GRHL2 Expression Functions in Breast Cancer Aggressiveness and Could Serve as Prognostic and Diagnostic Biomarker for Breast Cancer

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ABSTRACT

BACKGROUND: Breast cancer (BC) is the most frequent malignancy in women worldwide and the leading cause of female cancer–associated death in the world. Grainyhead-like 2 (*GRHL2*) is an important gene involved in human cancer progression. However, the role of GRHL2 in BC is unknown.

METHODS: In this study, we used in vitro experiments to verify the role of *GRHL2* expression in BC progression. We used 14 databases to analyse the expression level of GRHL2 in BC and its prognostic and diagnostic value. In addition, the correlation between GRHL2 expression and immune cell infiltration and DNA methylation was also analysed.

RESULTS: At the cellular level, overexpression of *GRHL2* induced E-cadherin expression in BC cells with a mesenchymal phenotype and resulted in a hybrid epithelial/mesenchymal (E/M) phenotype, which is more strongly correlated with tumour aggressiveness than a pure mesenchymal phenotype. Through analysis of various databases, we found that tumour tissue had a higher expression level of *GRHL2*. High expression of *GRHL2* was associated with worse prognosis of BC patients and indicated that GRHL2 had significant diagnostic value. Grainyhead-like 2 is also related to immune infiltration and regulated by DNA methylation. Furthermore, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses showed that *GRHL2*-related signalling pathways in BC were related to tumour cell proliferation, invasion, and angiogenesis.

CONCLUSIONS: In summary, evidence indicates that *GRHL2* can be used as a prognostic and diagnostic biomarker for BC.

KEYWORDS: GRHL2, breast cancer, EMT, immunotherapy, tumour aggressiveness

Introduction

Breast cancer (BC) is a global public health problem. It is currently the most common tumour in the world, surpassing lung cancer in 2020 with 2.26 million new BCs, and it is also the main cause of cancer deaths in women worldwide.[1,2] The improved mammography screening accuracy has led to a 20% to 40% overall reduction in BC mortality.[3] However, there is still a need for other ways to improve the diagnosis and survival rate of BC.

Grainyhead (GRH), the first member of the grainyhead-like (GRHL) transcription factor family, was found in the fruit fly *Drosophila melanogaster*.[4] Grainyhead-like 1 (GRHL1), grainyhead-like 2 (GRHL2), and grainyhead-like 3 (GRHL3) are 3 members of the grainyhead-like (GRHL) family of transcription factors found in mammals. In some studies, *GRHL* transcription factors were considered tumour suppressors.[5,6] However, under other conditions, they show carcinogenic activity. Grainyhead-like 2 factors are involved in many biological processes, including tumour epithelial–mesenchymal transition (EMT), invasion, and metastasis. Decreased *GRHL1* and *GRHL3* gene expression increases skin cancer risk.[7,8] Grainyhead-like is also a member of the GRHL family. The regulatory effect of GRHL2 in tumorigenesis and development is different in different types of cancer. For example, in BC, overexpressed GRHL2 was reported to induce apoptosis resistance by modulating death receptor ligands.[9] Conversely, it has been suggested that GRHL2 has a tumour suppressor role in gastric and colorectal cancer cells.[10,11] However, the efficacy of GRHL2 as a potential cancer prognostic biomarker has not been fully elucidated.

In the process of tumour metastasis, it is well known that cells with EMT lose their cell–cell adhesion and acquire migration and invasive properties to invade the basement membrane and enter blood vessels as circulating tumour cells (CTCs).[12] These CTCs flow with blood and usually undergo mesenchymal–epithelial transition (MET) to settle down at distant organs. However, the tumour metastasis process is very complicated. Epithelial–mesenchymal transition and MET are not

'all-or-none' processes. Epithelial–mesenchymal transition and MET themselves are also not enough for the accomplishment of tumour metastasis. Recently, a partial EMT or hybrid epithelial/mesenchymal (E/M) phenotype has been increasingly recognized, and it occurs between EMT and MET transition. The cells with this phenotype have mixed expression of epithelial and mesenchymal traits.[13,14] When compared with pure mesenchymal features, the hybrid E/M phenotype corresponds to higher invasive and metastatic potential and predicts worse outcomes regardless of BC subtype.[15]

A variety of cells make up the tumour microenvironment (TME). Infiltrating immune cells – such as tumour-associated macrophages (TAMs), B cells, CD8^{+} T cells, CD4^{+} T cells, neutrophils, natural killer (NK) cells, and dendrite-shaped cells (DCs) – make up a significant fraction of these cells.[16] In recent years, immunotherapy targeting the interaction between immune cells and tumour cells has been introduced to the clinical field, but only a limited number of cancer patients with certain molecular characteristics respond well to current immunotherapy.[17] Immune-related genes may influence the prognosis of cancer patients by altering the abundance of invading immune cells in several biological processes.[18] Therefore, exploring *GRHL2*-related immune cells could contribute to finding new therapeutic targets.

So far, GRHL2 has been poorly studied in BC. Therefore, in this study, we aimed to assess the role of GRHL2 in BC progression and investigate the potential mechanism of impact. We also attempted to determine whether GRHL2 has important implications for the prognosis of BC.

Materials and Methods

Cells

The human BC cell lines MDA-MB-231, MCF-7, 293T, and MCF10A were obtained from the American Type Culture Collection. Foetal bovine serum (FBS) was purchased from Invitrogen (Waltham, MA, USA). The Dulbecco Modified Eagle Medium (DMEM) was obtained from KeyGEN BioTECH (Jiangsu, China). All cells were cultured in DMEM supplemented with 10% FBS at 37°C and 5% CO_2 in an incubator.

