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CD2AP shapes a stromal reduced tumor microenvironment and contributes to immunotherapy in gastric cancer

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Abstract

Gastric cancer (GC) ranks as the fifth most prevalent malignant tumor and stands as the fourth leading contributor to cancer-related fatalities on a global scale. The specific link between CD2 Associated Protein (CD2AP) expression and the tumor microenvironment (TME) remains unclear, and further exploration is needed to understand its potential role in immune response and as a target for immunotherapy in GC. Utilizing RNA sequencing data acquired from The Cancer Genome Atlas (TCGA) for a pan-cancer analysis, a comprehensive evaluation was carried out to determine the expression pattern and immunological involvement of CD2AP. Systematic association of CD2AP with immunological features within the stomach adenocarcinoma (STAD) TME was subsequently performed, encompassing factors like cancer immunity cycles, immune checkpoints, immunomodulators, tumor-infiltrating immune cells (TIICs). We found that CD2AP was enhanced expression in the TME of a variety of malignancies. CD2AP contributes to forming a stromal reduced TME in GC and improve the efficacy of immunotherapy. It was observed that patients with elevated levels of CD2AP, along with high scores on their CD4, CD20, and CD57 immune markers, tended to experience the most favorable prognosis. Furthermore, an IRS was constructed to accurately assess the prognosis of STAD patients. Since CD2AP was associated with the formation of stromal reduced TME in STAD, the expression of CD2AP can improve the effect of immunotherapy of STAD. CD2AP could emerge as a novel prognostic biomarker for STAD, offering a fresh avenue for molecular targeted therapy.

Keywords Gastric cancer, Tumor microenvironment, Stromal, Immune cell, Immunotherapy

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Introduction

Gastric cancer (GC) ranks as the fifth most prevalent malignant tumor and stands as the fourth leading contributor to cancer-related mortality globally [1, 2]. The majority of individuals are diagnosed at advanced stages, characterized by severe lymph node and multi-organ metastasis, primarily owing to the absence of early, typical clinical symptoms [3, 4]. Despite the application of systemic chemotherapy, individuals with distant metastasis typically experience a median overall survival (OS) of merely 12 months [5]. Nonetheless, recent advances in treatment options such as targeted therapy or immunotherapy, which involve the use of human epidermal growth factor receptor 2 (HER2), programmed cell death ligand 1 (PD-L1), and microsatellite instability (MSI), have presented an opportunity for enhancing the treatment of individuals with advanced GC [6–8].

Tumor microenvironment is a very complex and dynamic system [9]. The main participants include tumor cells, immune cells and stromal cells. The tumor microenvironment is constantly changing during the course of the disease, and the understanding of this dynamic interaction makes it possible to find new treatment options and therapeutic targets at various stages of the disease. Tumor immunotherapy is a rapidly developing new generation of tumor therapy after traditional therapy, which has been proved to be effective in inhibiting or even curing tumors and has great clinical application prospects. Tumor immunotherapy includes Immune checkpoint inhibitors (ICIs) therapy, adoptive cellular immunotherapy, and tumor vaccine therapy. It is through enhancing the killing of immune cells to tumor cells, and finally eliminating the tumor. In this process, cytotoxic lymphocytes play a major role in killing tumors, including cytotoxic T lymphocytes (CTLs) and natural killer cells (NK cells), which are two of the most important and widely studied cytotoxic lymphocytes. The process of cytotoxic lymphocytes killing target cells is mainly divided into effect-target cell binding, cell polarization and lethal attack on target cells [10]. However, immunotherapy still faces the problem of low response rate and drug resistance. The effect of immunotherapy is influenced by the tumor microenvironment (TME) and the immune resistance of tumor cells [11]. The TME includes diverse cell populations, including inflammatory cells, stromal cells, immune cells, adipocytes, and pericytes [12]. They may contribute to the evolution of tumors in the initiation, progression, and metastasis stages and affect the persistence of inflammation [13–15]. Different changes in the expression of tumor-infiltrating immune cells (TIICs) may induce a potent anti-tumor response and enhance inflammation by secreting chemokines, cytokines, and matrix metalloproteinases [16–18]. TIIC markers can also predict the responsiveness and survival of individuals with GC to immunotherapy with PD-1/PD-L1 immune checkpoint blocks thereby enhancing the potential for immunotherapy in GC [19–21]. However, given the complexity of the GC microenvironment and the economic burden of immunotherapy, more effective biomarkers must be explored to predict and regulate the expression changes of TIICs and anti-tumor response. In addition, improving the effectiveness of ICIs therapies is critical.

CD2-associated protein (CD2 AP) has the capability to bind to CD2 and facilitate CD2 aggregation, thereby stabilizing the interaction between T cells and antigen-presenting cells [22]. It participates in signal transduction and cytoskeletal molecule interaction [23]. Previous reports have highlighted that CD2 AP may be linked to immune system dysfunction in Alzheimer's disease, promoting neurodegeneration through dysregulation of extracellular secretion and immune processes [24, 25]. However, the specific relationship between CD2 AP expression and the TME has not been elucidated, and its potential impact on immune responses and immunotherapeutic targets for GC requires further investigation. A recent study has revealed that an elevated CD2 AP expression level signifies a favorable prognosis for GC patients. Conversely, the reduction in CD2 AP expression level has been observed to enhance GC metastasis through its interaction with Capping Actin Protein of Muscle Z-Line Subunit Alpha 1 (CAPZA1), thereby improving intercellular adhesion and affecting cytoskeletal dynamics. This underscores the potential impact of CD2 AP as a crucial prognostic biomarker in GC [26]. Nevertheless, the immune-related role of CD2 AP in GC and its correlation with GC microenvironment require further investigation.

In this study, a comprehensive analysis of a variety of cancer types was conducted and focused on STAD detection of CD2 AP expression patterns and immune-related effects. The outcomes demonstrate that CD2 AP could potentially act as a promising biological marker for STAD immunotherapy. Furthermore, the study investigated the function of CD2 AP in prompting the evolution of a stromal reduced TME in stomach adenocarcinoma (STAD), and the immunological effects of CD2 AP in STAD were verified through the using of tissue chip technology. Finally, an immune risk scoring system with high clinical value was established, providing a reliable direction for the immunotherapy of STAD.

Methods

Data acquisition and processing

Expression profile data normalized as Fragments per Kilobase Million (FPKM) of pan-cancer, along with

information concerning somatic mutations and survival outcomes, were retrieved from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/). RNA sequencing data underwent log2 transformation, and tumor mutational burden (TMB) was computed for somatic mutation data using VarScan2 analysis. Microsatellite instability (MSI) data were obtained from the supplement to the Bonneville analysis [27]. Copy number variation (CNV) data underwent processing through the GISTIC algorithm and were acquired from the UCSC Xena data portal (https://xenabrowser.net/datapages/). Conversely, the methylation data were collected from the LinkedOmics data portal (https://www.linkedomics.org/login.php) [28].The STAD data queue, GSE84437, and GSE62254, containing detailed survival information, was acquired from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/). Expression data in its entirety, along with comprehensive clinical data for the IMherit210 cohort, were sourced from http://research-Pub.Gene.com/imvigor210corebiologies/ [29].

CD2 AP expression statistics in healthy tissues were obtained from the BioGPS data portal (http://biogps.org/#goto=welcome) and the Genotype-Tissue Expression (GTEx) project (https://www.genome.gov/Funded-Programs-Projects/Genotype-Tissue-Expression-Project). Similarly, the BioGPS data portal and the Cancer Cell Line Encyclopedia (CCLE) project (https://sites.broadinstitute.org/ccle/datasets) provided the CD2 AP expression data in cancer cell lines (CCLs). Drug sensitivity data of human CCLs were acquired from two sources: the Cancer Therapeutics Response Portal (CTRP v.2.0, released October 2015, https://portals.broadinstitute.org/ctrp) and the PRISM Repurposing dataset (19Q4, released December 2019, https://depmap.org/portal/prism/). A single-cell sequencing dataset was obtained from the Stanford DNA Discovery website (https://dna-discovery.stanford.edu/research/datasets/). The dataset consists of 56,167 cells, including 8 tumor tissues. This study selected 8 GC samples from this dataset.

Single-cell quality control and cell type annotation

In this study, quality control (QC) analysis was conducted utilizing the Seurat R package. Cells with mitochondrial gene expression percentages exceeding 20% were excluded. Additionally, low-quality cells were filtered out based on QC criteria, specifically nFeature-RNA values below 200 or exceeding 6000. The data normalization process involved the utilization of the"Harmony"function within Seurat. Adjustment was made to the number of principal components (PCs), setting it at 30 to facilitate the generation of cell clusters. These clusters were identified using the FindClusters function, employing a resolution of 1, and subsequently visualized using t-distributed stochastic neighbor embedding (tSNE). Within each cluster, the top 20 differentially expressed genes were identified. For the annotation of the single-cell RNA-seq data, the R"SingleR"was utilized. The analysis of cell communication was conducted utilizing the"Cellchat"R package.

Assessment of the immune properties of the TME in GC

The TME in STAD is characterized by the presence of immunomodulators, the activation of the cancer immunity cycle, the expression of inhibitory immune checkpoints, and the infiltration of TIICs To gather information on these characteristics, we initially gathered data on 122 immunomodulators, such as major histocompatibility complex (MHC) molecules, receptors, chemokines, and immune stimulators, sourced from the research conducted by Charoentong et al. (Table S1) [30]. The cancer immunity cycle represents the immune response against cancer and consists of seven stages: liberation of antigen found in cancer cells(Stage 1), presentation of cancer antigens (Stage 2), initiation and trigger (Stage 3), migration of immune cells towards tumors (Stage 4), permeation of immune cells into tumors (Stage 5), identification of tumor cells by T cells (Stage 6), and elimination of tumor cells (Stage 7) [31]. The operations involved in these stages significantly influence the outcome for tumor cells. To evaluate these activities, a Single Sample Gene Set Enrichment Analysis (ssGSEA) was executed. This evaluation was anchored on the gene expression data from individual specimens. The process within these stages greatly impacts tumor cell results. Xu and his team employed a ssGSEA to scrutinize these activities. Their study was fundamentally rooted in gene expression data from separate samples. In order to validate the influence of CD2 AP in regulating cancer immunity in STAD, an assessment was conducted to examine its correlation with the immune features of TME, considering the aforementioned factors (Table S2). Effector genes of TIICs were identified based on prior research (Table S3). Ultimately, a collection of 20 inhibitory immune checkpoints with therapeutic potential was gathered (Table S4) [32].

Patients and specimens

We collected 564 gastric cancer tissue samples from patients who underwent surgical resection for GC at the Second Affiliated Hospital of Wenzhou Medical University (Wenzhou, China) from December 2006 to July 2011. The gastric adenocarcinoma and 56 paired adjacent normal tissue samples (at least 10 cm from the negative margin) were retrieved from the patients, fixed in formalin, and embedded in paraffin. All tissues were confirmed by histopathological analysis for gastric cancer,

and no radiotherapy, chemotherapy or immunotherapy was performed prior to surgery. The patients comprised 382 males and 147 females ranging in age from 20 to 86 years (median, 59 years). The tissue microarray (TMA) was constructed as described previously. The study was approved by the Review Board of the Second Affiliated Hospital of Wenzhou Medical University. All patients were informed of the study and had signed informed consent.

