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EphB4 Inhibition Regulates Tumor Microenvironment by Promoting Immunogenic Cell Death Effector Mechanism in Breast Cancer

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Abstract

While combination chemo-immunotherapy has shown considerable potential in treating triple-negative breast cancer (TNBC), the inhibitory effects of the tumor microenvironment (TME) on cancer immunotherapy remain a significant challenge. Previous studies have demonstrated that EphB4 contributes to resistance against targeted therapies in breast cancer (BC). In our study, we observed that high EphB4 expression correlates with poor survival outcomes in BC patients. Using Single-Sample Gene Set Enrichment Analysis (ssGSEA) and unsupervised consensus clustering, we categorized BC patients into distinct immune subgroups. These subgroups were validated through analyses of immune cell infiltration, prognosis, immune characteristics, and their correlation with EphB4 expression. We discovered that inhibiting EphB4 enhances the chronic release of damage-associated molecular patterns, including calreticulin (CRT) translocation, the release of high-mobility group protein B1 (HMGB1), and adenosine triphosphate (ATP), which induces immunogenic cell death (ICD). Additionally, T-cell cytotoxicity was augmented when co-cultured with tumor cells treated with EphB4 inhibitors. We developed an EphB4-knockout MMTV-PyMT murine model of BC, which exhibited slower tumor growth, limited metastasis, and increased immune cell infiltration. Moreover, EphB4 inhibition altered glucose metabolism and ATP transport through the Src/STAT3/MYC signaling cascade. Collectively, our findings suggest that EphB4 inhibition promotes ICD and enhances immune cell infiltration in the TME, facilitating the transition of tumors from "cold" to "hot." This shift may improve the efficacy of immune checkpoint blockade, expanding the patient population that benefits from immunotherapy.

Keywords: EphB4, Immunogenic cell death, Tumor microenvironment, Immunotherapy

Introduction

Femal breast cancer is the most common malignant tumor and has become the primary problem that seriously threatens women's health¹.TNBC accounts for approximately 15-20% of all breast carcinomas, and has high rates of relapse, great metastatic potential and short overall survival time^{2, 3}.In recent years, chemotherapies combined with immune checkpoint inhibitors (ICIs) therapy have made remarkable achievements in advantaged TNBC staged treatment^{4, 5}.However, the reactivity of treatment is significantly affected because of the tendency of multidrug resistance and the induction of the TME against tumor immunosuppression^{6, 7}.

The interaction between tumor cells and the TME especially the tumor immune microenvironment (TIME) play a critical role throughout the course of tumor progression⁸.According to the spatial distribution of cytotoxic immune cells in the TME, tumors are divided into three basic immune phenotypes: immune-inflammatory, immune-excluded and immune-desert phenotypes⁹.Immune-inflamed tumors named "hot tumors" tend to be more responsive to ICIs, immune-excluded and immune-desert phenotypes described as "cold tumors" rarely respond to ICI monotherapy^{9, 10}.Tumor infiltrating lymphocyte (TIL) is an important component of tumor infiltrating immune cells (TIIC) and has been shown to be a potent independent prognostic predictor in TNBC, with higher levels of TIL associated with longer disease free

survival (DFS) and overall survival (OS)¹¹. We have reported that the strategy to improve the TME sensitizes the breast cancer cells to chemotherapy, and established an original immune-infiltrating cell breast cancer prognostic model based on 6 TILs significantly associated with OS¹². It indicates that the TIME is of great value in predicting prognosis and evaluating therapeutic efficacy.

Many studies have defined EphB4 as oncogene^{13, 14}, which is highly expressed in a variety of tumor tissues, and plays a role in promoting cancer. Some previous research has shown that knockdown of EphB4 or application of specific anti-EphB4 antibody and tyrosine kinase inhibitor (TKI) can reduce proliferation, migration, invasion and tumor angiogenesis of tumor cells^{15, 16}. We previously demonstrated that EphB4 promotes resistance to targeted therapy in breast cancer¹⁷. It has been reported that EphB4 targeted therapy induced ICD to stimulate antitumor immune response in prostate cancer¹⁸. However, the correlation between EphB4 and immunity and the underlying mechanism were not completely understood in advantaged breast cancer patients.

Although TNBC immunotherapy has made remarkable progress¹⁹, there are still some immune-desert or immune-excluded phenotypes insensitive to immunotherapy. Tumors lacking intrinsic antigen presentation or with no T cells reacted to antigens rarely respond to ICI²⁰. Some chemotherapeutics are known to induce ICD in tumor cells, thereby activating the body's anti-tumor immune system²¹. Tumor cells with ICD firstly show cell swelling and rupture, then display DAMPs features including of CRT translocation, release of HMGB1 and ATP, in turn to promote dendritic cell (DC) maturity which present antigens and activate specific T cells to kill cancer cells²². Some previous research has been shown that CDK12/13 inhibitor sensitizes antitumor effect of anti-PD-1 antibody via enhancing T cell activation, and CDK2 inhibitor enhances anti-PD1-mediated tumor suppression by inducing ICD^{23, 24}. Therefore, we hypothesized whether inhibition of EphB4 can promote the anti-tumor effect of immunotherapy and explore its potential mechanism.

The MMTV-PyMT model has been utilized together with either full-body knockouts or with mammary specific expression of Cre recombinase (MMTV-Cre) along with conditional alleles to knockout various proto-oncogenes and examine their effects on mammary tumorigenesis and metastasis²⁵. MMTV-PyMT mice spontaneously develop tumors where the oncogene of Polyomavirus Middle T (PyMT) is activated by the tissue-restricted MMTV promoter in an immunocompetent background²⁶. Accordingly, we established a MMTV- PyMT mouse model with EphB4 knockout mediated by Cre-loxP²⁷.

In this study, we investigate that EphB4 inhibition induces ICD and triggers ER stress, and combined with gene mouse model to verify that targeted EphB4 therapy improves tumor immune microenvironment and enhance immune-mediated antitumor activity.

Materials and Methods

Data acquisition and prognosis analysis

The TCGA database (<https://portal.gdc.cancer.gov/>), which is the largest database of cancer gene information, contains various types of data such as gene expression, miRNA expression, copy number variation, DNA methylation, SNPs, and more. In this study, we downloaded the raw mRNA expression data of treated breast cancers from the database. Gene Expression Profiling Interactive Analysis 2 (GEPIA2) (<http://gepia2.cancer-pku.cn/>) was used to analyze the expression and survival role of EphB4 in TNBC. Kaplan-Meier Plotter (www.kmplot.com) and were used for survival analyses. Univariate and multivariate cox regression analysis were performed to identify the proper terms to build the nomogram and used “forest plot” R package to show the P value, HR and 95% CI of each variable. Based on the results of multivariate cox proportional hazards analysis to predict overall recurrence using “rms” R package.

Immune infiltration analysis

The CIBERSORT method follows the principle of support vector regression and back-convolution analysis of the expression matrix of immune cell subtypes, and it contains 22 human immune cell phenotypes²⁸. In this study, it was used to analyze the immune infiltrating cells, and Pearson or Spearman correlation analysis was performed on gene expression and immune cell content. Differences in immune cells between different sample groups were observed using the TIMER algorithm²⁹. ConsensusClusterPlus is a stratification tool which uses consensus clustering to identify unsupervised intrinsic groups with similar biological features³⁰. Cellular compositions of immune infiltrates were computed using the metagene approach. We performed ssGSEA to derive the enrichment score of each immune-related term using a R package called "GSVA"³¹.

TMB, MSI, NEO data analysis

TMB is defined as total somatic gene coding errors, including base substitutions, insertions, or deletions, detected per million bases³². The study defined TMB by dividing the number of non-synonymous mutation sites by the total length of the protein coding region to calculate variant frequency and number of variants per exon length for each tumor sample. The value of MSI for each TCGA patient was derived from a previously published study³³. Neoantigen was evaluated for each patient using NetMHCpanv3.0³⁴.

Functional enrichment analysis

Gene sets were downloaded from the MSigDB database (<https://software.broadinstitute.org/gsea/downloads.jsp>). Each gene set was fully scored using GSVA algorithm. The "enrichplot" and "clusterProfiler" R packages³⁵ were used to perform GSEA analysis. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to evaluate relevant functional categories. GO and KEGG enriched pathways with both *p*-value and *q*-value less than 0.05 were considered as significant categories.

Co-expression and WGCNA analysis

We analyzed the co-expression of EphB4 genes using a correlation coefficient threshold of 0.3 and a significance level (*p*-value) of 0.05. Genes demonstrating significant correlation with EphB4 were identified. Visualization of these correlations was accomplished using circle plots and heatmaps generated by the "corrplot" and "circlize" software packages. Further, we constructed a weighted gene co-expression network using the WGCNA package in R, setting the soft-thresholding power to 4. This network allowed us to identify cooperatively expressed gene modules, explore correlations between the gene network and specific phenotypes, and determine core genes within the network.

Cell Culture and Reagents

Human breast cancer (BC) cell lines (MCF-7, MDA-MB-231, HCC-38, HCC-1187) and the mouse mammary carcinoma cell line (4T1) were sourced from ATCC (Manassas, VA, USA). The MDA-MB-231 and MCF-7 lines were cultured in DMEM medium (Gibco), while other lines were maintained in RPMI 1640 medium (Gibco), supplemented with 10% fetal bovine serum (FBS, Biological Industries) and 1% penicillin/streptomycin (HyClone) in a 37°C incubator with 5% CO₂. The EphB4 inhibitor NVP-BHG712 (Selleck Chemicals) was prepared in DMSO (Sigma). All cell lines used in this study were obtained from reputable cell banks and were authenticated by short tandem repeat (STR) profiling prior to use. All cell lines were tested and confirmed to be free of mycoplasma contamination before experimentation.

Cell transfection

Short hairpin RNAs (shRNAs) targeting EphB4 (shEphB4-1 and shEphB4-2) and a non-specific control shRNA (sh-NC) were synthesized by Sangon Biotech (Shanghai, China). These shRNAs were transfected into MDA-MB-231 and HCC-38 cells to assess gene silencing efficacy.

Cell viability and Cytotoxic Assay

Cell viability and cytotoxicity were evaluated using the CCK-8 assay (Bimake). Cells (2×10^3 cells/well) were seeded in 96-well plates and treated with varying concentrations of NVP-BHG712. Absorbance was measured at 450 nm using an xMark Microplate Spectrophotometer (Bio-Rad, USA) to determine optical density (OD).

Clonogenic Assay

Cells (10^3 cells/well) were plated in 12-well plates and cultured for 14 days, with media changes every 3 days. Post-culture, cells were stained with 0.5% crystal violet and solubilized in 10% glacial acetic acid. The resultant OD was measured at 590 nm using an xMark Microplate Spectrophotometer (Bio-Rad, USA).

Migration and Invasion Assay

Migration and invasion assays were conducted using a Corning Inc. transwell chamber. For invasion assays, chambers were coated with matrix gel (BD Biosciences, San Jose). Cells (5×10^4 cells/100 μ l of cell suspension) in serum-free medium were placed in the upper chambers, while the lower chambers were filled with medium containing 10% FBS. After 48 hours, non-invasive cells were removed, and invasive cells stained with 0.1% crystal violet were visualized and quantified using inverted phase-contrast microscopy (Olympus Corporation, Tokyo, Japan). Cell counts were determined from three random fields per group.

Western blotting

Cells were lysed in RIPA lysis buffer supplemented with phosphatase (1mM NaF, 1 mM Na_3VO_4 , 1mM $\text{C}_3\text{H}_7\text{Na}_2\text{O}_6\text{P}$, and 10mM $\text{Na}_4\text{O}_7\text{P}_2$), and protease inhibitors (Roche, USA). Protein concentrations were measured using BCA Protein Assay kit (Pierce). Samples were mixed with loading buffer and boiled for 10 minutes. Proteins were separated by SDS-PAGE and transferred to nitrocellulose blotting membranes (GE Healthcare Life Science, Germany). The membranes were blocked at room temperature in 5% skin milk powder in PBS for 1h and incubated at 4°C overnight. The following primary antibodies repaired in 5% PBST against EphB4 (D1C7N) (CST,#14960), vinculin (Abcam,ab129002), GAPDH (Wanleibio,WL0114), GLUT3 (Abcam,ab191071), c-Myc (CST,#5605), phospho-JAK2(Tyr1007/1008)(CST,#327),JAK2 (CST,#3230), eIF2 α (Affinity Biosciences, AF6087), phospho-eIF2 α (Ser51)(CST,#3398), PERK (Abcam,ab79483), phospho-PERK (Thr982) (Affinity Biosciences,DF7576), phospho-STAT3 Y705(CST,#9145), STAT3 (CST,#12640), IRE1(Wanleibio,WL02562),Bip (Wanleibio,WL03157). The membranes were incubated with secondary antibodies at room temperature for 1h goat anti-mouse IgG-horseradish peroxidase (HRP) (Abcam,ab205719) or goat anti-rabbit IgG-HRP (Abcam, ab6721).And the signals were analyzed using Fluorescence scanning imaging system(Odyssey, LI-COR) with Image Lab software.

