

Machine learning-based programmed cell death gene signature for prognosis and drug sensitivity in breast cancer

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Abstract. Breast cancer (BRCA) heterogeneity necessitates robust prognostic biomarkers. Programmed cell death (PCD) serves a key role in tumor progression and therapy response. However, the prognostic potential of PCD-related genes (CRGs) in BRCA remains to be fully elucidated. Therefore, the present study integrated transcriptomic data from The Cancer Genome Atlas, Molecular Taxonomy of Breast Cancer International Consortium and Gene Expression Omnibus databases. Differentially-expressed CRGs were identified in tumor tissues and subjected to univariate Cox regression analysis. A comprehensive machine learning framework, encompassing 101 algorithm combinations, was applied to construct an optimal PCD-based gene signature (CDS). The prognostic value of the CDS, and its association with the tumor immune microenvironment (TIME), predictive power for immunotherapy and drug sensitivity were systematically evaluated using the 'immunedeconv' and 'OncoPredict' R packages. A five-gene CDS (anoctamin 6, polo-like kinase 1, solute carrier family 7 member 5, tubulin α -1C chain and transcobalamin 1) was developed using the Stepwise Cox (both) + Elastic Net ($\alpha=0.9$) model, demonstrating notably increased predictive performance (concordance index=0.79). High CDS scores were found to be independent prognostic factors for inferior overall survival and were associated with an immunosuppressive TIME, characterized by reduced CD8⁺ T-cell infiltration and impaired immune activation. By contrast, low CDS scores predicted favorable responses to immune checkpoint inhibitors in three immunotherapy cohorts and increased sensitivity to chemotherapy, endocrine and targeted agents. Human Protein Atlas and reverse transcription-quantitative PCR validation were consistent with the dysregulation of CDS genes in BRCA

tissues and cell lines. In conclusion, the present study developed and validated a novel, machine learning-derived CDS that effectively stratified BRCA risk in patients. This signature provides insights into the immune landscape and serves as a valuable biomarker in predicting prognosis, immunotherapy efficacy and drug sensitivity, facilitating personalized treatment strategies in the future.

Introduction

Breast cancer (BRCA) remains one of the most prevalent and fatal malignancies affecting women worldwide, with >2.3 million new cases and 685,000 associated deaths every year worldwide, imposing notable psychological and economic burdens on both patients and their families (1,2). Despite advances in surgical techniques, radiotherapy, chemotherapy and targeted therapies, the heterogeneity of BRCA results in variable treatment responses and outcomes among patients (1). This clinical variability underscores the urgent need to identify novel biomarkers that can improve prognostic accuracy and guide personalized therapeutic strategies. In addition, the high incidence and mortality rates of BRCA continue to pose a notable challenge to global public health, highlighting the importance of refining prognostic tools to optimize patient management.

Programmed cell death (PCD) encompasses a spectrum of tightly regulated cellular processes, including apoptosis, necroptosis, pyroptosis, ferroptosis and autophagy, all of which collectively maintain tissue homeostasis and modulate immune responses (3,4). Emerging evidence has implicated PCD pathways as key modulators of tumor initiation, progression and response to therapy (5,6). However, the specific roles and regulatory mechanisms of PCD-related genes (CRGs) in BRCA remain poorly understood. Previous studies have demonstrated that alterations in PCD can contribute to tumor immune evasion and therapeutic resistance (6). However, to the best of our knowledge, comprehensive analyses integrating PCD gene signatures with BRCA prognosis and immunological context remain lacking. Therefore, elucidating the landscape of CRGs in BRCA may provide key insights into tumor biology and reveal novel prognostic biomarkers and therapeutic targets.

Advances in high-throughput sequencing technologies and bioinformatics have facilitated the exploration of transcriptomic

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alterations and their clinical relevance in oncology. In particular, the integration of RNA sequencing (RNA-seq) data with machine learning approaches has emerged as a powerful strategy to construct robust prognostic models, by capturing complex gene expression patterns. Whilst previous prognostic models in BRCA have mainly focused on limited gene sets or clinical parameters, targeting multi-cohort transcriptomic datasets and sophisticated computational algorithms can enhance predictive performance and clinical applicability. Huang *et al* (7) developed a nomogram using survival predictors (tumor grade, T-stage, N-stage, LNR, ER status, PR status, HER2 status) for predicting the overall survival of patients with breast cancer. Another palmitoylation-related gene model could be used for predicting the prognosis and treatment response in breast cancer (8). Multi-cohort and single-cell profiling of aging genes showed a good performance in predicting the prognosis and therapeutic response in breast cancer (9). Zeng *et al* (10) developed a lipid metabolism and ferroptosis-associated index for prognosis and immunotherapy response prediction in hormone receptor-positive breast cancer using multi-cohort data. Despite these methodological progresses, to the best of our knowledge, few studies have systematically developed and validated PCD gene-based signatures for BRCA prognosis (11,12). Furthermore, their potential association with the tumor immune microenvironment (TIME) and drug sensitivity remain unknown.

In the present study, multiple publicly available RNA-seq datasets encompassing BRCA tissues and normal controls were applied to identify differentially expressed genes (DEGs) associated with PCD. By integrating these data with curated CRG lists, the present study employed univariate Cox regression analysis to screen for prognostically significant genes. Subsequently, a comprehensive machine learning framework was implemented to evaluate the numerous algorithmic combinations, culminating in an optimal gene signature model with high concordance index (C-index) for survival prediction. This integrative approach enables the capture of key PCD-related molecular features underpinning BRCA heterogeneity.

Furthermore, the constructed PCD-based gene signature (CDS) was investigated for its association with immune cell infiltration and immune-related pathways within the tumor microenvironment. Understanding the interplay between tumor cell death programs and immune contexture is key to predicting immunotherapy responsiveness and developing combination treatment strategies (13). Additionally, the association between CDS and chemotherapeutic drug sensitivity was assessed using pharmacogenomic data, aiming to provide actionable information for individualized treatment selection. Through this multidimensional analysis, the present study aimed to establish a novel prognostic biomarker that can not only stratify patient risk but can also inform immunological and pharmacological aspects of BRCA management. Collectively, the present study aimed to fill the existing gap in BRCA prognostication by focusing on PCD gene networks and their clinical implications.

Materials and methods

Data acquisition. To validate the present findings, data were collected from various publicly available datasets. RNA-seq

and clinical data were sourced from The Cancer Genome Atlas (TCGA) database (<https://portal.gdc.cancer.gov/repository>), which offers a comprehensive molecular data repository for various cancer types, including BRCA. The present study specifically selected RNA-seq data for patients with BRCA, alongside corresponding clinical information, such as survival rates, tumor staging and other clinical variables.

Additionally, external validation cohorts were incorporated to strengthen the reliability of the present study model. These cohorts included the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC; <https://ega-archive.org/studies/EGAS00000000083>) dataset and datasets from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo>), namely GSE20685 (14), GSE20711 (15), GSE42568 (16), GSE58812 (17) and GSE96058 (18).

Inclusion criteria for the present study were as follows: i) A confirmed pathological diagnosis of invasive BRCA; and ii) complete clinical and survival data. Patients with <6 months of follow-up, previous malignancies or metastatic BRCA were excluded. This multi-cohort approach allowed for validation across different populations, enhancing the generalizability of the present study predictive signature. In addition, three immunotherapy-related datasets, namely, IMvigor210 (19), GSE91061 (20) and GSE78220 (21), were utilized to assess the ability of CDS in predicting the benefits of immunotherapy.