Microarray immunohistochemistry of GC

CD2 AP, CD4, CD20, and CD57 were detected in tissue microarrays (TMAs) which include healthy and tumor tissues. Immunohistochemical techniques were employed to visualize the expression of CD2 AP, CD4, CD20, and CD57 in tissues. Initially, TMAs were subjected to incubation in xylene and then immersed in distilled water for dewaxing, followed by treatment with a sodium citrate buffer solution (Zhongshan Golden Bridge Biotechnology, Beijing, China) for antigen recovery. Endogenous peroxidase activity was suppressed using a 0.5% hydrogen peroxide solution. Subsequently, a wash step with 0.01 M phosphate-buffered saline (PBS, pH 7.4) was performed, followed by blocking with 5% goat serum for 30 min. The tissue sections were then subjected to incubation with the following antibodies at 25 °C for 2 h in a humidified chamber: CD2 AP antibody (sc-25,272; Santa Cruz Biotechnology, Dallas, TX; 1:50 dilution), CD4 antibody (93,518; Dako), CD20 antibody (M0755; Dako), and CD57 antibody (GA647; Dako). Following the wash with PBS, staining was performed using DAB (Dako, Carpinteria, CA, USA) in accordance with the provided guidelines. The sections were subsequently restained with hematoxylin, hydrated using a gradient of alcohol, and sealed with neutral gum. Finally, slide images were obtained for the calculation of CD2 AP, CD4, CD20, and CD57 scores, utilizing the following scoring formula:

$$\mathbf{Log}_2\left(\frac{\mathbf{IOD}}{\mathbf{Arear}} \times 10^4 + 1\right) = \mathbf{score}$$

Verification of differentially expressed RNAs (DERs) related to the immune system

Individuals were divided into three different groups as per the median CD2 AP mRNA expression, immune scores, and stromal scores. The stromal and immune scores of STAD were computed utilizing the R"Estimate". DERs were identified from RNA-SEQ data using the R package"limma". DERs were determined as P value (adjust) <0.01, $|\log2\ FC|> 1$. The R package"VennDiagram"was engaged for intersection to identify DERs for subsequent analysis. Genetic Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were executed utilizing the R"ClusterProfiler".

Construction and validation of an immune risk score (IRS)

The TCGA-STAD cohort underwent division into training and validation sets in a 7:3 ratio, considering the chronological inclusion of patients in the study. Within the training set, a univariate Cox analysis was performed on common DERs utilizing the R"survival". Subsequently, the least absolute shrinkage and selection operator (LASSO) method was employed to identify the optimal candidate DERs (IRS RNA-expression profiles) with the highest discriminatory ability. An IRS was then constructed by employing the multivariate Cox regression coefficient as the weighting factor, utilizing the IRS RNA-expression data.

$$\mathbf{IRS} = \sum \boldsymbol{\beta}\mathbf{i} * \mathbf{RNAi}$$

In this instance, βi refers to the coefficient of the'i'th IRS RNA-expression profile. In particular, individuals were classified into high and low IRS groups as per the median IRS value. The statistical validity of the IRS was evaluated utilizing the R"tROC".

Cell lines and cell culture

MGC-803, SGC-7901 and NK-92 cell lines (purchased from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultivated in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS; Gibco). The cells were cultured at 37℃ in a humidified 5% CO2 incubator.

NK killing test

CD2 AP was overexpressed by transfection plasmid in gastric cancer cell lines BGC-823 and MGC-803, and NK92 cells were added 24 h later at ratio of 1:3, and co-cultured at 37℃. The cytotoxic effect of NK92 cells on gastric cancer cells was examined by LDH Assay Kit (DOJINDO, Japan) cytotoxicity test.

Statistical analysis

Associations among different variables were examined utilizing Pearson or Spearman coefficients. When continuous variables conformed to a normal distribution among binary groups, a t-test was employed for comparison. Survival curves were plotted utilizing the K-M method for prognostic analyses involving categorical variables. The statistical significance threshold was established at $P< 0.05$ for all statistical tests, with a two-sided approach applied. Statistical data analyses were conducted utilizing R, v 4.2.2 (Fig. 1).

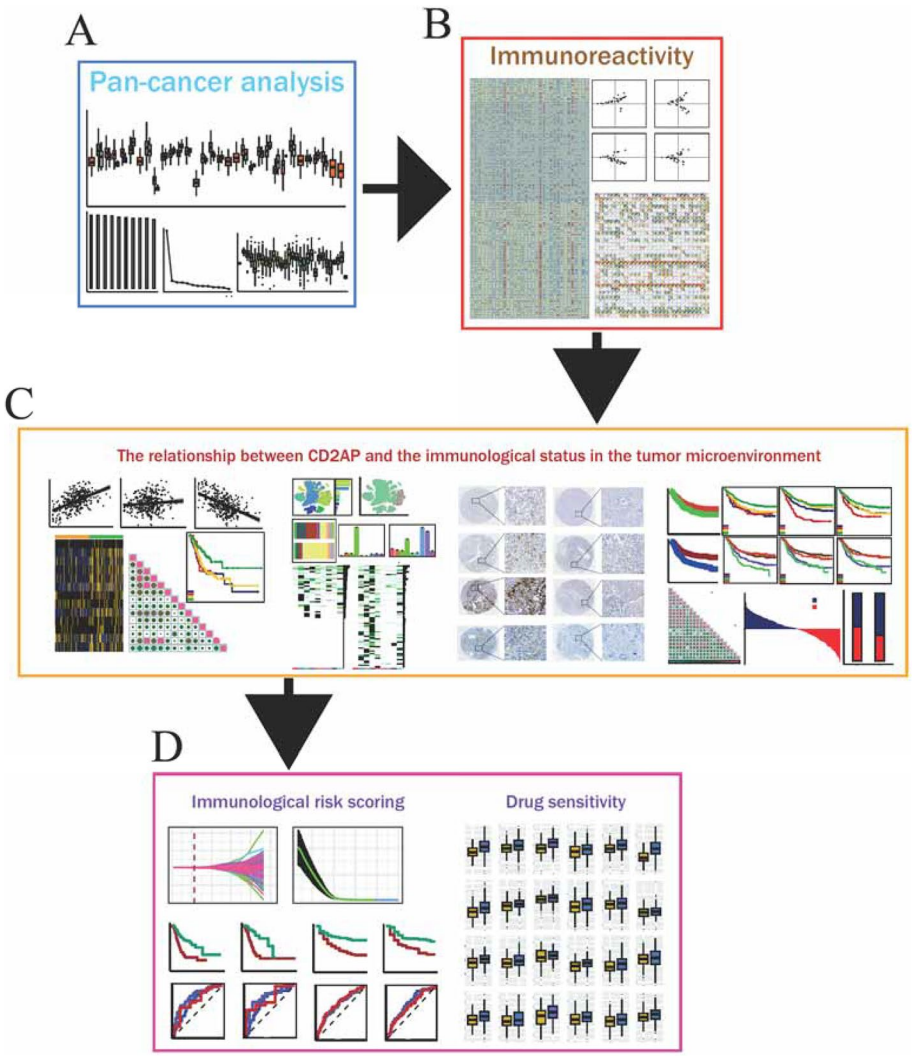


Fig. 1 The overall design of the research. **A** The pan-cancer expression pattern of CD2 AP. **B** The correlation between CD2 AP and immunological factors. **C** The association between CD2 AP and tumor microenvironment. **D** The clinical significance of CD2 AP in gastric cancer patients

Results

Expression level, prognosis-predictive value, and immunological correlation of CD2 AP in Pan-cancer

After analyzing the expression data in the TCGA and GTEx databases, it was revealed that CD2 AP exhibited elevated expression levels in most primary tumor tissues compared to normal solid tissues. This trend was significant in STAD but was only lowly expressed in certain tumors, such as colon adenocarcinoma (COAD) (Fig. 2A). According to the data analysis in the BioGPS database, the expression of CD2 AP was the highest in the gastrointestinal tumor cell line HT29 (Fig. 2B). Combined with the expression data from the CCLE database, the findings demonstrated that CD2 AP was expressed in all CCLs, including stomach CCLs (Fig. 2D). In addition, the expression of CD2 AP was high in macrophages (Fig. 2C). Given the consistently high expression of CD2 AP across pan-cancer, further exploration of its prognosis-predictive value was conducted. Hence, comprehensive analysis of multiple cancer types for overall survival (OS) and progression-free survival (PFS) was carried out utilizing COX regression analysis, K-M analysis, and LOG-RANK tests. The investigation revealed that CD2 AP can serve as a prognostic biomarker in diverse cancer types, but with varying prognostic values (Figure S1A, B). The K-M curve demonstrated that the expression of CD2 AP was different in the prognosis of individuals with gastrointestinal tumors, and its high expression was better in the prognosis of patients with STAD according to the

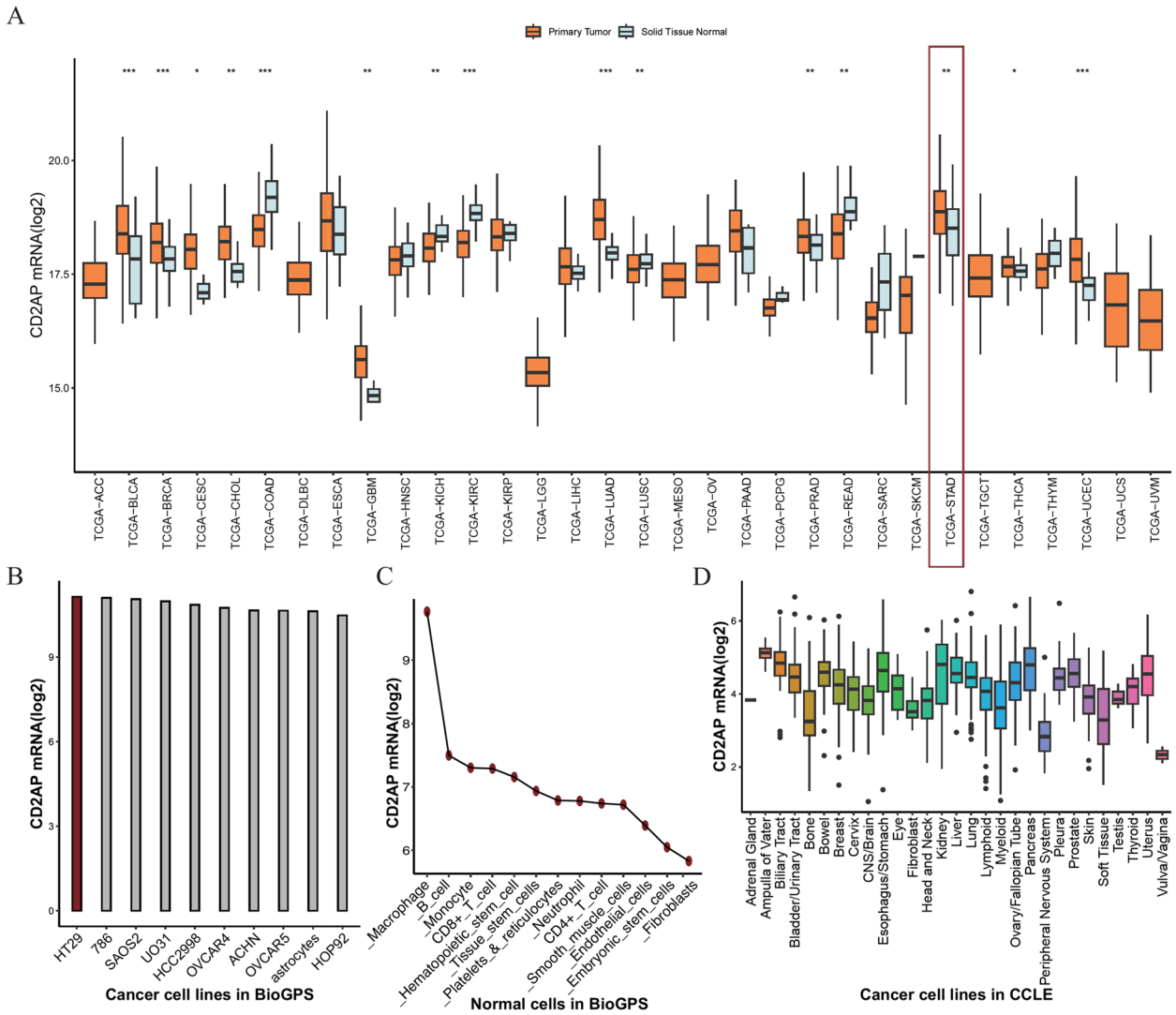


Fig. 2 Expression pattern of CD2 AP across all cancer types. **A** The CD2 AP expression pattern of pan-cancers in the TCGA dataset. Significant statistical differences are denoted by asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). **B**-**C** CD2 AP expression in cancer cell culture compared to healthy tissues, as observed in BioGPS. **D** CD2 AP expression in cancer cell culture, as observed in the CCLE(Cancer Cell Line Encyclopedia) dataset

median CD2 AP expression grouping (Figure S2 A) and the optimum cutoff value of CD2 AP expression (Figure S2B).

