Glucocorticoid receptor regulates the epithelial-mesenchymal transition process through GR/ZEB1/E-cad and is involved in breast cancer endocrine drug resistance—a bioinformatics analysis

Yuhan Tang[1#^], Jianli Ma[2#], Han Zhang[1], Weiwei Ma[3], Wenjie Ma[1^], Thomas J. O'Keefe[4], Akshay Pratap[5], Akimitsu Yamada[6], Lu Wang[1], Yuan Gao[1], Qingyuan Zhang[1], Wenhui Zhao[1^]

[1]Department of Medical Oncology, Harbin Medical University Cancer Hospital, Harbin Medical University, Harbin, China; [2]Department of Radiotherapy, Harbin Medical University Cancer Hospital, Harbin Medical University, Harbin, China; [3]Department of Medical Oncology, Xiaogan Central Hospital, Xiaogan, China; [4]Division of Breast Surgery and the Comprehensive Breast Health Center, University of California San Diego, La Jolla, CA, USA; [5]Division of Gastrointestinal Tumor and Endocrine Surgery, University of Colorado Anschutz Medical Campus, Aurora, CO, USA; [6]Department of Gastroenterological Surgery Yokohama City University Graduate School of Medicine, Yokohama, Japan

Contributions: (I) Conception and design: Y Tang, J Ma; (II) Administrative support: Q Zhang, W Zhao; (III) Provision of study materials or patients: H Zhang, Weiwei Ma, Wenjie Ma; (IV) Collection and assembly of data: Y Tang, L Wang, Y Gao; (V) Data analysis and interpretation: J Ma, TJ O'Keefe, A Pratap, A Yamada; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Wenhui Zhao, PhD; Qingyuan Zhang, PhD, Department of Medical Oncology, Harbin Medical University Cancer Hospital, Harbin Medical University, 150 Haping Road, Harbin 150081, China. Email: 0187@hrbmu.edu.cn; 0566@hrbmu.edu.cn.

Background: Studies have shown that there is a connection between estrogen receptor (ER) and glucocorticoid receptor (GR), which can impact the epithelial-mesenchymal transition (EMT) process and contribute to endocrine resistance in breast cancer. However, the specific mechanism is unclear. It is crucial to investigate this mechanism further.

Methods: This study aimed to confirm the role of GR in breast cancer endocrine resistance. Based on our hypothesis, GR is linked to a gene involved in the EMT process, and thus contributes to endocrine resistance in breast cancer. We obtained survival data and GR expression data from Molecular Taxonomy of Breast Cancer International Consortium (METABRIC). Additionally, we gathered GR expression data from Gene Expression Omnibus (GEO). Using Cytoscape, we constructed a protein-protein interaction (PPI) network and identified key genes. Data of Vimentin, E-cad, and Wnt/β-catenin expression were obtained from The Cancer Genome Atlas (TCGA). We used the co-expression method to identify key proteins. UALCAN and cBioPortal were utilized to verify the function of the key protein.

Results: In ER+ breast cancer, GR (P=3.12780899271121E−08) and zinc finger E-box binding homeobox 1 (ZEB1) (P=1.716157E−01) were lowly expressed and down-regulated genes of GR differentially expressed genes were enriched in cell adhesion molecules. We screened for the key protein ZEB1 and found high levels of it was positively associated with prolonged recurrence-free survival (RFS) in patients receiving endocrine therapy (P=0.0024), while high levels of E-cad were negatively associated (P=0.0038). GR expression was positively associated with ZEB1 (Spearman =0.29, P=8.50e−21), negatively associated with E-cad (Spearman =−0.13, P=5.17e−5), and negatively associated with the SETD1B (Spearman =−0.14, P=1.527e−5), a gene downstream of ZEB1. In contrast, ZEB1 expression was negatively correlated with E-cad (Spearman =−0.081, P=3.132e−3) and negatively correlated with SET domain-containing 1B (SETD1B) (Spearman =−0.177, P=9.07e−11).

Conclusions: In ER+ breast cancers, GR expression is suppressed, and the EMT process is inhibited by suppressing ZEB1 expression and thus promoting E-cad expression. For the investigation of endocrine

^ ORCID: Wenhui Zhao, 0000-0003-0387-2704; Wenjie Ma, 0009-0009-5657-0356; Yuhan Tang, 0000-0003-0966-616X.

medication resistance in breast cancer, it is crucial to identify the mechanisms by how GR participates in the EMT process.

Introduction

Background

According to the latest cancer research data, breast cancer has replaced lung cancer as the most common malignant tumor worldwide (1). About 70% of diagnosed breast cancer patients are estrogen receptor (ER)-positive (ER+). Endocrine therapy that blocks the ER signaling pathway is widely used in the treatment of hormone receptor-positive breast cancer (2). However, about 30% of ER+ patients have primary resistance to endocrine therapy, and almost all advanced patients who respond to initial treatment will develop secondary resistance after a period of treatment (3). The development of endocrine resistance (either *de novo* or acquired) is a clinically challenging issue in the management of ER+ breast cancer. Although new agents have emerged in recent decades, such as CDK4/6 inhibitors [previous studies have demonstrated that cyclin-dependent kinase 4/6 (CDK4/6) inhibitors combined with endocrine therapy

are able to effectively improve the prognosis of hormone receptor positive (HR+)] (4). Also, endogenous exRNAs are also hot topics in the study of drug resistance in tumor cells (5). endocrine resistance remains an unresolved and demanding problem (6).

The response elements of glucocorticoid receptor (GR) and ER have a high degree of DNA overlap (7). Region-specific GR promoter methylation is an independent prognostic marker for patient survival and identifies a subset of patients with poor prognosis, particularly those without tamoxifen treatment (8). Liganded GR can suppress ER chromatin occupancy at shared ER-regulated enhancers, including *Cyclin D1* (*CCND1*), regardless of whether the ligand is a classic GR agonist or antagonist. The resulting GR-mediated suppression of ER+ breast cancer proliferative gene expression and cell division suggests that a selective GR modulator (SGRM) could decrease ER-driven gene expression (9). Previous studies have shown that ER, progesterone receptor (PR), androgen receptor (AR), and GR have similar properties on the promoters of target genes. The binding sequence (a couple of the binding sites) of several repeating sequences such as 5'-AGGTCA and 5'-AGAACA are separated by three nucleotides, as in IR3, an inverted repeat sequence separated by three base pairs (bps), or DR3, a direct repeat sequence separated by three bps (10,11). GR promotes the expression of *activating protein-1* (*AP1*) and *forkhead box A1* (*FoxA1*), which bind to the ER promoter and inhibit the expression of ER, indicating that there may be cross-regulation between the GR and ER pathways. However, GR appears to play opposing roles in ER+ and ER-negative (ER–) breast cancers. Treatment with physiologic concentrations of GC reduces the sensitivity of triple-negative breast cancer (TNBC) to chemotherapy both *in vitro* and *in vivo*, and the subsequent administration of a GR antagonist increases the cytotoxicity of paclitaxel, which is not induced by a GR antagonist alone (12,13). In early ER– breast cancer patients, the activation of GR is associated with the expression of a series of genes related to tumor survival and chemotherapy resistance, such as

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Highlight box

Key findings
- Glucocorticoid receptor (GR) participates in endocrine resistance by GR/ZEB1/E-cad regulation of epithelial-mesenchymal transition (EMT).

What is known and what is new?
- GR is involved in breast cancer endocrine therapy resistance by regulating the EMT process.
- GR is involved in endocrine resistance to breast cancer through the EMT-related gene, *ZEB1*. *ZEB1* is involved in the EMT process through downstream *SETD1B*.

What are the implications, and what should change now?
- We investigated the role and mechanism of GR in the EMT process and endocrine resistance of estrogen receptor positive/ human epidermal growth factor receptor-2 negative breast cancer.
- This study mostly used data from public databases, but this data may be incomplete, such as the via omission of relevant genes and proteins.

apoptosis inhibition pathways and epithelial-mesenchymal transition (EMT) pathways (7).

Glucocorticoids induce effects that are dependent upon the GR-mediated transcriptional regulation of specific genes known to play key roles in cellular/tissue functions, including growth, apoptosis, differentiation, metastasis and cell survival. Glucocorticoids, like dexamethasone, can increase the expression of *c-fms* in breast cancer cells in a laboratory setting, through a pathway that depends on the GR. This is associated with an increase in the invasiveness of the cancer. The research suggests that glucocorticoids regulate the growth and differentiation of cancerous breast cells, at least partially, by controlling the activity of the *c-fms* gene. The *c-fms* gene encodes a receptor called CSF-1 receptor (CSF-1R), which is a growth factor. Abnormal expression of *CSF-1* and *CSF-1R* is linked to higher risk of disease and poorer outcomes. On the other hand, introducing *c-fms* into normal breast cells promotes cellular invasion and growth without the need for anchorage. In a mouse model of experimental metastasis, injecting human breast cancer cells with high levels of *c-fms* into the spleen has resulted in increased tumor growth and spread compared to control breast cancer cells (14). Active *RAS* proteins activate multiple signaling pathways that regulate various cellular processes such as proliferation, differentiation, migration, metastasis, senescence, and survival. These pathways include *RAF-MAPK*, *PI3K-AKT*, *RASSF5-MST1/2*, *TIAM1-RAC*, and *ASPP1/2-p53*. These pathways are crucial for tumor development. Recent evidence suggests that GR influences *RAS*-dependent signaling and *RAS* activation. When unliganded, GR reduces *RAS* activation. However, upon ligand binding, GR dissociates from *RAS* complexes, moves into the nucleus, and triggers the activation of *RAS* and its downstream pathways. GR interacts with *RAS* and *RAF1*, as well as their associated proteins, such as members of the 14-3-3 family of adapter proteins (15).