Detection of ICD indicators

Cells treated with EphB4 inhibition for 48h, were fixed with 95% ethanol, permeabilized with PBS containing 1% Triton X-100 and blocked using 1% BSA. Cells were incubated with antibody against Calreticulin (CST, #12238) at 4°C overnight with avoiding light and followed with Alexa Fluor 647 goat anti-rabbit antibody (Beyotime) for 30 minutes. Nucleus was stained with DAPI for 15 minutes. Fluorescent images were observed using fluorescent microscope (OLYMPUS). Collected the cell supernatant and the extracellular HMGB1 and ATP level was evaluated by ELISA kit (Elabsience) and ATP Kit (Solarbio).

RNA Sequencing and Data Analysis

RNA sequencing of MDA-MB-231 cells (vector vs. EphB4 knockdown) was carried out by Novogene Corporation (Beijing, China). The sequencing libraries were created by employing the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, Ipswich, MA, USA) according to the manufacturer's instructions. FPKM (expected number of

Fragments Per Kilobase of transcript sequence per Million base pairs sequenced) was used to estimate gene expression levels. We tested the statistical enrichment of differentially expressed genes in KEGG pathways by using the ClusterProfiler R package.

EphB4-CKO/PyMT mouse model

Transgenic mice were from Shanghai Model Organisms Center, Inc., and were all housed in the Research Institute of University Animal Experiment Centre. Mammary gland-specific EphB4 knockout mice was constructed by CRIASP-CAS9 technique, and the EphB4-CKO/PyMT mouse model was obtained by hybridization with MMTV-PyMT breast cancer mice, the genotype was identified by PCR. The model was used to verify the effect of EphB4 on the growth and metastasis of breast cancers. When the tumor volume reached 100-150mm³, tumor dimensions were measured using a caliper, and the volume was determined as per the formula: Volume (mm³) = $L \times W^2 / 2$, where L and W indicate the length and width of the tumor (both in mm), respectively. At the same time, the changes of tumor size and number were observed. Gene knockout mice were randomly divided into two groups and treated with PBS and anti-PD-L1 antibody for one month. Mice were anesthetized by intraperitoneal injection when necessary during the experimental procedures. At the experimental endpoint, mice were euthanized by cervical dislocation. A tumor diameter of approximately 3 cm was defined as the humane endpoint.

Immunohistochemistry (IHC)

Mouse tumor samples were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue slices were deparaffinized, rehydrated, and antigen retrieval was performed using 10mM citrate buffer (pH6.0). Slices were blocked with goat serum for 30 min and with the following antibodies against CD3 (Abcam, ab5690), CD4 (Abcam, ab18368), CD8 (Abcam, ab209775), CD80 (Abcam, ab209775), CD86 (Abcam, ab243887), CD11c (Abcam, ab219799), CD69 (Abcam, ab202909), incubated overnight at 4°C. Slices were washed and incubated with goat anti-rabbit biotinylated secondary antibody (Abcam, ab6720) and visualized using a horseradish peroxidase (HRP)-conjugated ABC system (ab7403, Abcam). Additionally, some slices were stained with hematoxylin and eosin (H&E) and quantified under a microscope magnification.

Statistical Analysis

All statistical analyses were performed using R language (version 4.2.2). Univariate Cox regression analysis was used to calculate hazard ratios (HRs) and 95% confidence intervals. GraphPad Prism 8 software was used for two-tailed Student's *t*-test and one-way ANOVA were used for statistical analyses. The results were presented 3 times as the means ± SD (standard deviation). *p* < 0.05 was considered statistically significant.

Results

Expression and prognostic values of EphB4 in breast cancer

In this study, we investigated the differential expression and prognostic significance of EphB4 in breast cancer. Analysis of EphB4 expression in normal and tumor tissues demonstrated a significant upregulation in tumor samples, indicating a potential oncogenic role in breast cancer progression (Fig. 1A). Specifically, EphB4 expression was significantly elevated in triple-negative breast cancer (TNBC), as shown by survival analysis using GEPIA2 (Fig. 1B).

We further examined the clinical correlation between EphB4 expression and nodal involvement (N stage). Our findings revealed a strong association between increased EphB4 expression and advanced nodal stage, suggesting that EphB4 could serve as a marker for tumor aggressiveness (Fig. 1C).

Prognostic analysis based on EphB4 expression levels identified a significant disparity in outcomes. Patients with high EphB4 expression had considerably worse prognosis compared to those with low expression, using an optimal

survival cutoff derived from the data (Fig. 1D). Survival analysis confirmed that high EphB4 expression was associated with poor overall survival (OS) in both the GEPIA2 dataset (Fig. 1E) and according to K-M plotter online analysis for breast cancer overall and specifically in TNBC (Figs. 1F-I). This correlation extended to distant metastasis-free survival (DMFS), emphasizing the negative impact of high EphB4 levels on survival.

To refine the prognostic utility of EphB4, we performed univariate and multivariate Cox regression analyses to identify significant predictors for inclusion in a prognostic nomogram (Figs. 1J-K). This nomogram was developed to enhance the prediction of patient outcomes based on clinical and molecular data, including EphB4 expression (Fig. 1L).

Validation of the nomogram's accuracy was conducted through logistic regression analysis, which confirmed significant differences in EphB4 expression across different prognostic categories within breast cancer samples. Calibration curves for 3-year and 5-year survival periods were constructed to assess the consistency and reliability of the nomogram's predictions, showing good alignment with observed outcomes (Fig. 1M).

Overall, our results highlight the importance of EphB4 as a prognostic biomarker in breast cancer, particularly in predicting aggressive disease course and poorer survival, thus providing a valuable tool for tailored therapeutic strategies.

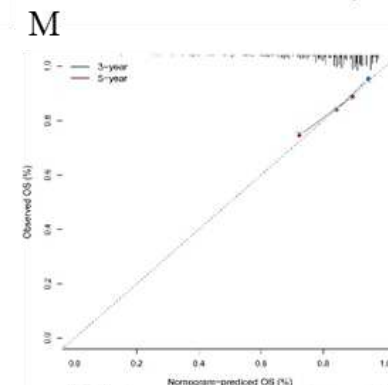
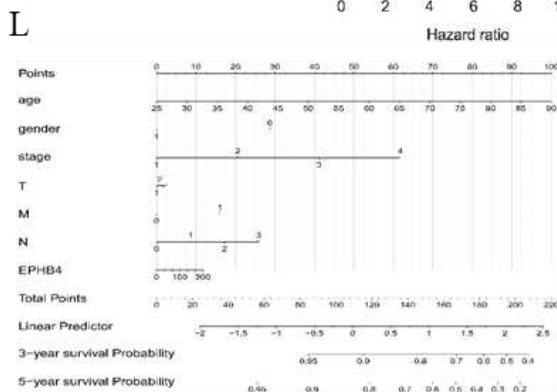
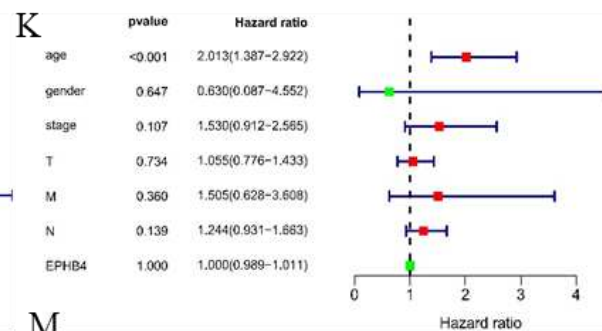
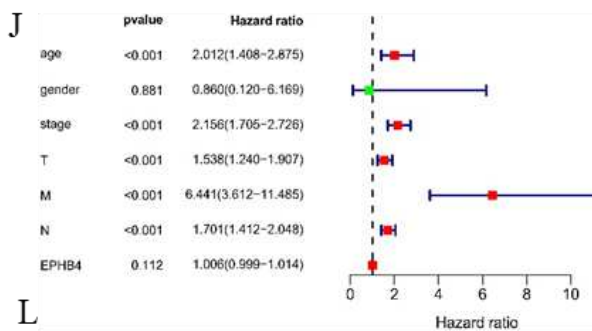
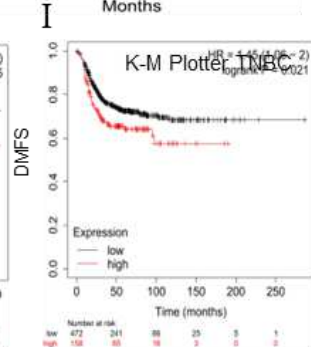
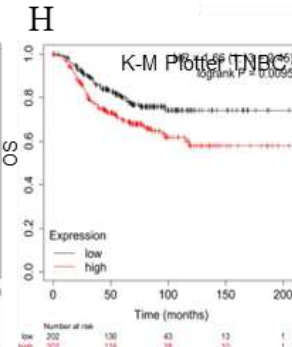
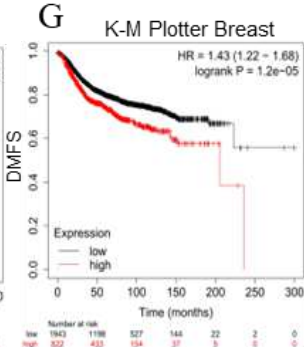
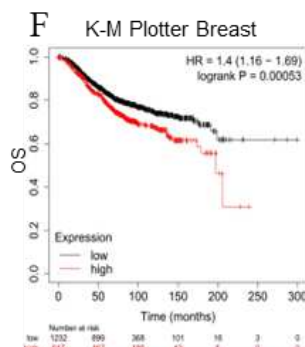
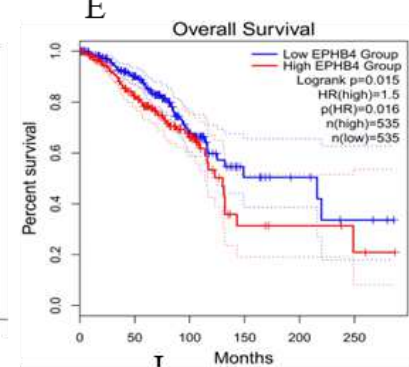
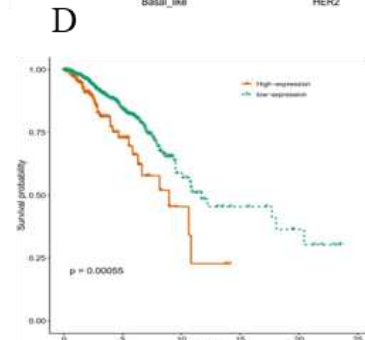
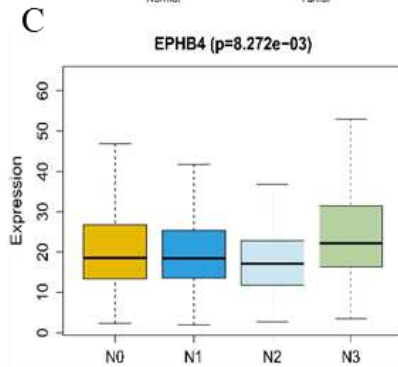
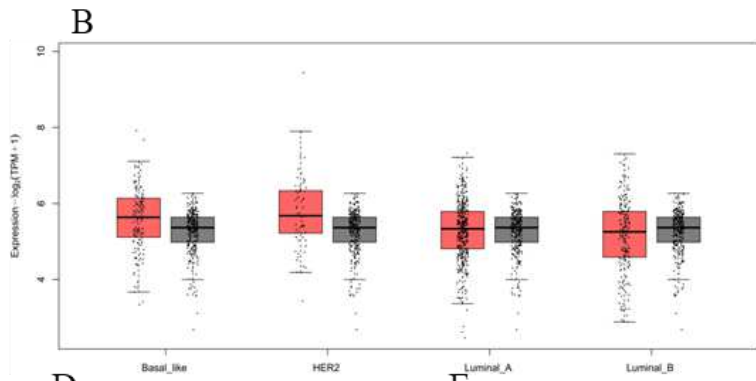
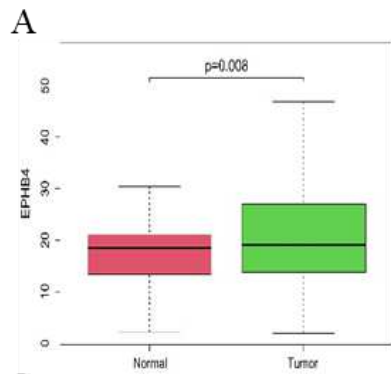


Fig.1 Expression and prognostic values of EphB4 in BRCA. A: Expression of EphB4 between normal and tumor groups on TCGA database. B: Expression of EphB4 in different cancer types. C: Clinical correlation between EphB4 expression and patients in N stage. D: Correlation of EphB4 expression with OS in TCGA. E: Using GEPIA2 for survival analysis. F-I: Correlation of EphB4 expression with OS (F), DMFS (G) in BRCA and OS (H), DMFS (I) in TNBC determined using Kaplan-Meier Plotter. J-K: The univariate and multivariate forest plots of EphB4 expression and clinical symptoms in TCGA-BRCA cohort. L: Nomogram prediction model of EphB4 in BRCA and different peers represent the different clinical characteristics of the sample. M: The correction curve of the model correlation diagram was 3 years in blue and 5 years in red.