Conducting a comprehensive analysis of multiple cancer types to examine the immune-related function of CD2 AP is crucial in identifying the cancer types that may effectively respond to CD2 AP immunotherapy. The results showed that CD2 AP is negatively or very weakly associated with most immunomodulators in STAD (Fig. 3A). In addition, the ssGSEA algorithm was utilized for estimating the infiltration degree of TIICs in TME, and the findings also highlighted that CD2 AP exhibited a negative correlation with most TIICs in STAD (Fig. 3C). In gastrointestinal tumors, apart from STAD, a negative immune correlation between CD2 AP and COAD was also observed. The expression level of CD2 AP was inversely associated with NRP1, CD200, and CD28 in STAD and COAD, showing a significant negative correlation in STAD and a significant positive correlation in COAD. Interestingly, the expression of CD2 AP exhibited a negative association with PDCD1 in both STAD and COAD, which may be a potential immune checkpoint therapeutic target for the prevalence of gastrointestinal tumors (Fig. 3B). Furthermore, in gastrointestinal tumors such as STAD and COAD, a positive association was observed between CD2 AP, TMB and MSI. This association indicates the potential role of CD2 AP in immunotherapy (Figure S3).

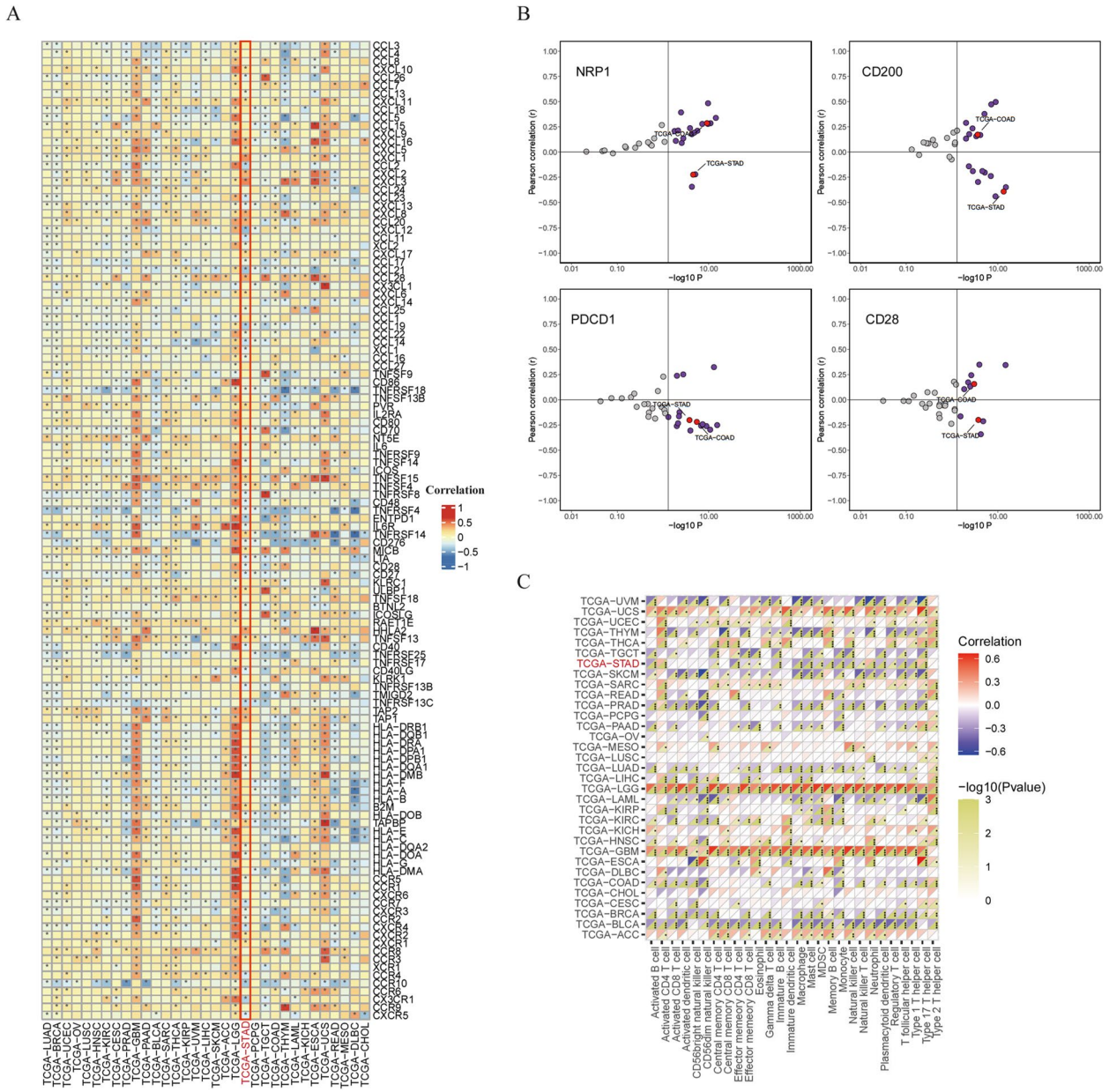


Fig. 3 The impact of immunological status across all cancer types. **A** Association between cancer types and 122 immunomodulators. **B** Association between cancer types and four immune checkpoints, namely NRP1, CD200, PDCD1, and CD28. **C** Association between cancer types and 28 tumor-associated immune cells computed with the ssGSEA algorithm. (**P* < 0.05; ***P* < 0.01; ****P* < 0.001)

In conclusion, it was demonstrated that the expression of CD2 AP shows good prognostic value in gastrointestinal tumors. The expression of CD2 AP in STAD was negatively correlated with the expression of immune checkpoint, indicating that CD2 AP may become a new marker for tumor immunotherapy.

CD2 AP is involved in shaping the tumor microenvironment characterized by reduced stromal cells

The TMEscore was found to be a robust prognostic biomarker and a predictive factor for the response to immune checkpoint inhibitors [33]. To evaluate the influence of CD2 AP expression on immunotherapy, we

analyzed the STAD TMEscore and compared it with the CD2 AP expression level. The results showed a significant positive correlation between CD2 AP expression level and TMEscore (Fig. 4A). TMEscore is the sum of TMEscore A and TMEscore B. TMEscore A was significantly associated with immune-relevant signatures, while TMEscore B was associated with stromal-relevant signatures [18]. Furthermore, we assessed the correlation between CD2 AP expression and TMEscore A and B. We observed a significant negative correlation between CD2 AP expression and TMEscore B (Fig. 4A). These results indicate that CD2 AP expression is negatively correlated with the stromal component of the immune microenvironment. It is worth noting that TMEscoreB negatively regulates TMEscore. Stromal cells promote tumor development and can inhibit the effects of immunotherapy. The shaping of a stromal-reduced tumor microenvironment by CD2 AP may be the reason for the favorable prognosis observed in patients with high expression of CD2 AP. We divided the expression level of CD2 AP and the expression level of TMEscore into groups and analyzed the prognosis of each group. The results showed that patients with high expression of CD2 AP and high TMEscore had the best prognosis (Fig. 4B). The high expression of CD2 AP in STAD was significantly reduced in stromal cells, and was negatively correlated with all stromal cells, among which fibroblasts and endothelial cells were the most negatively correlated (Fig. 4C, D). We analyzed the correlation between CD2 AP expression and TME in the GSE62254 database, and the results showed that CD2 AP was significantly positively correlated with TME, and negatively correlated with TMEscore B (Fig. 4E). Only patients with high CD2 AP expression and high TME score had the best prognosis (Fig. 4 F). The content of stromal cells was negatively correlated with CD2 AP (Fig. 4G, H). It is consistent with the results of TCGA STAD. These findings indicate that CD2 AP is involved in shaping the tumor microenvironment characterized by reduced stromal cells.

The relationship between TMEscore A and CD2 AP expression was not significant, so we further used six independent algorithms calculated TIIC penetration levels (Figure S6-S10). We found a weak negative correlation between CD2 AP and the level of immune cell infiltration (Fig. 5A, B). At the same time, we found that CD2 AP was significantly negatively correlated with CAFs, endothelial cells, fibroblasts and other stromal cells (Fig. 5B). This is consistent with the previous conclusion. Within the high-CD2 AP group, downregulation was observed in activity for most stages in the cancer immunity cycle (Fig. 5C), such as cancer antigen presentation (stage 2), initiation and activation (stage 3), trafficking of immune cells to tumors (stage 4) which encompasses T cell recruitment, CD4 T cell recruitment, dendritic cell recruitment, eosinophil recruitment, and B cell recruitment. Reduced activity of these steps may downregulate the level of effector TIIC penetration in TME. Therefore, the infiltration of immune cells into tumors (stage 5) was reduced in the high-CD2 AP group. It is noteworthy that in the high-CD2 AP group, there was an upregulation in the activity of eliminating cancer cells (stage 7), which could be associated with the immune effects of CD2 AP itself [22]. The effect of CD2 AP on immunotherapy effect may not be achieved by promoting immune cell infiltration, but by stromal cells.

Single cell sequencing data analysis

Various GC samples were integrated by analyzing the single-cell sequencing dataset available on the Stanford DNA Discovery website. Their expressions were examined across various clusters, ultimately identifying nine cell types: NK cells, epithelial cells, T cells, B cells, dendritic cells, endothelial cells, tissue stem cells, macrophages, and fibroblasts, in that order (Fig. 6A). The cells were separated into two groups based on the CD2 AP expression. (Fig. 6B). The analysis results indicate that cells expressing CD2 AP are mostly distributed in epithelial cells, while cells lacking CD2 AP expression are mostly distributed in NK cells and T cells (Fig. 6C-D). Cell communication analysis relies on the information of ligand-receptor pairs expressed between two cell types, which indicates the molecular interactions involved in cell-to-cell communication. The samples of patients with STAD exhibited intricate and frequent communication processes through complex connection graphs representing the interaction strength between the two cell groups (Fig. 6E). To be specific, in the CD2 AP expression group, the interaction between fibroblasts and epithelial cells, endothelial cells, DCs and tissue stem cells was enhanced, and the interaction between tissue stem

(See figure on next page.)

