohort (Fig. 5C and D). The high CR2 expression group exhibited higher TIDE scores (Fig. 5E and F), indicating a more robust immune response, validated in two melanoma cohorts (Fig. 5G and H), showcasing CR2's strong predictive capability for immune response.

3.5. Immune landscapes and cellular communication at the single-cell level

In the GSE139324 cohort, UMAP analysis revealed a cluster of 11 distinct cell types (Fig. 6A). Cell lines were annotated based on gene expression, referencing TISCH human primary cell atlas data (Fig. 6A). CD8 T cells and B cells were significantly more prevalent in healthy donors than in HNSC patients (Fig. 6B). Expression levels of each cell type in HNSC are shown in Fig. 6C. CR2 expression was significantly reduced in HNSC, confirmed by the cellular localization map (Fig. 6D–F). Functional exploration identified CR2 expression in the FCRE2A - CR2 ligand receptor pair, mediating communication among B cells, Mono/Macro cells, and DC cells (Fig. 6G). Pathway analysis revealed enriched chemokine and IL17 related pathways of high- and low-CR2 DEGs, indicating diverse immune responses among risk groups (Fig. 6H).

4. Discussion

Our study unveils a potential causal link between immune phenotypes and HNC, shedding light on their pivotal role in disease development. Comprehensive analyses identify immune phenotypes with both risk-promoting and protective effects, offering novel perspectives on the immune regulatory mechanisms in HNC. CR2 emerges as a noteworthy mapping gene, exhibiting significant expression differences in HNC patients and suggesting its potential as a biomarker. High CR2 expression links to improved survival, superior immune predictions, and heightened immunotherapy responsiveness in head and neck cancer. The potential of CR2 as a prognostic and therapeutic guide, particularly in immunotherapy, is underscored. Its association with the immune microenvironment implies a role in HNC immune escape and impact on responsiveness.

The immune cell transcription profiles revealed by single-cell transcriptomics are related to the prognosis of HNSCC patients,