The PCD patterns included in the present study were pyroptosis, ferroptosis, necroptosis, autophagy, immunological cell death, entotic cell death genes, cuproptosis, parthanatos, lysosome-dependent cell death, intrinsic apoptosis, extrinsic apoptosis, necrosis, anoikis, apoptosis-like and necrosis-like morphology. The present study obtained a set of genes associated with PCD from multiple resources, including the Molecular Signatures Database (22), Kyoto Encyclopedia of Genes and Genomes database (23), articles (24,25) and the GeneCards (26) database (Table SI) (26,27).

Preprocessing of RNA-seq data. RNA-seq data from TCGA and GEO were preprocessed to standardize and ensure consistency across datasets. Key preprocessing steps included: i) Normalization, where RNA-seq data was normalized using ‘DESeq2’ to adjust for sequencing depth differences (27); ii) batch effect correction, where the ‘ComBat’ method from the ‘sva’ R package (version 3.46.0; R Development Core Team) was applied to correct for batch effects (28); and iii) log₂ transformation, which was used on the normalized counts to stabilize variance and prepare the data for subsequent analysis.

Construction of CDS. A CDS was developed using a multi-step machine learning framework. First, univariate Cox regression analysis identified CRGs significantly associated with overall survival (OS) in BRCA. Subsequently, 101 distinct model combinations derived from 10 machine learning algorithms were evaluated, including Random Survival Forests (29), least absolute shrinkage and selection operator (30), Ridge (31), Elastic Net (Enet) (32), CoxBoost (33), stepwise Cox regression (34), partial least squares Cox models (35), supervised principal component analysis (36), generalized boosted regression (37) and survival support vector machines (38). Detailed information about the operation of these machine learning programs is presented in Data SI.

Leave-one-out cross-validation was applied to TCGA cohort to determine the optimal model. The accuracy of its prediction of the overall survival rate of patients for each model was assessed using Harrell's C-index (39). The combination designated as Stepwise Cox (StepCox) (both) + Enet ($\alpha=0.9$) demonstrated the highest average C-index and was therefore selected as the final CDS model. This model incorporated five genes and the risk score for each patient was computed as follows: CDS score=[0.0135x anoctamin 6 (ANO6)level] + [0.079x polo-like kinase 1 (PLK1)level] + [0.1241x solute carrier family 7 member 5 (SLC7A5)level] + [0.0615x tubulin α -1C chain (TUBA1C)level] + [-0.165x transcobalamin 1 (TCN1)level].

Patients were then stratified into high- and low-risk subgroups based on the median CDS score.

Nomogram construction. To further improve the clinical value, a nomogram was constructed based on age, sex, T stage, N stage, clinical stage, estrogen receptor, progesterone receptor and CDS-based risk score using 'nomogramEx' R package (version 3.0; R Development Core Team) (40). A calibration curve was constructed to show the relationship between the actual and predicted probabilities for the 1-, 3- and 5-year OS (41).

Immune microenvironment analysis. The relationship between the CDS-based risk score (ANO6, PLK1, SLC7A5, TUBA1C and TCN1) and the TIME was investigated using several computational approaches. The 'immunedeconv' R package (version 2.0.3; R Development Core Team), which incorporated multiple deconvolution algorithms, including Cell-type Identification By Estimating Relative Subsets Of RNA Transcripts (CIBERSORT), Estimating the Proportions of Immune and Cancer Cells (42), xCell (43), Microenvironment Cell Populations-counter (44) and Tumor Immune Estimation Resource (45), was used to estimate the abundance of various immune cell types (46). CIBERSORT is a versatile computational method for quantifying cell fractions from bulk tissue gene expression profiles (47). The Estimation of Stromal and Immune cells in Malignant Tumor tissues using Expression (ESTIMATE) algorithm was used to compute stromal, immune and ESTIMATE scores, reflecting the non-tumor cellular components in the tumor microenvironment (48). Additionally, single-sample gene set enrichment analysis (ssGSEA) through the 'Gene Set Variation Analysis' R package (version 1.46.0; R Development Core Team) was performed to quantify the activity of immune-related pathways and functions. Several computational tools were used to evaluate the role of CDS in predicting immunotherapy benefit. Intratumor heterogeneity (ITH) scores for BRCA were calculated using the 'DEPTH2' algorithm (49). Tumor Immune Dysfunction and Exclusion (TIDE) scores were obtained from the TIDE database (<http://tide.dfci.harvard.edu>; version 1.0) (50). Immunophenoscores (IPS) for patients with breast cancer were retrieved from The Cancer Immunome Atlas database (<https://tcia.at/home>; version 1.0) (51). Tumor mutation burden (TMB) scores for patients with BRCA were collected from TCGA database (52).

Drug sensitivity prediction. Drug sensitivity predictions were generated using the 'OncoPredict' R package (R Development

Core Team, version no.0.2) (53), which leverages gene expression profiles and pharmacogenomic data from the Genomics of Drug Sensitivity in Cancer database (54) to estimate the half-maximal inhibitory concentration (IC_{50}) values. The 'OncoPredict' R package was utilized to predict IC_{50} of various chemotherapeutic and targeted agents based on gene expression profiles. Differences in drug sensitivity between the high and low CDS score groups were compared to assess the potential of the signature for the prediction of therapeutic response.

Protein expression data from the Human Protein Atlas (HPA). Protein expression levels of the CDS genes were examined using immunohistochemistry data from the HPA (<https://www.proteinatlas.org/>) (55), which provides protein localization and expression information in both normal and cancerous tissues. Representative immunohistochemical images of CDS genes of in normal and cancerous tissues was obtained from the 'TISSUE' resource and 'CANCER' resource of HPA, respectively.

Cell culture. Human BRCA cell lines (T47D, BT549, SKBR3, MCF-7 and MDA-MB-468) and a normal breast epithelial cell line (Bst578Bst) were acquired from the Shanghai Institute of Biochemistry and Cell Biology. These cells were cultured in RPMI-1640 medium (Procell Life Science & Technology Co., Ltd.) enriched with 10% FBS (Procell Life Science & Technology Co., Ltd.) and 1% antibiotic solution (Procell Life Science & Technology Co., Ltd.). Cells were maintained at 37°C in an atmosphere containing 5% CO₂. The use of these cell lines follow the guidelines of Shanghai Institute of Biochemistry and Cell Biology.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the aforementioned cells (T47D, BT549, SKBR3, MCF-7, MDA-MB-468 and Bst578Bst) using Triquick® Reagent (Beijing Solarbio Science & Technology Co., Ltd.). According to the manufacturer's protocol, complementary DNA (cDNA) was synthesized from the isolated RNA employing the SweScript RT I FTTSt Strand cDNA Synthesis Kit (cat. no. G3330-100; Wuhan Servicebio Technology Co., Ltd.). The resulting cDNA was then used as a template for qPCR analysis performed on a PCR system using 2X Fast SYBR Green qPCR Master Mix (Low ROX) (cat. no. G3321-05; Wuhan Servicebio Technology Co., Ltd.). The thermocycling conditions were as follows: Initial denaturation at 95°C for 5 min; followed by 35 cycles at 95°C for 30 sec, 55°C for 30 sec and 70°C for 30 sec. Gene expression levels were quantified using the $2^{-\Delta\Delta C_q}$ method (56) and normalized to GAPDH expression. Primer sequences used were as follows: GAPDH forward (F), 5'-GTCTCCTCTGACTTCAACAGC G-3' and reverse (R), 5'-ACCACCCTGTTGCTGTAGCCA A-3'; anoctamin 6 (ANO6) F, 5'-ATGAAAAGATGAGC AGGAATG-3' and R, 5'-TTATTCTGATTTTGGCCGTA-3'; Polo-like kinase 1 (PLK1) F, 5'-GGCAACCTTTTCTCTGAAT GA-3' and PLK1 R, 5'-AATGGACCACACATCCACCT-3'; solute carrier family 7 member 5 (SLC7A5) F, 5'-GTCCAA TCTAGATCCCAACTTCTC-3' and R, 5'-ATTCCATCCTCC ATAGGCAAAG-3'; tubulin α 1C (TUBA1C) F, 5'-GCTTCA AGGTTGGCATTAA-3' and R, 5'-GAGCAATACCACAGC

TGTT-3'; transcobalamin (TCN) F, 5'-CATCCGCCTAAA ACCTCTGTT-3' and TCN R, 5'-CCGAGCTTACATCTG ACAATCTG-3'.