Plasmids and transfection

Overexpression, shRNA, and negative control plasmids for GRHL2 were constructed by GeneCopoeia (Guangzhou, China) and used to transfect MDA-MB-231 and MCF-7 cells using Lipofectamine 2000 (Invitrogen). Puromycin (Sigma, St.Louis, USA) was used to screen stably transfected cells.

Western blot analysis

Protein was extracted from the cell lysate and electrophoresed, membrane with polyvinylidene difluoride (PVDF) membrane for 90 minutes, blocked in 5% skimmed milk powder for 1 hour. The primary antibody was added, and the membrane was incubated on a shaker at room temperature for 1 hour and overnight at 4°C. On the second day, the corresponding secondary antibody was added and incubated at room temperature for 2 hours. The grey values of the protein bands were analysed with ImageJ. Primary antibodies against GRHL2 (1:500, Sigma) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000, Santa Cruz Biotechnology, Dallas, TZ, USA) were used according to the manufacturers' guidelines. Rabbit or mouse horseradish peroxidase (HRP)–conjugated secondary antibodies diluted with antibody diluent at a ratio of 1:1000 were purchased from Santa Cruz Biotechnology.

Wound-healing assay

The wound-healing experiment was carried out according to Li et al.[19]

Invasion assay and migration assays

Cell invasion and migration abilities were detected using a Transwell assay (Corning Inc., Corning, NY, USA). The cells were digested, and a serum-free cell suspension adjusted to 5×10^4 cells/mL was prepared. Twenty microliters of Matrigel were placed in the Transwell chamber for the invasion experiment. Matrigel was not used for migration experiments. Five hundred microliters of complete medium were added to the upper chamber of the 24-well plate and 200 μL of serum-free cell suspension to the lower chamber. After 24 hours of culture in the migration experiment and 48 hours in the invasion experiment, the cells were fixed with cold methanol for 20 minutes and stained with crystal violet for 30 minutes. Five visual fields in the chamber were randomly selected and imaged using a Nikon Eclipse TS100 microscope. The assay was independently performed in triplicate.

Immunofluorescence staining

When the cells were overgrown to 70% confluence on the slide, they were removed and fixed with cold methanol for 30 minutes, permeabilized with 1% Triton for 30 minutes, washed 3 times with phosphate-buffered saline (PBS), and blocked with 5% FBS for 30 minutes. The slides were incubated with primary antibodies against E-cadherin (1:200, Cell Signaling Technology, Danvers, MA, USA) and vimentin (1:200, Santa Cruz Biotechnology) overnight at 4°C. The next day, the slides were rewarmed at room temperature for 1 h, and the secondary antibody conjugated with fluorescent dyes was incubated with the slide at 37°C for 1 h in darkness. After counterstaining with 4′,6-diamidino-2-phenylindole (DAPI), 20× images were acquired using a Nikon Eclipse TS100 microscope (Nikon Corporation, Tokyo, Japan). The mean fluorescence intensity was detected by ImageJ software.

Database

The following databases were used: Oncomine (www. oncomine.org),[20] TIMER (http://cistrome.org/TIMER/),[21] CCLE (https://portals.broadinstitute.org/ccle/about),[22] GEPIA (http://gepia.cancer-pku.cn/),[23] The Human Protein Atlas (HPA https://www.proteinatlas.org/),[24] UALCAN (http://ualcan.path.uab.edu/),[25] TCGA database (https://www.cancer.gov/tcga), Kaplan–Meier Plotter (https://kmplot.com/analysis/),[26] PrognoScan (http://dna00.bio.kyutech.ac.jp/PrognoScan/index.html),[27] LinkedOmics (http://www.linkedomics.org/),[28] Metascape (http://metascape.org/),[29] cBioPortal (https://www.cbioportal.org/),[30] DiseaseMeth version 2.0 (http://bio-bigdata.hrbmu.edu.cn/diseasemeth/),[31] MEXPRESS (https://mexpress.be),[32] and MethSurv (https://biit.cs.ut.ee/methsurv/)[33] (see Supplementary Materials for instructions on using the databases).

Statistical analysis

SPSS 25.0 (SPSS Inc., USA) was used to perform statistical analysis of the obtained data. A receiver operating characteristic (ROC) curve was generated to evaluate the diagnostic value expressed by GRHL2, and the area under the curve represents the diagnostic value. $P < .05$ was considered statistically significant.

Results

GRHL2 mRNA expression across cancers

To determine the expression of *GRHL2* in all cancer types, we analysed the expression level of *GRHL2* mRNA in the Oncomine database. The results showed that the expression of *GRHL2* was higher in bladder cancer, BC, colorectal cancer, lung cancer, and ovarian cancer tissues than in their corresponding normal tissues (Supplemental Figure S1A). We also examined 33 different tumour types from the TCGA database. Grainyhead-like 2 was overexpressed in 18 different types of malignancies (Supplemental Figure S1B). Furthermore, the Cancer Cell Line Encyclopedia (CCLE) database revealed elevated expression of *GRHL2* mRNA in 28 cancer cell lines, particularly in BC cell lines (Supplemental Figure S1C). Thus, our findings suggest that GRHL2 may play a significant role in BC.