Fig. 4 The connections between CD2 AP and stromal cells in the tumor microenvironment across all populations with gastric cancer. **A**, **E** The relationship between CD2 AP and scores assessing the tumor microenvironment (A for TCGA-STAD and D for GSE62254). **B**, **F** The influence of CD2 AP and tumor microenvironment scoring on the outcomes for patients with gastric cancer (B for TCGA-STAD and F for GSE62254. **C**, **G** A heatmap depicting the discrepancies in xCELL scores between the high-CD2 AP and low-CD2 AP groups (C for TCGA-STAD and G for GSE62254). **D**, **H** Correlation plots showing the interrelationship between CD2 AP and stromal cells (D for TCGA-STAD and H for GSE62254)

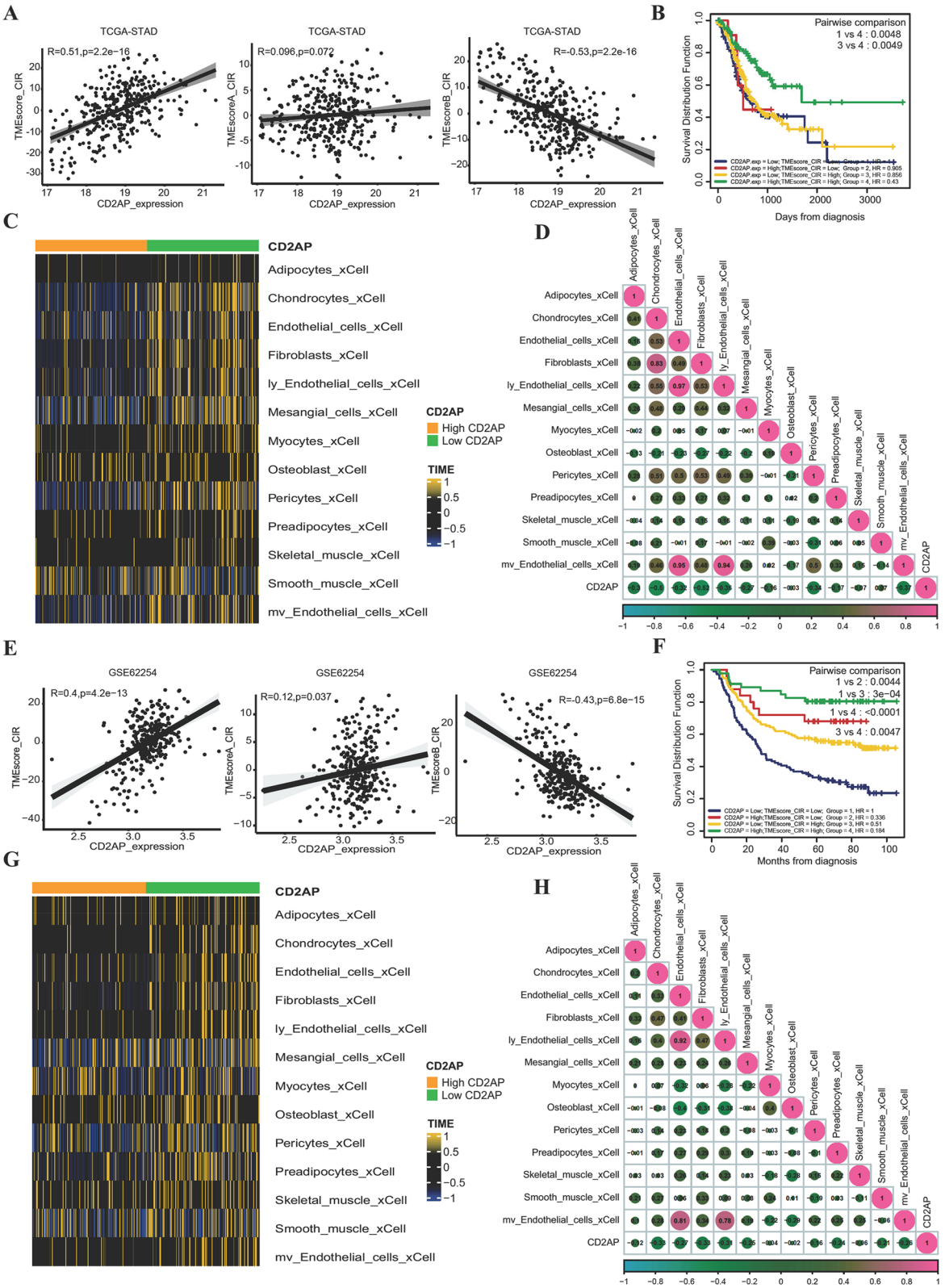
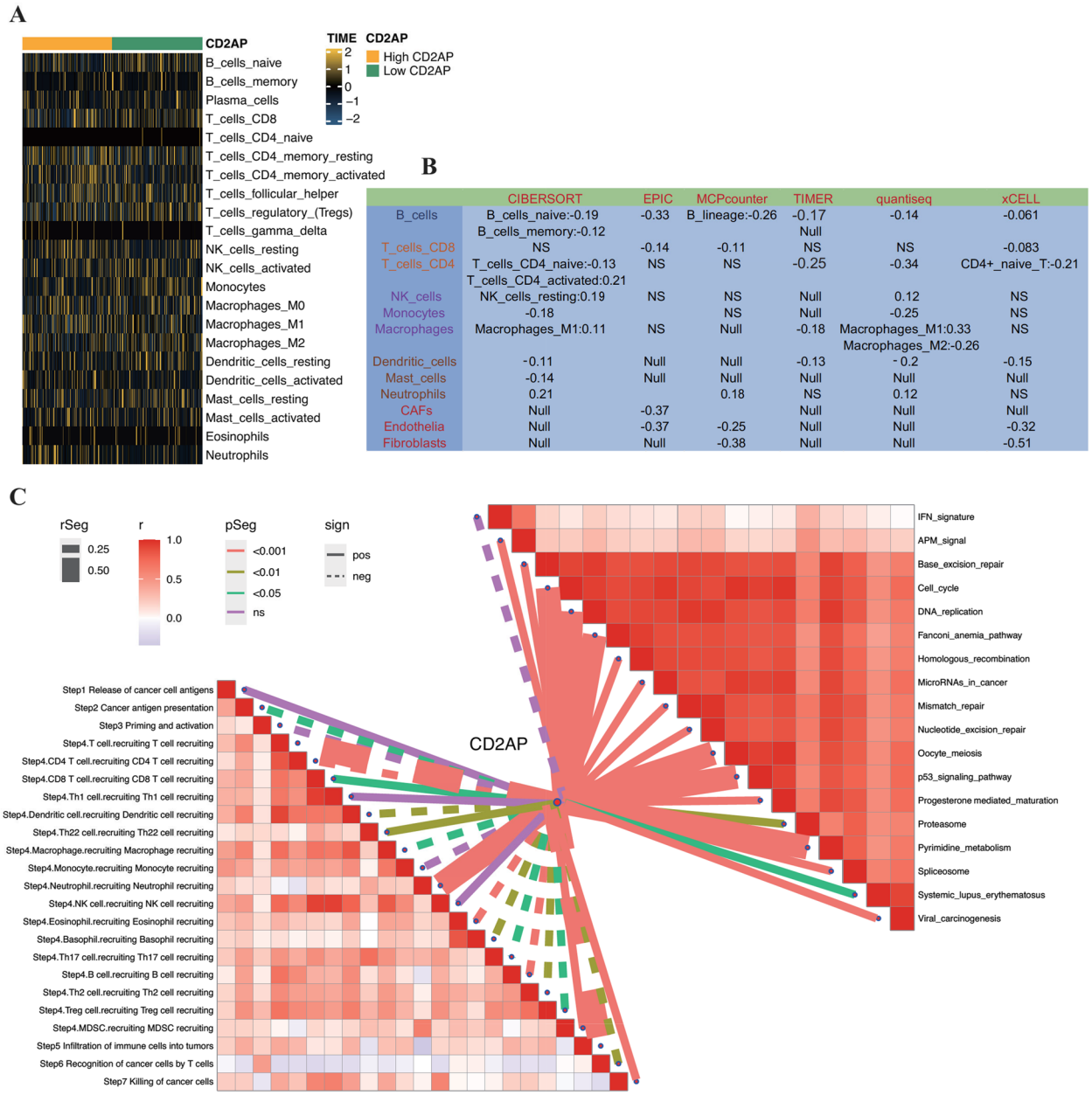


Fig. 4 (See legend on previous page.)



	CIBERSORT	EPIC	MCPcounter	TIMER	quantiseq	xCELL
B_cells	B_cells_naive:-0.19 B_cells_memory:-0.12	-0.33	B_lineage:-0.26	-0.17 Null	-0.14	-0.061
T_cells_CD8	NS	-0.14	-0.11	NS	NS	-0.083
T_cells_CD4	T_cells_CD4_naive:-0.13 T_cells_CD4_activated:0.21	NS	NS	-0.25	-0.34	CD4+_naive_T:-0.21
NK_cells	NK_cells_resting:0.19	NS	NS	Null	0.12	NS
Monocytes	-0.18		NS	Null	-0.25	NS
Macrophages	Macrophages_M1:0.11	NS	Null	-0.18	Macrophages_M1:0.33 Macrophages_M2:-0.26	NS
Dendritic_cells	-0.11	Null	Null	-0.13	- 0.2	-0.15
Mast_cells	-0.14	Null	Null	Null	Null	Null
Neutrophils	0.21		0.18	NS	0.12	NS
CAFs	Null	-0.37		Null	Null	Null
Endothelia	Null	-0.37	-0.25	Null	Null	-0.32
Fibroblasts	Null	Null	-0.38	Null	Null	-0.51

Fig. 5 The association between CD2 AP and the infiltration of immune cells in TCGA-STAD. **A** A heatmap illustrating the relationship between CD2 AP and immune cell infiltration as analyzed by the CIBERSORT algorithm. **B** The relationship of CD2 AP with immune cell infiltration across the six algorithms: CIBERSORT, EPIC, MCPcounter, TIMER, quanTIseq, and xCELL. **C** The correlation within the different stages of the cancer immunity cycle between groups with high and low CD2 AP expression

(See figure on next page.)