Statistical analysis. Statistical analyses were conducted in R software (version 4.2.1; R Development Core Team). Survival differences between risk groups were assessed using Kaplan-Meier analysis and the log-rank test. For survival analysis, right-censoring was handled using the Cox Proportional Hazards Model, accounting for censored observations. The proportional hazards assumption was evaluated using Schoenfeld residuals. The independent prognostic value of the CDS was evaluated using univariate and multivariate Cox proportional hazards models. Multicollinearity was assessed using correlation matrices and variance inflation factor (VIF). Genes with high pairwise correlations ($r > 0.8$) or VIF values > 10 were excluded to prevent redundancy in the multivariate analysis. Time-dependent receiver operating characteristic (ROC) curves were plotted to determine the predictive accuracy of the signature at 1, 3 and 5 years. GSEA was performed to identify biological pathways associated with CDS risk groups. Comparisons between two groups for continuous variables were conducted using the unpaired Student's t-test or Wilcoxon rank-sum test. One-way ANOVA (parametric) followed by a post-hoc test (Bonferroni) was performed to compare differences between three or more groups, whilst correlations were analyzed using Pearson's or Spearman's rank correlation analysis. The data were presented as mean $\pm$ standard deviation. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Identification of prognostic CRGs. Comparative transcriptomic analysis between BRCA and normal breast tissues identified 2,404 DEGs in the TCGA dataset (Fig. S1A and C). Intersection with a curated list of CRGs yielded 373 candidate genes implicated in PCD (Fig. S1C). Univariate Cox regression analysis further refined this set to 40 CRGs by being significantly associated with OS, representing potential prognostic biomarkers for BRCA (Fig. S1B).

Development and validation of a machine learning-based CDS. The present study constructed a prognostic CDS using a comprehensive machine learning framework. Among the 101 algorithm combinations evaluated, the StepCox (both) + Enet ($\alpha = 0.9$) model achieved the highest average C-index of 0.79 (Fig. 1A) and was selected for subsequent analysis. This model incorporated five genes to calculate a CDS risk score for each patient.

Stratification of patients into the high- and low-risk groups based on the median CDS score revealed significant survival disparities. Survival analysis pertaining to the composite signature risk groups demonstrated that patients with a high CDS score exhibited significantly shorter OS across all datasets tested (Fig. 1B-H). The time-dependent ROC analysis confirmed the robust predictive power of the model, with area under the curve (AUC) values for 3-, 5- and 7-year survival reaching 0.837, 0.815 and 0.799, respectively, in TCGA cohort (Fig. 1B). Consistent performance was observed in

the METABRIC and GEO validation cohorts, where AUC values ranged from 0.749 to 0.838 across various time-points (Fig. 1C-H).

Previous studies also explored the functional roles of ANO6, PLK1, SLC7A5, TUBA1C and TCN1 within cellular and molecular processes associated with PCD and tumor-immune interactions. PLK1 is a key regulator of the cell cycle, particularly during mitosis. PLK1 governs various stages of cell division and has been implicated in apoptosis resistance. PLK1 upregulation has been associated with decreased apoptosis and increased tumor progression, suggesting a role in immune evasion (57). SLC7A5 is a transporter of essential amino acids, such as leucine, serving a key role in metabolic reprogramming. The transporter is key to nutrient sensing and mTOR activation, processes that are closely associated with cell survival. Furthermore, recent studies have suggested that SLC7A5 may modulate ferroptosis (58,59). ANO6 is involved in phospholipid scrambling and membrane dynamics. This process is central to the execution of apoptosis and ferroptosis, since the exposure of phosphatidylserine on the cell surface is a hallmark of early apoptotic events (60,61). TUBA1C is a structural component of the microtubule network that is key to mitosis, cell division and apoptosis pathways (62). TCN1 is a vitamin B12-binding protein, which facilitates cellular uptake of cobalamin. Previous studies have suggested that TCN1 is involved in regulating immune responses and may influence cell survival under metabolic stress conditions. TCN1 has been implicated in tumor progression and metastasis, potentially through its effects on cellular metabolism and DNA synthesis, both of which can influence cell death pathways, including apoptosis (63,64).

CDS as an independent risk factor for BRCA. The CDS demonstrated notably increased prognostic performance compared with conventional clinical variables, including age, tumor grade, stage and estrogen receptor/progesterone receptor status, as indicated by a higher C-index (Fig. 2A). Both univariate and multivariate Cox regression analyses confirmed that the CDS score served as an independent prognostic factor in TCGA, METABRIC and all GEO datasets (Fig. 2B and C). This independent predictive capacity was consistently observed across diverse clinical subgroups.

Construction of a clinical nomogram. To facilitate clinical translation, the present study developed a nomogram integrating the CDS score with a number of key clinical parameters, such as age, tumor grade and clinical stage (Fig. 2D). Calibration curves demonstrated notable agreement between predicted and observed survival probabilities in both training and validation sets (Fig. 2E), supporting the utility of the nomogram for individualized risk assessment and treatment planning.

Correlation between CDS score and immune micro-environment. Further analysis revealed a significant negative correlation between the CDS and immune contexture. Immune deconvolution indicated that high CDS scores were weakly associated with reduced infiltration of dendritic cells, macrophage M1 and CD8⁺ T cells (Fig. 3A-D). Based on the results of the CIBERSORT method, CDS score indicated significant weak negative correlations with the abundance of