Immune microenvironment landscape of breast cancer

The TME consists of tumor-associated fibroblasts, immune cells, extracellular matrix, multiple growth factors, inflammatory factors, and special physicochemical features, as well as cancer cells themselves. It has a significant impact on diagnosing tumors, predicting survival, and determining clinical sensitivity. To investigate the molecular mechanisms of EphB4 in BC progression, we used Spearman's correlation analysis to describe the correlation between EphB4 expression and immune infiltrates in TCGA-BRCA cohort (Fig.2A-F). The results showed that EphB4 was negatively correlation with T cell CD8⁺ and positively correlation with T cell CD4⁺, Neutrophil and Myeloid dendritic cell. Pearson analysis indicated that EphB4 had a positive correlation with M0 macrophages, regulatory T cells (Tregs), follicular helper T cells and NK cells (Fig.2G). It was indicated that BC would be in an immunosuppressive state due to EphB4 high expression. ssGSEA was used for transcriptomic immunoassay of TCGA breast cancer samples (Fig.2H). Immune-related genes were included to assess breast cancer immunocompetence. The TCGA population was clustered into four subgroups(G1-G4) using consistent analysis (Fig.2I-K). Using the CIBERSORT algorithm to evaluate the immune cells in each subpopulation of samples by the R package "immunedeconv"³⁷ that integrates six algorithms (Fig.3A-B). SIGLEC15, TIGIT, CD274, LAG3, HAVCR2, PDCD1, CTLA4 and PDCD1LG2 were selected to be ICP-relevant transcripts and extracted these genes expression values (Fig.3C). ICPs expressed on immune cells can prevent the body from mounting an effective anti-tumor immune response, thereby allowing tumor evasion. Most of ICPs were high expression in G2 and G4 except SIGLEC15 with EphB4 high expression, preventing immunization evasion. R package "ggplot2" was used to compare the expression of EphB4 in different subgroups (Fig.3D). EphB4 expression in G1 and G4 was weakly higher than G2 and G3. Potential ICB response was predicted with TIDE algorithm³⁸ (Fig.3E). TIDE used an array of gene expression markers to assess 2 distinct mechanisms of immune escape, namely CTL dysfunction and CTL rejection, and was associated with poorer response to immune checkpoint blockade (ICB) and shorter survival following ICB. The results showed low EphB4 expression group G3 could be more suitable for ICB treatment. Interestingly, the G2 group had lower EphB4 expression than G1 and G3, but had the worst response to ICB treatment, which may be related to the different staging of breast cancer.

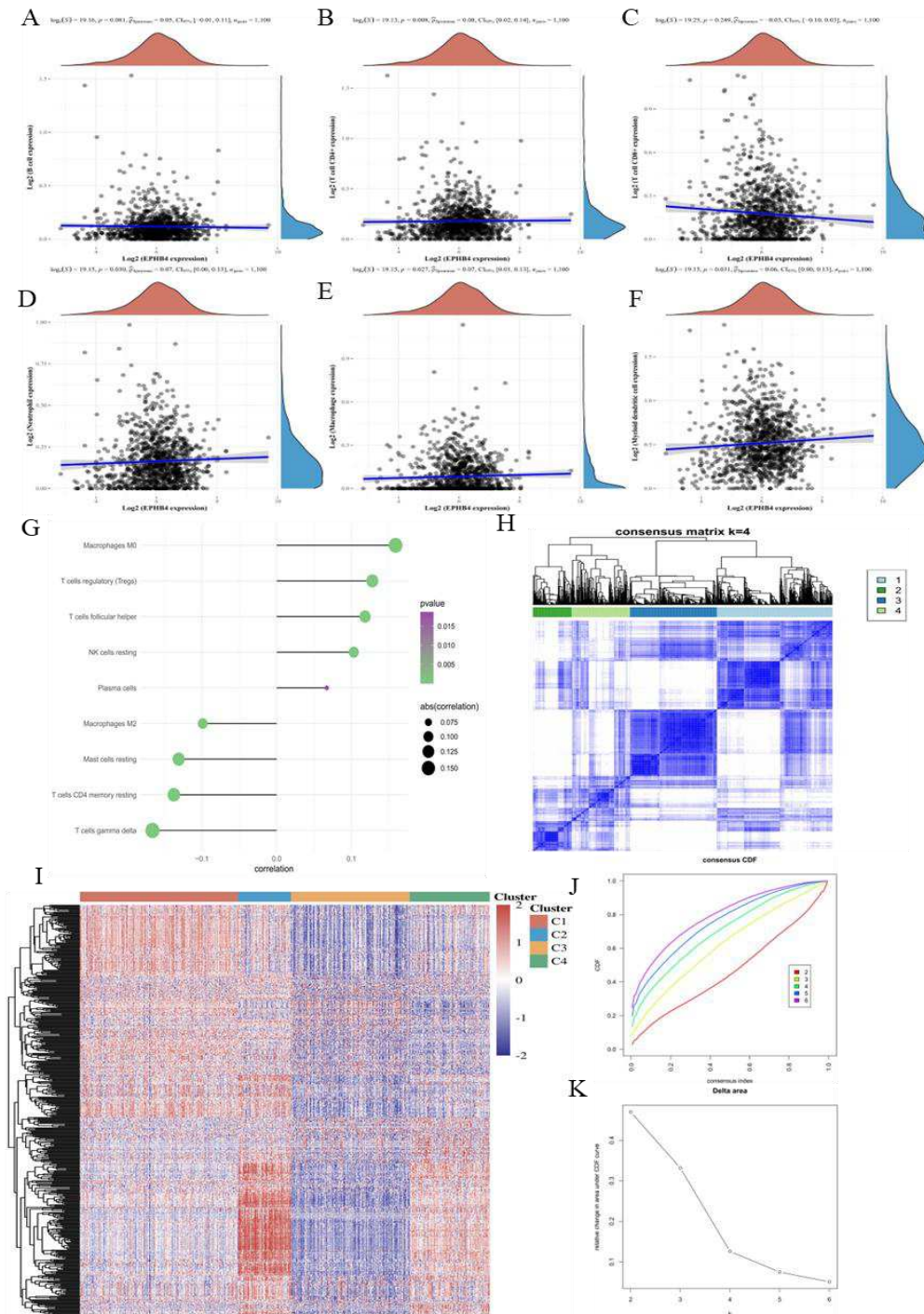


Fig.2 Identification and validation of breast cancer immune subgroups. A: The correlations between EphB4 expression and immune score in BC was analyzed with Spearman. (A) B cell, (B) T cell CD4+, (C) T cell CD8+, (D) Neutrophil, (E) Macrophage, (F) Myeloid dendritic cell. G: Pearson analysis of correlations between EphB4 expression and immune cells. H: Heat map of sample clustering at consensus k=4. I: Heat map of immune genes in different subgroups on TCGA-BRCA cohort. J: Consensus clustering cumulative distribution function (CDF) for k=2 to 6. K: Relative change in area under CDF curve for k=2 to 6.

chose $k=4$, resulting in four subtypes where immune cells appeared stably clustered. into four subtypes and analyze immune cell infiltration (Fig.4D). By analyzing the expression level of EphB4 in A-D subtypes, we found that EphB4 expression was higher in C subtype (Fig.4E). We also assessed the relationship between EphB4 expression and TIICs, indicated that EphB4 was negatively correlated with, such as CD8 T cells, DCs and macrophages (Fig.4F-H). The above results further suggested that EphB4 expression was lower in hot tumors and higher in cold tumors, revealing that EphB4 may play a more important role in a specific kind of TNBC.

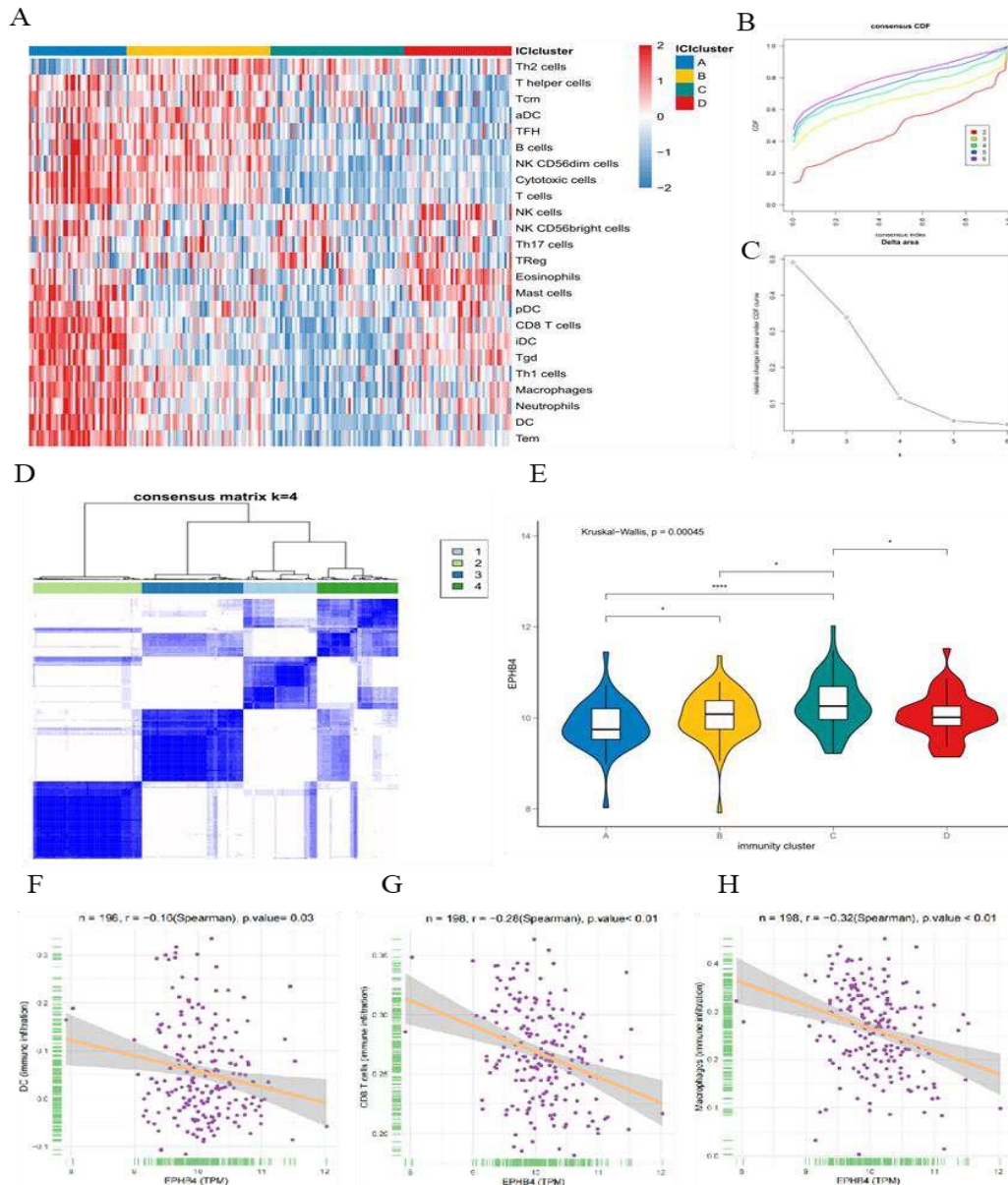


Fig. 4 Identification and validation of immunity-related subgroups in TNBC using unsupervised consensus clustering. A: Heat map of 24 tumor infiltrating cells in different subtypes in TNBC cohort. B: Unsupervised consensus clustering CDF for $k=2$ to 6 . C: Relative change in area under CDF curve for $k=2$ to 6 . D: Heat map of sample clustering at consensus $k=4$. E: EphB4 expression levels in different subtypes. F-H: Correlation between the expression levels of EphB4 and infiltration of DCs(F), CD8+ T cells(G) and macrophages(H) in TNBC. $*p < 0.05$ and $***p < 0.001$.

Relationship between UBE2T and TMB, MSI, NEO Genes

We further explored the correlation between EphB4 and common immunotherapy-related tumor markers. The higher TMB with the higher tumor immunogenicity will more sensitive to the immunotherapy. There were differences with

tumor mutation load TMB, MSI, Neoantigen between high and low EphB4 expression groups (Fig.5A-C). We further explored the mutation profiles of patients in the high and low expression groups and showed that the proportion of TP53 gene mutations in patients in the high expression group was significantly higher than that in the low expression group (Fig.5D).