Expression of GRHL2 in BC

Further investigation using the HPA database revealed that GRHL2 was expressed at low levels in normal breast tissues (Supplemental Figure S2A) and overexpressed in cancer tissues (Supplemental Figure S2B). It was also confirmed from the GEPIA database that *GRHL2* was more highly expressed in cancer tissues (n = 1085) than in normal tissues (n = 291) (Supplemental Figure S2C). Immunohistochemical staining obtained from HPA also confirmed that GRHL2 protein

expression was higher in tumour tissues than in normal tissues (Supplemental Figure S2D).

Next, we further verified the correlation between *GRHL2* mRNA levels and clinical data of BC patients, including age, sex, and cancer stage. The expression of *GRHL2* was not correlated with age, cancer stage, or nodal metastasis status ($P > .05$) but was significantly correlated with sex (Figure 1A to D; $P < .05$).

The prognostic value of GRHL2

We used the Kaplan–Meier plotter to assess the prognostic value of *GRHL2*. *GRHL2* can predict poorer overall survival (OS) in kidney renal clear cell carcinoma (KIRC) ($P < .05$); however, it could not predict relapse-free survival (RFS) ($P = .05$) (Supplemental Figure S3A and B). For pancreatic ductal adenocarcinoma (PDA), *GRHL2* had a predictive effect on OS and RFS (Supplemental Figure S3C and D; $P < .05$). In a total of 1643 and 1089 BC patients, higher *GRHL2* was associated with poorer OS and RFS ($P < .05$; Supplemental Figure S3E and F).

To further verify the prognostic role of *GRHL2*, the PrognoScan and GEPIA databases were used. The data in PrognoScan mainly come from the Gene Expression Omnibus (GEO) database. Overexpression of *GRHL2* in 3 BC data sets and 1 bladder cancer data set was associated with poorer survival (DMFS – distant metastasis-free survival and OS) (Supplemental Figure S4A to D). The GEPIA database also showed that high GRHL2 expression was related to poorer OS in BC (Supplemental Figure S4E).

GRHL2 expression is a diagnostic biomarker for BC

To evaluate the diagnostic value of *GRHL2*, an ROC curve was generated from TCGA database data. The results here are part based upon data generated by the TCGA Research Network:https://www.cancer.gov/tcga. The area under the ROC curve was 0.818, indicating a high diagnostic value of *GRHL2* for BC (Figure 2).

Hybrid EMT can be induced in vitro in MDA–MB–231 cells

GRHL2 expression levels were detected by western blot in different cell lines, and there was slightly higher expression in MCF-7 cells (Figure 3A), suggesting that GRHL2 functions in maintaining the epithelial characteristics of MCF-7 cells, a widely studied epithelial cancer cell line. Next, we investigated the effect of GRHL2 overexpression or silencing in MDA-MB-231 and MCF-7 cells and characterized their EMT status by western blot and immunofluorescence (IF) staining with the canonical EMT markers E-cadherin and vimentin. The upregulation of *GRHL2* obviously increased E-cadherin expression in MDA-MB-231 cells (Figure 3B
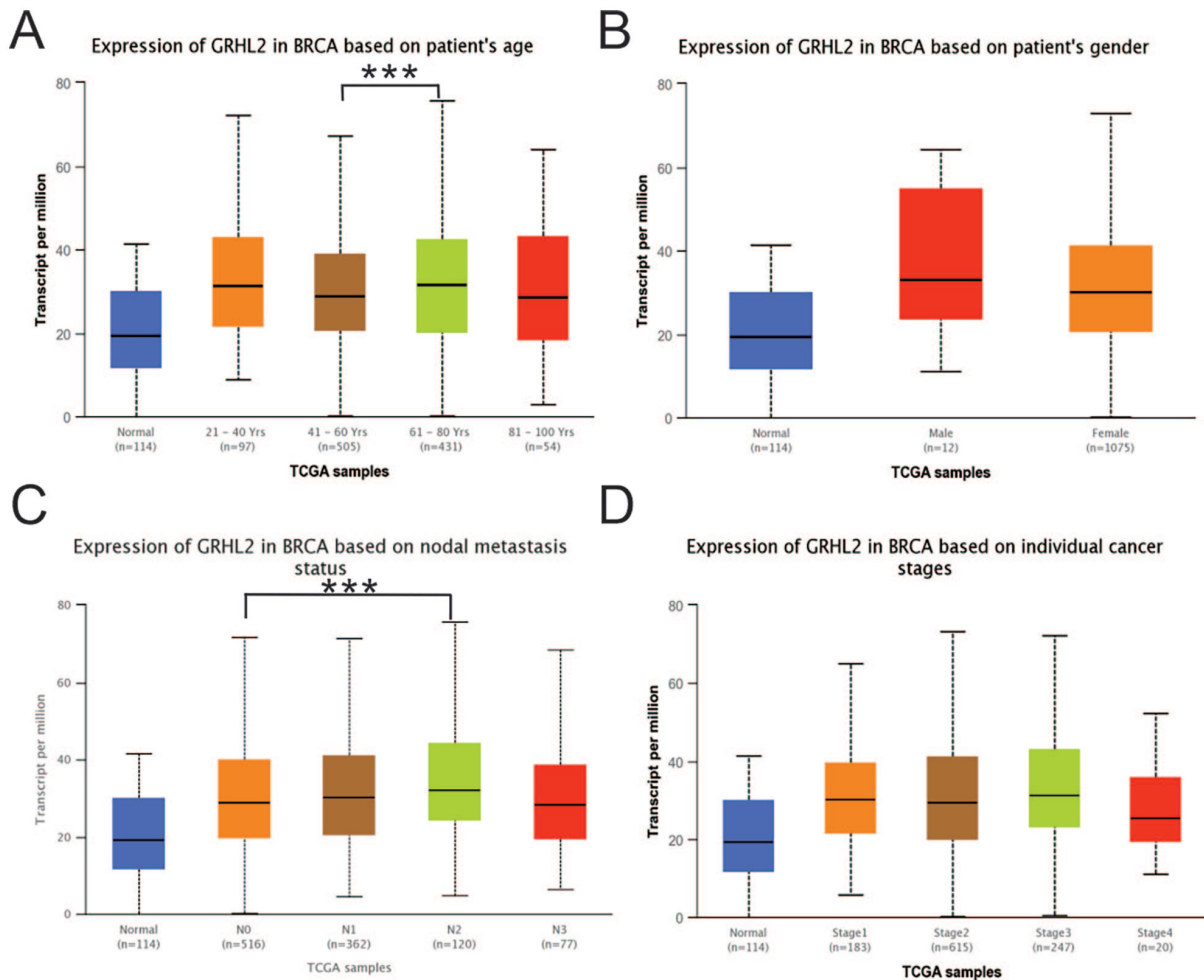
Figure 1. GRHL2 expression is correlated with clinicopathologic characteristics. (A) Age (21-40 [n=97], 41-60 [n=505], 61-80 [n=431], and 81-100 [n=54]). (B) Sex (men [n=12] and women [n=1075]). (C) Clinical stage (Stage 1 [n=183], Stage 2 [n=615], Stage 3 [n=247], and Stage 4 [n=20]). (D) Nodal metastasis status (N0 [n=516], N1 [n=362], N2 [n=120], and N3 [n=77]).
GRHL2 indicates grainyhead-like 2. BRCA indicates breast invasive carcinoma.
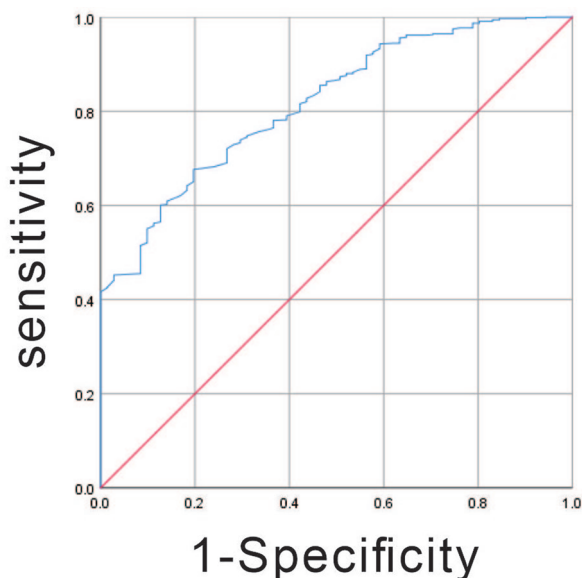