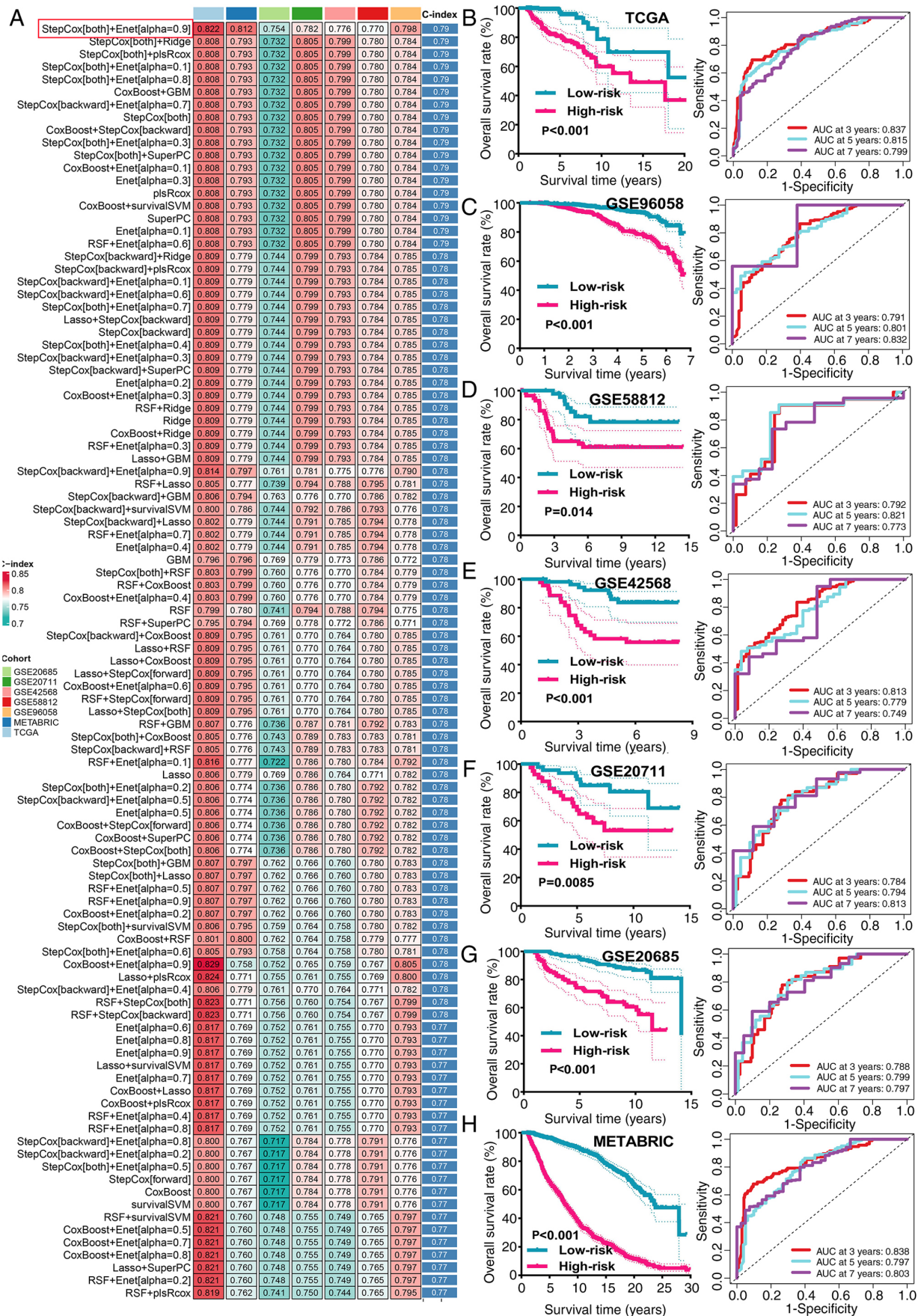


Figure 1. Machine learning develops a prognostic CDS. (A) C-index of 101 prognostic models of TCGA and all included GEO datasets. Kaplan-Meier survival analysis for patients stratified by the median CDS risk score and their corresponding receiver operating characteristic curve in (B) TCGA, (C) GSE96058, (D) GSE58812, (E) GSE42568, (F) GSE20711, (G) GSE20685 and (H) METABRIC datasets. The darker the red, the larger the C-index; the darker the green, the smaller the C-index. CDS, programmed cell death-based gene signature; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; AUC, area under the curve; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; C-index, concordance index.

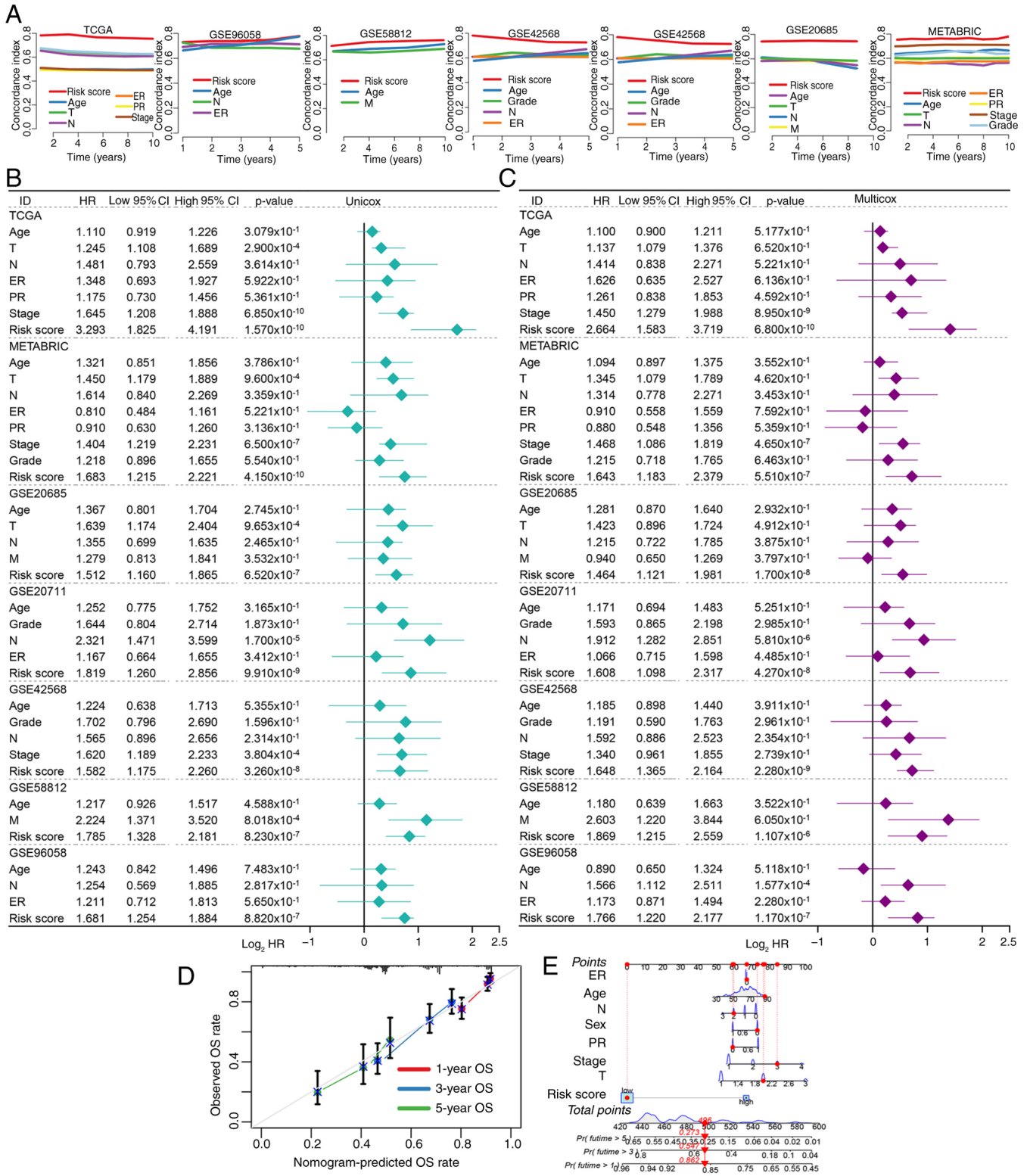


Figure 2. Evaluation of the predictive performance of CDS in patients with BRCA. (A) Concordance-index of CDS and clinical characteristics in TCGA, METABRIC and the 5 included GEO datasets. Prognostic risk factors identified by (B) univariate and (C) multivariate Cox regression analysis. (D) Nomogram developed based on CDS and clinical characters. (E) Calibration evaluated the role of the nomogram in evaluating the prognosis of patients with BRCA. CDS, programmed cell death-based gene signature; BRCA, breast cancer; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; ER, estrogen receptor; PR, progesterone receptor; OS, overall survival; HR, hazard ratio.