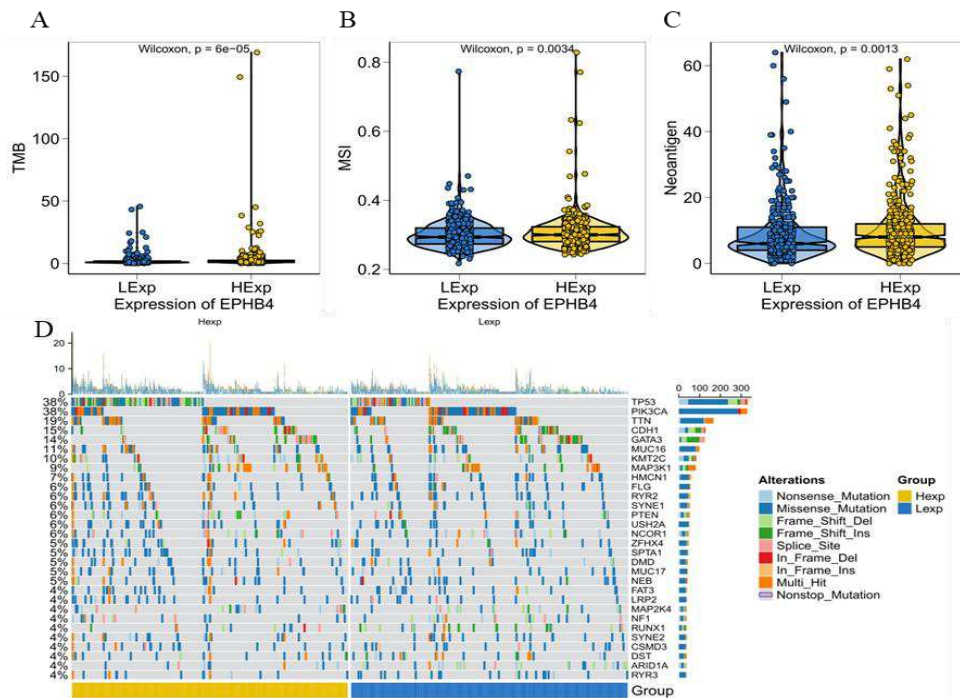


Fig.5 The correlation of TMB(A), MSI(B), Neoantigen(C) between high and low expression groups of EphB4, respectively. D: The mutation profiles of patients in the high and low expression groups.

Co-expression of EphB4 with WGCNA analyses

The co-expression network of EphB4 was analyzed in depth through correlation analysis based on the breast cancer expression profiles on the TCGA database, and a correlation coefficient filter condition of 0.3 and a p -value of 0.05 were obtained. A total of 69 genes were screened and significantly correlated with EphB4 expression presents a heatmap of the TOP10 genes with the most pronounced correlation coefficients (Fig.6A), and depicts the co-expression correlation circle diagram (Fig.6B). We further constructed a WGCNA network based on the expression profile data of BRCA to explore the EphB4-related co-expression network in BRCA. The soft threshold β was set to 4 (Fig.6C-D). Based on the tom matrix detection of gene modules, and a total of 14 gene modules were detected in this study (Fig.6E-F), which were black (193), blue (659), brown (537), green (492), greenyellow (92), grey (49), magenta (139), pink (171), purple (136), red (255), salmon (74), tan (83), turquoise (1626) and yellow (494) with the brown module having the highest correlation ($r=0.24$, $p<0.01$). We next investigated the specific signaling pathways involved in EphB4 to explore the potential molecular mechanisms by which EphB4 affects tumor progression. GSEA results indicated that patients with high expression of EphB4 were enriched in signaling pathways such as MYC_TARGETS_V2, UNFOLDED_PROTEIN_RESPONSE and other signalings (Fig.6G). GSEA results suggested that EphB4 could enrich Central carbon metabolism in cancer, Galactose metabolism, mRNA surveillance pathway signaling pathways (Fig.6H). The network of molecular interactions between the pathways is shown in the figure (Fig.6I), suggesting that EphB4 may affect breast cancer progression through these pathways.

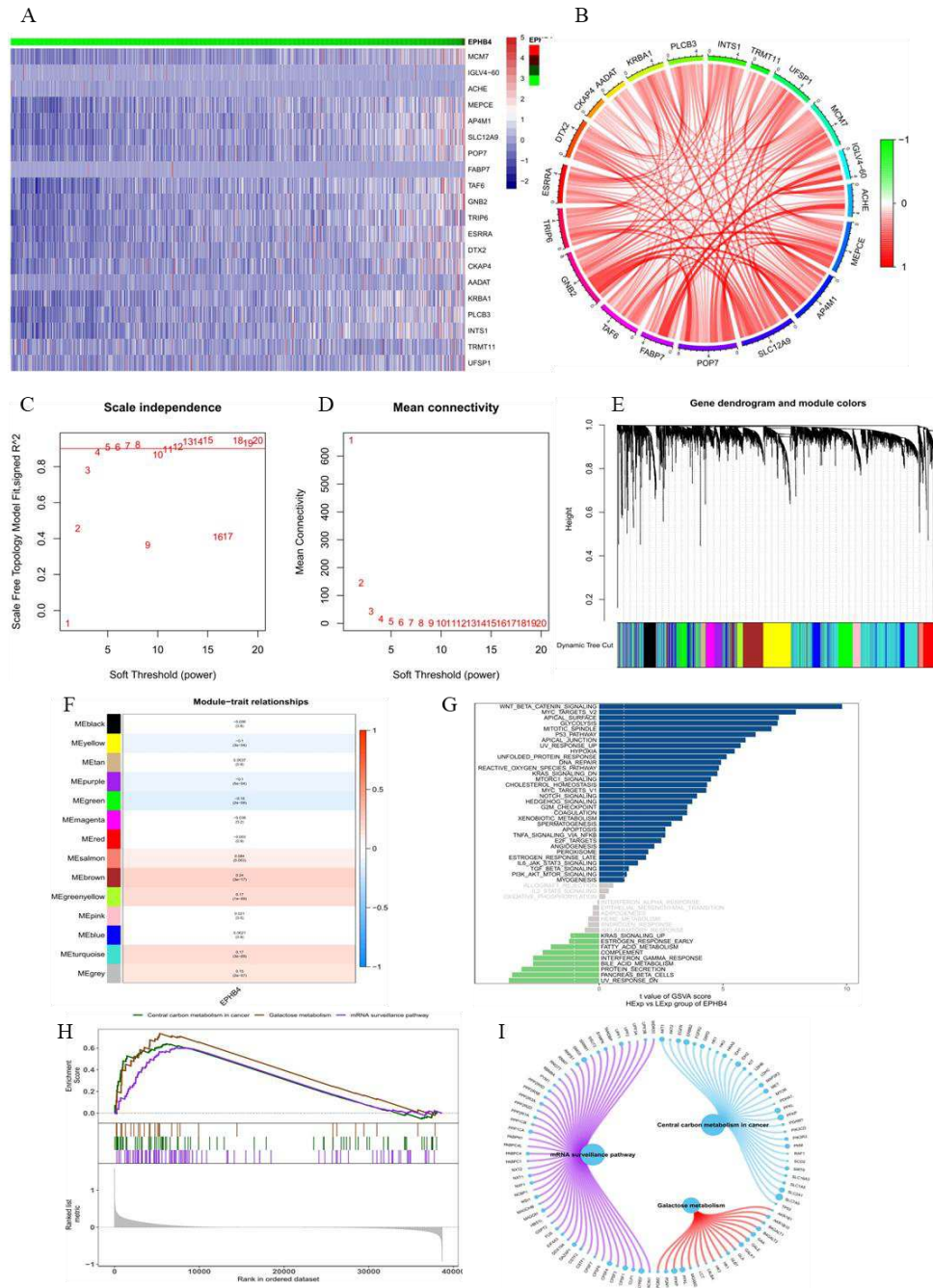


Fig.6 Co-expression of EphB4 with WGCNA analysis. A: Heatmap of the TOP10 genes with the most significant correlation coefficients with EphB4 expression. B: Co-expression correlation circle diagram. C: The optimal soft-threshold was estimated with the scale-free fit index and the mean connectivity for various soft thresholding powers. B-C: Analysis of the correlation between module and gene expression/clinical symptoms. G: Enrichment analysis of EphB4 related GSVA pathway. H: Enrichment analysis of EphB4 related GSEA pathway. I: The network of molecular interactions between the pathways.

EphB4 inhibition TNBC cell viability

To further investigate the regulatory role of EphB4 on the function of BC cells. In this study first applied Western Blot to detect EphB4 expression at the protein level in different cell lines. The results showed that there were differences in EphB4 protein expression levels in different BC cell lines (Fig.7A). There were also differences in the expression of EphB4 protein in TNBC cell lines, with higher levels of EphB4 protein expression in MDA-MB-231 and HCC38 cell lines, and lower levels of EphB4 expression in HCC1187 and 4T1 cell lines, reflecting the heterogeneity

between tumors of different subtypes. We constructed stable shEphB4 cells and their control cells in MDA-MB-231 and HCC38 cells by lentivirus system, and verified the expression level of EphB4 by western blot (Fig.7B-C). The effect of knockdown EphB4 on the proliferation and growth of MDA-MB-231 and HCC38 cells was detected by CCK-8 cell proliferation test (Fig.7D-E) and plate cloning test (Fig.7H-I). The CCK-8 cytotoxicity test was used to determine the IC₅₀ of EphB4 inhibitor NVP-BHG712 in MDA-MB-231 and HCC38 cells and the best time of drug action (Fig.7F-G). MDA-MB-231 and HCC38 cells were treated with BHG712, and the same results were obtained in plate colony formation assay (Fig.7J-K). The above results show that targeted inhibition of EphB4 can significantly inhibit the proliferation and growth of TNBC cells.

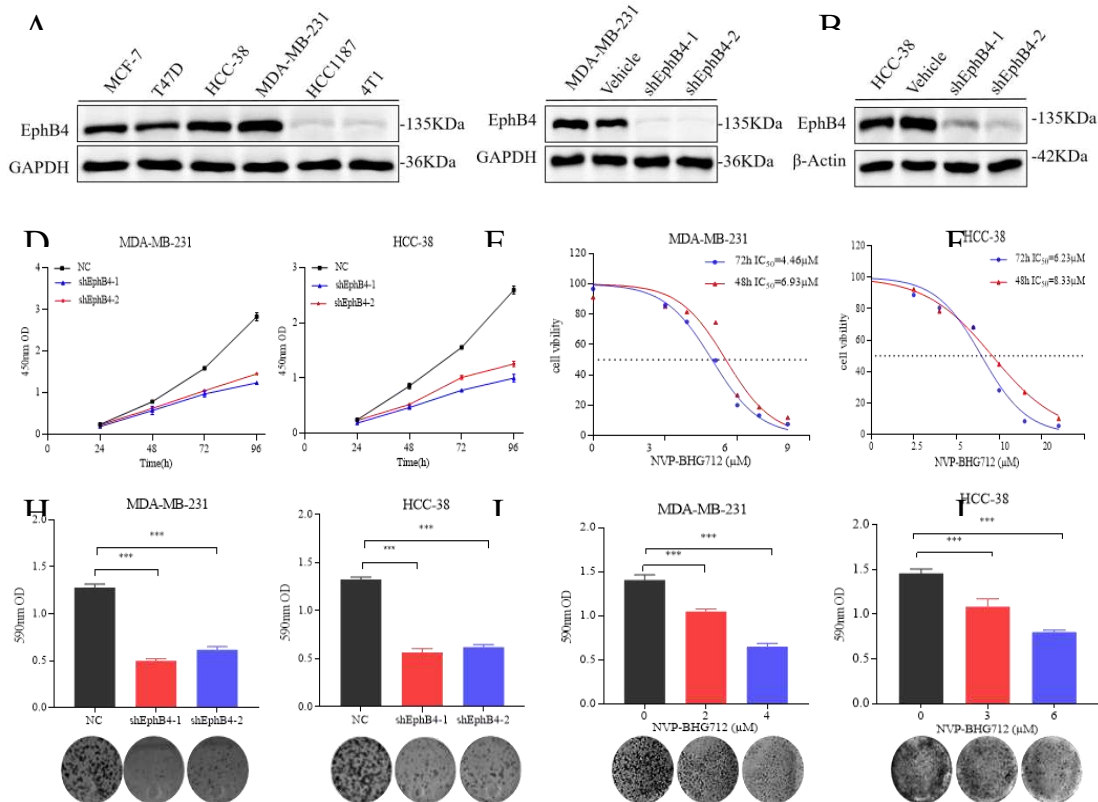


Fig.7 Targeting EphB4 inhibit TNBC cells proliferation growth. A: Western Blot detected the protein expression level of EphB4 in different BC cell lines, and GAPDH was the internal reference protein control. B-C: Western Blot to verify the expression level of EphB4 protein in MDA-MB-231 and HCC-38 cells. D-E: CCK-8 cell experiment reduced the effect of EphB4 on the proliferation. H-I: Plate cloning experiment and staining with crystal violet and determined the OD value at the wavelength of 590nm. F-G: CCK-8 to determine the IC₅₀ values of NVP-BHG712 with the treatment of MDA-MB-231 and HCC-38 cells. J-K: MDA-MB-231 and HCC-38 cells were administrated with 72h IC₅₀ and BHG712 at half IC₅₀. Plate cloning experiment and staining with crystal violet, we determined the OD value at the wavelength of 590nm. The average value was expressed as $\pm$ S.D. (Student's *t*-test). ****p*<0.001

We verified the effect on the migration ability of MDA-MB-231 cells after inhibiting EphB4 by cell scratch and transwell migration and invasion assays to clarify the effect of EphB4 on the migration and invasion ability of TNBC cells. The results suggested that knockdown of EphB4 diminished the motility of MDA-MB-231 cells (Fig.8A). The same inhibition was obtained with a specific concentration of EphB4 inhibitor NVP-BHG712 (Fig.8B). Likewise,

knockdown or inhibition of EphB4 affected the migration and invasion ability of TNBC cells. Inhibition of EphB4 effectively suppressed the migration (Fig.8C) and invasion (Fig.8D) abilities of MDA-MB-231 cells and HCC-38 cells.