Fig. 6 Single-cell sequencing analysis of dataset on the Stanford DNA Discovery website. **A** Cell type classification in GC integrated samples and t-SNE plot of 9 cell clusters (NK cell, epithelial cells, T cell, B cell, DC, endothelial cells, tissue stem cells, macrophages, and fibroblasts). **B** A t-SNE plot is generated based on the expression status of CD2 AP in each cell. **C**-**D** There is a chart illustrating the proportion of CD2 AP expression in two cell groups. **E**–**F** A connection graph depicting the level of interaction strength between different cell types from two groups of cells **G** The relationship between signal pathways and CD2 AP

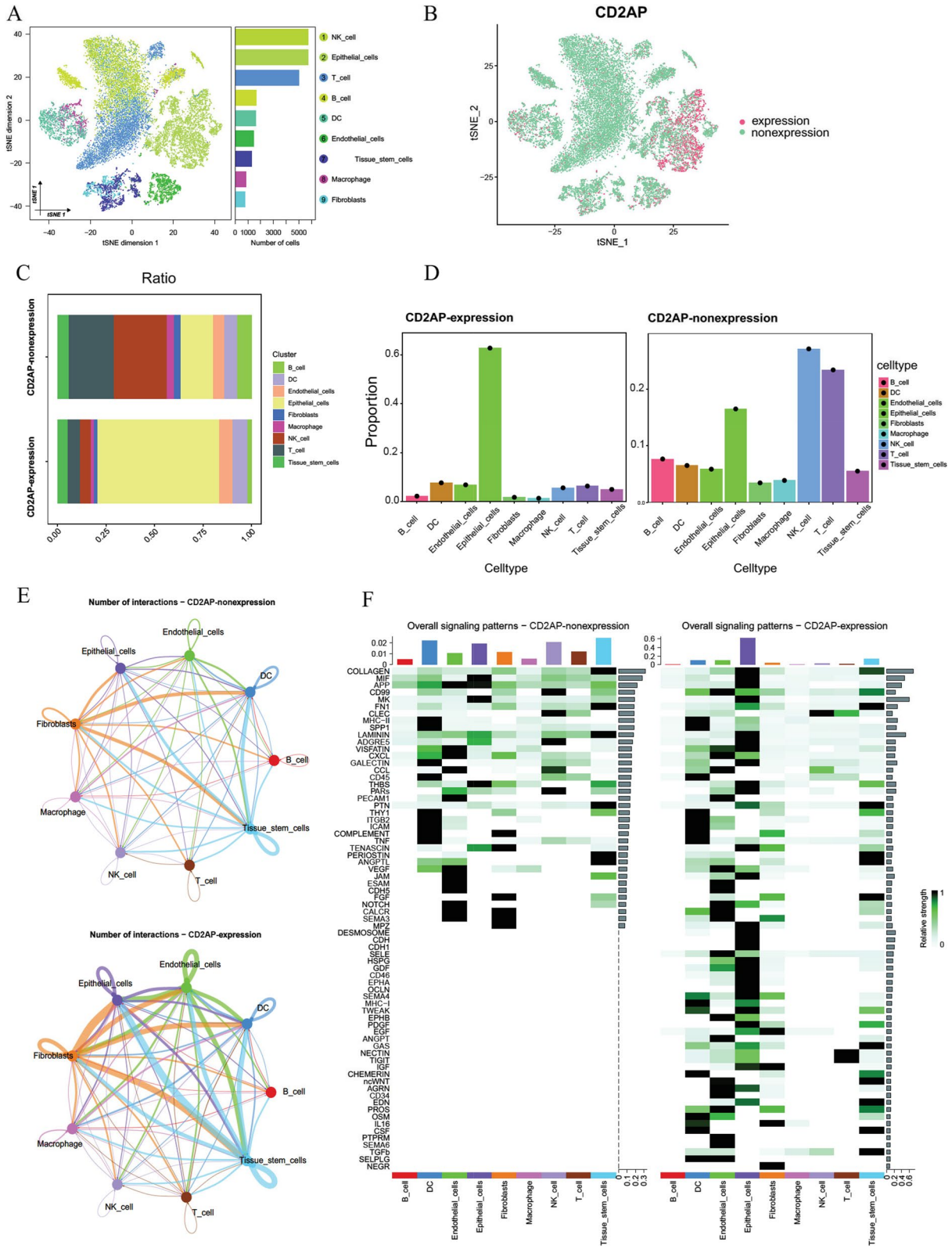


Fig. 6 (See legend on previous page.)

cells and Fibroblasts, epithelial cells, endothelial cells and DCs was upregulated. The signaling pathways affected by the two groups of cells also exhibit significant differences (Fig. 6F).

CD2 AP is positively correlated with immunotherapy of STAD

Subsequently, further analyses were carried out to delve deeper into the relationship between CD2 AP and immunomodulators, as well as their impacts on the immune processes in tumorigenesis. The result found that CD2 AP was negatively associated with most immunomodulators (Fig. 7A). Consequently, a correlation analysis between CD2 AP and ICPs was conducted, revealing that CD2 AP exhibited negative correlations with the major of ICPs (Fig. 7B). These include CD86, LAG3, PDCD1, LAIR1, TIGIT, CD276, CD200, CD200R1, KIR3DL1, CTLA4, BTLA and ADORA2 A. These results suggest that CD2 AP expression may contribute to the benefit of immunotherapy. In addition, the tumor immune dysfunction and exclusion (TIDE) algorithm was used to assess patient response to immunotherapy and showed better responsiveness in the high CD2 AP group (Fig. 7C). Additionally, it was observed that patients with high CD2 AP expression demonstrated improved responses to immunotherapy (Fig. 7D, E). Individuals with elevated CD2 AP scores exhibited the best prognosis when they also scored high on the CD4, CD20, and CD57 immune

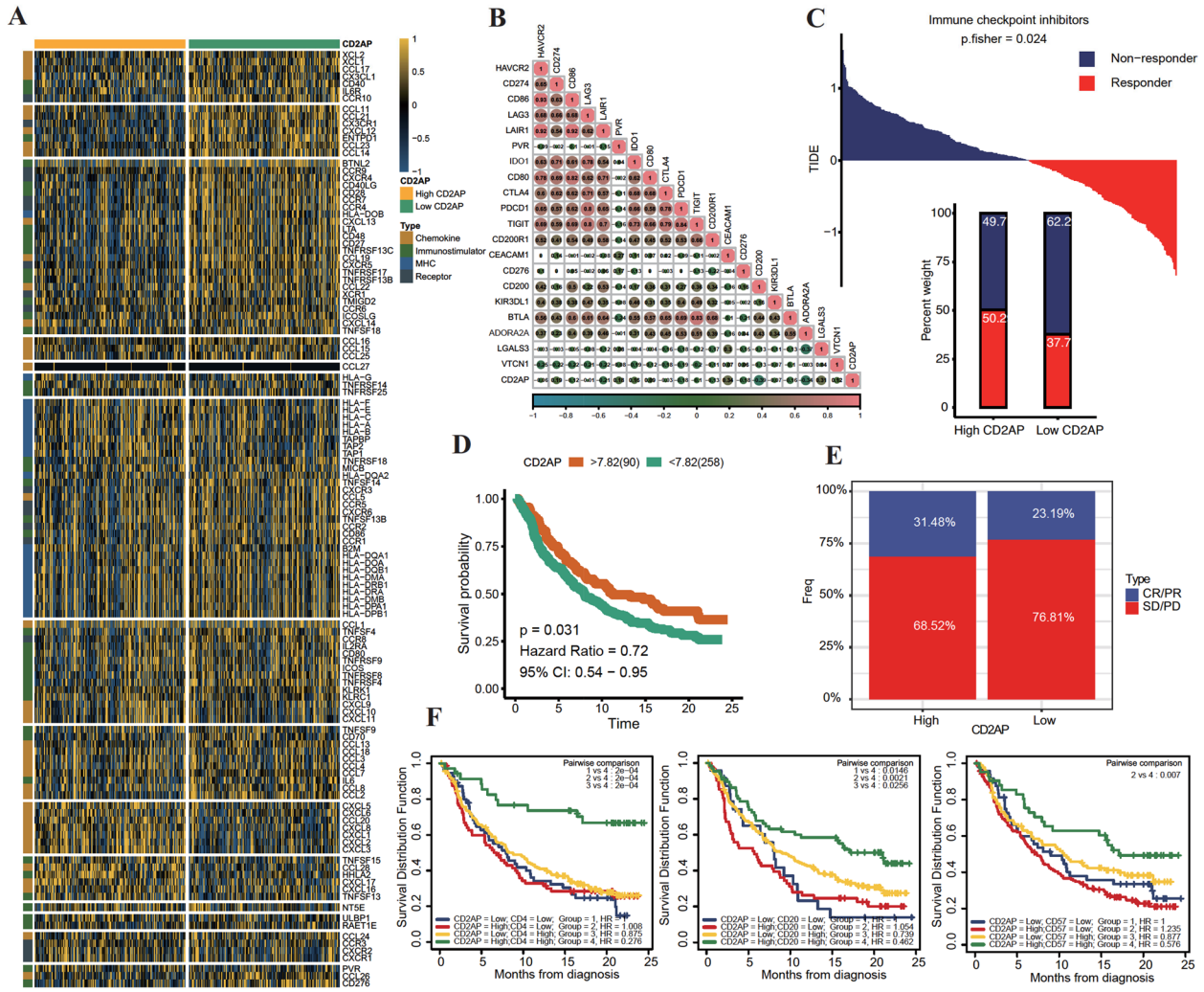


Fig. 7 The influence of CD2 AP and immune cell infiltration on the immunotherapy outcomes for patients with gastric cancer. **A** 122 immunomodulators between high- and low-CD2 AP groups in TCGA-STAD. **B** Association between CD2 AP and 20 inhibitory immune checkpoints. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). **C** The TIDE algorithm forecasts immunotherapy responses in groups with high and low CD2 AP levels. (**D**-**E**) Individuals with elevated expression levels of CD2 AP exhibit better prognosis and are more responsive to immunotherapy. **F** The impact of changes in CD2 AP and the expression levels of CD4, CD20, and CD57 on the prognosis of patients undergoing immunotherapy in IMvigor210

indicators (Fig. 7F). This provides positive implications and insights for the immunotherapy of GC patients.

CD2 AP is positively correlated with the killing effect of immune cells in STAD

Initially, immunohistochemistry was employed to examine CD2 AP, CD4, CD20, and CD57 expression in tissues from a cohort of 506 individuals with GC (Fig. 8G-8H). The K-M survival analysis revealed that groups exhibiting reduced scores of CD2 AP experienced inferior OS rates (Fig. 8B). Moreover, an analysis was conducted on the scores of CD4, CD20, and CD57 marker indices in the corresponding cohort of GC patients (Fig. 8A-F). High or low scores were classified based on the optimal cutoff value. Individuals with elevated CD2 AP scores exhibited the best prognosis when they also scored high on the CD4, CD20, and CD57 immune indicators. This result was validated using the GEO public dataset GSE62254 (Fig. 9A-D).