A panel of TNBC breast cancer cell lines was treated with cortisol, which resulted in epigenetic alteration characterized by the loss of methylation on the promoter regions of tumor suppressor genes including *estrogen receptor 1* (*ESR1*) and the loss of methylation on the *long interspersed nuclear elements-1* (*LINE-1*) repetitive element, which is used as a surrogate marker for global methylation (16,17). Studies have shown that in patients with ER+ breast cancer, high GR levels can improve the recurrence-free survival (RFS) of patients, while low GR levels can shorten the RFS (18,19). Mechanistic studies have shown that in ER+/

GR+ mouse breast cancer cells, ER and GR can mutually enhance each other's chromatin openness; at the same time, the high expression of GR can induce ER activation (20,21). In the human luminal breast cancer cell line MCF-7, co-activation of ER and GR increased the expression of genes associated with cell differentiation while inhibiting the Wnt/β-catenin signaling pathway, as compared to ER activation alone (22,23). Moreover, compared with the activation of GR alone, the co-activation of GR and ER can down-regulate the expression of GR-regulated EMT-related genes and increase the phosphorylation level of β-catenin, but the specific mechanism remains unknown. Endocrine resistance to ERα confers increased invasive proliferation and induced cell proliferation to epithelial cells. ERα signaling may affect transcription factors that govern EMT. Knockout or silence the ERα and ERβ in MCF-7 breast cancer cells, respectively, lead to changes in phenotype, cellular function, transcription and protein levels of EMT epithelial markers (21). ER binding sites are not found on the target gene promoters of core transcription factors of EMT, such as *Snail*, *Slug*, *zinc finger E-box binding homeobox* 1 (*ZEB1*), and *ZEB2*, but there are GR binding sites on them, accounting for the largest number among steroid receptors (24). There is evidence that breast cancer endocrine therapy resistance may be related to EMT-related genes, such as *Slug*, *Snail*, *Twist*, and *ZEB* (12).

Our theory states that GR is connected to a gene involved in the EMT process via a gene or a pathway, and this connection is related to the endocrine resistance of breast cancer. To identify associated genes, we used bioinformatics analysis for gene enrichment analysis and retrograde correlation analysis to draw scientific conclusions. The majority of current research on the sex hormone receptors ER, PR, AR, PI3K pathway, MAPK pathway, cell cycle, and other intra- and extracellular signaling pathways is focused on the resistance of breast cancer to endocrine therapy. A significant problem in the treatment of breast cancer is figuring out the regulatory processes that lead to resistance to endocrine therapy and developing strategies to overcome them. This study aimed to confirm the role of GR and EMT in ER+/Her2– breast cancer and provide favorable evidence supporting GR as a new target of endocrine drug resistance.

Rationale and knowledge gap

GR, ER, PR, and AR all belong to the steroid receptor family and have similar structures, including a hormone-

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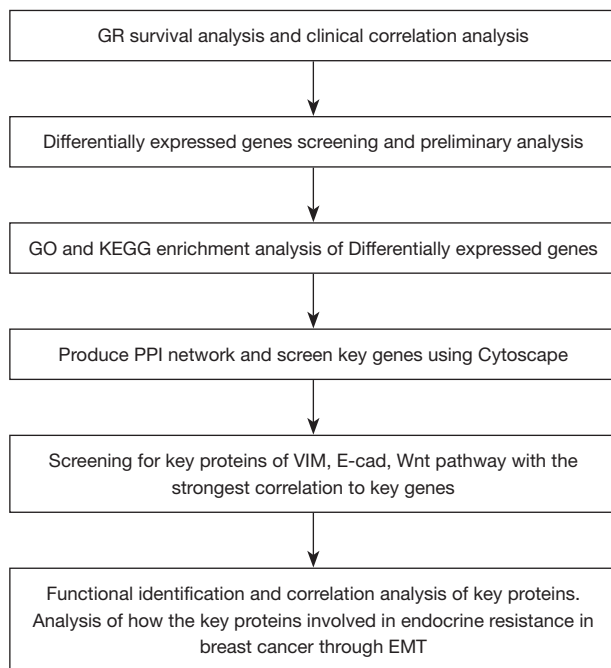Tang et al. GR regulates EMT via ZEB1 involved in endocrine resistance

Figure 1 Study flow chart. GR, glucocorticoid receptor; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction; VIM, Vimentin; EMT, epithelial-mesenchymal transition.

binding region, a DNA-binding region, and a nuclear transcription region. Different steroid receptors interact in the promoter region of target genes due to the nuclear co-localization mechanism. It has been shown that there is a cross-regulation between ER and GR, which can affect the expression of GR-regulated EMT genes. We have previously found that GR expression was significantly upregulated in recurrent or metastatic foci of endocrine-resistant breast cancer patients, and the expression of the epithelial-like cell marker E-cad was significantly downregulated, while that of the mesenchymal-like cell marker Vimentin was upregulated, suggesting the mesenchymal transformation of tumors. We hypothesized that the suppression of ER activation during endocrine therapy mediates the upregulation of GR-regulated EMT-related gene expression through Wnt/β-catenin, leading to EMT in breast cancer cells. However, the exact mechanism involved is unclear.

Objective

This study will explore possible molecular mechanisms of endocrine therapy resistance in ER+/Her2– breast

cancer and attempt to identify any targets for this pathway. We present this article in accordance with the STREGA reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1628/rc).

Methods

In step 1, we screened and analyzed genes to find the ones that are expressed differently. We repeated this process in step 2. In step 3, we performed enrichment analysis on these genes using GO and KEGG. For step 4, we created a PPI network and used Cytoscape to find important genes. In step 5, we focused on finding key proteins associated with specific pathways that have a strong correlation with the important genes. Finally, in step 6, we conducted functional identification and correlation analysis of these key proteins to understand their role in endocrine resistance (*Figure 1*).

Data acquisition and processing

We obtained ER+/Her2– breast cancer data from METABRIC (https://www.cbioportal.org/study/summary?id=brca_metabric), used the impute package for data cleaning and gap-filling, merged the *NR3C1* [*NR3C1* encodes GRs, is located on chromosome 5 (region 5q31.3) (25)] gene expression data with ER+/Her2– breast cancer patient survival data, and obtained 1,475 gene identity document (ID) numbers. We also obtained a preprocessed dataset (GSE139870) from the GEO (https://www.ncbi.nlm.nih.gov/geo/) database, which was established based on the anti-proliferative transcriptional effects of medroxyprogesterone acetate in ER+ breast cancer cells predominantly mediated by the progesterone receptor (HG-U133A) (26). This dataset contains three low GR expression samples and three high expression samples of the ER+/Her2– breast cancer cell lines. Moreover, we obtained all of the related proteins of Wnt/β-catenin, E-Cad, and Vimentin from TCGA (https://www.cancer.gov/ccg/research/genome-sequencing/tcga) and screened them using P<0.05 and correlation >0.2 (Pearson test), finally obtaining a total of 18,303 proteins. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

GR correlation analysis

We obtained the ER+/Her2– breast cancer data from METABRIC, merged the *NR3C1* (GR) gene expression data with the survival data of ER+/Her2– breast cancer

patients, drew the survival curve by binary classification of *NR3C1* (GR) expression, and observed the survival relationship between the two curves. Survival analysis was performed using the Kaplan-Meier Plotter (https://kmplot. com/analysis/index.php?p=service) to verify the OS of ER+/ Her2–. A total of 603 patients were screened to verify the *NR3C1* gene. Clinical correlation analysis was performed in UALCAN (http://ualcan.path.uab.edu/analysis.html) to observe the expression of *NR3C1* in different types of breast cancer (normal, luminal, HER2, TNBC).

Next, we obtained the uniform standardized pan-cancer dataset, TCGA Pan-Cancer (PANCAN, N=10,535), from the University of California Santa Cruz Genome Database (UCSC) (https://genome.ucsc.edu), from which we further extracted the ENSG00000139718 (*SETD1B*) gene expression data in each sample (27). We further screened the samples from solid tissue normal, primary blood-derived cancer-peripheral blood, and primary tumor, and performed log2 transformation for each expression value. Finally, we also eliminated cancer types with less than three samples and obtained the expression data for 26 types of cancer.

Screening of differentially expressed genes

We used the limma package for data preprocessing, setting P<0.05 for screening, and obtained a gene expression matrix containing a total of 1,767 genes. We then used the ggplot2, clusterProfiler, and heatmap packages and classified the original data according to the GR expression level to construct volcano maps and heat maps (28,29).

Functional enrichment and pathway analysis of differentially expressed genes

We used the ggplot2 and clusterProfiler packages to perform GO and KEGG enrichment analysis on the differentially expressed genes, selecting P<0.05 as the cutoff value. We also identified the biological processes (bps), cellular components (CCs), and molecular functions (MFs) of the differential genes. Next, we performed a KEGG pathway enrichment analysis of the differential genes to analyze the biological metabolic and identified pathways of differential gene enrichment.

PPI network construction and screening of core genes

The Search Tool for the Retrieval of Interacting Genes/ Proteins (STRING) database (https://string-db.org/) was used to evaluate PPIs in the functional protein association networks. A PPI network of differentially expressed genes was constructed using the STRING database. The node genes downloaded from the PPI network were screened according to logFC >2 (log fold change) or logFC <–2. Visualization was performed to hide the unconnected genes by Cytoscape (30). Molecular Complex Detection (MCODE) for Cytoscape to find gene functional clusters with a degree cutoff =2, Node Score Cutoff =0.2, k-core =2, and Max Depth =100 in the PPI network. Then, we filtered out the modules with scores >5 and employed CytoHubba in Cytoscape to identify the top 10 key genes (31).