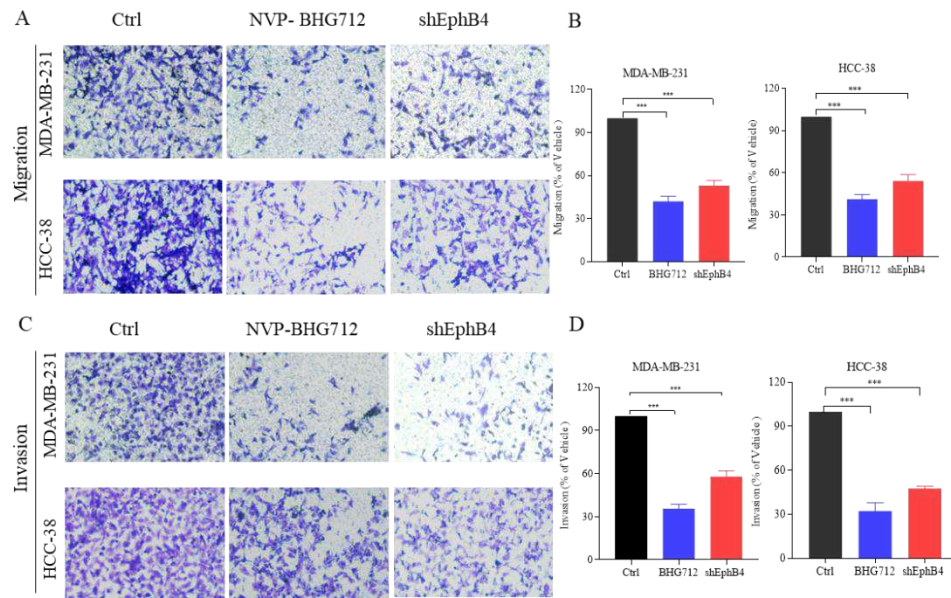
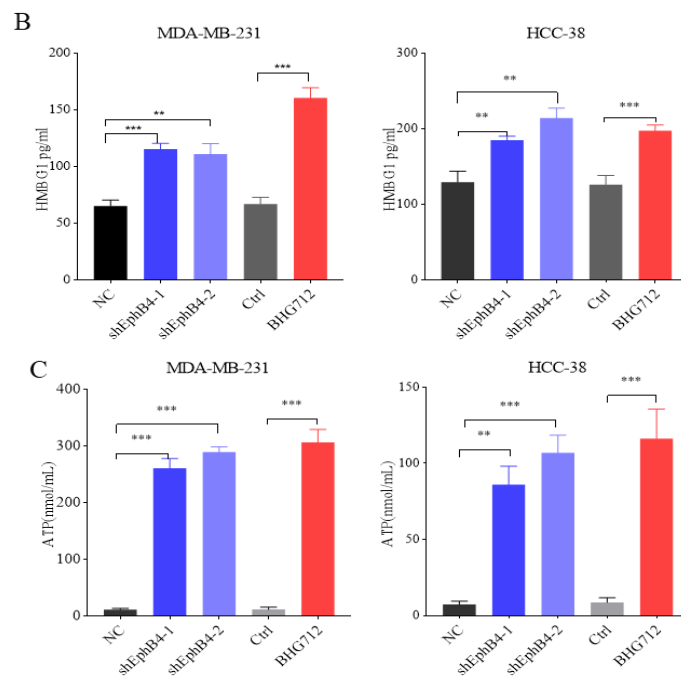
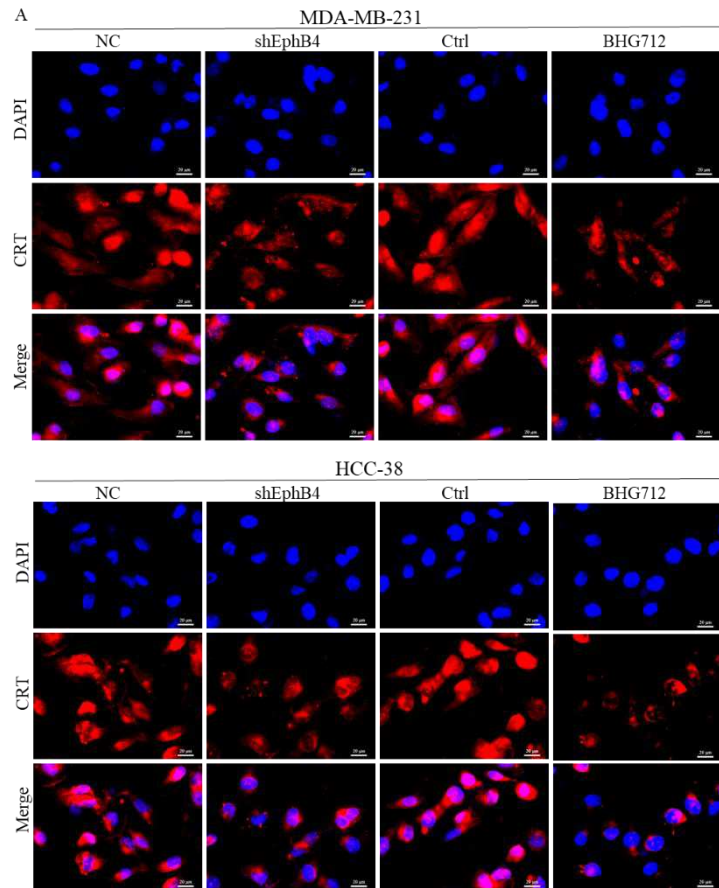


Fig.8 Targeting EphB4 inhibit TNBC cells migration and invasion. Transwell chamber test was used to detect the level of cell migration (A, B) and invasion (C, D), crystal violet was used to quantify the migrated cells. The average value was expressed as $\pm$ S.D. (Student's *t*-test). ** p < 0.01, *** p < 0.001

EphB4 inhibition induce ICD

To further investigate whether inhibition of EphB4 could induce TNBC immunogenic cell death (ICD). Recognition of ICD occurring in tumor cells is largely dependent on the detection of DAMPs, with three main markers, namely CRT, HMGB1 and ATP. We treated the cells with NVP-BHG712 for 48h and silenced EphB4. Applying immunofluorescence to detect the location of CRT expression on cell surface (Fig.9A). The secretion level of HMGB1 was detected by ELISA kit (Fig.9B), and the secretion level of extracellular ATP was detected by ATP content kit (Fig.9C). The results showed that after inhibition of EphB4, CRT reversed from the cytoplasm to the cell membrane, the release of HMGB1 and the exocrine level of ATP also increased, indicating that targeted inhibition of EphB4 could induce immunogenic cell death of TNBC cells. To further investigate the changes in the regulation of antitumor immune response of T cells after EphB4-induced ICD in TNBC cells, Jurkat T cells were co-cultured with MDA-MB-231 and HCC38 cells, control and inhibition of EphB4 groups were set up. After 48h of treatment factors in each group, the complete medium was replaced and the culture was continued until 7 days. Crystalline violet staining and quantitative analysis of the cells showed that targeted inhibition of EphB4 significantly enhanced the regulatory ability of T cells, enabling them to kill more tumor cells (Fig.9D-E)



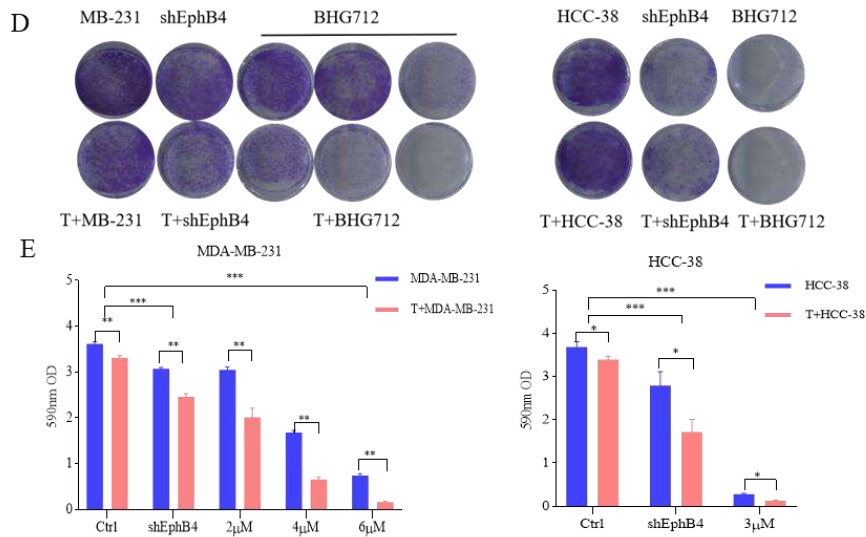


Fig.9 Targeting EphB4 induce ICD and enhance T cell toxicity. A: The expression of CRT in MDA-MB-231 and HCC38 cells was detected by immunofluorescence. B: The expression level of HMGB1 was detected by ELISA KIT. C: The expression of ATP was detected by ATP content detection kit. EphB4 silencing cells and cells treated with BHG712 for 48h by 3 μ M. Ctrl was wild type cell and Vehicle unloaded plasmid cell serves as control group. D: MDA-MB-231 and HCC38 cells treated with silenced EphB4 and BHG712 were co-cultured with T cells for 48h, MB-231: 2 μ M, 4 μ M and 6 μ M, HCC-38: 3 μ M. E: Clone forming crystal violet staining and determination of OD value at 590nm. The average value was $\pm$ S.D., * p <0.05, ** p <0.01, *** p <0.001 (Student's t -test).

Inhibition of EphB4 activate ER stress

Endoplasmic reticulum stress is the main mechanism of ICD. Endoplasmic reticulum stress response begins after the phosphorylation of eIF2 α ³⁹. Endoplasmic reticulum stress response involves three receptors, namely, activating transcription factor 6 (ATF6), eIF2 α kinase PERK and IRE1 located by PKR-like receptors related to the etiology of immunogenic cell death, forming a stable complex with molecular chaperone immunoglobulin (BiP) under non-stress state⁴⁰. When the protein is misfolded or unfolded, BiP is dissociated from the complex. Therefore, the activation site of the receptor is exposed and the downstream signal is activated. We evaluated the protein expression of endoplasmic reticulum related receptors in cells with silenced EphB4 or treated with inhibitor NVP-BHG712 (Fig.10A and B), and found that up-regulated the phosphorylation levels of eIF2 α and PERK, as well as the levels of IRE1 and BiP, indicating that inhibition of EphB4 can trigger endoplasmic reticulum stress response, which may be related to ICD in BC cells. However, there was no significant change in the expression of endoplasmic reticulum receptor-related proteins ATF6 and ATF4 and their chaperone protein CHOP. Therefore, we considered that EphB4 inhibition might trigger endoplasmic reticulum stress response through other pathways, and then induce ICD.