Gastric cancer cells BGC-823 and MGC-803 were transfected with CD2 AP plasmid and overexpressed CD2 AP (Fig. 9E). After transfected 24 h, NK-92 cells were added in a ratio of 1:3 for co-culture with gastric cancer cells. The NK-92 cells could kill gastric cancer

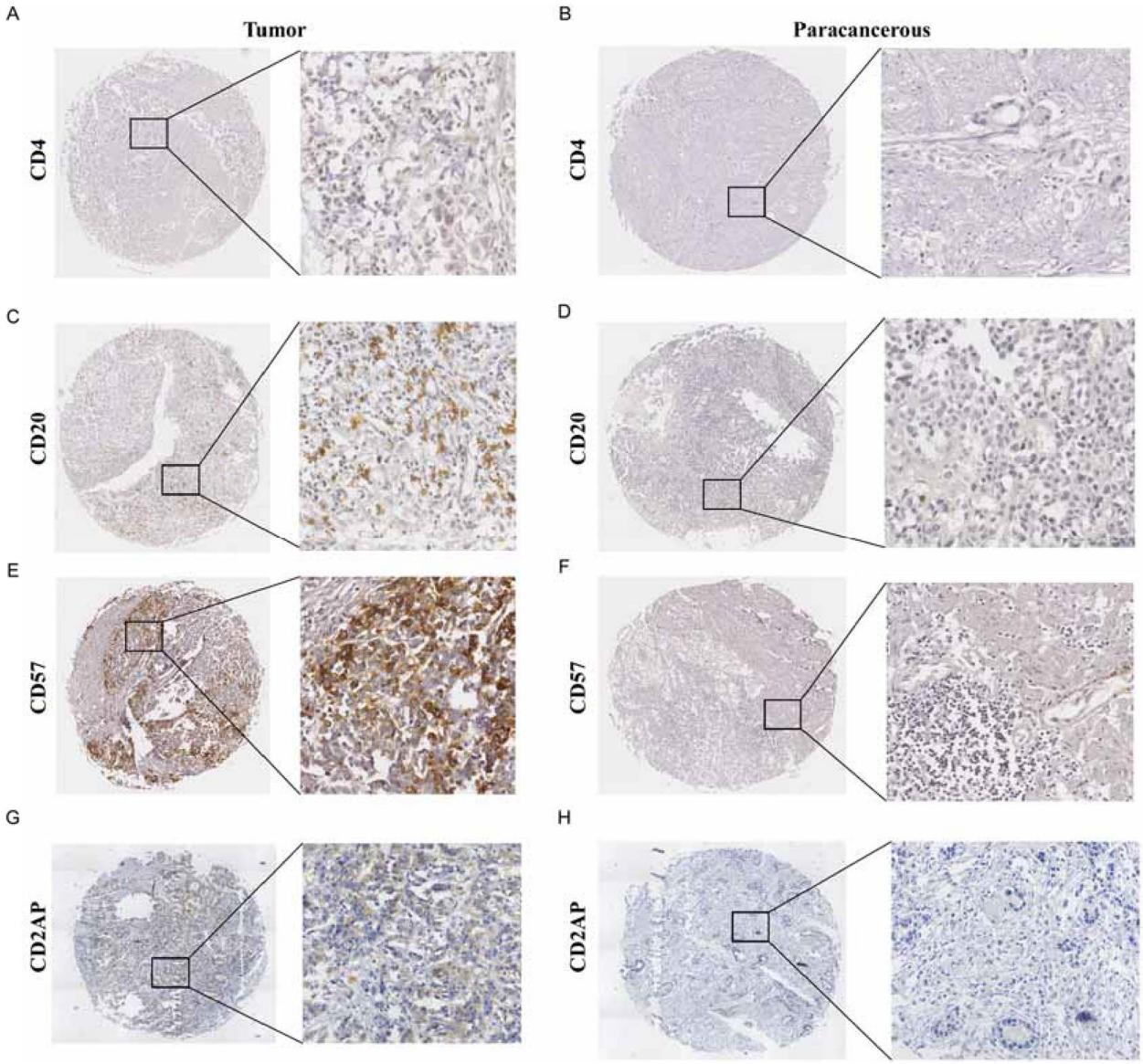


Fig. 8 Tissue microarray from gastric cancer patients. **A**-**B** CD4(A for Tumor and D for Paracancerous). **C**-**D** CD20(C for Tumor and D for Paracancerous). **E**–**F** CD57(E for Tumor and F for Paracancerous). **G**-**H** CD2 AP (G for Tumor and H for Paracancerous)

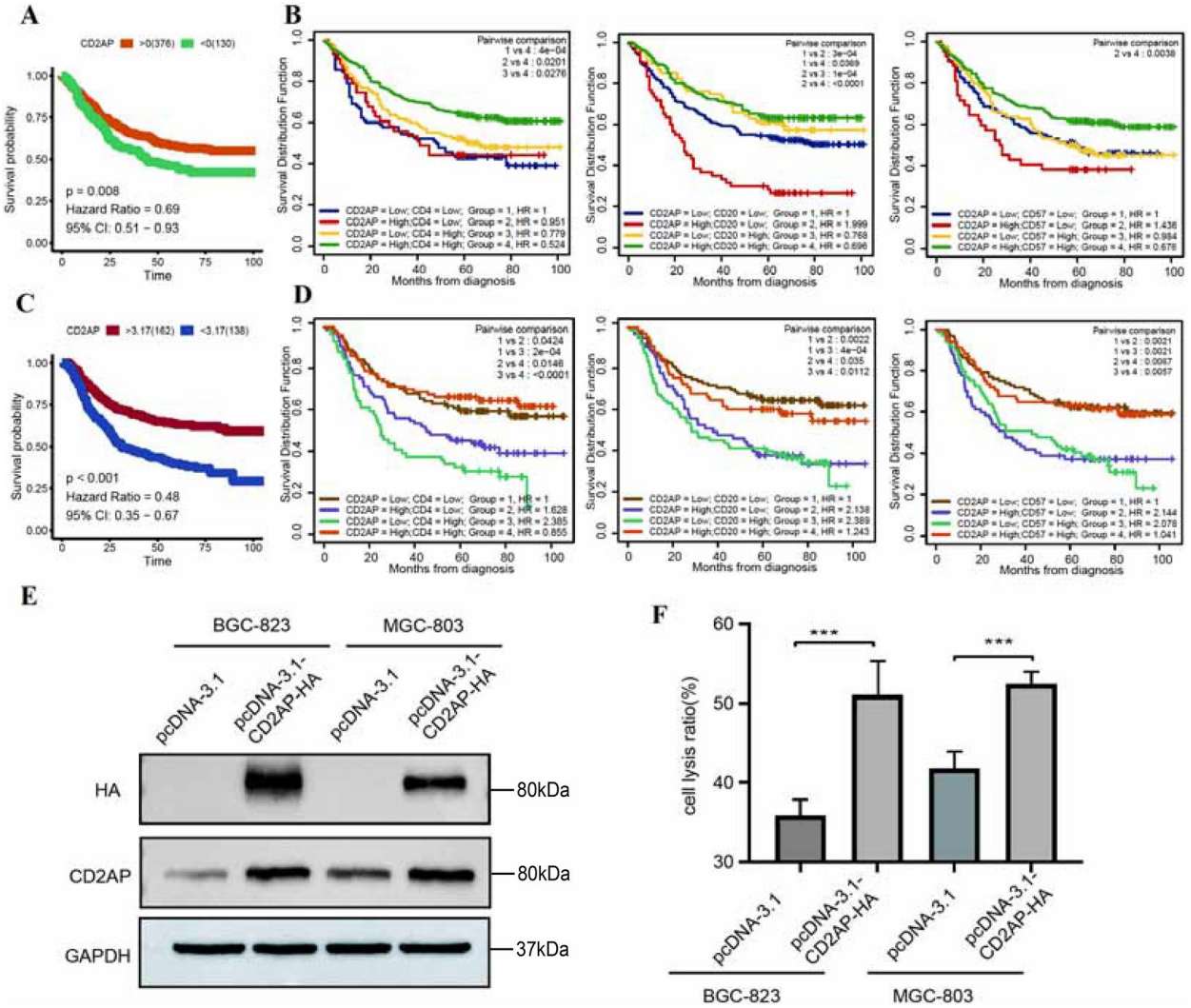


Fig. 9 The association between the expression levels of CD2 AP, CD4, CD20, and CD57 and the prognosis of individuals with gastric cancer. **A**-**B** The cohort of Tissue Microarray: Patients with high expression of both CD2 AP and immune markers have a better prognosis compared to other groups. **C**-**D** GSE62254 (**E**) Western blot analysis to detect CD2 AP-HA expression in BGC-823 and MGC-803 cells. **F** The cytotoxic activity of NK cells against gastric cancer cells with varying levels of CD2 AP expression

cells. LDH detection reagent was added to detect the percentage of lysated cells after 8 h of culture. The results of cell killing experiment showed that gastric cancer cells expressing CD2 AP were more likely to be killed by NK-92 cells (Fig. 9F). These results suggest that gastric cancer cells with high expression of CD2 AP are easy to be killed by immune cells.

CD2 AP expression predicts the efficacy of targeted drug therapy for STAD

CD2 AP expression affects patients'responsiveness to immunotherapy, and some patients are resistant to immunotherapy if CD2 AP expression cannot be altered. To further enhance the survival time of these patients, targeted drugs were analyzed based on CD2 AP expression in patients, aiming to identify effective targeted drugs for immunotherapy-resistant patients. We analyzed the genetic variation of STAD. Notably, copy number gain and demethylation of CD2 AP increased CD2 AP mRNA expression (Figure S4). Figure S5 A-S5B showed the top 30 genes with mutation rates in the high- and low-CD2 AP expression group. It was found that TTN and TP53 were the top two in both groups. At the same time, the mutation profile of STAD was also analyzed. TTN exhibited the highest mutation rate among the genes, with the most prevalent mutation type being missense mutation (Figure S5 C).

The mutational profiles of neoadjuvant chemotherapy-related genes in low CD2 AP and high CD2 AP groups were further studied. The results demonstrated that the neoadjuvant immunotherapy-related gene mutations were higher in the group with high CD2 AP expression and the highest mutation rate gene was ARID1 A (Fig. 10A-B). Additionally, for a more comprehensive prediction of therapeutic prospects and prognosis in STAD subtypes, the Prognosis-Associated Signature (PPS) was employed to conduct drug sensitivity analysis based on the common mutant gene ARID1 A. Drug response data for CTRP and PRISM-derived compounds were included in the analysis. Our analyses obtained six CTRP-derived compounds (including KX-2391, SB-743921, BI-2536, CR-1-31B, methotrexate, and paclitaxel) and four PRISM-derived compounds (including ispinesib, volasertib, epothilone-b, and dolastatin-10). All of these compounds exhibited a negative association with CD2 AP expression (Fig. 10C-D). It is suggested that immunotherapy combined with neoadjuvant chemotherapy can achieve better therapeutic effect in patients with high CD2 AP expression.

To find effective targeted drugs for gastric cancer patients with low CD2 AP expression, the Genomics Sensitivity in Cancer (GDSC) of 36 anti-tumor drugs was utilized. The findings indicated that the IC50 values of most tumor drugs in the high-CD2 AP groups were lower than those in the low-CD2 AP groups. We were surprised to find that ZM.447439 has a lower IC50 value for patients with low CD2 AP expression, suggesting that patients with low CD2 AP expression can be targeted with ZM.447439 (Figure S11).

Development and validation of an IRS

First, in this study, 2312 DERs were identified across three patient groups (Fig. 11A). Interestingly, very few common denominators existed between upregulated DERs in the high-CD2 AP group and the high stromal score group. Likewise, there were very few intersections between downregulated DERs in the down-CD2 AP group and the down-stromal score group. (Fig. 11B). This demonstrated a negative correlation between CD2 AP and the stromal score within the TME. Additionally, simultaneous GO and KEGG pathway analyses were executed. The findings indicated the enrichment of these DERs in various immune-related functions, including negative regulation of the immune system process and regulation of the immune effector process (Fig. 12). Subsequently, 471 DERs with prognostic significance were further identified by means of univariate Cox regression analysis. LASSO-COX analysis was then conducted to identify the 18 best DERs with the lowest λ value (Fig. 13A-B) (Table S5). In addition, using the IRS median as the risk threshold, 245 individuals in the TCGA training set were separated into high-IRS group (n = 122) and low-IRS group (n = 123) (Table S6-1). As shown in Fig. 13C, the survival probability of the low-IRS group was considerably elevated relative to the high-IRS group. ROC analysis demonstrated that the 1, 3, and 5-year AUC values were 0.72, 0.8, and 0.72, respectively. Finally, the IRS model was evaluated in both the TCGA internal testing set and GEO external validation set GSE84437(Table S6-2), and the model was proved to have good accuracy and validity (Fig. 13D).