Screening key proteins in Wnt/β-catenin, E-Cad, Vimentin

We obtained the E-Cad, Vimentin, and Wnt/β-catenin pathway-related proteins in breast cancer from TCGA database, set P<0.05 and correlation >0.15 for screening, and then analyzed the co-expression of the key genes and all proteins. According to the analysis results, we screened out two proteins that are both related to the core genes and have a high degree of correlation: *ZEB1* and *GLI family zinc finger 2* (*GLI2*).

Clinical data demonstration

We used UALCAN for further clinical correlation analysis. We used METABRIC to download the processed ER+/ Her2– breast cancer survival data, GR expression data, and *ZEB1* and *GLI2* expression data. Next, we drew a receiver operating characteristic (ROC) curve.

Statistical analysis

All statistical analyses were conducted using R (version 4.3). We considered P values less than 0.05 as statistically significant, using a two-sided approach. Descriptive statistics were employed to analyze data from ER-positive/ HER-2-negative breast cancer patients in TCGA, Metabric, and TCGA. Categorical variables were presented using frequencies and proportions, while continuous variables were reported as means plus standard deviations.

Results

Validation of GR's clinical relevance

A positive correlation between high GR levels and longer OS

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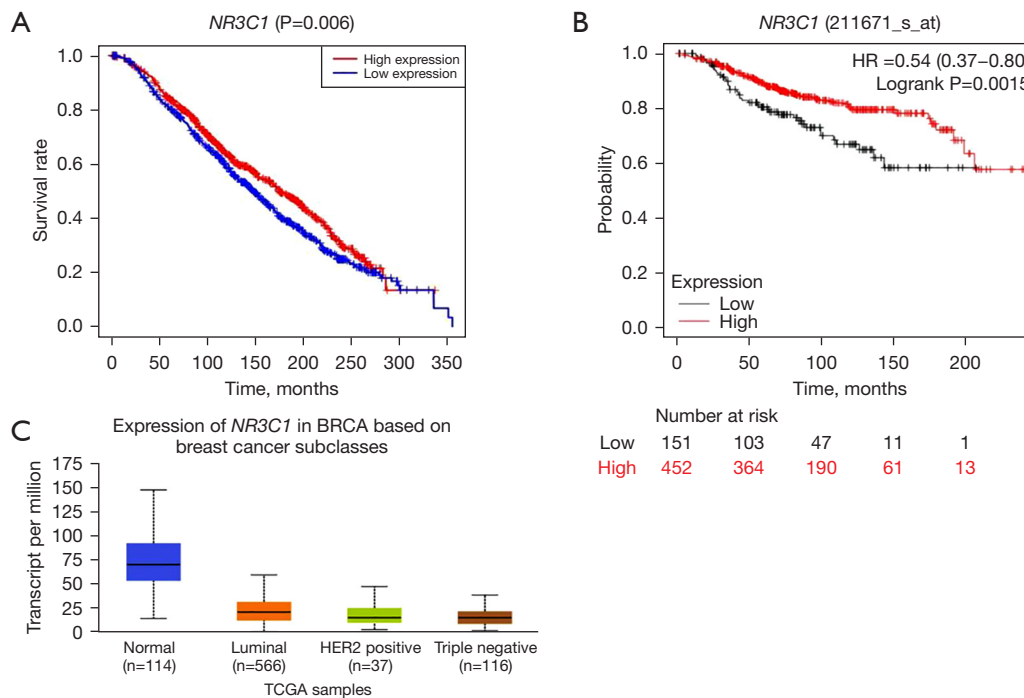

Figure 2 Expression of *NR3C1* in breast cancer. METABRIC survival analysis showed that high GR levels were positively associated with longer OS in patients with ER+/Her2− breast cancer (A). Kaplan-Meier Plotter survival analysis showed that high GR levels were positively associated with longer OS (B). Validation of GR's clinical relevance. The UALCAN clinical correlation analysis showed that GR expression was highest in patients with luminal breast cancer relative to that in patients with HER2 or TNBC, and the comparison between GR expression in TNBC, HER2, and luminal breast cancer was statistically significant (C). METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; OS, overall survival; GR, glucocorticoid receptor; HER2, human epidermal growth factor receptor 2; TNBC, triple-negative breast cancer.

was identified on analysis of *NR3C1* expression. On analysis with the Kaplan-Meier Plotter and UALCAN databases, 2,241 patients with ER+/Her2− receiving endocrine therapy and chemotherapy. The conclusions obtained were similar as those from the METABRIC database, with a positive correlation between high GR levels and longer OS.

Using UALCAN for clinical correlation analysis, we found that the GR expression was highest in patients with luminal-type breast cancer, as compared to those with HER2 and TNBC breast cancer (*Figure 2*). To further investigate the correlation between GR and the efficacy of endocrine therapy for ER+/Her2− breast cancer, we performed survival analysis using the Kaplan-Meier Plotter database for patients with ER+/HER2− breast cancer treated with endocrine therapy and screened the pathological staging of ER+/Her2− patients treated with endocrine therapy. In total, 1,174 patients were analyzed for RFS survival and 708 patients for DMFS (distant metastasis-free survival), and the results again showed that

high GR levels were positively correlated with longer RFS and DMFS (*Figure 3*).

Differentially expressed genes screening and preliminary analysis

We created volcano plots and heat maps to compare the differentially expressed genes between the low-expression group (t) and the high-expression group (n) screened from the GEO database. Analysis of the differentially expressed genes was based on the condition of P<0.05; a total of 1,767 differential genes were finally screened out and a heat map generated (*Figure 4*).

GO and KEGG enrichment analysis of differentially expressed genes

We also performed GO and KEGG enrichment analyses to study the function of the differentially expressed genes. The
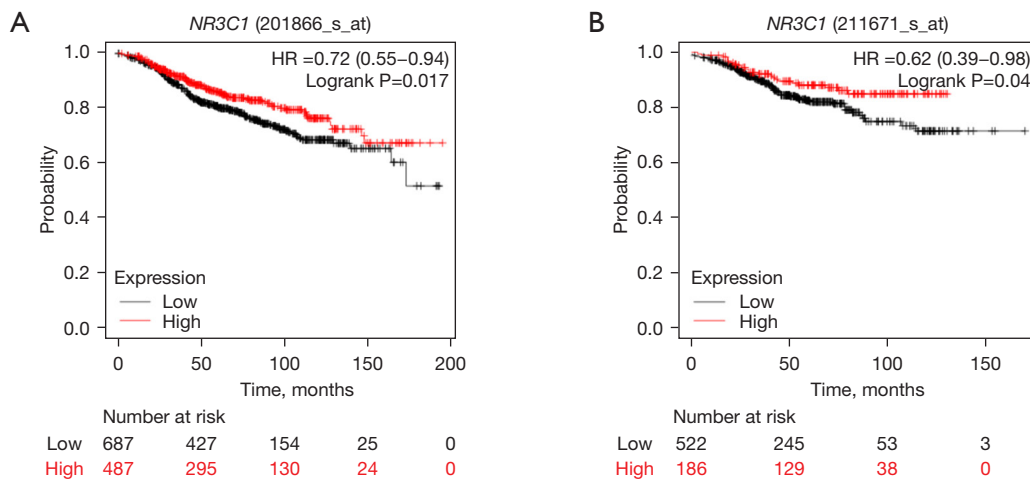
Figure 3 Prognosis of GR in patients resistant to endocrine therapy. The Kaplan-Meier Plotter survival analysis showed that (A) high GR levels were positively associated with longer RFS and (B) DMFS in ER+/HER2– breast cancer patients receiving endocrine therapy. GR, glucocorticoid receptor; RFS, recurrence-free survival; DMFS, distant metastasis-free survival; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2.
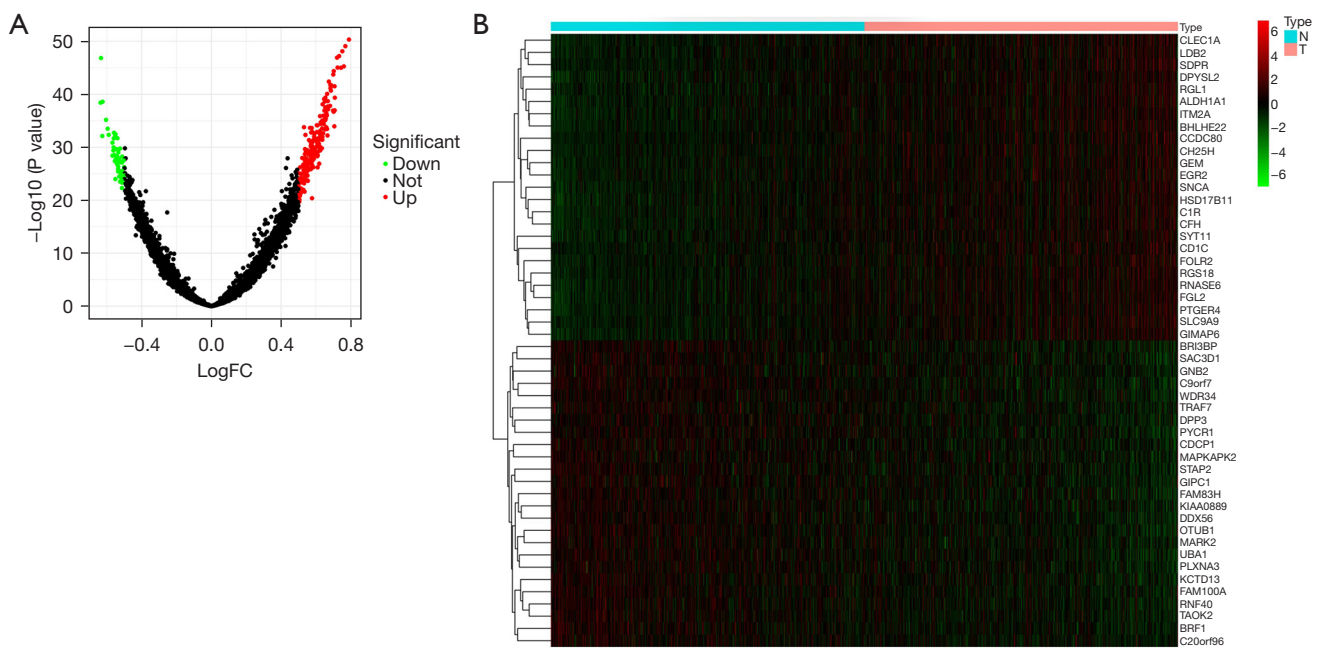