Figure 2. The diagnostic value of GRHL2 expression in breast cancer. Receiver operating characteristic curve for GRHL2 expression in normal tissues (n=71) and breast cancer tissues (n=701) in TCGA. GRHL2 indicates grainyhead-like 2.

and C). Western blot and IF results demonstrated that MDA-MB-231 cells, as a mesenchymal cell line, contained cell subpopulations expressing E-cadherin and vimentin jointly or separately, indicating that a hybrid E/M or partial EMT phenotype was induced by *GRHL2* overexpression. As a control, *GRHL2* overexpression increased E-cadherin expression and decreased vimentin expression in MCF-7 cells (Figure 3B). Accordingly, *GRHL2* silencing caused a decrease in E-cadherin expression and an increase in vimentin expression in both MCF-7 and MDA-MB-231 cells (Figure 3B and C).

Next, we conducted a scratch assay for MDA-MB-231 and MCF-7 cells with GRHL2 overexpression or silencing, and they showed different cell motility patterns. In MCF-7 cells, *GRHL2* overexpression resulted in slower wound healing. However, in MDA-MB-231 cells, control cells moved largely individually, but *GRHL2* overexpression cells moved collectively and formed finger-like projections (Figure 4A, black arrow). These finger-like projections are the hallmarks of collective migration[34] and the hybrid E/M phenotype. We observed that collective migration was not observed in
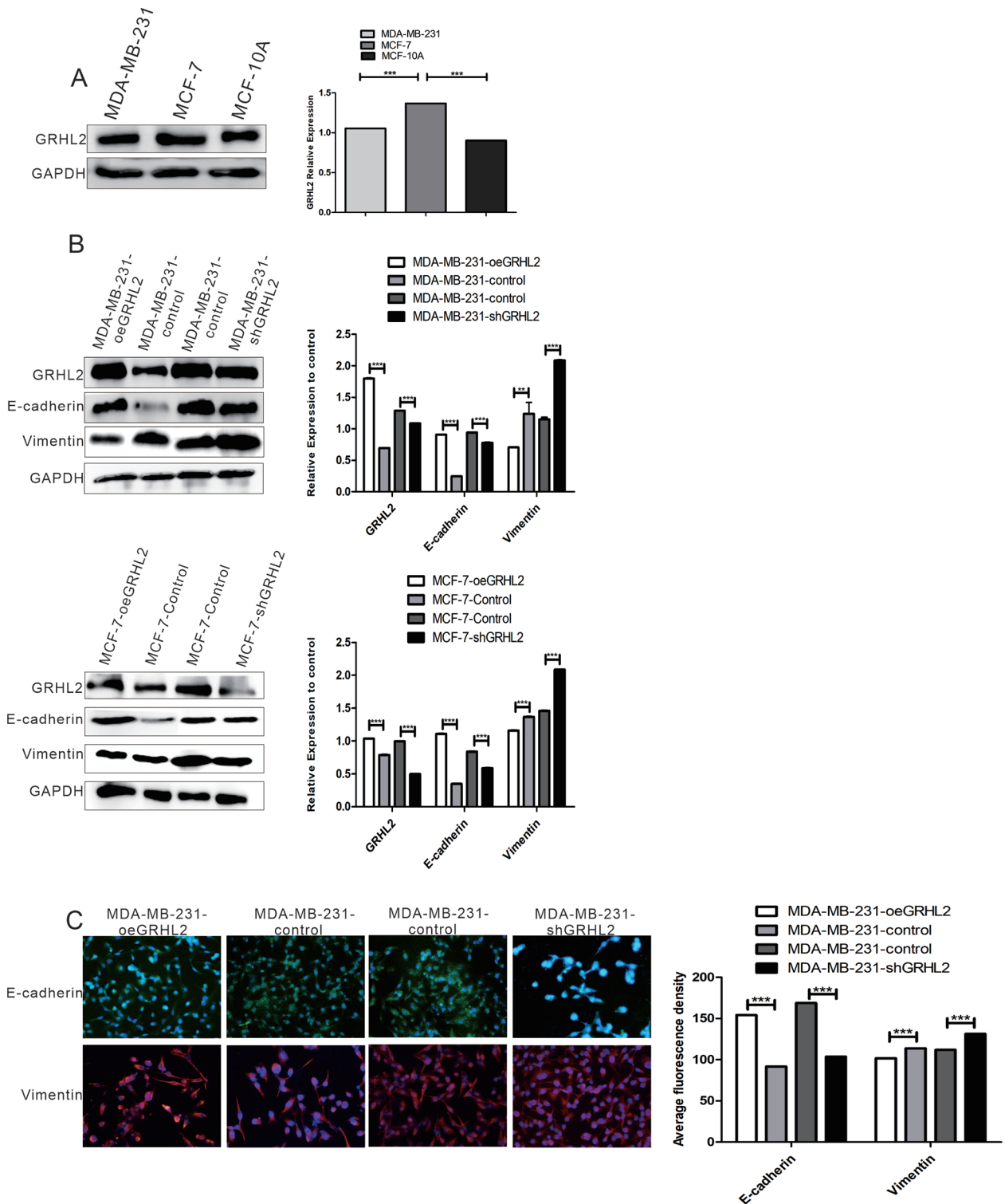