CD4⁺ memory resting and CD4⁺ memory activated T cells, memory B cells, macrophages M1, monocytes, resting myeloid dendritic cells, and activated and resting mast cells (Fig. S2A-H). Furthermore, these CDS genes (ANO6, PLK1,

SLC7A5, TUBA1C and TCN1) also demonstrated significant correlations with CD8⁺ T-cell infiltration, M1 macrophage abundance and immune score (Table SII). ssGSEA further confirmed that the high-risk group exhibited multiple immune

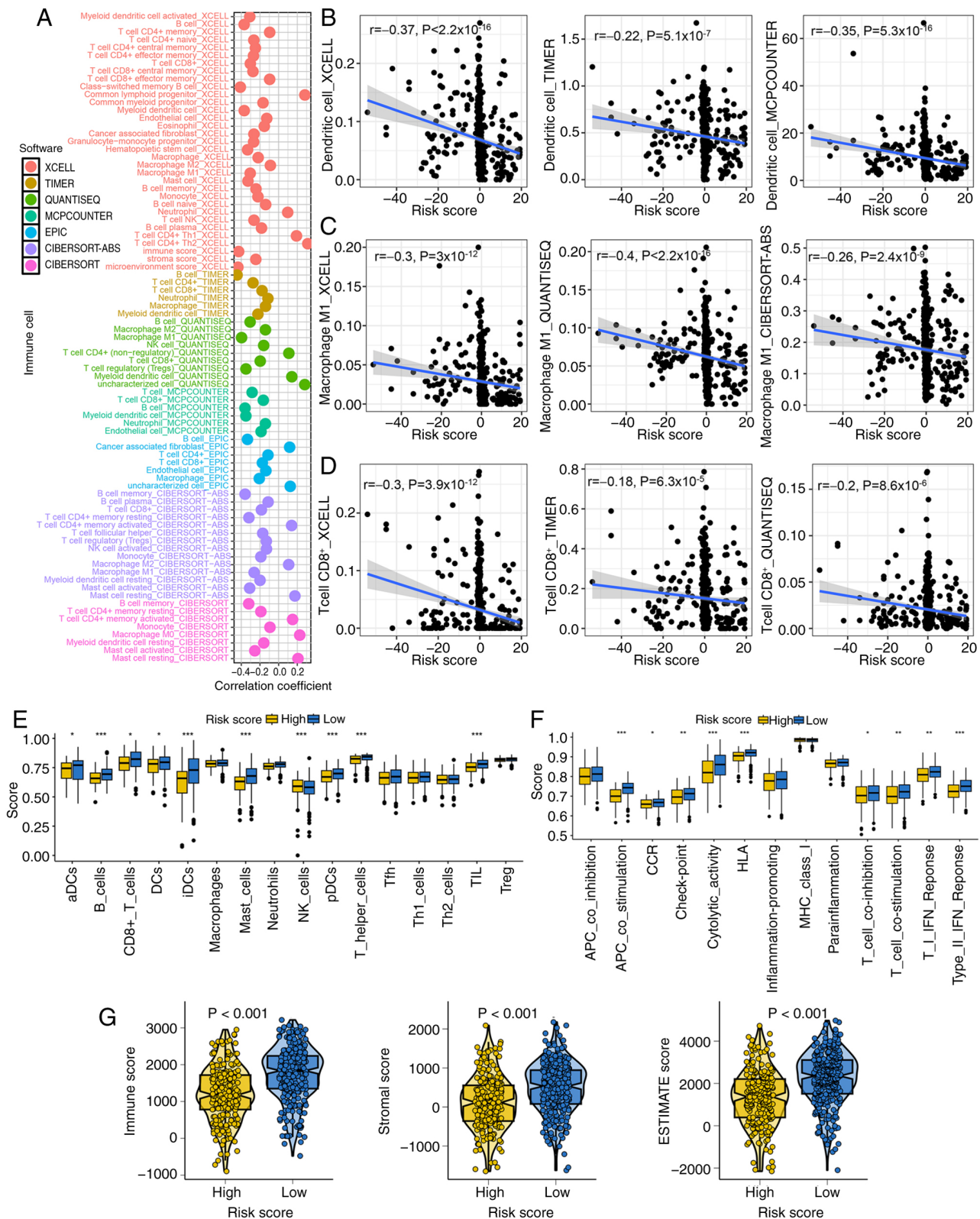


Figure 3. Association between CDS score and the immune microenvironment. (A) Correlation between CDS score and the abundance of various immune cell types based on seven algorithms. Level of (B) dendritic, (C) macrophage M1 and (D) CD8⁺ T cells, was negatively correlated with the CDS score. Level of (E) immune cells and (F) immune-related function in the different CDS score groups. (G) Immune score and ESTIMATE score in BRCA cases with different CDS scores. *P<0.05, **P<0.01 and ***P<0.001. CDS, programmed cell death-based gene signature; BRCA, breast cancer; ESTIMATE, Estimation of Stromal and Immune cells in Malignant Tumor tissues using Expression.

cell subsets, including B and mast cells, and higher enrichment scores for natural killer cells (Fig. 3E). By contrast, low CDS scores were associated with enhanced activity of

immune activation pathways, such as antigen-presenting cell and T-cell co-stimulation, cytolytic activity and type II IFN response (Fig. 3F). Consistently, ESTIMATE, immune

and stromal scores were significantly lower in the high CDS score group, indicating an immunologically ‘cold’ tumor microenvironment (Fig. 3G).

CDS acts as a biomarker in predicting immunotherapy response. Increased expression levels of human leukocyte antigen (HLA)-related genes suggest a broader range of antigen presentation, which could lead to the presentation of more immunogenic antigens and potentially enhance the success of immunotherapy (65,66). A low TIDE score and ITH score indicate a reduced likelihood of immune escape and an improved response to immunotherapy (67). The present study next explored the potential of the CDS to predict response to immune checkpoint inhibition. Patients with low CDS scores exhibited elevated expression of most of the immune checkpoint molecules (Fig. 4A) and HLA-related genes (Fig. 4B). This group also demonstrated markedly higher TMB and IPS (Fig. 4C and D), alongside lower T-cell dysfunction (TIDE), immune escape and ITH scores (Fig. 4E-G), all indicators of favorable immunotherapy response. Furthermore, patients with BRCA with a low CDS score indicated higher antigen presentation gene set score (Fig. S3A) and inflammatory response gene set score (Fig. S3B), and lower T-cell exhaustion gene set score (Fig. S3C).

Validation in three independent immunotherapy cohorts (IMvigor210, GSE91061 and GSE78220) confirmed that patients with low CDS scores experienced significantly improved OS and higher response rates following anti-PCD protein-1/PCD-ligand 1 treatment (Fig. 4H-J).

CDS and drug sensitivity. Drug sensitivity analysis revealed that patients in the low CDS score group were significantly more sensitive to a range of chemotherapeutic, endocrine and targeted agents, including fulvestrant, oxaliplatin, gemcitabine, epirubicin, cisplatin, lapatinib, ribociclib, dinaciclib, palbociclib and tamoxifen (Fig. 5A and B). These findings position the CDS as a potential biomarker in guiding personalized therapy selection beyond immunotherapy.

Biological pathways associated with CDS. ssGSEA revealed that high CDS scores were significantly enriched in oncogenic and metabolic pathways, including ‘p53 pathway’, ‘EMT signaling’, ‘NOTCH signaling’, ‘mTORC1 signaling’, ‘IL2-STAT5 signaling’, ‘hypoxia’, ‘glycolysis’, ‘DNA repair’, ‘angiogenesis’ and E2F targets (Fig. 6). These pathways are collectively associated with tumor progression, treatment resistance and immune suppression.