Figure 4 Screening and preliminary analysis of differentially expressed genes. GR expression was grouped according to high and low expression, and the ggplot2 and clusterProfiler packages were used to draw a volcano map and a heat map. (A) The differentially expressed genes were continuously up-regulated or down-regulated in the experimental group, and (B) there were significant differences between the high and the low expression groups of *ZEB1*. FC, fold change; GR, glucocorticoid receptor; ZEB1, zinc finger E-box binding homeobox 1.

ggplot2 and clusterProfiler packages were used to determine the enriched differential genes in terms of BP, CC, and MF, respectively. The differentially expressed genes were mainly enriched in the extracellular matrix, tumor necrosis factor signaling pathway, and PI3K signaling pathway (*Figure 5*). The down-regulated and up-regulated genes

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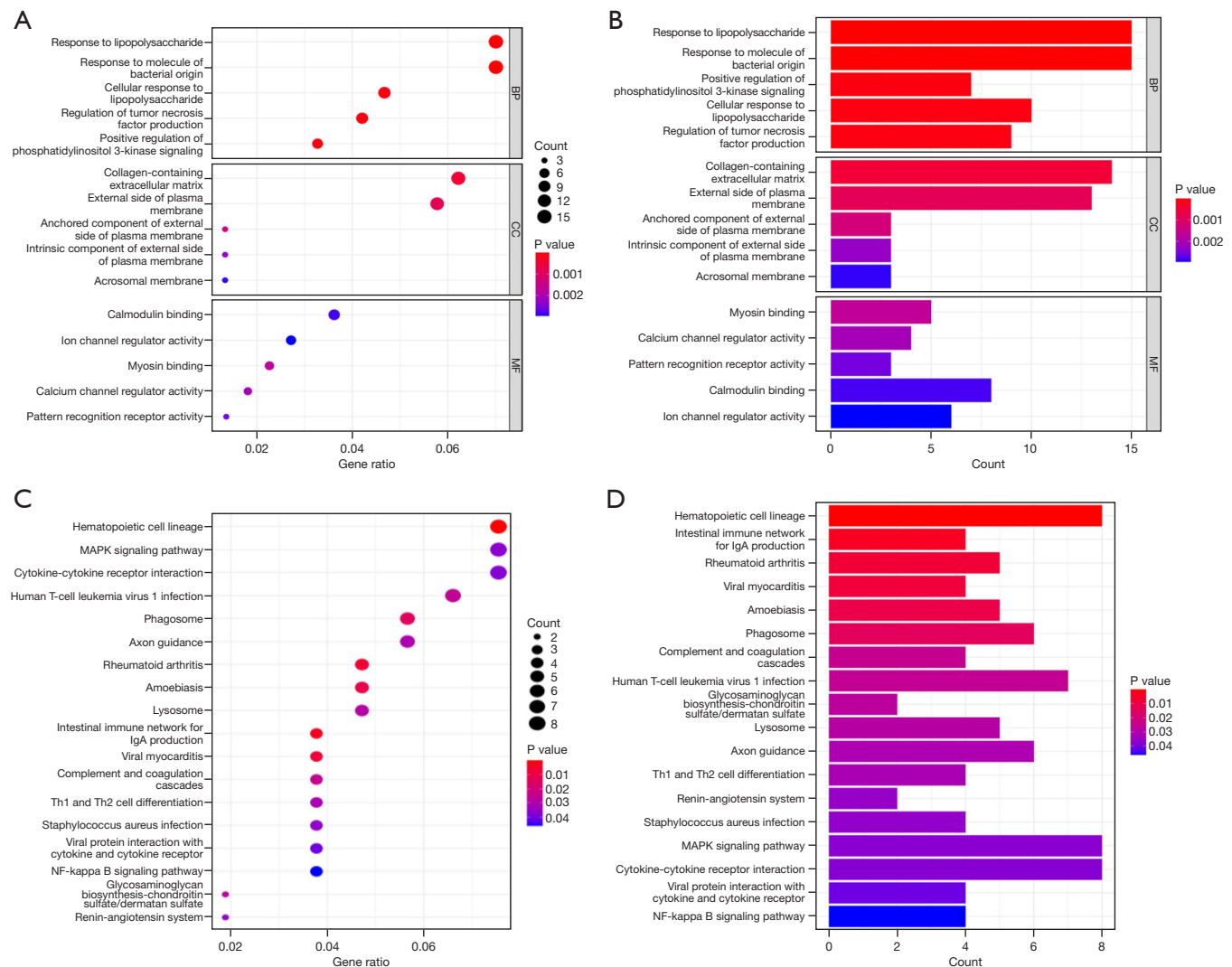Tang et al. GR regulates EMT via ZEB1 involved in endocrine resistance

Figure 5 GO and KEGG enrichment analyses of differentially expressed genes. The GO enrichment analysis of differentially expressed genes showed that in terms of the biological process, the differentially expressed genes were mainly involved in the response to lipopolysaccharide and bacteria-derived molecules, the regulation of tumor necrosis factor, and the PI3K pathway. In terms of cellular component, the differentially expressed genes were mainly enriched in the extracellular matrix and external side of the plasma membrane when performing their functions (A,B). The KEGG analysis of differentially expressed genes showed that the ordinate was the name of the KEGG metabolic pathway, the abscissa was the number of proteins in the pathway, and the differentially expressed genes were mainly enriched in inflammatory responses, complement, and the coagulation factor cascade (C,D). BP, biological process; CC, cellular component; MF, molecular function; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

were subjected to KEGG enrichment analysis, respectively, and the results showed that the down-regulated genes were mainly enriched in neuroactive ligand-receptor interactions, the PI3K pathway, cell adhesion molecules, and cytokine-cytokine receptor interactions; while the up-regulated genes were mainly enriched in cancer microRNAs including breast cancer, immune-related pathways (*Figure 6*).

Establishing a PPI network and screening for key genes

A PPI network was constructed using the STRING bioinformatics database to explore the interaction between the proteins corresponding to the differentially expressed genes. The node genes obtained from the PPI network were screened according to logFC>2 or logFC<–2, and 78
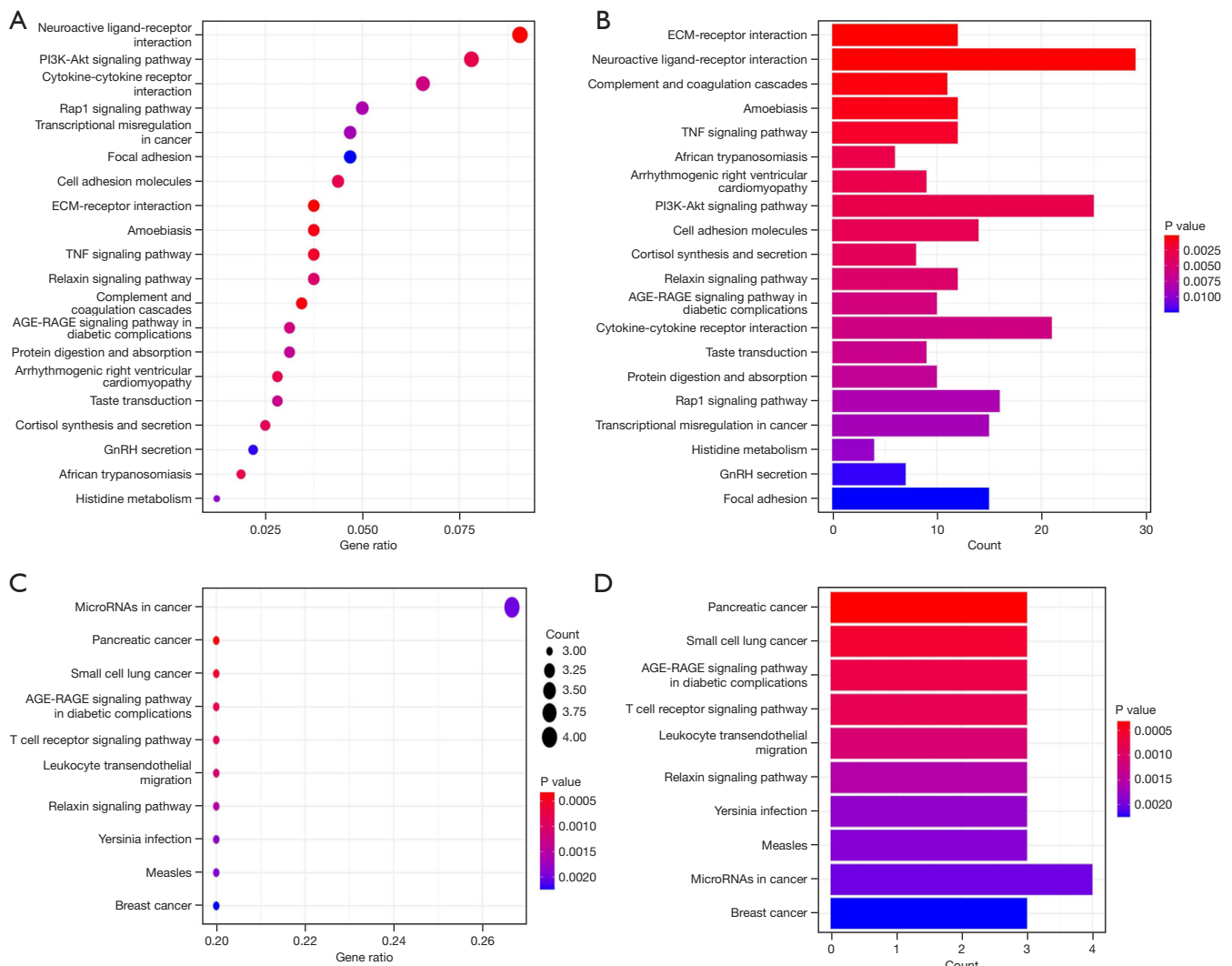
Figure 6 KEGG enrichment analysis of the up-regulated and down-regulated differentially expressed genes. The down-regulated differentially expressed genes were mainly enriched in neuroactive ligand-receptor interactions, the PI3K pathway, cell adhesion molecules, cytokine-cytokine receptor interactions (A,B); the up-regulated genes were mainly enriched in cancer microRNAs, immune-related pathways, breast cancer, etc. (C,D). KEGG, Kyoto Encyclopedia of Genes and Genomes.