Figure 3. GRHL2 expression in breast cancer cells. (A) GRHL2 expression in MDA-MB-231, MCF-7, and MCF-10A. (B) Effect of overexpression or downregulation of GRHL2 on E-cadherin and vimentin as analysed by western blot. The results are from 3 repeated experiments (***$P < .001$). (C) E-cadherin and vimentin expressions in breast cancer cells after the downregulation or overexpression of GRHL2 by immunofluorescence. The results are from 3 repeated experiments (***$P < .001$).
GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase; GRHL2, grainyhead-like 2;.

MDA-MB-231 cells with GRHL2 silencing, and these cells migrated more individually (Figure 4A). Grainyhead-like 2 overexpression did not lead to increased migratory and invasive cell numbers in Transwell assays (Figure 4B). Increased migratory and invasive abilities are cellular traits usually associated with EMT occurrence. This effect was demonstrated in
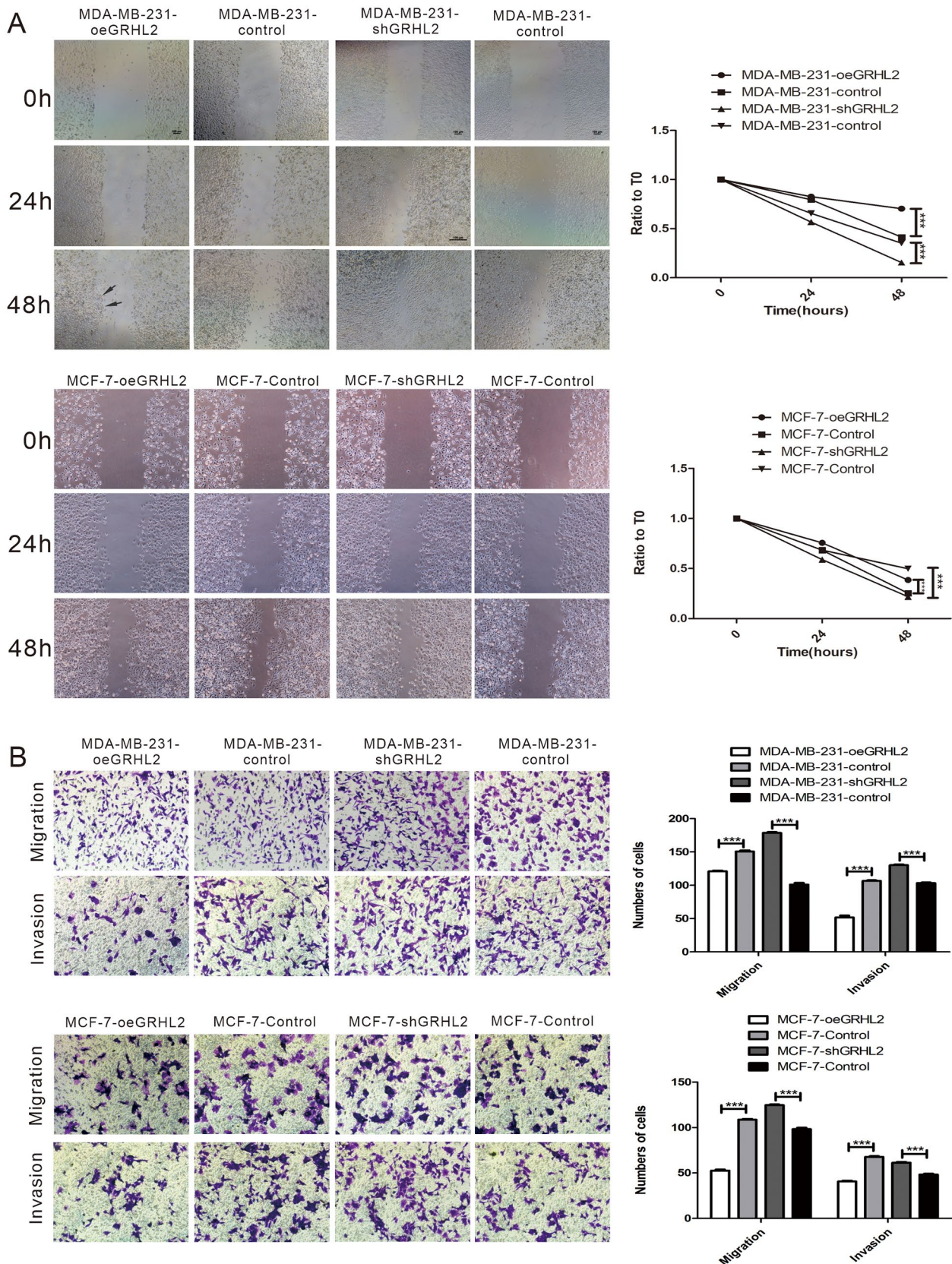
Figure 4. GRHL2 promotes hybrid E/M phenotype in MDA-MB-231 cells. (A) In MCF-7 and MDA-MB-231 cells, *GRHL2* overexpression resulted in slower wound healing (***P < .001). However, in MDA-MB-231 cells, control cells moved largely as single cells, but *GRHL2* overexpression cells moved collectively and formed finger-like projections (black arrow). (B) GRHL2 overexpression did not lead to an increased migratory and invasive cell numbers by Transwell assays (***P < .001).
GRHL2 indicates grainyhead-like 2.