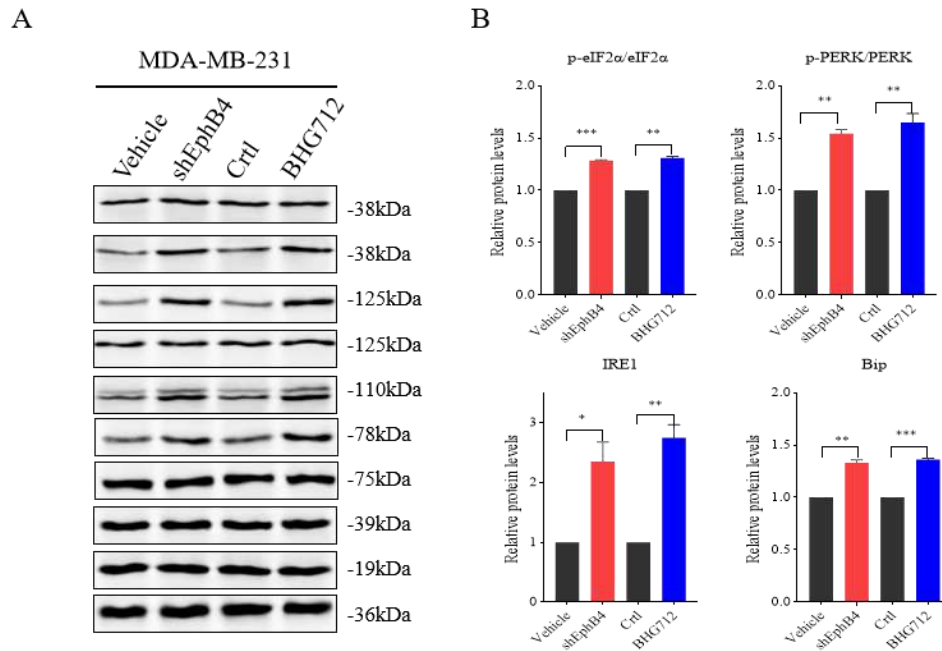
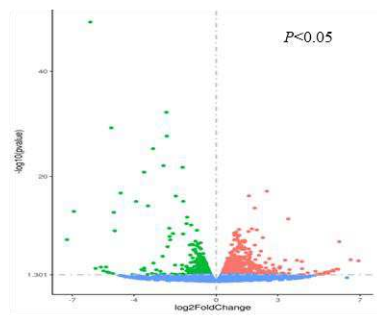


Fig.10 Inhibit the effect of EphB4 on endoplasmic reticulum related pathway proteins. A: Western Blot was used to detect the expression of endoplasmic reticulum-related proteins in MDA-MB-231 cells silenced by EphB4 and treated with 3 μ M inhibitor BHG712 for 48h, including p-eIF2 α , p-PERK, IRE1, BiP, ATF6, ATF4 and CHOP proteins, GAPDH as internal reference protein, Vehicle and Ctrl as control group. B: to show the quantitative results of the expression abundance of each protein. The average value was $\pm$ S.D., * p <0.05, ** p <0.01, *** p <0.001

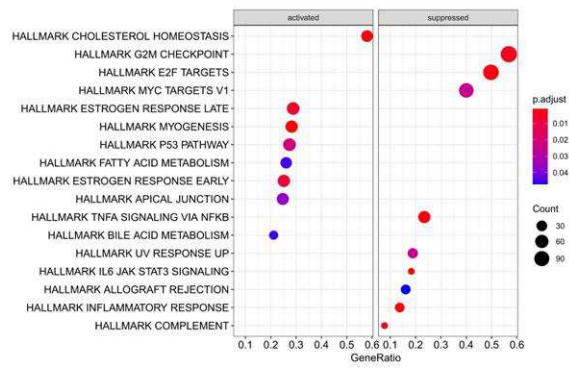
EphB4 mediates by regulating Src/STAT3/MYC signal pathway

In order to deeply investigate the mechanism of EphB4 regulating the growth of TNBC cells, we performed transcriptome sequencing (RNA-seq) on MDA-MB-231 cells silenced by EphB4 and control cells. The results of differential expression profile analysis showed that silent EphB4 could induce gene expression profile changes, including up-regulation and down-regulation of gene expression (Fig.11A) and KEGG pathway enrichment scrutiny of target genes (Fig.11B), suggesting that EphB4 did affect the changes of TNBC cells at the molecular level. GSEA analysis indicated that GOBP_GLUCOSE_CATABOLIC_PROCESS and GOBP_ATP_TRANSPORT gene sets were reported through functional enrichment analysis, and MYC_TARGETS and IL6-JAK-STAT3 signal pathways were significantly negatively enriched in pathway enrichment analysis (Fig.11C). Furthermore, we used STRING to analyze the protein interaction between EphB4 and STAT3, and discovered interactive proteins Src and JAK2 (Fig.11D). Based on the above-mentioned results, we speculated that the signal pathway between EphB4 and STAT3 may be mediated by the above gene molecules. Through TCGA database analysis, we found that EphB4 was positively correlated with STAT3 (Fig.11E). Using Cancer Cell Line Encyclopedia, CCLE analysis of tumor cell lines, we found that EphB4 showed a positive correlation with STAT3 in TNBC cell lines, instead of in other breast cancer cell lines (Fig.11F), which validated the association between EphB4 and STAT3 and also demonstrated the accuracy of our RNA-seq sequencing data.

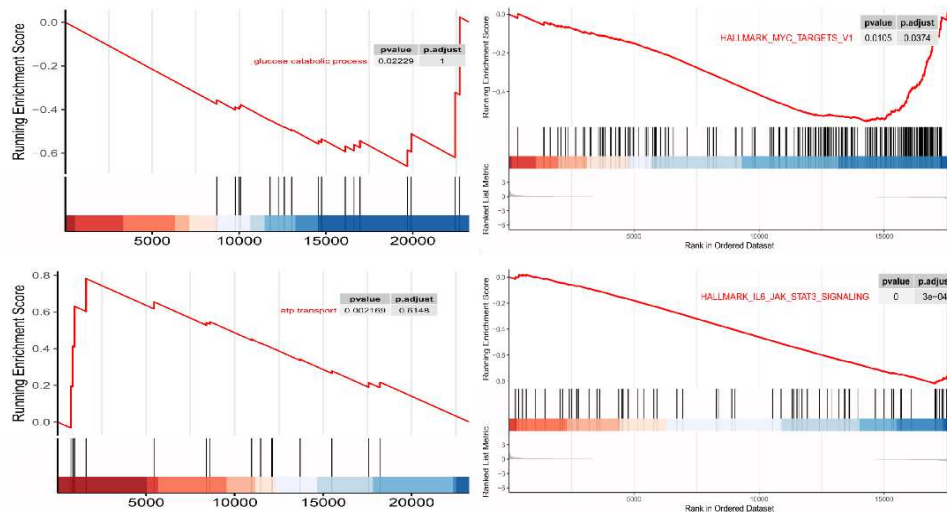
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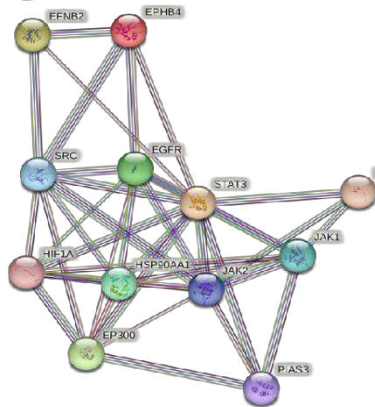
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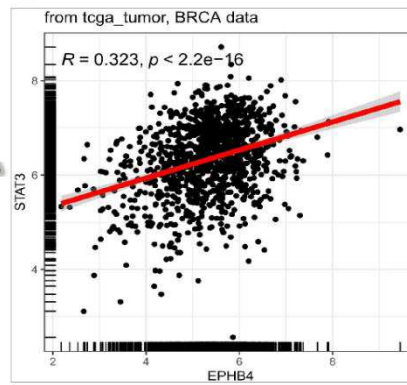
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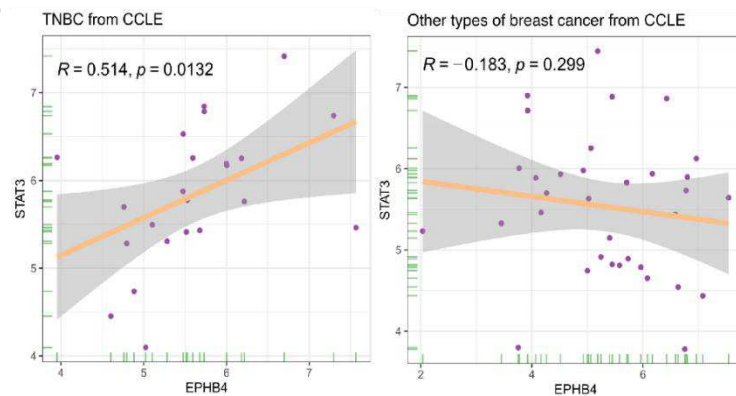


Fig. 11 A: Volcano Plot shows the changes of gene expression levels. B: KEGG pathway enrichment scrutiny of target genes and transcriptome analysis. C: GSEA enrichment analysis indicated the p values of four gene sets. D: STRING database to analyze the protein interaction. E: TCGA database analysis of the correlation between EphB4 and STAT3. F: CCLE analysis of the correlation between EphB4 and STAT3.

The process of ER stress response depends on the external energy sources provided by glycolysis or oxidative phosphorylation, combined with the results of bioinformatics analysis, considering the effect of glucose metabolism changes. The c-MYC oncoprotein is a known regulatory protein of glucose metabolism that has previously been correlated with EphB4 in a variety of cancer cells⁴¹. We found that GLUT3 and c-MYC protein expression was downregulated and the phosphorylation level of eIF2 α was upregulated in cells treated with silent or inhibition of EphB4, suggesting that altered regulation of glucose metabolism induces an endoplasmic reticulum stress response (Fig.12A). STAT3, as a signal transducer and transcription factor, plays an important role in signal transduction, which can be activated or phosphorylated by its upstream regulatory factor protein kinase Src⁴², while Src can be activated by EphB4 to up-regulate MYC levels⁴³. Western Blot results also indicated that of EphB4 inhibited down-regulation the phosphorylation of STAT3 and Src, whereas it did not pronouncedly inhibit the phosphorylation of JAK2 (Fig.12B). Therefore, we consider that EphB4 inhibition can downregulate the expression of c-MYC by reducing the phosphorylation level of STAT3, which leads to the decrease of GLUT3 expression and the decrease of intracellular ATP level by affecting glucose metabolism, which triggered with ER stress.

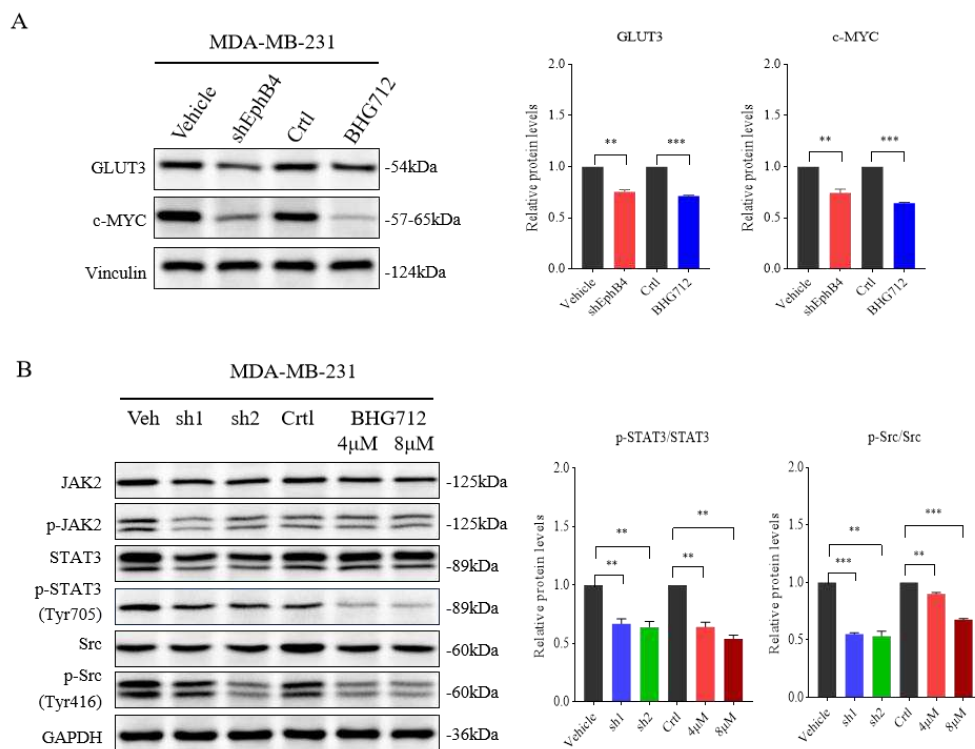


Fig.12 A-B: Western Blot was used to detect the changes of related protein expression with the designated antibodies, including GLUT3, c-Myc, p-eIF2 α , JAK2, p-JAK2, STAT3, p-STAT3, Src, p-Src proteins, Vinculin and GAPDH as internal reference protein. MDA-MB-231 cells silenced by EphB4 and treated with 4 μ M or 8 μ M inhibitor BHG712 for 48h, Veh and Ctrl as control cells. The average value was expressed as $\pm$ S.D. * p <0.05, ** p <0.01, *** p <0.001

Regulatory effect of EphB4 on the growth and metastasis of in PyMT mice

Mammary-specific EphB4 knockout mice were hybridized with MMTV-PyMT spontaneous breast cancer transgenic mice to obtain mammary-specific EphB4 knockout spontaneous breast cancer model mice, namely EphB4-CKO/PyMT model mice (Fig.13A- B). We are the first to study this kind of model mice. Gene mice started to grow mammary tumors at around 9 weeks and develop lung metastases around 12 weeks. The results indicated that the tumor growth rate of EphB4-CKO/PyMT group was slower than that of EphB4/PyMT group, and the tumor volume was relatively smaller (Fig.13C). Although there was no significant difference between the two groups, it can be seen objectively that EphB4 knockout can inhibit the growth of mammary tumors in mice. To further observe the growth of mice, we dissected the late model mice to take tumors, and observed the tumor tissues and lung tissues morphology under microscope after H&E staining (Fig.13D-E). The tumor cells of EphB4/PyMT mice compared with EphB4-CKO/PyMT mice, they have faster proliferation, deeper nucleus staining, more mitotic phase, and more metastases, suggesting that EphB4 can regulate the growth and metastasis of mammary tumors.