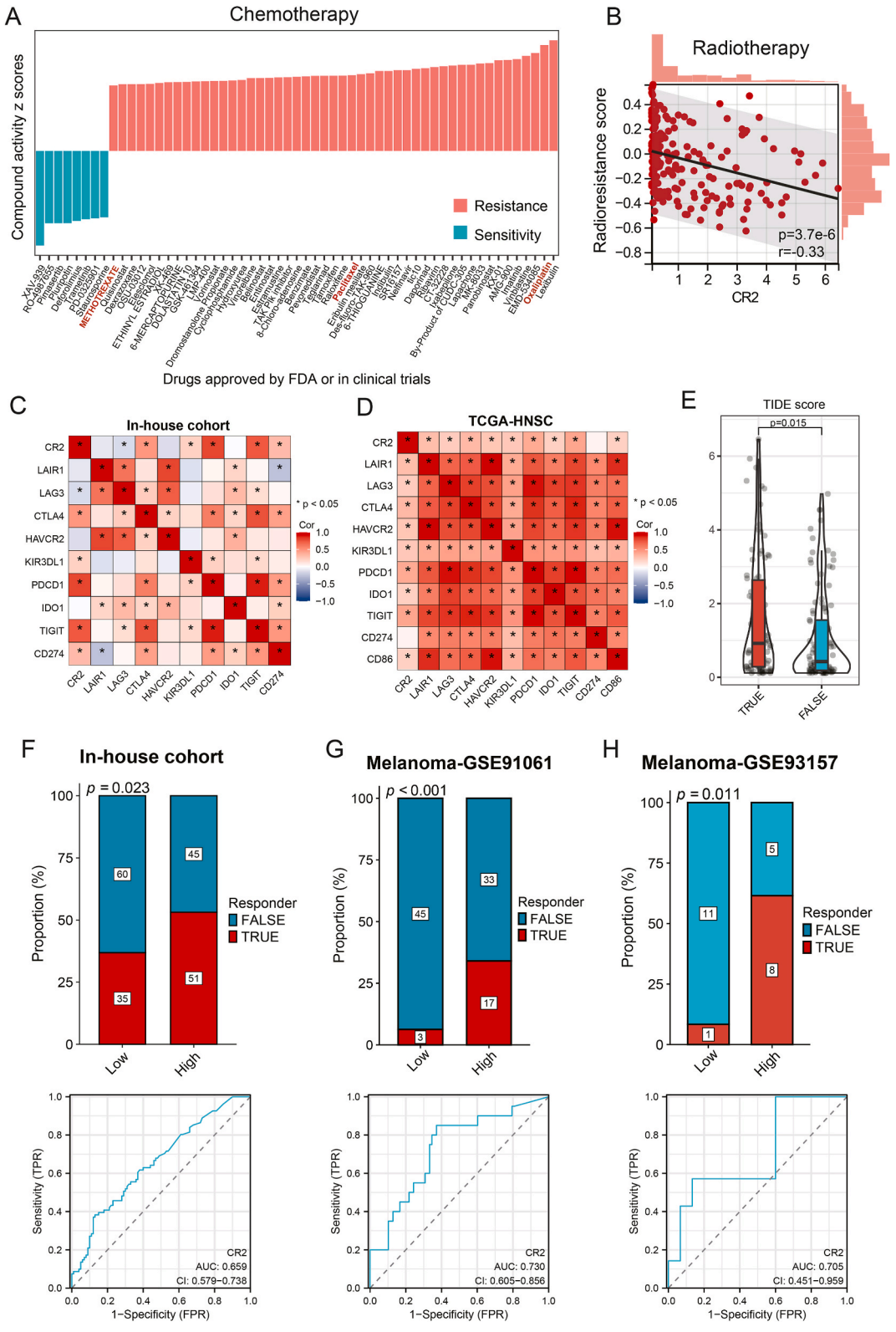

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Fig. 5. CR2 predicts the response of chemotherapy, radiotherapy and immunotherapy. A. The relationship between CR2 expression and chemotherapy drug sensitivity was evaluated. **B.** The correlation between CR2 expression levels and radiation therapy resistance scores. **C–D.** The correlation between CR2 and immune checkpoint expression in the in-house cohort **(C)** and TCGA-HNSC cohort **(D)**. **E.** Difference in TIDE scores between high and low CR2 expression groups. **F–H.** The proportion of immune therapy responses in high and low CR2 expression groups and the predictive ability of CR2 for immune therapy response.