differentially expressed genes were identified. Cytoscape software was employed to visualize the entire PPI network. The PPI network was drawn using node and logFC, consisting of 274 nodes and 647 edges. Cytoscape was also applied to construct a protein interaction network, and we introduced betweenness centrality (BC) to draw the graphics (*Figure 7*). The MCODE plugin in Cytoscape was used to build the functional modules; the results showed that there were three modules with a specific score >6.

The top 10 key genes were obtained by filtering and intersecting with the genes of the core module using

the topology method of the CytoHubba plugin in Cytoscape. The key genes were as follows: *CFP*, *SEMA5A*, *ADAMTS15*, *ADAMTSL1*, *ADAMTSL4*, *THSD7B*, *ADAMTS6*, and *ADAMTS13* (*Figure 8*). *ADAMTS6* suppresses tumor progression via the ERK (extracellular regulated protein kinases) signaling pathway and serves as a prognostic marker in human breast cancer (18). Germline variation in *ADAMTSL1* is associated with prognosis following breast cancer treatment in young women (19). *ADAMTSL1* and *ADAMTS6* were found to be associated with ER+/Her2– breast cancer; the *CFP*, *SPON2*, and

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SEMA5A genes had the highest core scores; and finally, five genes (*ADAMTSL1*, *ADAMTS6*, *CFP*, *SPON2*, and *SEMA5A*) were screened as key genes.

Screening for key proteins

To investigate how the GR key genes participate in the EMT process of ER+/HER2– breast cancer by regulating the epithelial marker E-cad, the mesenchymal marker Vimentin, and the core pathway Wnt/β-catenin during EMT, we downloaded all related proteins of Wnt/β-catenin, E-Cad, and Vimentin in breast cancer from TCGA, set P<0.05 and correlation degree >0.15 for screening, and obtained a total of 18,303 proteins. We then correlated the five GR key genes with these 18,303 proteins and found that the *ZEB1* and *GLI2* proteins were related to the five key genes and were highly correlated according to the analysis (*Figure 9*). *ZEB1* and *GLI2* are key proteins involved in the EMT process, and thus, we speculated that GR is involved in ER+/Her2– breast cancer EMT process through *ZEB1* or *GLI2*.

Key protein function validation and survival analysis

We performed survival and clinical correlation analyses of *ZEB1* and *GLI2* to demonstrate the function and efficacy of key proteins. In the METABRIC analysis, we screened 1,475 patients aged 30–90 years who were pathologically typed as ER+/Her2– and were receiving endocrine therapy and chemotherapy. By constructing ROC curves, we found that *ZEB1* (AUC =0.633) was more clinically relevant than that of *GLI2* (AUC =0.552). We considered that *ZEB1* is an EMT core transcription factor that could directly repress E-cad expression and participate in the EMT process. Validation using the UALCAN database on 833 breast cancer patients in TCGA revealed that *ZEB1* was most expressed in ER+/HER2– breast cancers, as compared to other types of breast cancer.

Further validation using the Kaplan-Meier Plotter database identified 1,174 patients with ER+/Her2– breast cancer who were receiving endocrine therapy. The results showed a positive correlation between high *ZEB1* levels and longer RFS in patients with ER+/Her2– breast cancer treated with endocrine therapy, and a negative correlation between high *CDH1* levels in ER+/Her2– breast cancer patients receiving endocrine treatment and longer RFS. Also, *NR3C1* expression was positively correlated with *ZEB1* expression and negatively correlated with *CDH1*

expression (*Figure 10*).

ZEB1 participates in the EMT process through its downstream SETD1B

The EMT transcription factor, *ZEB1*, alters the epigenetic landscape of colorectal cancer cells (20). A positive feedback loop between *ZEB1* and its downstream *ZEB1* in colorectal cancer has bene identified that maintains the EMT regulatory state. We further analyzed *ZEB1*, the gene downstream of *ZEB1*. TCGA showed that the expression of *ZEB1* was significantly upregulated in breast cancer, and the cBioPortal showed that the expression of the *ZEB1* mutant type was higher than that of the wild type in breast cancer. These results suggest that *ZEB1* may be associated with breast cancer. We also used the Kaplan-Meier Plotter database to perform a survival analysis of *ZEB1* expression in ER+ breast cancer patients receiving endocrine therapy and screened 1,174 patients with a pathological staging of ER+/Her2– receiving endocrine therapy. The results showed that high *ZEB1* levels were positively associated with longer RFS in patients with ER+/Her2– breast cancer treated with endocrine therapy.

Correlation analysis revealed that *SETD1B* expression was negatively correlated with *ZEB1* expression, positively correlated with *CDH1* expression, and negatively correlated with *NR3C1* expression in ER+ breast cancer treated with endocrine therapy (*Figure 11*), which is opposite to the role of *ZEB1* in colorectal cancer. Considering that *ZEB1* plays a contrasting role in ER+ breast cancer relative to colorectal cancer, GR may affect EMT by regulating its downstream *ZEB1* through *ZEB1*.

Discussion

Key findings

GR is involved in endocrine therapy resistance in breast cancer

GR has a similar structure to ER, PR, and AR. Previous studies have shown that the response elements of GR and ER have highly overlapping DNA sequences, and there is cross-regulation between ER and GR (12,13). Research has also found that GR may regulate the EMT process through the Vimentin, E-cad, and Wnt/β-catenin pathway and thus be involved in secretory drug resistance in breast cancer (19). In ER+ early-stage breast cancer patients, the high expression of GR is associated with improved RFS. This is
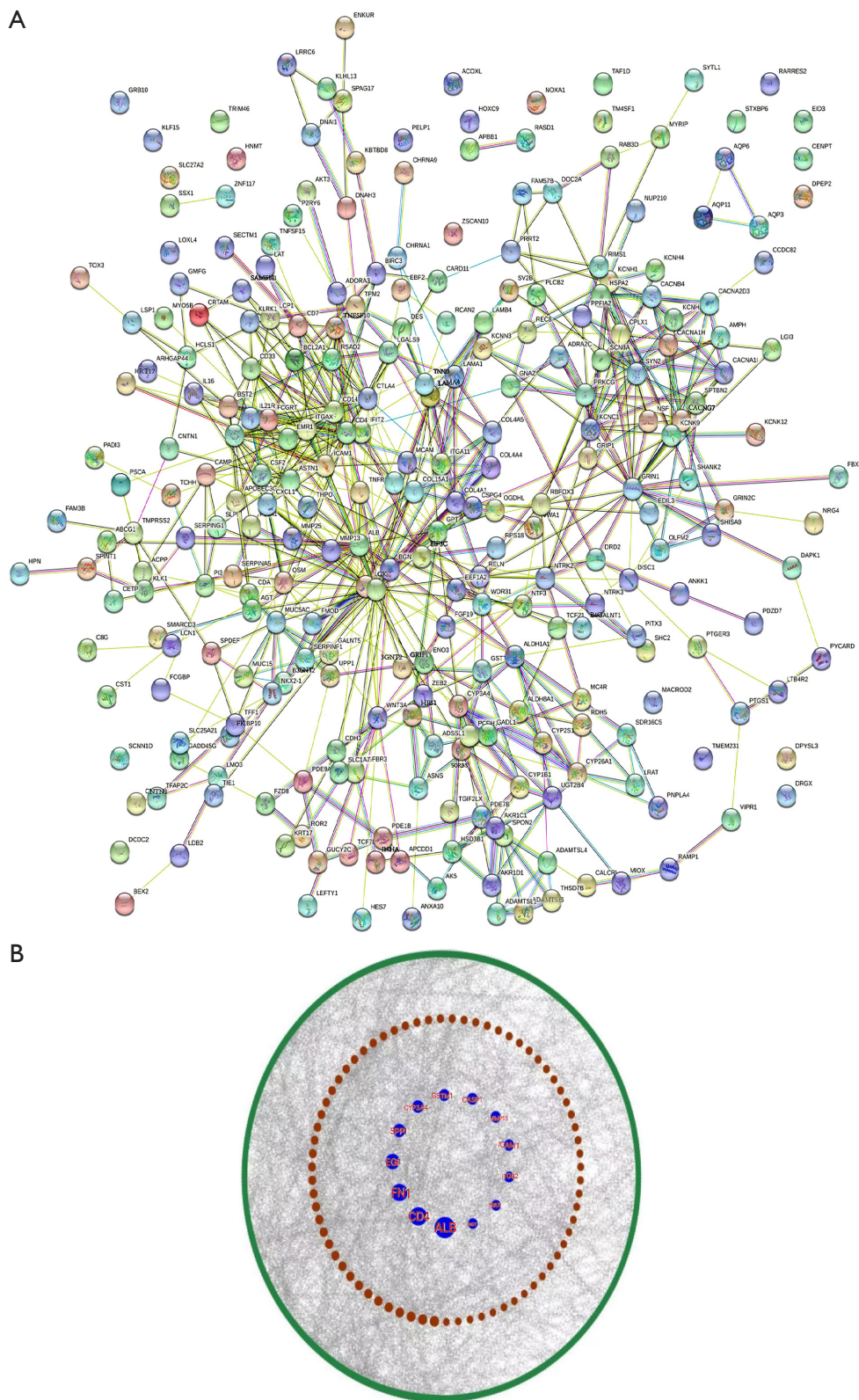
A