Verification of the expression of CDS genes. mRNA and protein expression analyses corroborated the present bioinformatic findings. Expression levels of ANO6, PLK1, SLC7A5, and TUBA1C were found to be significantly upregulated in BRCA tissues and cell lines, whereas TCN1 was downregulated compared with those in normal breast tissues and normal human breast cells (Fig. 7A and B).

Discussion

BRCA remains one of the most prevalent and lethal malignancies affecting women worldwide, posing notable challenges

to public health due to its high incidence and mortality rates. Despite advances in surgical interventions, radiotherapy, chemotherapy and targeted therapies, treatment outcomes typically vary markedly among patients, reflecting the heterogeneity of the disease and its complex biological underpinnings. Furthermore, conventional therapies are frequently limited by adverse effects and the emergence of resistance, underscoring an urgent need for more precise prognostic tools and therapeutic strategies to improve patient management and survival.

In the present study, integrated transcriptomic datasets and advanced machine learning techniques were applied to develop a novel gene signature based on CRGs for prognostic evaluation in BRCA. This signature demonstrated robust discrimination between patient risk groups and revealed significant associations with immune cell infiltration and drug sensitivity profiles, suggesting its potential utility in guiding personalized treatment decisions. The present study findings establish a foundation for the further exploration of PCD pathways in BRCA progression and therapeutic response, offering novel insights into the molecular mechanisms that may underlie tumor immune evasion and chemoresistance.

The five genes constituting the CDS, namely, ANO6, PLK1, SLC7A5, TUBA1C and TCN1, serve distinct but interrelated roles in tumor biology and immune regulation, offering biological plausibility for the observed correlation between the CDS score and immune infiltration patterns. ANO6 encodes a calcium-activated phospholipid scramblase that mediates the externalization of phosphatidylserine during apoptosis and other forms of PCD. This process is key to the recognition and clearance of dying cells by phagocytes and antigen-presenting cells (68). Aberrant ANO6 expression can impair efferocytosis, leading to accumulation of apoptotic bodies and secondary necrosis, which in turn promotes chronic inflammation and immunosuppression within the tumor microenvironment. PLK1 is a master regulator of mitotic progression and DNA damage repair. Upregulation of PLK1 in BRCA promotes uncontrolled proliferation and genomic instability, conditions that are frequently accompanied by immune evasion (69). Mechanistically, PLK1 can suppress type I IFN signaling and reduce antigen presentation, thereby blunting cytotoxic T-cell activation (70,71). SLC7A5 encodes a large neutral amino acid transporter that facilitates leucine uptake, activating the mTORC1 pathway (72). Hyperactivation of mTOR signaling drives metabolic reprogramming toward an immunosuppressive phenotype by increasing lactate production and depleting nutrients in the tumor microenvironment (73). Elevated SLC7A5 expression has been associated with the reduced infiltration of CD8⁺ T cells and impaired antitumor immunity due to competition for essential amino acids between tumor and immune cells (74). The present study findings highlight the importance of a biologically grounded approach to predictive signature construction, wherein gene selection is informed by established roles in PCD and immune modulation. The five-gene signature identified in the present study, consisting of PLK1, SLC7A5, ANO6, TUBA1C and TCN1, captures key aspects of cell cycle regulation, metabolic reprogramming and membrane remodeling, all of which are key determinants of cell survival and death

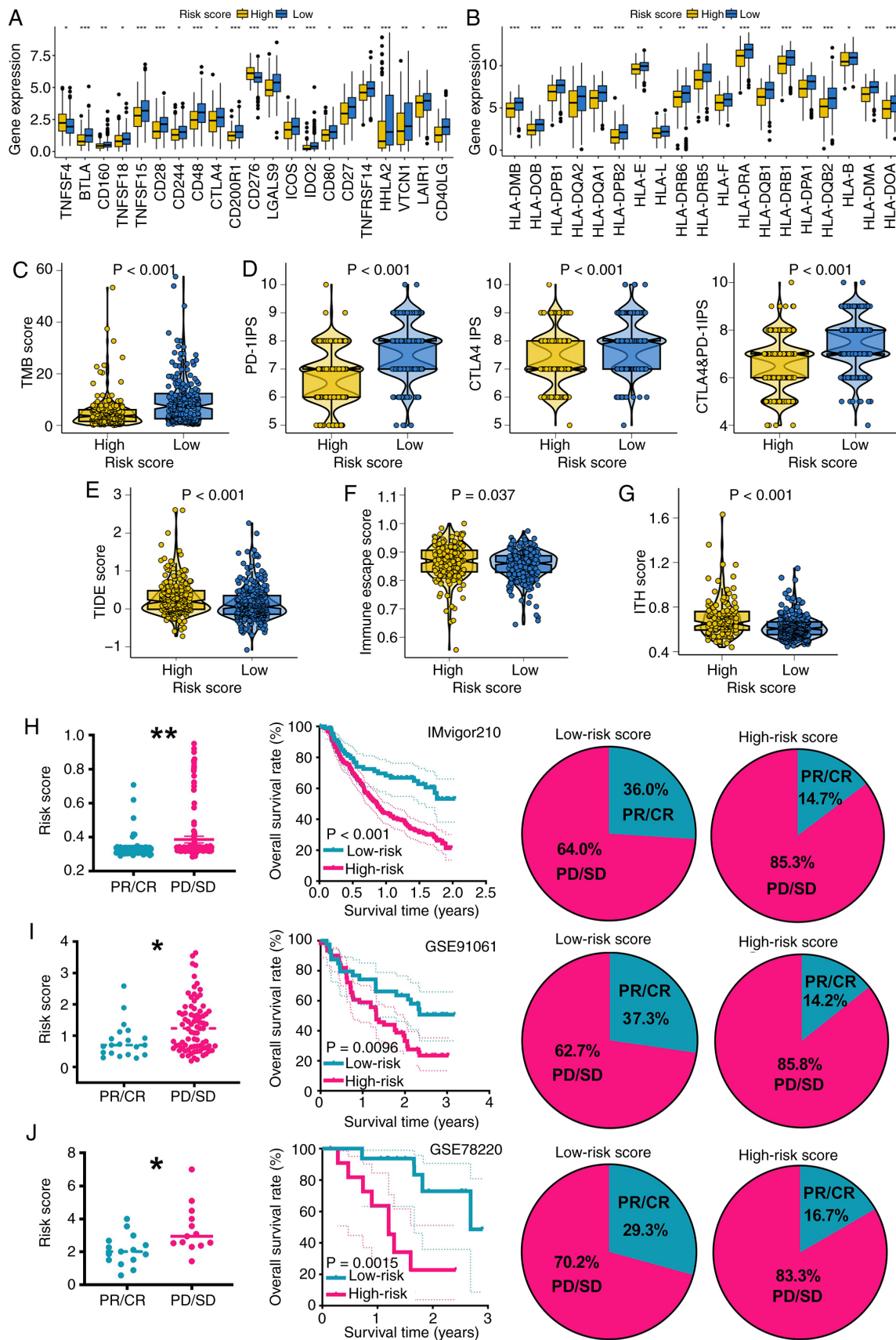


Figure 4. CDS score acts as an indicator for immunotherapy benefits in BRCA. Level of (A) immune checkpoint genes and (B) HLA-related genes, (C) TMB score, (D) PD-1 and CTLA4 immunophenoscore, (E) TIDE, (F) immune escape and (G) ITH scores in the different CDS score group. Overall rate and immunotherapy response rate in the different CDS score groups in (H) IMvigor210, (I) GSE91061 and (J) GSE78220 cohort. (A-G) Analyses are performed in The Cancer Genome Atlas-BRCA cohorts to predict potential immunotherapy response, which is then validated in (H-J) independent immunotherapy cohorts. *P<0.05, **P<0.01 and ***P<0.001. CDS, programmed cell death-based gene signature; BRCA, breast cancer; HLA, human leukocyte antigen; TMB, tumor mutational burden; TIDE, Tumor Immune Dysfunction and Exclusion; ITH, intratumor heterogeneity; PR, partial response; CR, complete response; PD, progressive disease; SD, stable disease; PD-1 programmed cell death protein 1; CTLA-1, cytotoxic T-lymphocyte-associated antigen 4.