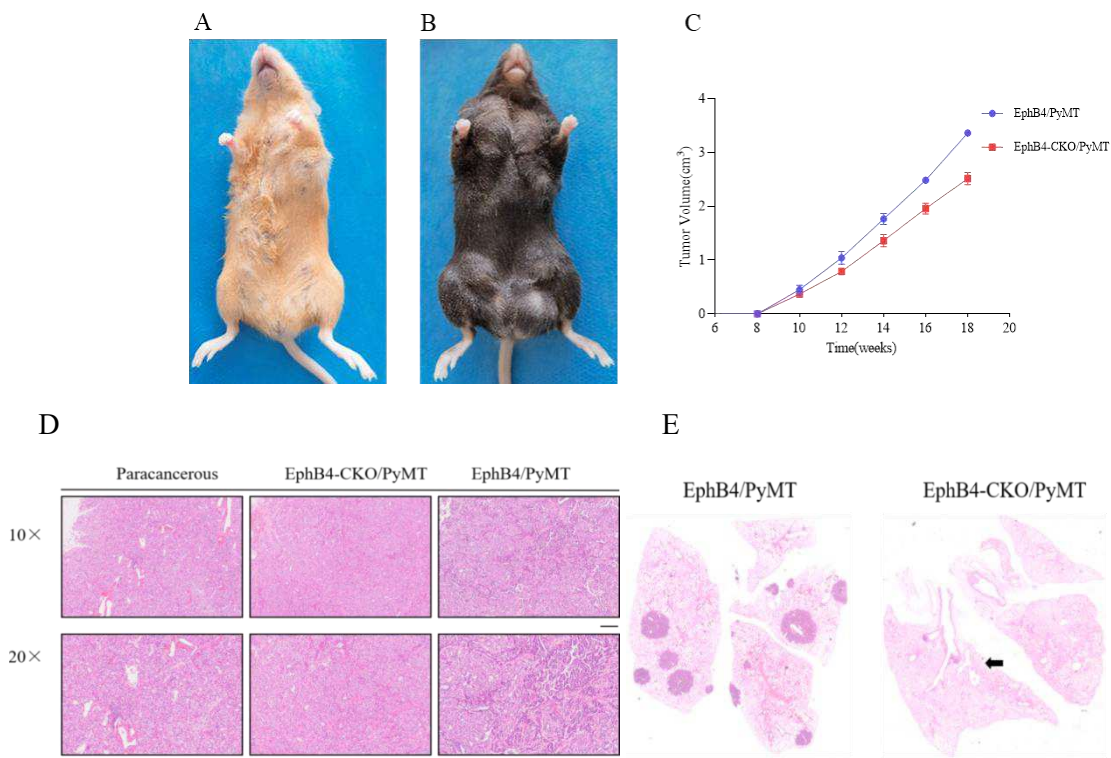
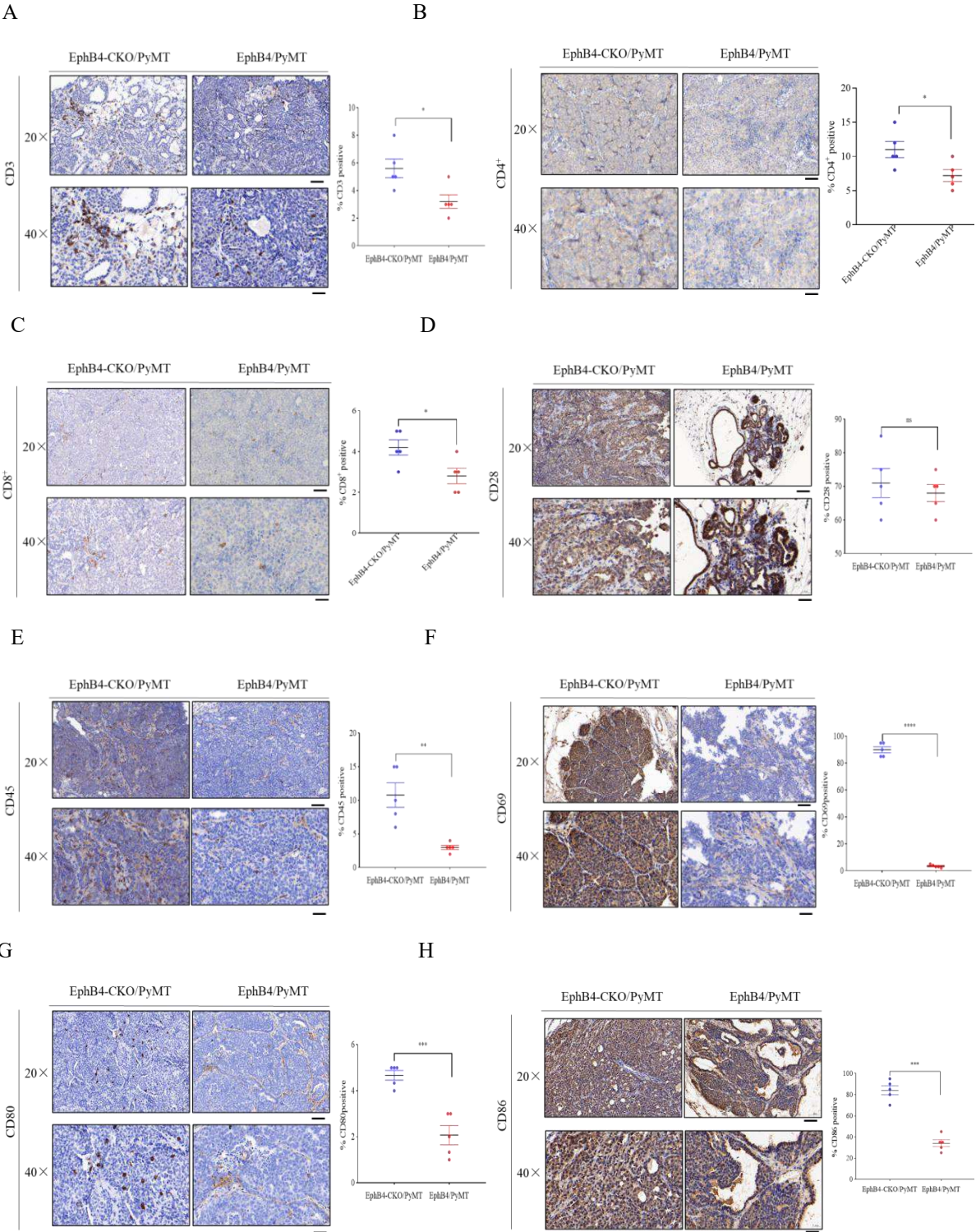


Fig. 13 EphB4-CKO/PyMT mice(A) and Control group EphB4/PyMT mice(B). C: 6 mice in each group and the tumor growth was observed. Student's *t*-test showed that there was no significant difference between the two groups. D: In the paracancerous tissues, the breast glands were arranged regularly, and a small number of inflammatory cells could be seen, the tumor cells of EphB4/PyMT mice showed adenoid growth with different sizes and large nuclei and the nucleoli of tumor cells in EphB4-CKO/PyMT mice were clear under 10-fold and 20-fold microscope. (scale 200μm, 100μm). E: The morphological changes of lung tissues (scale 1mm)

Regulatory effect of EphB4 on immune microenvironment of breast tumor

To further explore the regulatory effect of EphB4 on mammary tumor immune microenvironment, we used IHC to observe the expression of immune infiltrating cells in mammary tumor tissues of model mice. The results suggested that in experimental mice, inhibition of EphB4 could increase the infiltration degree of CD3, CD4, CD8, CD45 and CD69 in T cells or the expression of CD28 in tumor tissue, and the difference was statistically significant (Fig.14A-F). The degree of infiltration of CD80, CD86 and CD11c, the markers of DC cell maturation, also increased (Fig.14G-I), the difference was statistically significant. It reflects the regulation of EphB4 on mammary tumor microenvironment. We

also examined the IHC of CTLA4 and PD-L1 in the same tumor tissue. The results indicated that inhibition of EphB4 could increase the expression of CTLA4 and PD-L1, and both TPS and CPS increased (Fig.14K-L). Although the difference did not have statistical significance, it objectively reflected that inhibition of EphB4 could increase the possibility of benefit from immunotherapy and might be effective for immunosuppressive therapy. We also evaluated in vivo whether there was a synergistic effect between anti-PD-L1 and EphB4. EphB4-CKO/PyMT mice were treated with PBS and anti-PD-L1 antibody respectively, and the therapeutic effects were observed (Fig.14L). The size of tumor in the treatment group decreased slightly, but there was no significant difference, which objectively indicated that inhibition of EphB4 may enhance the sensitivity of immunotherapy for breast cancer, but more verification is needed.



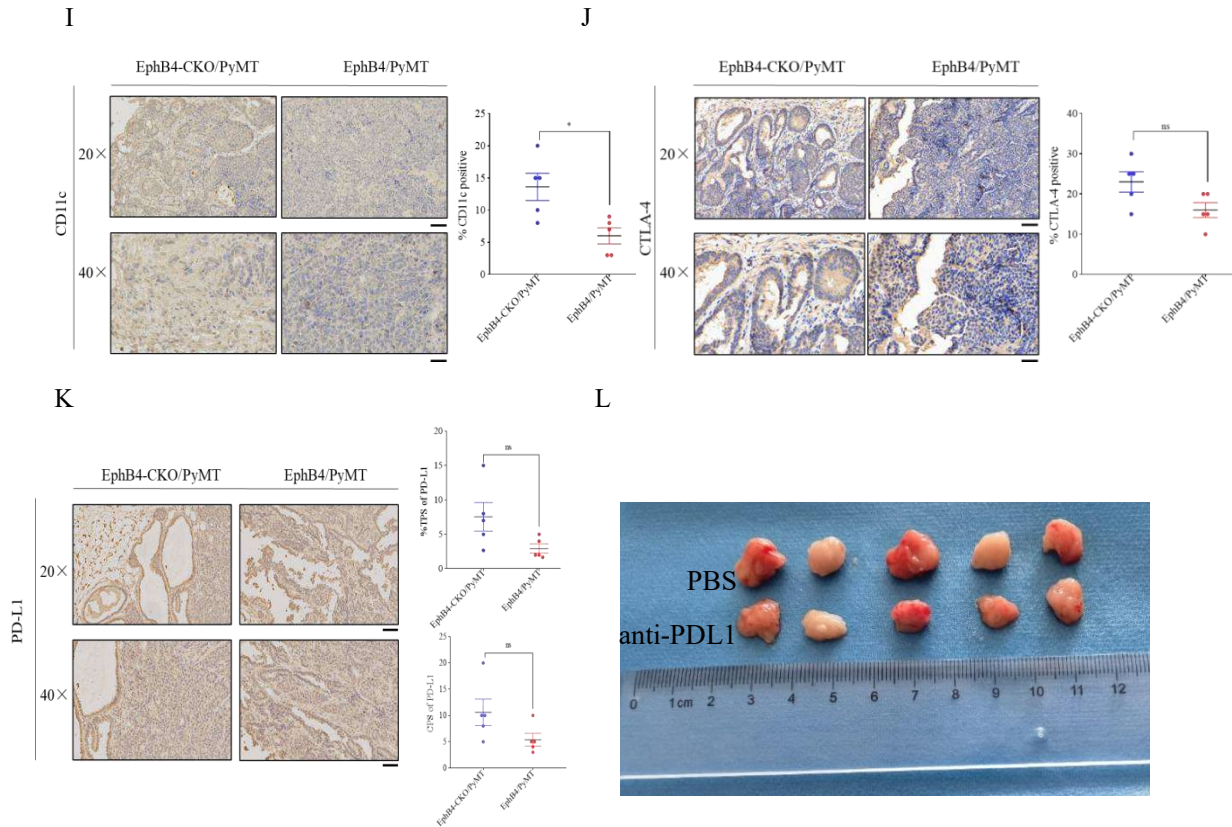


Fig. 14 A-F: IHC was used to detect the expression of related immune indexes in mammary tumor tissues of EphB4-CKO/PyMT mice and EphB4/PyMT mice, including CD3, CD4, CD8, CD28, CD45, CD69, CD11c, CD80 and CD86 under 20-fold and 40-fold microscopes. The average values of three visual fields were computed and statistically analyzed. We divided K and L into the expression of CTLA4 and PD-L1 in tumor tissues. The expression level of PD-L1 was evaluated based on TPS (tumor cell positive fraction) and CPS (combined positive score). CPS refers to a numerical value. The evaluation criteria are: any intensity of membrane staining of tumor cells, membrane/cytoplasmic staining of lymphoid/macrophages directly correlated with tumor cells, the ratio of these cells to tumor cells. (n=5) (scale 100 μ m, 50 μ m). The average value was expressed as $\pm$ S.D. (Student's *t*-test). I: When tumors reached about 200 mm³, mice were then randomized to two treatment groups with PBS and anti-PDL1 antibody, once a week for a month. (n=5).