Figure 5. Correlation between GRHL2 expression and immune infiltration in breast cancer. (A) Correlation of GRHL2 expression level with immune cell infiltration levels in breast cancer. (B) Correlation between *GRHL2* gene copy number and immune cell infiltration levels in breast cancer. GRHL2 indicates grainyhead-like 2. BRCA indicates breast invasive carcinoma.

MCF-7 cells with EMT induction by GRHL2 silencing (Figure 4B).

Correlation between GRHL2 expression and immune cell infiltration in BC

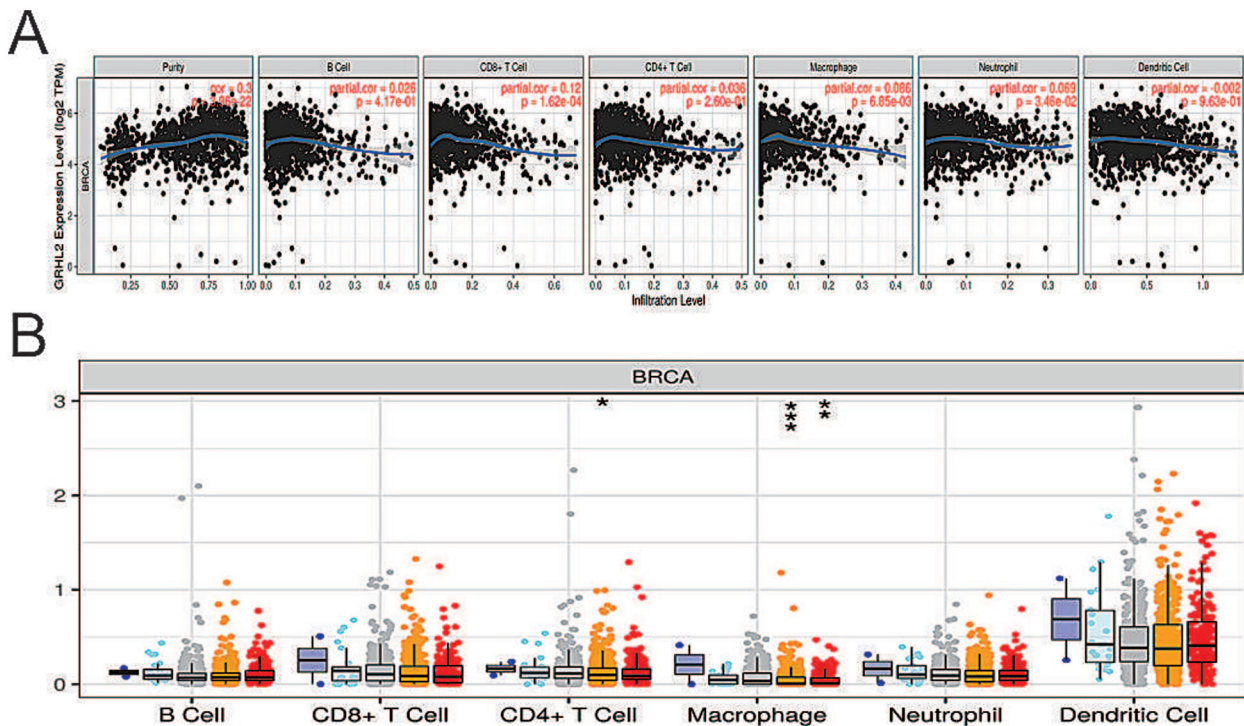To evaluate the correlation between *GRHL2* expression and immune cell infiltration in BC, we used the TIMER database for analysis. The *GRHL2* expression level was significantly correlated with tumour purity, positively correlated with CD8+ cell, macrophage, and neutrophil infiltration, negatively correlated with DC infiltration, and not significantly correlated with B cells and CD4+ cells (Figure 5A). We further evaluated the relationship of several immune cell infiltration levels with *GRHL2* gene copy number and found that CD4+ cell and macrophage infiltration were related to *GRHL2* gene copy number in BC (Figure 5B).