Discussion

ICIs, such as PD-1, PD-L1, and CTLA-4 inhibitors, were the main force in immunotherapy [34]. They work by blocking the immunosuppressive mechanism. However, due to the heterogeneity of the tumor microenvironment, clinical findings have shown that the efficacy of immunotherapy can vary significantly within the same indication [35]. There is growing evidence indicating that the"hot"or"cold"nature of a tumor directly determines the effectiveness of immunotherapy [36]. Factors such as immune score, stromal score, and tumor purity in the tumor immune microenvironment jointly determine the"cold"or"hot"classification of tumors, and the regulation of these immune system networks has a complex interaction with the tumor, forming a complex immunotherapy response system. Therefore, the search for effective prognostic markers of immunotherapy efficacy has become the focus of attention in the field of immunotherapy.

In this study, by comparing the prognosis and immune effects of STAD, COAD and esophageal cancer (ESCA), CD2 AP expression in STAD can guide immunotherapy. Illustrating the negative association between CD2 AP and immunoinfiltration within the TME in STAD, it was further demonstrated that CD2 AP contributes to the development of a stromal reduced TME. In addition, IRS was constructed based on CD2 AP expression levels, immune scores, and stromal scores to assess prognosis effectively and accurately in the high- and low-CD2 AP expression groups. Finally, an investigation was conducted into the efficacy of targeted therapy for CD2 AP, encompassing ICIs and targeted anti-tumor drug therapy. The present study highlighted the potential of CD2 AP as a target for normalizing cancer immunotherapy, with a specific focus on its immune role in STAD. The suitability of a molecule as a target for normalized cancer immunotherapy hinges on two crucial attributes: TME-specific overexpression and immunosuppressive function [37]. Prior reports have indicated that CD2 AP was identified as a mutated gene through a genome-wide association study (GWAS) of Alzheimer's disease and had an impact on changes in the immune system [38]. However, little research has been

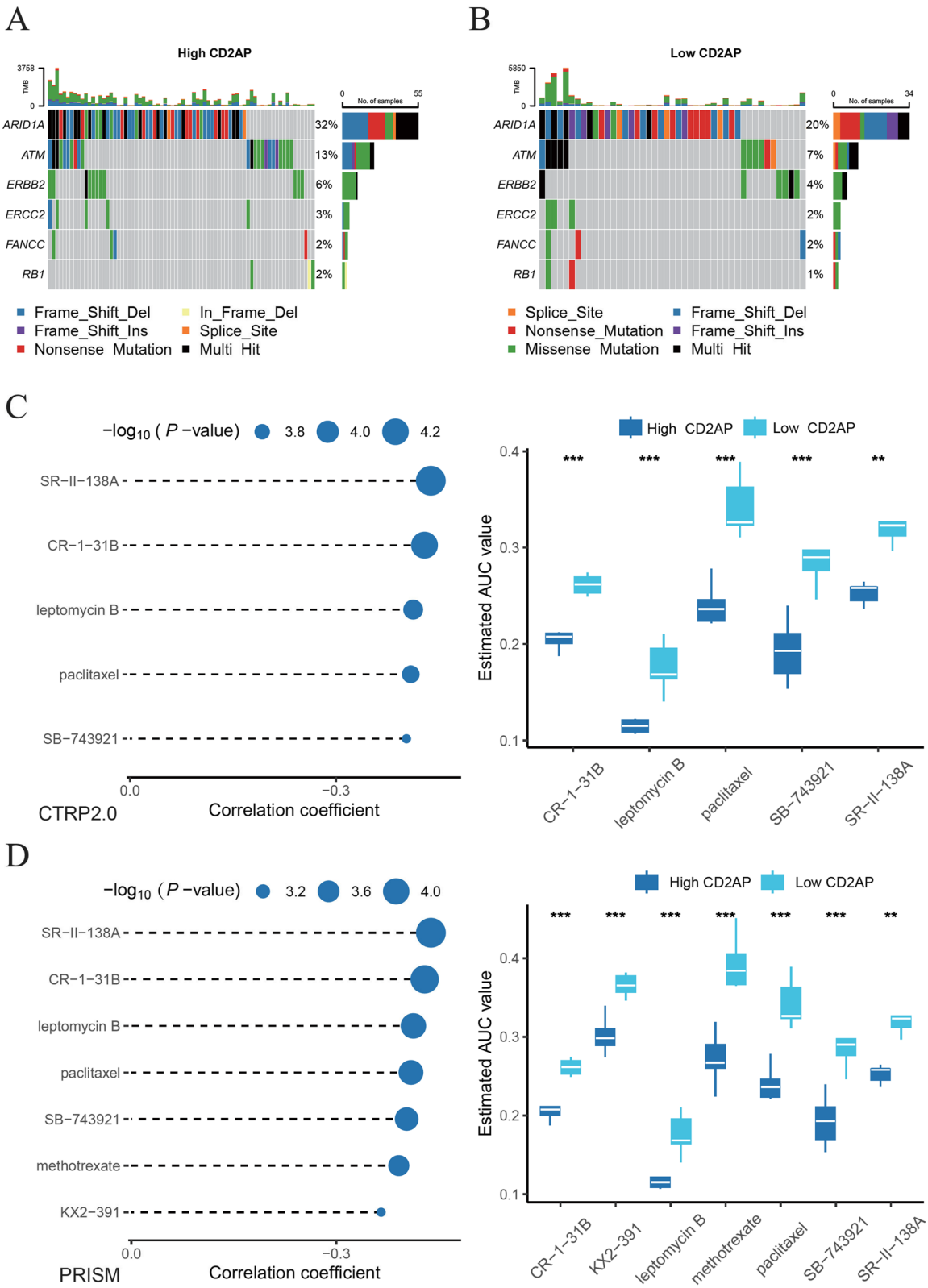


Fig. 10 CD2 AP expression predicts the efficacy of targeted drug therapy for TCGA-STAD **A**-**B** Mutational profiles of neoadjuvant chemotherapy-related genes in low- and high-CD2 AP groups. **C**-**D** In the TCGA-STAD dataset, select gastric cancer patients with ARID1 A mutations and screen for potential drug sensitivities in groups with high and low CD2 AP expression through correlation and differentiation analysis of drugs (C for CTRP 2.0 and D for PRISM)

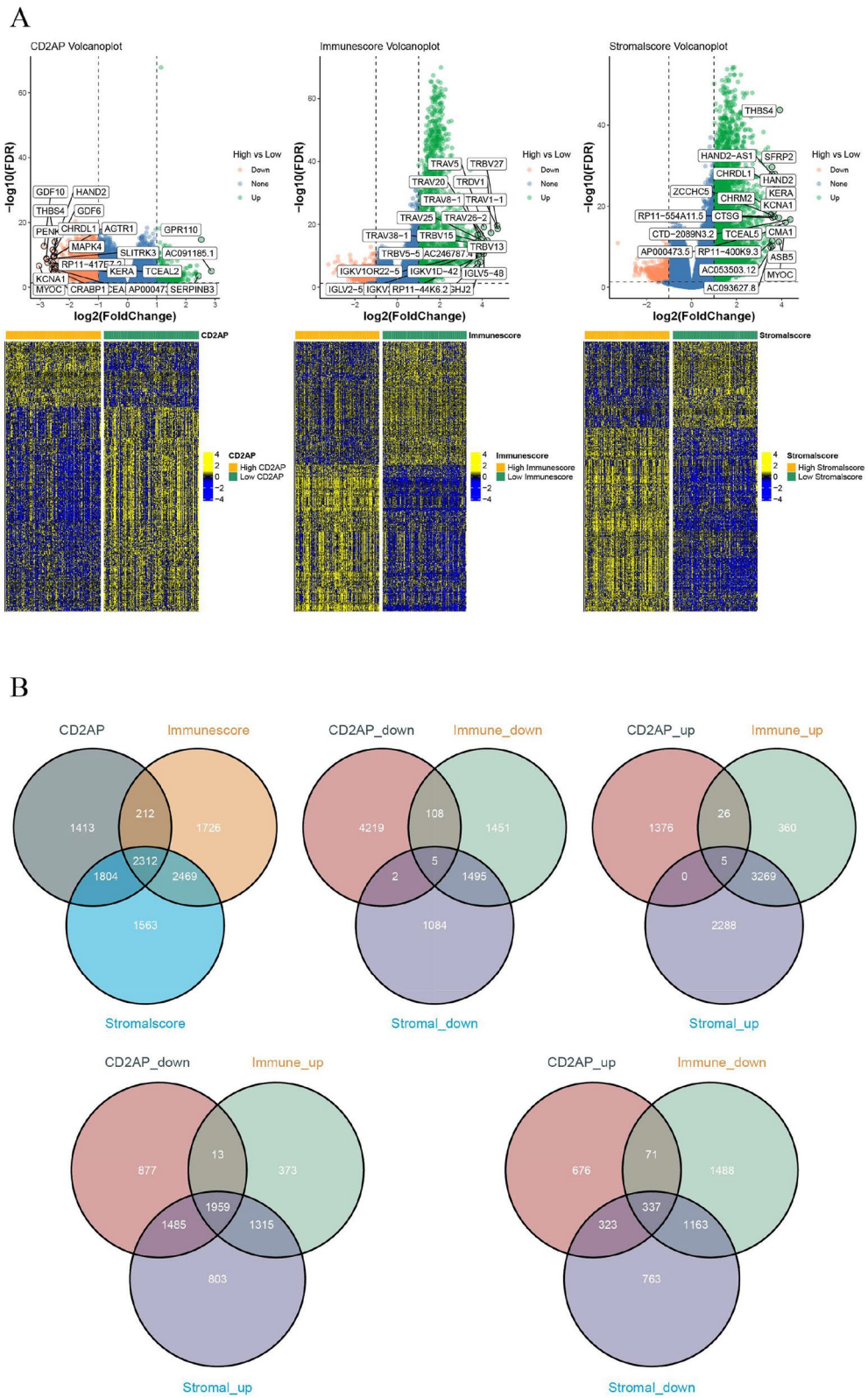


Fig. 11 **A** Differential analysis was performed on individuals with gastric cancer as per the CD2 AP expression levels, immune scores, and stromal scores. **B** Immune-related genes: the intersection of differentially expressed genes in three groups