B

Figure 7 PPI network. A PPI network containing 275 genes was constructed using the Search Tool for the Retrieval of Interacting Genes/ Proteins database. It consisted of 274 nodes and 647 edges (A,B). PPI, protein-protein interaction.
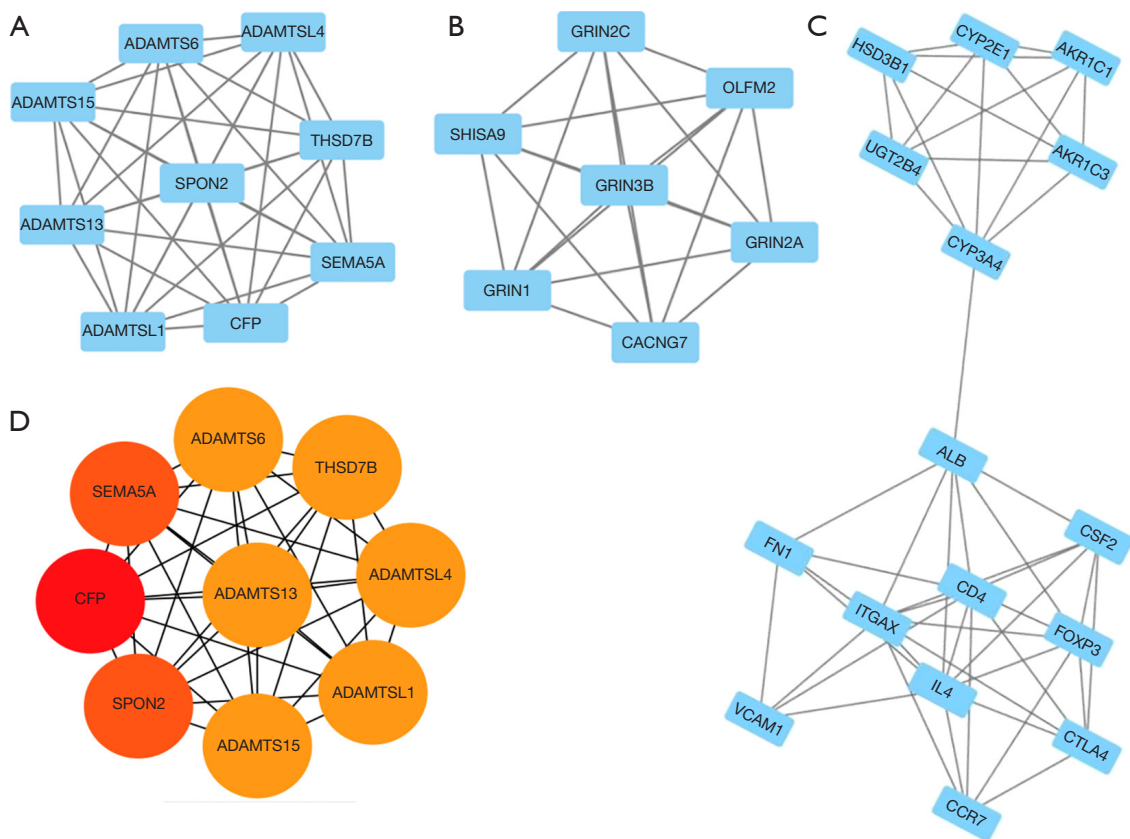
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Figure 8 Screening of key genes. Cytoscape software was used to visualize the entire PPI, draw the PPI network using node and logFC, and introduce BC to draw the protein interactions. The MCODE plugin in Cytoscape was used to build the functional modules. The results showed that three modules had a specific score >6 (A-C). The core genes of the three modules were *ADAMTSL1*, *GRIN2A*, and *ITGAX*, and the genes filtered by the CytoHubba plugin in Cytoscape were intersected with the genes of the three core modules to obtain the top 10 key genes (D). PPI, protein-protein interaction; BC, betweenness centrality; MCODE, Molecular Complex Detection; FC, fold change.

consistent with the positive correlation between high GR levels and longer OS in ER+/Her2− breast cancer patients observed in our study, the improvement of OS in ER+/Her2− breast cancer patients by GR, and the improvement of RFS and DMFS in ER+/Her2− breast cancer patients treated with endocrine therapy by GR.

GR is involved in breast cancer endocrine therapy resistance by regulating the EMT process
Previous studies have shown that the cross-regulation between ER and GR can influence the expression of GR-regulated EMT genes and that GR may be involved in breast cancer endocrine resistance by regulating the EMT process through the mesenchymal marker Vimentin, the epithelial marker E-cad, and the Wnt/β-catenin pathway (18,19). This is consistent with the results of the retrograde correlation analysis between the core genes screened in this

study and genes related to the Vimentin, E-cad, and Wnt/β-catenin pathways in ER+/Her2− breast cancers.

GR may mediate endocrine resistance to breast cancer via the EMT-related gene, ZEB1
Previous studies have shown that endocrine therapy resistance in breast cancer is associated with EMT-related genes, such as Slug, Snail, Twist, and ZEB. *ZEB1* is overexpressed in breast cancer, regulates cell adhesion and polarity, regulates chemoresistance and radioresistance, and promotes breast cancer stem cell generation (21-24). *ZEB1* expression in a miR (microRNA)-200-dependent or miR-200-independent manner leads to E-cad transcriptional silencing and cancer progression.

This is consistent with the results of the retrograde correlation analysis of the GR core gene with Vimentin, E-cad, and Wnt/β-catenin pathway genes in this study.
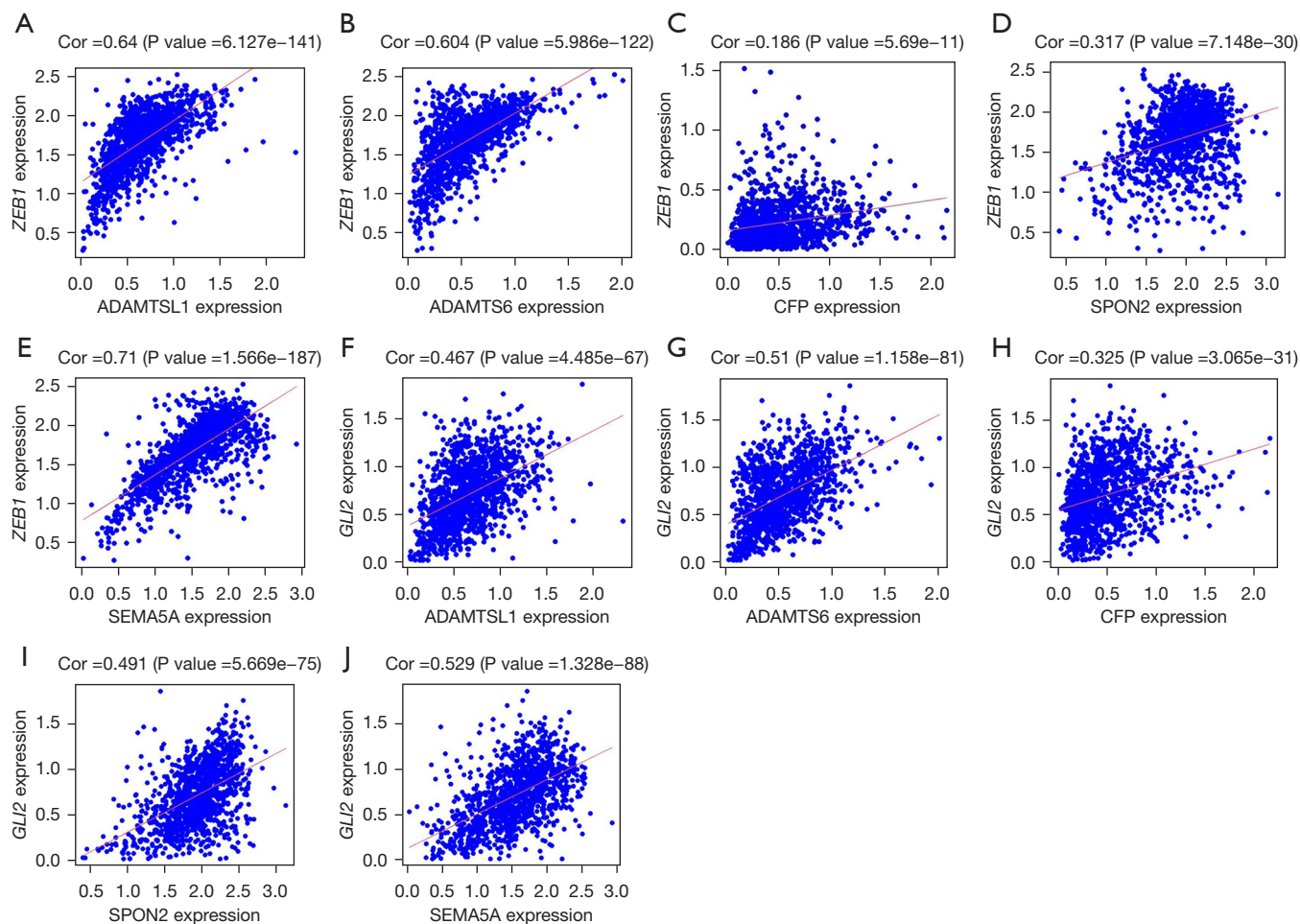
Figure 9 Screening of key proteins. Correlation of ZEB1 with five key GR proteins (A-E), GLI2 with five key GR proteins (F-J). ZEB1, zinc finger E-box binding homeobox 1; GR, glucocorticoid receptor.