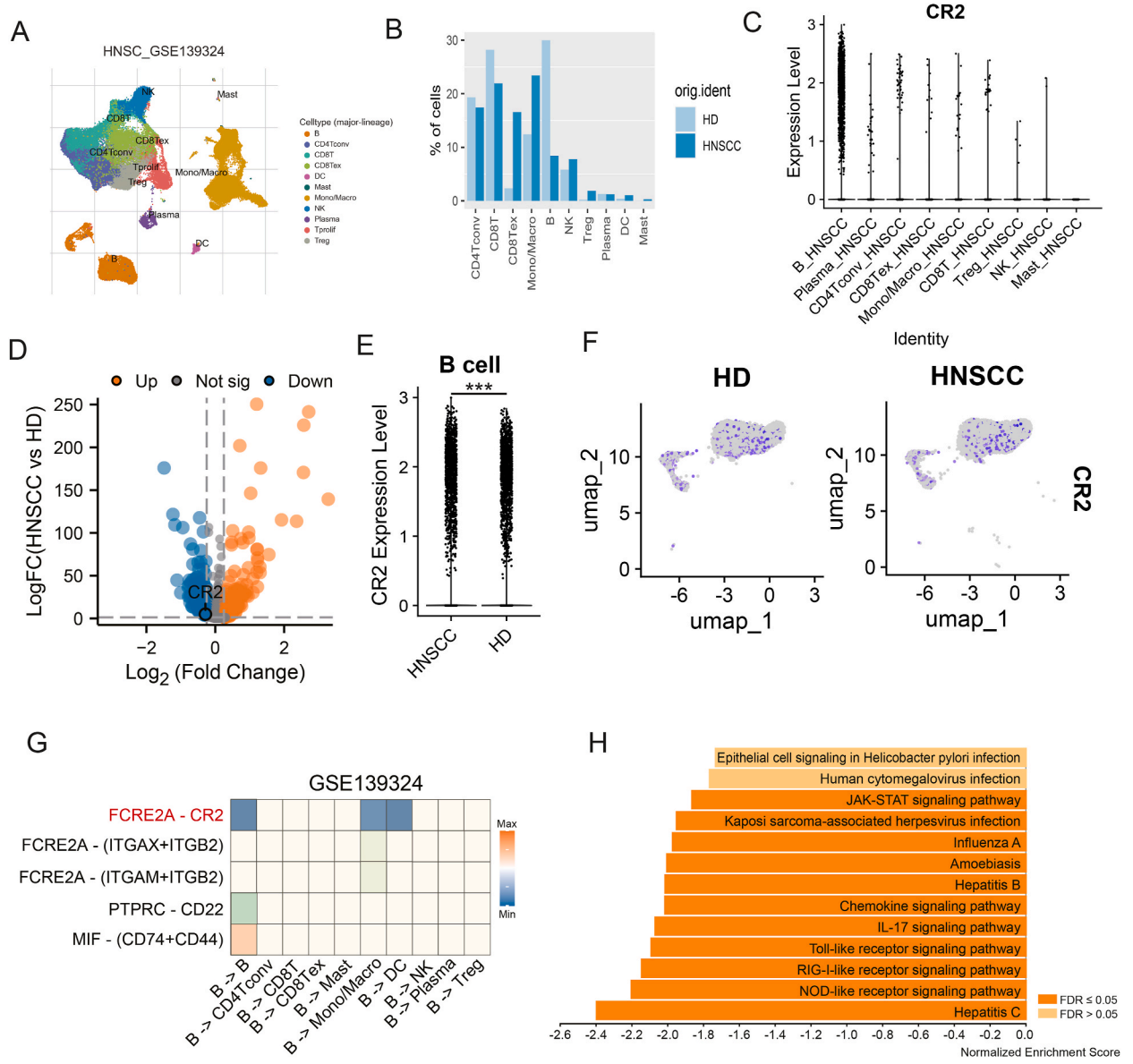

Fig. 6. Exploring CR2 expression and functional patterns at the single-cell level. A. Single-cell dimensionality reduction annotation map. **B.** The difference in proportions of various cell types between healthy donors (HD) and HNSC. **C.** The expression levels of CR2 in different cell types within HNSC. **D.** Differential genes between HD and HNSC, with CR2 expression downregulated. **E.** The difference in CR2 expression in B cells between HD and HNSCC, with CR2 expression downregulated in HNSC. **F.** Cellular localization map of CR2 in HD and HNSC. **G.** Intercellular communication, the role played by CR2 on its receptor pairs. **H.** Differential gene enrichment pathways in high and low CR2 groups.

emphasizing the key role of immune cells in tumor evolution [32,23]. Analysis of these profiles reveals that the characteristics of specific immune cell subgroups are closely related to patient outcomes. Factors such as activation status and the dichotomy between immune suppression and immune activation in specific T cells or macrophages can affect the proliferation, spread of the tumor, and the patient's lifespan [33].

The discussion extends to the prevalent immune suppressive environment in HNC patients, characterized by tumor cells inhibiting

immune activity by releasing immunosuppressive agents or expressing immune checkpoint molecules. Tumor cells achieve this through multiple mechanisms, especially by releasing immunosuppressive factors such as TGF-β and interleukin-10 (IL-10), and expressing immune checkpoint molecules including PD-L1 [34]. In the tumor microenvironment, these factors and molecules directly act on immune cells, inhibiting their activity and proliferation, thereby weakening the host's immune surveillance. For example, they can inhibit the proliferation and cytokine production of effector T cells or enhance the proliferation and function of regulatory T cells (Tregs), further suppressing the immune response [35].

Research on HNC has revealed a complex immune regulatory network within the tumor microenvironment. This network encompasses the diversity of immune cells, the role of the complement system, and the regulation of immune checkpoint molecules. Specifically, CR2, complement C3d receptor 2, as a cornerstone molecule in the complement system, has garnered widespread attention for its critical role in regulating immune responses [36]. The role of CR2 is worth further exploration. Literature suggests that CR2 can regulate immune cell dynamics by affecting the activation of the complement system [37]. As a component of innate immunity, the complement system triggers inflammatory responses and aids in the mobilization and activation of immune cells through a series of complex pathways. Therefore, the interaction between CR2 and the complement system's byproduct C3d may play a key role in regulating the responses of immune cells such as B and T cells in the tumor environment. Its activation catalyzes the activation and proliferation of B cells, highlighting its importance in adaptive immune responses [38]. In the tumor microenvironment, the role of CR2 is not limited to the regulation of B cells. It may also affect T cells and other immune components, particularly by coordinating local immune responses through interactions between the complement system and immune cells. Thus, CR2 becomes an important link between innate and adaptive immunity, guiding the behavior of immune cells in the tumor environment. Consequently, CR2's ability to regulate the complement system makes it a key modulator of the tumor immune microenvironment, providing a theoretical basis for developing therapeutic strategies targeting CR2.

Despite our study offering valuable insights into immunophenotyping and the role of CR2 in head and neck cancer, it does present certain limitations. For instance, our study extensively relied on retrospective data and samples drawn from specific populations, which might have influenced the generalizability of our findings. Moreover, the discovery cohort of this study was limited to Fujian Provincial Cancer Hospital and was solely validated using the TCGA database.

5. Conclusion

In conclusion, our study provides fresh insights into the immunobiology of head and neck cancer, spotlighting CR2's potential as a biomarker. Its application holds promise in enhancing diagnostics, prognostic assessments, and therapeutic decision-making for HNC patients.

Ethics statement

This study was authorized by the ethics committee of Fujian Cancer Hospital (Fuzhou, China; numbers K2022-074-01). Each patient was asked to grant their written and

informed consent before participating in any study-specific research.

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Data availability

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to their containing information that could compromise the privacy of research participants.

Consent for publication

All authors confirm their consent for publication of the manuscript.

CRediT authorship contribution statement

Qin Ding: Writing – original draft, Methodology. **Wenqian Xu:** Methodology. **Hanxuan Yang:** Investigation. **Wenxi Wu:** Investigation. **Lishui Wu:** Writing – original draft. **Xin Chen:** Writing – review & editing. **Hui Liu:** Supervision. **Sufang Qiu:** Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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The flowchart presented in this article was created using Figdraw, for which we extend our sincere gratitude.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29312.

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