DISCUSSION

Breast cancer is the most common malignant tumor in women with significant heterogeneity. The emergence of immunotherapy has shown promising results in several disease models, although the response rate has not been optimal⁴⁴. In addition, the development of immune-related adverse toxicity and drug resistance are the main factors affecting the efficacy of immunotherapy drugs^{45, 46}. Therefore, it is particularly important to find new treatment strategies and reshape the TIME to produce effective anti-tumor immune response.

As an important member of the Eph family, EphB4 is often overexpressed in colon cancer⁴⁷, prostate⁴⁸, and breast cancer. However, the mechanism of EphB4 promoting or inhibiting tumor is not clear, which is closely related to its environment in various types of tumors. Our study confirmed the carcinogenic role of EphB4 in mammary tumors. We analyzed the difference of mRNA expression in samples from the original mRNA expression data of breast cancer in TCGA database. The results showed that BC with high expression of EphB4 has a poorer prognosis, and we also developed a nomogram model for predicting patient prognosis. By regulating EphB4 expression and then affecting the

proliferation, invasion and migration of TNBC cells, as well as the occurrence and development of mammary tumors, suggesting that EphB4 can become a new target for clinical targeted therapy.

We observed that the expression of EphB4 increased the proliferation of breast tumor cells and the increase of cell death in vitro. Cells that die in the form of apoptosis are immune silent and cannot stimulate the immune response⁴⁹. However, some specific chemotherapeutic drugs can induce cell death through ICD⁵⁰. ICD refers to the process of anti-tumor immune response mediated by the transformation from non-immunogenicity to immunogenicity when tumor cells die under external stimulates⁵¹. It is characterized by the release or exposure of a series of DAMPs when tumor cells die, such as CRT, HMGB1, ATP. Innate and adaptive immune responses are then activated by the immune system and alerted by DAMPs⁵². These DAMPs can activate antigen-presenting cells (APCs), such as dendritic cells (DCs), promote their phagocytosis of dead tumor cells, and present tumor antigens to T lymphocytes, thus forming an effective anti-tumor immune response chain⁵³. ICD has great significance and potential in tumor therapy. ICD can enhance the killing effect of traditional therapy and reduce its toxic and side effects, on the other hand, ICD can cooperate with new immunotherapy methods to improve its clinical effect. Chemotherapeutic drugs have certain side effects, so it is very important to choose safe and effective drugs with few side effects. Some drugs have been proved to have the ability to induce ICD^{23, 24}, but it has not been reported that EphB4 can induce ICD in BC. Our study confirmed that inhibition of EphB4 can reverse the membrane of CRT, increase the secretion of HMGB1 and ATP, also enhanced the killing ability of T cells by co-culture experiment, which indicates that EphB4 inhibition could induce ICD of TNBC cells.

ICD induction can make the tumor immunogenic, enhance DC cell activity and maturation, and present antigen to activate specific T cells to kill tumor cells²². We found that the degree of immune cell infiltration in different TNBC patients also varied by ssGESA and unsupervised consistency clustering analysis, and EphB4 was negatively correlated with CD8⁺T cells, DCs and macrophages among the immune infiltrating cells. There were significant differences between EphB4 high and low expression groups and TMB, MSI and Neoantigen. This suggests that EphB4 may be lowly expressed in hot tumors and highly expressed in cold tumors. TNBC can be classified into four molecular phenotypes, including immunomodulatory (IM), so we speculate that EphB4 may play a greater role in immunomodulatory TNBC, and that inhibition of EphB4 could potentially improve the TME and modulate the immune response by facilitating the transition between hot and cold tumors.

Endoplasmic reticulum emergency is the mainly mechanism of ICD. Some studies have found that anthracycline-induced ICD in cancer cells will preferentially enhance the phosphorylation of eIF2 α rather than activate other stress response factors. We found that after inhibition of EphB4, the expression of IRE1 and the phosphorylation of eIF2 α and PERK increased in TNBC cells, while the expression levels of ATF6, ATF4 and their CHOP did not change significantly, which was similar to that reported before. Inhibition of EphB4 can induce ICD in prostate cancer cells by inhibiting glucose uptake and reducing intracellular ATP levels⁵⁴. Because of protein assembly, folding and glycosylation require energy, ATP consumption may lead to endoplasmic reticulum stress. The decreased glucose uptake after EphB4 inhibition could be attributed to the decreased expression of the glucose transporter, GLUT3. Down-regulation of GLUT3, which is mainly involved in the transport of glucose from the extracellular to the intracellular compartment, can lead to intracellular glucose deficiency, which in turn leads to cellular metabolic abnormalities. MYC is known to enhance glycolysis, regulating glucose transporters and uptake in many cells⁵⁵. Therefore, that reduced glycolysis in EphB4 depleted cells can be explained by the decrease of MYC levels. Our data demonstrated that EphB4 inhibition decreased the levels of GLUT3 and c-MYC, and lead to the phosphorylation of eIF2 α .

We performed RNA-seq sequencing of EphB4 knockdown to further explore the mechanism to induce ICD in TNBC cells. Combined with previous data analysis, indicating that targeted MYC mediated by EphB4 expression to regulate glucose catabolism, resulting in ATP transport to extracellular. Src and JAK2 were interactive proteins between EphB4 and STAT3 by using STRING. There is a positive correlation between EphB4 and STAT3. SRC family kinases can up-regulate MYC levels through cap-dependent translation through ERK pathway in BC⁴³. It is known that Src is the downstream factor of EphB4, and it reported that Src is activated by EphB4 in ovarian cancer model, suggesting the role of Src kinase in regulating MYC. Other studies show that the CSF2/p-STAT3 signaling pathway upregulates MYC expression and enhances the stemness of epithelial ovarian cancer cells ⁵⁶, CSF2, p-STAT3 and MYC maybe involved in the regulation of cell phenotypic plasticity⁵⁷. Combined with pre-analysis, we found that inhibition of EphB4 reduced the phosphorylation of STAT3 and Src, and down-regulated c-MYC expression, whereas the phosphorylation of JAK was not significantly inhibited, indicating that inhibition of EphB4 may regulate TME and change immune response through SRC/STAT3/c-MYC pathway, but the specific mechanism still needs to be further explored.

MMTV-PyMT model was adopted to investigate several aspects of breast cancer. In terms of immune activity, the high penetration of PyMT model transfer is helpful to study the interaction between innate immune system and adaptive immune system^{58, 59}. We hybridized homozygous breast-specific knockout EphB4 mice with MMTV-PyMT transgenic mice, and continued to hybridize the offspring of mice containing PyMT gene with homozygous breast-specific knockout EphB4 mice to form an EphB4-CKO/PyMT spontaneous breast cancer gene mouse model. Through this model, we found the regulatory effect of EphB4 on the growth and metastasis of breast tumors. At present, there is no study on breast specific knockout EphB4 spontaneous tumor formation in mice. To further study the regulatory effect of EphB4 on TIME, as a response to the application of antineoplastic drugs, the changes of tumor immune infiltration components may be very important to the treatment outcome. DCs play a key role in anti-tumor immune response. Tumor-associated antigens are presented to T cells through MHC- II and recognized by T cell surface receptors, supplemented by costimulatory molecules and cytokines, thus activating the anti-tumor immune response, which is also the key to limit the efficacy of anti-PD1/PD-L1 drugs^{60, 61}. CD80 and CD86 are markers of DC cell maturation. They are expressed on the surface of DC and can bind to the receptor CD28 on the surface of T cells, thus activating T cell better⁶². We used IHC to detect the expression of related immune infiltration indexes in the tumor tissues of EphB4-CKO/PyMT mice and control EphB4/PyMT mice. Our results showed that inhibition of EphB4 could increase the expression of CD3, CD4, CD8, CD45 and CD69 in immune infiltrating cells, as well as the expression of DC cell maturation markers CD11c, CD80 and CD86. The expression of CTLA-4 and PD-L1 in tumor tissue also increased, and gene knockout mice were treated with anti-PD-L1 mAb therapy, although the tumor shrank, but the difference was not statistically significant, it also objectively indicated that inhibition of EphB4 may increase the sensitivity of breast cancer to ICIs therapy, which still needs a large number of treatment data to verify. Our study shows that targeted inhibition of EphB4 can promote the maturation of DC cells and enhance T cell infiltration, combined with immunotherapy may improve the clinical benefit population. However, clinical trial results are still needed to determine the effectiveness of ICD induction and immune checkpoint blockade in cancer.

Conclusions

Our study elucidates that inhibition of EphB4 significantly contributes to the immune-mediated destruction of TNBC cells via immunogenic cell death (ICD) and enhances immune cell infiltration within the tumor microenvironment (TME). This process facilitates the conversion of tumors from an immunologically "cold" to a "hot"

state, subsequently improving the efficacy of immune checkpoint blockade and broadening the population of patients who may benefit from immunotherapy.

It is critical to acknowledge the limitations of our study. Not all TNBC cell lines exhibit high EphB4 expression, which may restrict the generalizability of our findings to all cases of TNBC. This highlights the need for further investigations to comprehensively understand the mechanisms of ICD induction and the potential for immune evasion in EphB4-regulated TNBC cells. Additional studies, particularly those involving in vivo models such as genetically engineered mice, are essential to validate our in vitro results and to further delineate the role of EphB4 as a regulator of the immune system in breast cancer.

The identification of EphB4 as a modulator of immune responses in breast cancer provides a critical foundation for developing targeted clinical immunotherapy strategies. Further exploration of this pathway holds the promise of enhancing therapeutic outcomes for patients with TNBC, particularly those with tumors initially classified as immune-resistant.

Declarations

Abbreviations

ATP, adenosine triphosphate; BC, breast cancer; CRT, calreticulin; DAMPs, damage-associated molecular patterns; DC, dendritic cell; DFS, disease-free survival; DMFS, distant metastasis-free survival; GEO, Gene Expression Omnibus; GEPIA2, Gene Expression Profiling Interactive Analysis 2; GO, Gene Ontology; GSEA, Gene Set Enrichment Analysis; GSVA, Gene Set Variation Analysis; HMGB1, high-mobility group box 1; ICB, immune checkpoint blockade; ICD, immunogenic cell death; ICIs, immune checkpoint inhibitors; IHC, immunohistochemistry; KEGG, Kyoto Encyclopedia of Genes and Genomes; MSI, microsatellite instability; OS, overall survival; ssGSEA, single-sample Gene Set Enrichment Analysis; TCGA, The Cancer Genome Atlas; TIICs, tumor-infiltrating immune cells; TILs, tumor-infiltrating lymphocytes; TMB, tumor mutational burden; TME, tumor microenvironment; TIME, tumor immune microenvironment; TNBC, triple-negative breast cancer; WGCNA, weighted gene co-expression network analysis.

Ethical approval

Publicly available datasets used in this study, including TCGA and GEO (GSE76124), were analyzed in accordance with their data access policies, and no additional ethical approval was required for these de-identified data. All animal experiments were approved by the Ethics Committee of Dalian Medical University (Approval No. AEE19026) and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The public datasets used and/or analyzed during the current study are available from the following resources: The Cancer Genome Atlas breast cancer cohort (TCGA-BRCA), <https://portal.gdc.cancer.gov/>; Gene Expression Omnibus (GEO), accession number GSE76124, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76124>; Gene Expression Profiling Interactive Analysis 2 (GEPIA2), <http://gepia2.cancer-pku.cn/>; Kaplan–Meier Plotter, <https://kmplot.com/analysis/>; CIBERSORT, <https://cibersort.stanford.edu/>; TIMER/TIMER2.0, <http://timer.cistrome.org/>; Molecular Signatures Database (MSigDB), <https://www.gsea-msigdb.org/gsea/msigdb>; NetMHCpan, <https://services.healthtech.dtu.dk/>; STRING, <https://string-db.org/>; Cancer Cell Line Encyclopedia (CCLE), <https://sites.broadinstitute.org/ccle/>; and TIDE, <http://tide.dfci.harvard.edu/>.

The RNA-seq data generated in the current study have been deposited in the GEO repository under accession number GSE333268.

All other data generated or analyzed during this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Y.W. and J.L. performed the bioinformatics analysis and conducted the in vitro experiments. Y.W. drafted the manuscript. J.L. contributed to data analysis and interpretation. M.L. conceived and designed the study, supervised the project, and revised the manuscript. All authors read and approved the final manuscript.

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