Immune markers and GRHL2 expression relationships

We used TIMER and GEPIA to examine B cells, CD8+ T cells, M1/M2 macrophages, TAMs, monocytes, NK cells, neutrophils, and DC indicators in BC to determine whether there was a link between GRHL2 and immunologic markers. Follicular helper T cell (Tfh), T helper cell (Th)1, Th2, Th9, Th17, Th22, regulatory T cell (Treg), and T-cell exhaustion were among the functional T cells studied (Table 1 and Figure 6). The GRHL2 expression level was substantially linked with 22 of the 45 immune cell markers in BC in TIMER after adjusting for

tumour purity (Table 1). The results also showed that macrophage subgroup M2 marker ARG1 and MRC1 expression was positively related to GRHL2 (Table 1).

As shown in Figure 6, CD8+ T cells, B cells, TAMs, and monocytes in BC have a close relationship with *GRHL2* expression. The CD8+ T-cell marker was negatively correlated with GRHL2 (Figure 6 and Table 2). Interestingly, the B-cell markers CD19, CD38, and MS4A1 were negatively correlated to *GRHL2* in BC but not in normal tissue. These results indicate that the different immune cells related to *GRHL2* might be involved in BC aggressiveness in different microenvironments.

Functional enrichment analysis

To clarify the genes and signal transduction pathways related to *GRHL2*, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses. We first used the LinkedOmics database to analyse the upstream and downstream genes co-expressed with *GRHL2* in the volcano map (Figure 7A to C). Kyoto Encyclopedia of Genes and Genomes and GO analyses identified 3 main groups related to tumour aggressiveness (Figure 7D and E). The first group included lymphocyte activation and Th1, Th2, and Th17 cell differentiation. This further verified the analysis results of TIMER and GEPIA, which demonstrated that *GRHL2* could regulate immune cell infiltration in tumour tissue. The second group included the establishment or maintenance of cell polarity, regulation of actin filament length and polymerization,

Table 1. Correlations between *GRHL2* and gene markers of immune cells in TIMER.

CELL TYPE	GENE MARKER	BREAST CANCER			
		NONE		PURITY	
		COR	*P*	COR	*P*
B cell	*CD19*	−0.159	***	−0.02	.484
	CD38	−0.048	.11	0.088	*
	MS4A1	−0.109	**	0.057	.0729
CD8+ T cell	*CD8A*	−0.126	.684	0.04	.211
	CD8B	−0.2	***	−0.054	.0901
Tfh	*CXCR5*	−0.14	***	0.015	.629
	ICOS	−0.045	.133	0.103	**
Th1	*IL12RB2*	−0.002	.947	0.081	*
	TBX21	−0.2	***	−0.059	.0979
Th2	*CCR3*	−0.056	.0631	0.014	.653
	STAT6	0.111	**	0.158	***
	GATA3	0.292	***	0.024	***
Th9	*TGFBR2*	−0.029	.336	0.145	***
	IRF4	−0.057	.0605	0.122	**
	SPI1	−0.331	***	−0.209	***
>TH17	*IL21R*	−0.116	**	0.034	.286
	IL23R	0.015	.631	0.105	**
	STAT3	0.312	***	0.358	***
Th22	*CCR10*	−0.253	***	−0.205	***
	AHR	0.163	***	0.244	***
Treg	*FOXP3*	−0.033	.267	0.116	**
	CCR8	0.14	**	0.252	***
T-cell exhaustion	*PDCD1*	−0.234	***	−0.107	**
	CTLA4	−0.135	***	0.002	.954
Macrophage	*CD68*	−0.079	*	0.029	.359
	ITGAM	−0.07	.02	0.028	.370
M1	*NOS2*	−0.017	.569	0.003	.925
	ROS1	0.022	.466	0.047	.140
M2	*ARG1*	0.036	.236	0.087	**
	MRC1	−0.059	.0486	0.088	**
TAM	*HLA-G*	−0.192	***	−0.144	***
	CD80	0.069	.022	0.156	***
	CD86	−0.085	**	0.036	.254
Monocyte	*CD14*	−0.307	***	−0.234	***
	FCGR3A	0.042	.168	0.13	***

(Continued)

Table 1. (Continued)

CELL TYPE	GENE MARKER	BREAST CANCER			
		NONE		PURITY	
		COR	*P*	COR	*P*
NK	*XCL1*	−0.144	***	0.002	.939
	KIR3DL1	−0.099	*	−0.017	.588
	CD7	−0.312	***	−0.197	***
Neutrophil	*FUT4*	−0.086	*	0.036	.254
	MPO	−0.11	**	−0.009	.774
DC	*CDIC*	−0.21	***	−0.075	.0184
	THBD	−0.109	**	−0.026	.410