GR and *ZEB1* were lowly expressed in ER+ breast cancer. Through further correlation analysis of *ZEB1*, we found that GR was positively correlated with *ZEB1* expression, negatively correlated with *ZEB1* expression, a downstream protein of *ZEB1*, and negatively correlated with E-cad. In contrast, *ZEB1* expression was negatively correlated with E-cad and negatively correlated with *ZEB1*. High *ZEB1* levels in ER+/Her2– breast cancer treated with endocrine therapy were positively correlated with longer RFS in ER+/Her2– breast cancer treated with endocrine therapy, and high *CDH1* levels were negatively correlated with longer RFS in ER+/Her2– breast cancer treated with endocrine therapy. This is consistent with the speculation that GR may inhibit the expression of *ZEB1*, via GR/ZEB1. Notably, it is known that *ZEB-1* is a transcriptional regulator of epithelial-mesenchymal transformation which translates to the more malignant behavior of cancer and therefore shortened survival. We hypothesize that there may be a negative feedback mechanism between GR and *ZEB1* in ER+ breast cancer, and that when *ZEB1* is highly expressed, it will suppress GR expression and ultimately inhibit the EMT process, improving patient prognosis. Recently, a study showed that hsa-miR-497-3p controls EMT in TNBC cells by targeting *ZEB1*. This was proven by the changes in the expression of *vimentin*, *N-cadherin*, *and E-cadherin* due to *ZEB1* inhibition. The study suggests that hsa-miR-497-3p can reduce the growth and invasion of TNBC cells by regulating EMT through *ZEB1* targeting (32).

ZEB1 may be involved in the EMT process through its downstream gene SETD1B

Previous studies have shown that there is a positive

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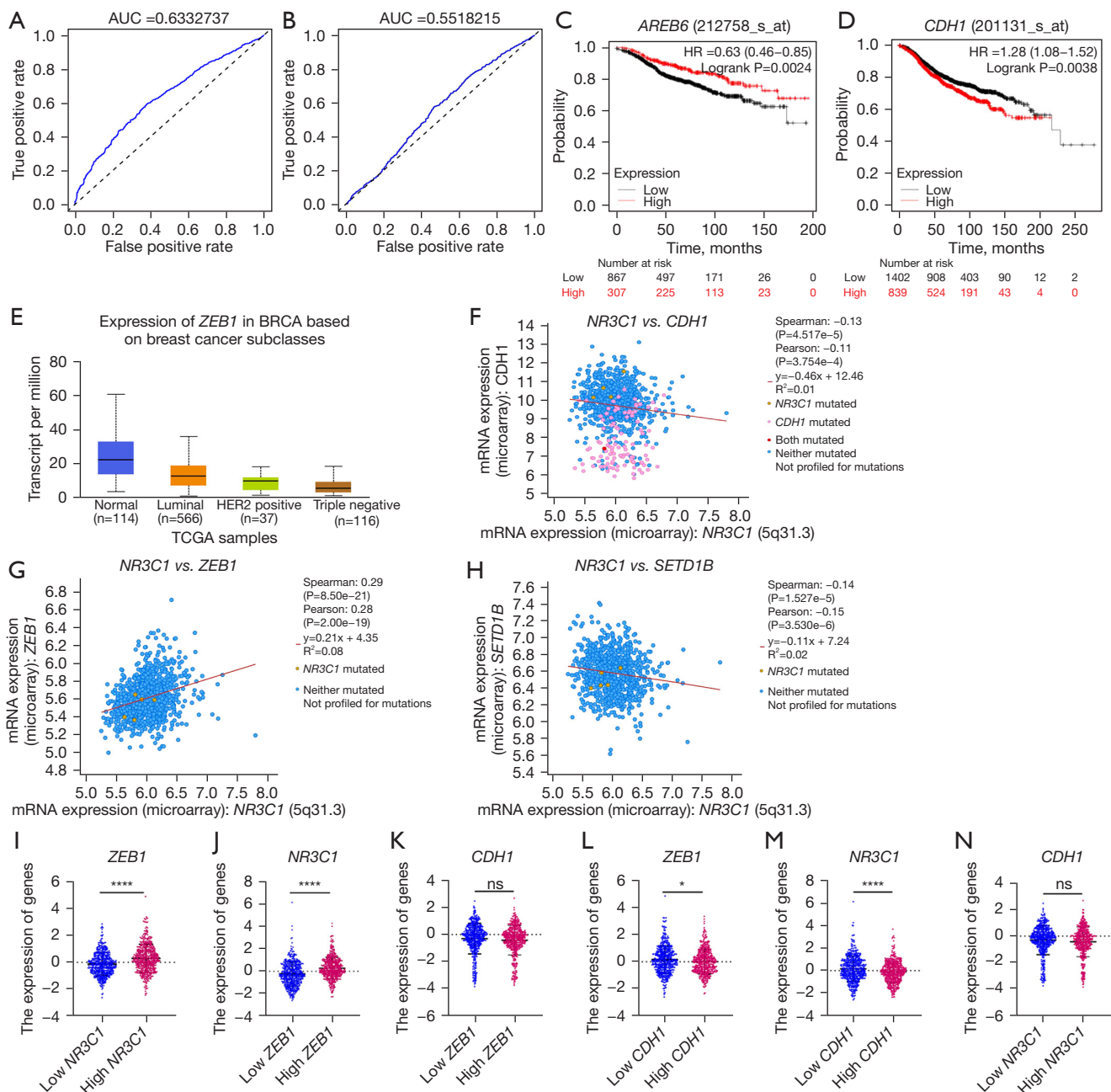Tang et al. GR regulates EMT via ZEB1 involved in endocrine resistance