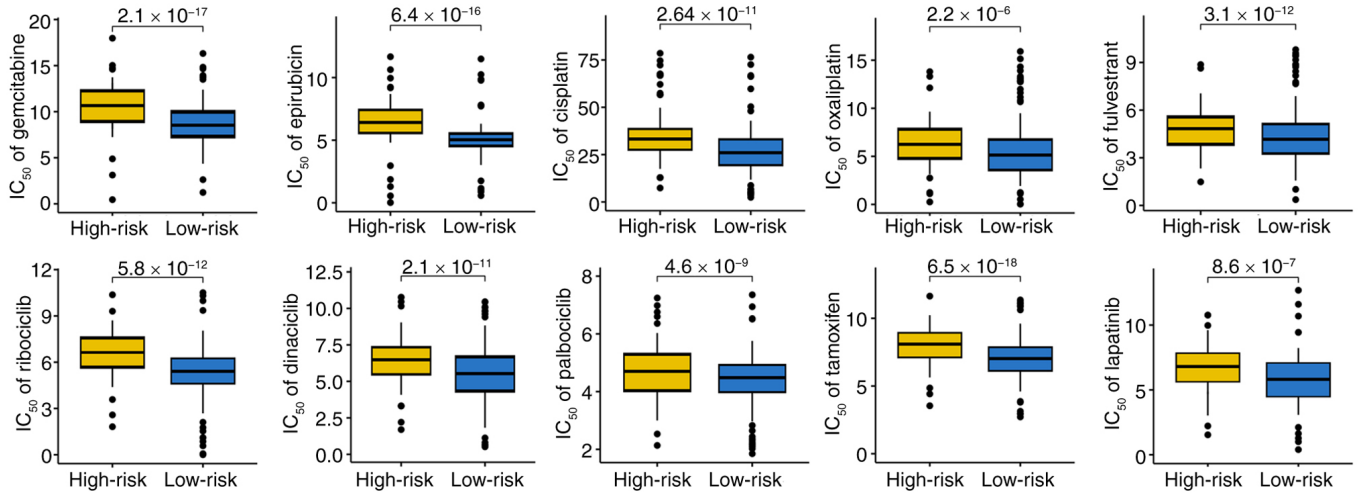


Figure 5. IC₅₀ value of drugs in different CDS score groups. IC₅₀ value of drugs involved in chemotherapy, target therapy and endocrinotherapy in the different CDS score groups. CDS, programmed cell death-based gene signature; IC₅₀, half maximal inhibitory concentration.

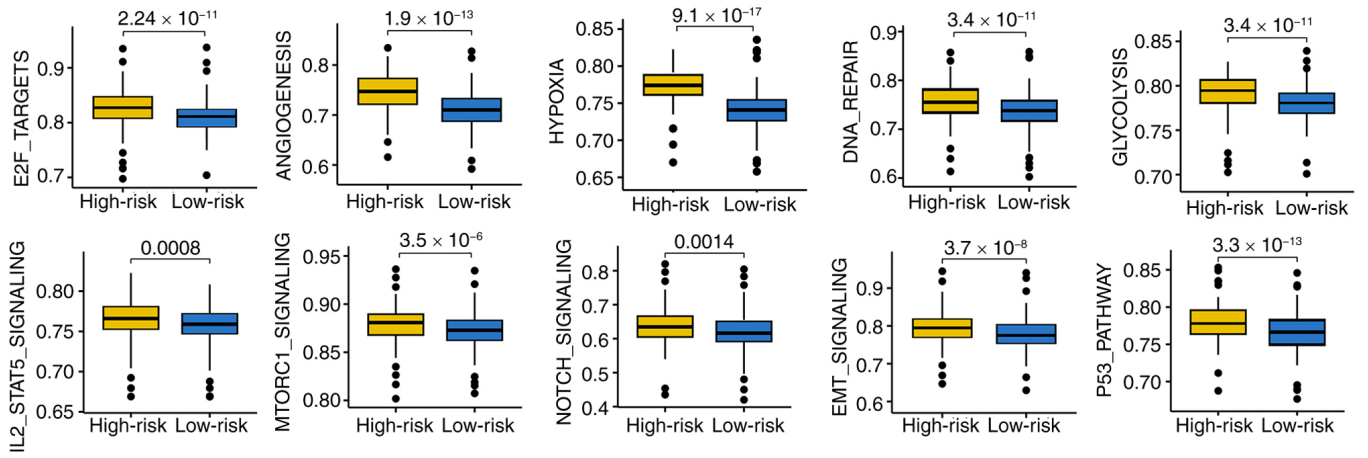


Figure 6. Association between cancer-related hallmarks and programmed cell death-based gene signature in breast cancer in The Cancer Genome Atlas dataset. The gene set score of cancer-related hallmarks in breast cancer cases was calculated with single sample gene set enrichment analysis. E2F, early region 2 factor.

under immune pressure. Although the present study model demonstrated strong predictive performance (C-index), it is key to emphasize that these genes were not selected based purely on statistical associations, but rather due to their biological relevance to PCD-related processes. The ability of these genes to influence tumor cell survival and immune modulation underscores the biologically plausible nature of this signature. However, the present study acknowledges that whilst the computational model demonstrates promise, the exact molecular interactions between these genes and their role in immunotherapy response should be further explored using experimental and clinical validation.

PCD pathways, including apoptosis, necroptosis and ferroptosis, intersect with both tumor immunity and metabolic regulation, serving key roles in cancer progression, immune evasion and therapy resistance. Apoptosis is a fundamental mechanism for eliminating damaged cells, including tumor cells. Dysregulation of apoptosis allows tumors to evade immune surveillance, as apoptotic cells release signals that promote immune clearance. Genes, such as PLK1

regulate mitosis and apoptosis, where its upregulation in tumors leads to apoptosis resistance, promoting immune evasion and chemotherapy resistance (75,76). Necroptosis is triggered when apoptosis is inhibited, often due to stress or inflammatory signals. Necroptotic cell death can release damage-associated molecular patterns, which activate the immune system (77). However, uncontrolled necroptosis can also enhance tumor progression through inflammation (78,79). Ferroptosis is an iron-dependent, oxidative cell death pathway that is closely associated with metabolic regulation. Ferroptosis is influenced by the availability of amino acids and lipids, as well as the cellular redox state. SLC7A5, by controlling nutrient uptake, serves a notable role in modulating oxidative stress and ferroptosis susceptibility (80). Ferroptotic cells can influence the immune system by affecting cGAS-STING signals that activate both innate and adaptive immune responses (80,81).