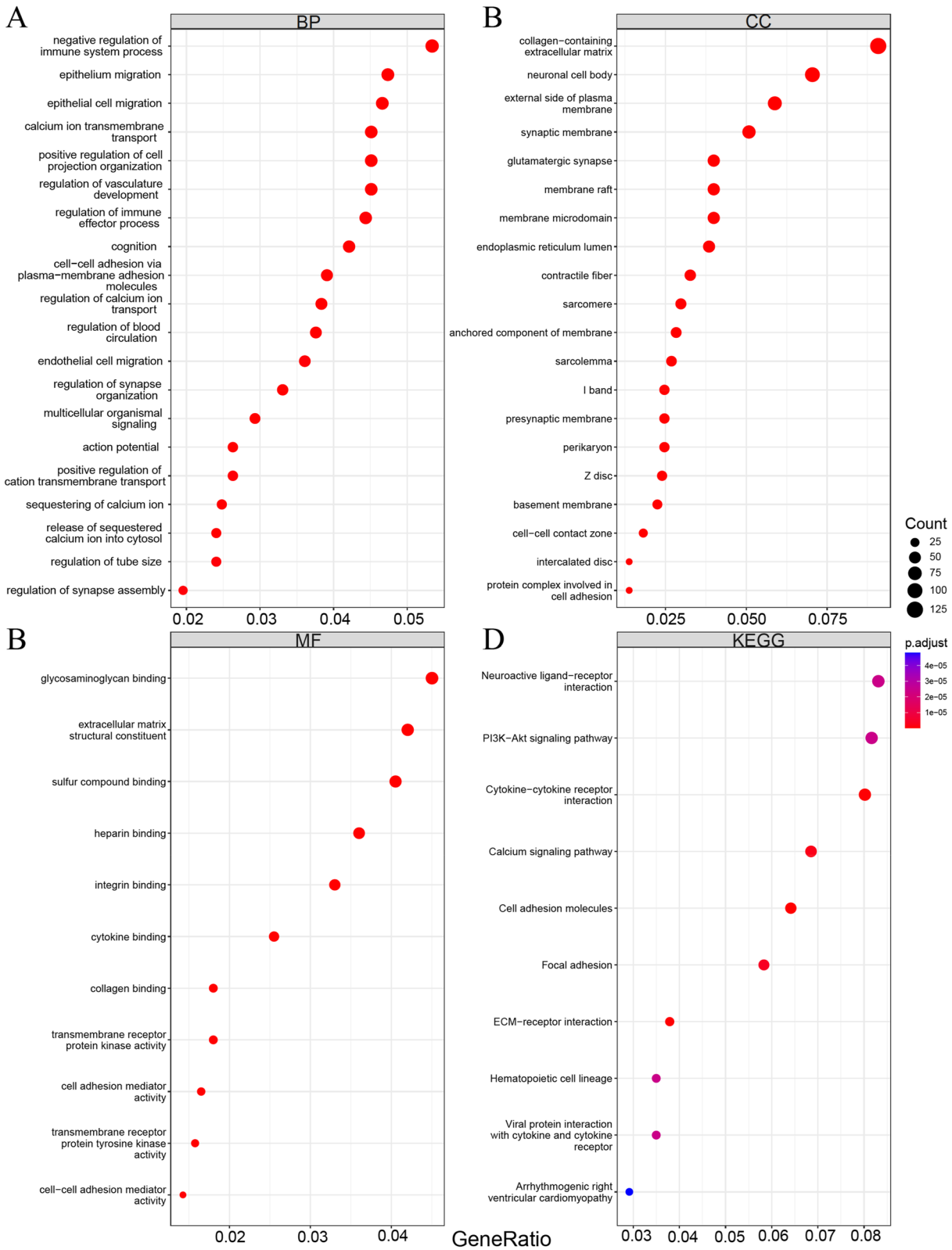


Fig. 12 Enrichment analysis of immune-related genes. **A**-**C** GO **D** KEGG

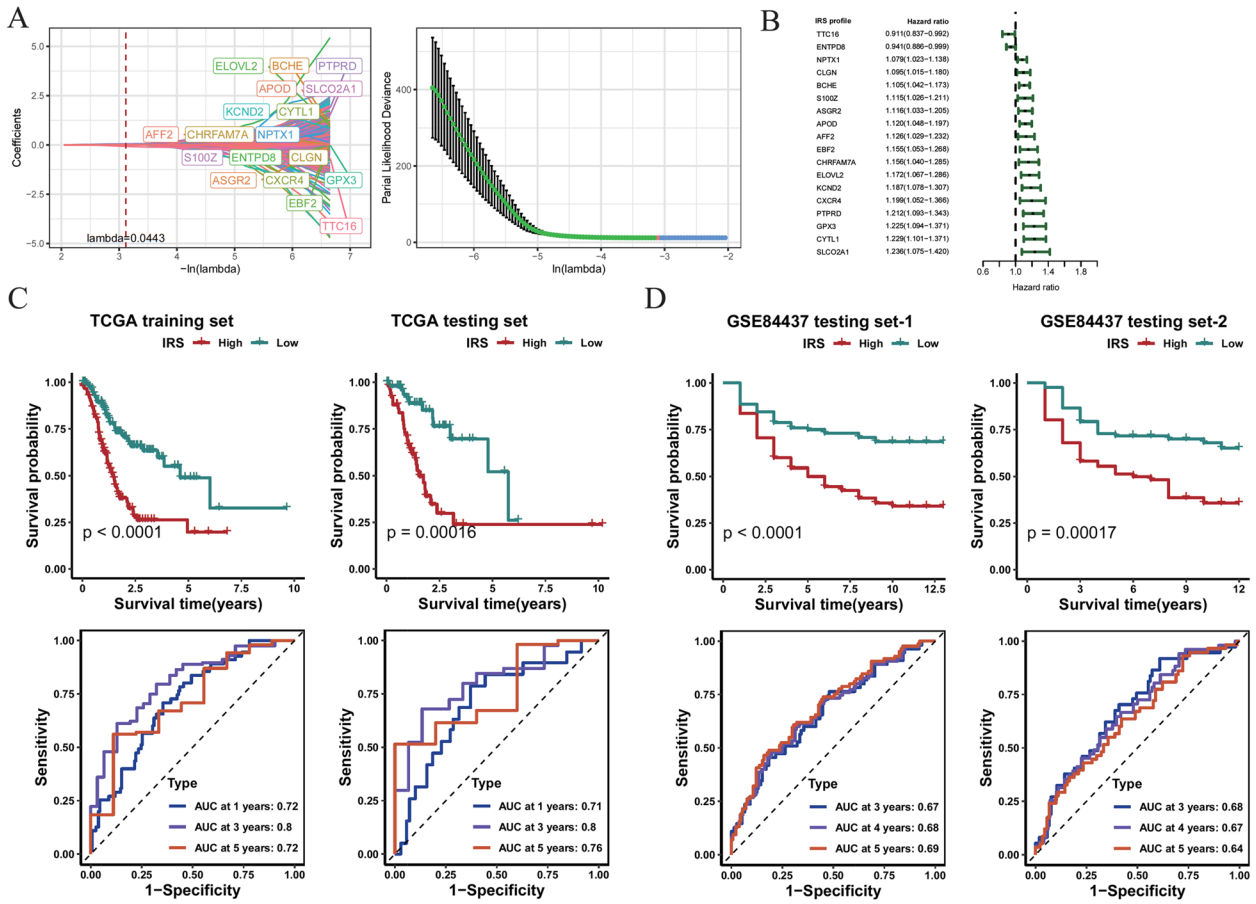


Fig. 13 Creating IRS RNA-expression profiles through the utilization of LASSO Cox regression. **A** In the training set of TCGA-STAD, LASSO-COX analysis was conducted on 471 immune-related genes. **B** Forest plot: univariate Cox analysis was performed on the filtered immune-related genes. **C** The TCGA dataset was classified into training and validation sets to develop the IRS, and its predictive accuracy for survival was evaluated. **D** Validation of the IRS in GSE84437

conducted on the immune role of CD2 AP in cancers and its potential immunotherapeutic targets. The results of this study suggested that CD2 AP exhibited TME-specific overexpression in various cancer types, especially in gastrointestinal tumors. In addition, pan-cancer analyses showed that CD2 AP had immunosuppressive effects in various cancers, including uveal melanoma (UVM), testicular germ cell tumors (TGCT), STAD, prostate adenocarcinoma (PRAD), lung adenocarcinoma (LUAD), and COAD. At the same time, CD2 AP was negatively correlated with NRP1, CD200, CD28, and PDCD1 in STAD. These findings collectively support the potential of CD2 AP as a target for normalized cancer immunotherapy.

Cutting-edge diagnostic techniques and drugs are essential to identify potential new therapeutic targets, and molecular targeted and ICIs therapy are at the forefront of GC treatment [8, 39–41]. ICIs, either as monotherapies or in combination with other treatments, have shown anti-tumor effects in a range of solid tumors, including gastrointestinal tumors [8, 42, 43]. This research delved into the involvement of CD2 AP in the treatment of GC and found a positive association between the expression of CD2 AP and TMEscores. This suggests that CD2 AP may become a new marker for tumor immunotherapy.CD2 AP shapes a TME, aiding in the immunological normalization of GC patients. By analyzing the tissue immunohistochemistry results, it becomes evident that GC patients who exhibit high co-expression of CD2 AP and our immune markers experience a more favorable prognosis in comparison to patients in other groups. Combining multiple immunotherapy strategies will be an extremely important immunotherapeutic approach in the future. In addition, targeted anti-tumor drugs such as novel anti-HER2 therapeutics like T-DXd and disentomb vedotin (RC48) have made substantial breakthroughs in the treatment of GC [44]. An analysis of 36 anti-tumor drugs in the Genomics Sensitivity in Cancer (GDSC) database

revealed that individuals in the high-CD2 AP group displayed increased sensitivity to these drugs, suggesting that they may gain greater benefits from chemotherapy. Finally, an IRS was constructed and validated, exhibiting strong clinical prognostic value. The results indicated a notably elevated survival probability in the low-IRS group relative to the high-IRS group.

The cancer-immune cycle is a series of progressive events that must be initiated in order for the anti-cancer immune response to effectively kill cancer cells and is repeated and expanded to achieve anti-cancer goals [31, 45]. Meanwhile, the cancer-immune cycle activity also reflects the results of the complex immunomodulatory interaction in TME [46]. Cancer immunotherapy accomplishes its immunomodulatory role by initiating or reinitiating the cancer-immune cycle, causing it to expand and repeat. It can be targeted at each step of the cycle to either suppress or halt the anti-cancer immune response [47]. It was observed that CD2 AP expression showed significant positive correlation with TMEscore, but no significant correlation with immune cell infiltration or weak negative correlation. The effect of CD2 AP on immunotherapy effect may not be achieved by promoting immune cell infiltration, but by stromal cells.

Conclusion

This study suggests that CD2 AP expression in gastric cancer could serve as a potential marker for immunotherapy. It was found that CD2 AP can form a thermal tumor immune microenvironment in STAD, which can improve the effect of immunotherapy, and the constructed IRS can effectively predict the prognosis of STAD patients. Future studies should explore the specific mechanism of CD2 AP on immune microenvironment and its related clinical application. In addition, this study confirmed that CD2 AP is beneficial for molecular targeted therapy of STAD.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-025-14248-z.

Supplementary Material 1.

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Authors' contributions
Study conception and design, S.M., X.S.,X.X., M.L.; experiment, H.L.,P.D., H.L., J.D., W.Z; analyze bioinformatics data T.Z.,J.F.; data collection, Z.H., D.X.; writing and original draft preparation, H.C, W.X.All authors reviewed the manuscript.

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Data availability
Publicly available datasets were analyzed in this study. These data can be found in the TCGA (https://portal.gdc.cancer.gov/), GEO (https://www.ncbi.nlm.nih.gov/), and IMvigor210 CoreBiologies (http://research-pub.gene.com/IMvigor210 CoreBiologies) databases. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Approval of the research protocol by an Institutional Reviewer Board: the study was approved by the Review Board of the Second Affiliated Hospital of Wenzhou Medical University (permit and approval number: 2021-K-06–02). All patients were informed of the study and had signed informed consent. All experiments were performed in accordance with relevant guidelines and regulations.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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