Figure 10 Key protein function validation and survival analysis. The METABRIC ROC curve showed that the area under the curve of *ZEB1* (AUC =0.633) (A) was higher than that of *GLI2* (AUC =0.552) (B). The Kaplan-Meier Plotter database showed that high *ZEB1* levels were positively correlated with longer RFS in patients with ER+/Her2− breast cancer treated with endocrine therapy (C), and high *CDH1* levels were negatively correlated with longer RFS in patients treated with endocrine therapy for ER+/Her2− breast cancer (D). For clinical correlation and survival analyses of *ZEB1*, the UALCAN database showed that *ZEB1* expression was higher in luminal-type breast cancer than in other types of breast cancer (E), the cBioPortal showed that *NR3C1* negatively correlated with *CDH1* expression (F), expression was positively correlated with *ZEB1* expression (G), and negatively correlated with *SETD1B* expression (H). The Gradph prism software showed that the expressions of *NR3C1* and *ZEB1* were positively correlated (I,J), *ZEB1* and *CDH1* expressions were negatively correlated (K,L), and *NR3C1* and *CDH1* expressions were negatively correlated in ER-positive breast cancer treated (M,N) (*, P<0.05; ****, P<0.0001; ns, non-specific). METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; ROC, receiver operating characteristic; ZEB1, zinc finger E-box binding homeobox 1; RFS, recurrence free survival; AUC, area under curve; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2.
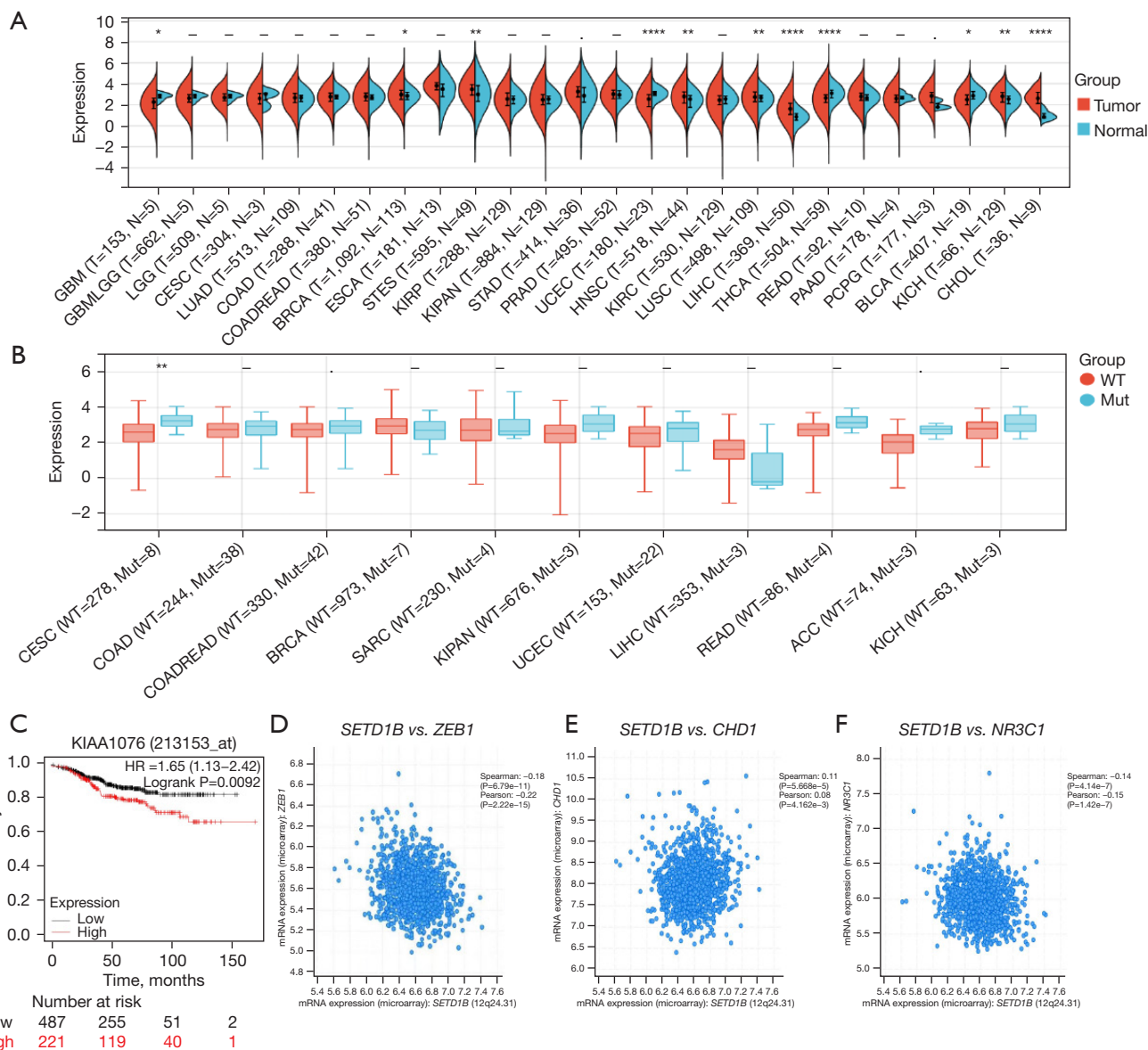
Figure 11 SETD1B function validation. TCGA showed that the expression of *ZEB1* was significantly upregulated in breast cancer (A); the cBioPortal showed that the expression of the *ZEB1* mutant type was higher than that of the wild type in breast cancer (B) (*, P<0.05; **, P<0.01; ****, P<0.0001; –, non-specific). Using the METABRIC database for analysis, high *SETD1B* levels were negatively correlated with longer RFS in patients with ER+/Her2– breast cancer treated with endocrine therapy (C), and correlation analysis revealed that *SETD1B* expression was negatively correlated with *ZEB1*, positively correlated with *CDH1* expression, and negatively correlated with *NR3C1* expression in ER-positive breast cancer treated with endocrine therapy (D-F). TCGA, The Cancer Genome Atlas; ZEB1, zinc finger E-box binding homeobox 1; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; RFS, recurrence-free survival; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2.

feedback loop between *ZEB1* and its downstream gene *ZEB1*, maintaining the EMT regulatory state in colorectal cancer. This is consistent with our finding that *ZEB1* gene expression is correlated with E-cad and *ZEB1* in the present study. However, we also found that the negative regulatory relationship between *ZEB1* and *ZEB1* in ER+ breast cancer is different from the positive feedback regulatory relationship between them in colorectal cancer. Considering

that *ZEB1* plays an opposite role in ER+ breast cancer that that in colorectal cancer, GR may affect EMT through *ZEB1* regulation of its downstream gene *ZEB1*.

In the tumor microenvironment, *ZEB1* has been found to play an increasingly important role. The study has shown that *ZEB1* is a key factor in the tumor microenvironment and for maintaining tumor-associated macrophages (TAMs)' tumor-promoting functions (33). The fibrotic microenvironment promotes the spread of tumor cells to the lungs by mediating the ZEB1-AS1/miR-200b-3p/ZEB1 signaling pathway (34). Removing *ZEB1* reduces the ability of breast cancer cells to grow, spread, and change their characteristics. Additionally, we have shown that endothelial cells derived from the tumor microenvironment provide the *Notch ligand Jagged1* (*Jag1*) to neighboring breast cancer stem cells through direct cell-cell contact. This leads to an increase in *ZEB1* levels through the Notch1 pathway. In response, tumor cells with ectopic *ZEB1* produce more *VEGFA* and induce *Jag1* in endothelial cells in a paracrine manner. Removing *ZEB1* disrupts this positive feedback loop in the tumor perivascular niche, which ultimately reduces tumor growth and progression both *in vivo* and *in vitro* (35). A study observed that the conditioned medium from ZEB1-expressing MDA-MB-231 cells increased the formation of capillary tubes in human umbilical vein endothelial cells (HUVECs). On the other hand, when ZEB1 was knocked down using RNA interference, it had the opposite effect. ZEB1 also increased the expression of vascular endothelial growth factor A (*VEGFA*) at both the mRNA and protein levels. When HUVECs were pre-incubated with an anti-VEGFA neutralized antibody, it reduced the tube formation caused by *ZEB1*. In breast cancer tissues, the expression of *ZEB1* was positively correlated with the expression of *VEGFA* and *CD31*. At the molecular level, *ZEB1* activated the transcription of *VEGFA* by increasing the recruitment of *SP1* to its promoter, which was mediated through the activation of the PI3K and p38 pathways. In a nude mouse xenograft model, study demonstrated that elevated expression of *ZEB1* promotes tumorigenesis and angiogenesis in breast cancer (36). *ZEB1* has significant potential in tumor microenvironment and endocrine therapy, which brings new opportunities for developing targeted agents.

If the role of the cross-regulation between ER and GR in endocrine therapy resistance of breast cancer is confirmed, GR is expected to become a new target for breast cancer endocrine therapy and will provide a novel treatment idea for endocrine-resistant breast cancer patients.

This study explored the molecular mechanisms of ER+/Her2– endocrine therapy resistance via the effects of GR on EMT. This study is important for identifying potential strategies to combat resistance in ERα+/Her2– breast cancer to endocrine therapy, and suggests a new therapeutic target for improving the clinical prognosis and minimizing medical costs for breast cancer patients.

Since continuous cell and clinical experiments have not been carried out to verify this hypothetical GR/ZEB1/E-cad pathway, more rigorous data supporting this concept is lacking, and cell experiments in ER+/Her2– breast cancer cell line mice will be required in the future. This study mostly used data from public databases, which may be incomplete, such as via the omission of relevant genes and proteins. Exploring the potential mechanism of how GR affects endocrine therapy is crucial for the targeted creation of effective treatment options, enhancing patient resistance to treatment, and extending the duration of time before a relapse occurs.

Implications and actions needed

This study is important for overcoming the common clinical phenomenon of drug resistance in ERα+/Her2– breast cancer endocrine therapy, and suggests a new therapeutic target for improving the clinical prognosis and minimizing medical costs for breast cancer patients. However, more rigorous data supporting this concept is lacking, and cell experiments in ER+/Her2– breast cancer cell line mice will be required in the future.

Conclusions

In this study, we first validated the involvement of GR in endocrine resistance in breast cancer. We screened *ZEB1*, a key protein of GR associated with the EMT process in ER+/Her2– breast cancer, and found that high *ZEB1* levels were positively correlated with longer RFS in ER+/Her2– breast cancer patients receiving endocrine therapy, and high *CDH1* levels were negatively correlated with RFS in patients receiving endocrine therapy for ER+/Her2– breast cancer. Also, *NR3C1* expression was positively correlated with *ZEB1* expression, negatively correlated with *CDH1* expression, and negatively correlated with *ZEB1*, the downstream gene of *ZEB1*. High *ZEB1* levels were negatively correlated with RFS in ER+/Her2– breast cancer receiving endocrine therapy. Furthermore, *ZEB1* expression was negatively correlated with *ZEB1*, while *CDH1*

expression was positively correlated with *CDH1* expression and negatively correlated with *NR3C1* expression. The role of *ZEB1* in the endocrine resistance of ER+/Her2− breast cancer was verified, further validating the involvement of GR in the endocrine resistance of breast cancer by regulating the EMT process.

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Footnote

Reporting Checklist: The authors have completed the STREGA reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1628/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1628/coif). A.Y. received consulting fees from Daiichi Sankyo and honoraria from Asahi ELLES, AstraZeneca, Chugai, Daiichi Sankyo, Eisai, Eli Lilly, Kyowa Kirin, Nihon Medi-Physics, Pfizer, Taiho and Takeda. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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