The inverse correlation between high CDS scores and immune cell infiltration, particularly cytotoxic CD8⁺ T cells and dendritic cells, underscores a potential immunosuppressive

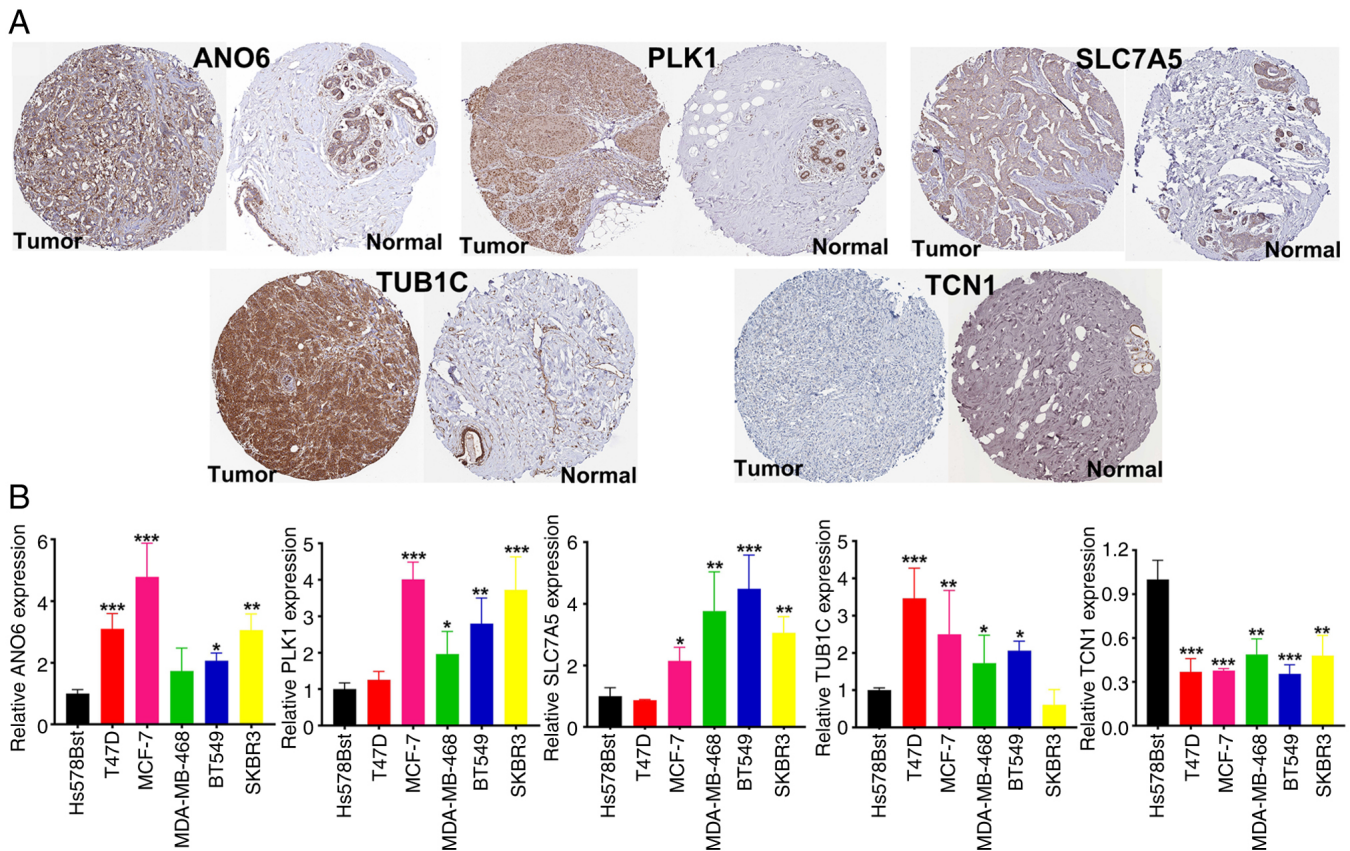


Figure 7. Verification of the expression levels of CDS genes. (A) Immunohistochemical staining from the Human Protein Atlas indicating CDS gene expression in BRCA and normal tissues. (B) Expression levels of CDS genes in a normal mammary epithelial cell line (Hs578Bst) and multiple BRCA cell lines. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, BRCA cells vs. Hs578Bst cells. CDS, programmed cell death-based gene signature; BRCA, breast cancer; ANO6, anoctamin 6; PLK1, polo-like kinase 1; SLC7A5, solute carrier family 7 member 5; TUBA1C, tubulin α -1C chain; TCN1, transcobalamin 1.

tumor microenvironment. This observation parallels findings that high-risk patients, as defined by PCD signatures, exhibit diminished immune activation and increased immune escape mechanisms. Mechanistically, PCD may influence antigen presentation and immune surveillance through mitochondrial dysfunction and altered metabolic states, leading to reduced recruitment or function of effector immune cells (82). In the present study, the ssGSEA analysis corroborates this by revealing decreased expression levels of immune activation-related gene sets in high CDS groups, indicating a suppression of key immune pathways, such as IFN signaling and T-cell cytotoxicity. These results suggested that CDS not only prognosticates survival but also reflects the immunological landscape, providing a rationale for the integration of CDS assessment in immunotherapy stratification. Targeting the interplay between PCD and immune modulation may thus represent a novel therapeutic avenue in BRCA.

The differential sensitivity to chemotherapeutic agents observed between patients with high and low CDS scores reveals a clinically relevant association between CRG expression and drug response. The increased sensitivity of patients with low CDS to agents such as oxaliplatin and gemcitabine, evidenced by significantly lower IC_{50} values, aligns with previous studies associating PCD pathways to chemosensitivity (83,84). PCD may modulate drug efficacy by affecting mitochondrial metabolism and reactive oxygen species generation, which are key mediators of chemotherapy-induced

cell death (85). Furthermore, machine learning-derived prognostic models incorporating CRGs have demonstrated promise in predicting chemotherapy response in breast cancer, suggesting that CDS can serve as a biomarker for personalized chemotherapy regimens (86). These findings advocate for the prospective clinical validation of CDS-guided treatment strategies and highlight the potential of integrating molecular signatures with pharmacogenomics to optimize therapeutic outcomes in BRCA.

The limitations of the present study primarily stem from the lack of experimental validation, which raises concerns about the robustness of the identified association between CRGs and BRCA prognosis. Furthermore, the relatively small sample size may hinder the generalizability of the results, potentially limiting the applicability of the CDS model across diverse patient populations. Additionally, the batch effects inherent in the datasets utilized could introduce variability that may confound the analysis, emphasizing the necessity of cautious interpretation of the results in clinical contexts. Notably, the immunotherapy response predictions are based on computational analyses derived largely from cancer cell line-based datasets and public databases. Therefore, these findings should be interpreted with caution and require further validation in experimental models and well-designed clinical cohorts.

Future studies should include experimental validation of the core CDS genes (ANO6, PLK1, SLC7A5, TUBA1C and TCN1) and signature scores using techniques such as western

blotting and reverse transcription-quantitative PCR in independent collections of human BRCA tissues to confirm their protein and mRNA expression patterns and solidify their clinical relevance. Furthermore, the functions and potential molecular mechanism of CDS genes in BRCA will be explored in future research.

In summary, the present study successfully identified multiple CRGs associated with BRCA prognosis and constructed a CDS model grounded in gene expression data, demonstrating its potential in predicting patient outcomes and treatment responses. This approach not only enhances prognostic capabilities but also potentially lays the groundwork for personalized treatment strategies. Future research should aim to validate these findings through larger, multicentric studies and investigate the clinical utility of the CDS model, ultimately striving to integrate these biomarkers into routine clinical practice to improve therapeutic decision-making.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

NW prepared the original draft and conducted the bioinformatics and experimental investigation. ZF designed the present study, provided supervision and reviewed the manuscript. NW and ZF confirm the authenticity of all the raw data. Both authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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