Abbreviations: Cor, *R* value of the Spearman correlation; DC, dendritic cell; NK, natural killer cell; none, correlation without adjustment; purity, correlation adjusted for tumour purity; TAM, tumour-associated macrophage; Tfh, follicular helper T cell; Th, T helper cell; Treg, regulatory T cell.
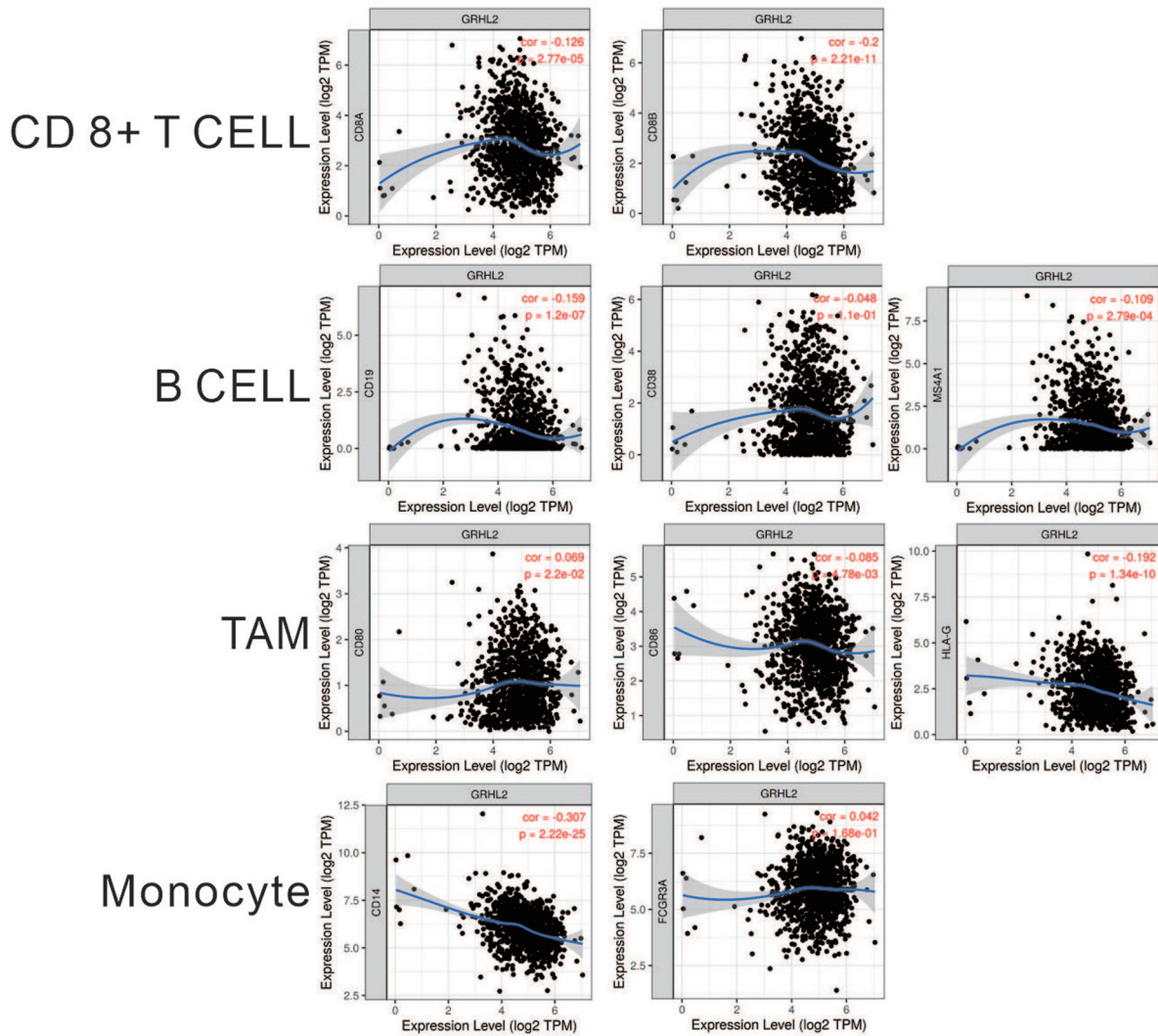*$P < .01$; **$P < .001$; ***$P < .0001$.

Figure 6. Markers include CD8A, CD8B of CD8+ T cell; CD19, CD38, MS4A1 of B cell; CD80, CD86, HLA-G of TAM; CD14, FCG13 of monocyte. TAM indicates tumour-associated macrophage.

Table 2. Correlations between GRHL2 and genes markers of CD8+ T cells, B cells, macrophages, and monocytes in GEPIA.

CELL TYPE	GENE MARKER	BREAST CANCER			
		TUMOUR		NORMAL	
		R	P	R	P
CD8+ T cell	CD8A	−0.17	***	0.51	***
	CD8B	−0.18	***	0.53	***
B cell	CD19	−0.17	***	0.029	.76
	CD38	−0.01	**	−0.081	.39
	MS4A1	−0.11	**	0.032	.74
Monocyte	CD14	−0.15	***	−0.32	**
	FCGR3A	0.021	.5	−0.017	.86
TAM	CCL2	−0.15	***	−0.23	.013
	CD68	−0.031	.31	−0.38	***
	IL10	−0.023	.45	−0.5	***
M2	CD163	−0.12	***	−0.43	***
	VSIG4	−0.01	**	−0.48	***
	MSA4A	−0.1	**	−0.54	***
M1	NOS2	−0.012	.69	0.27	*
	ROS1	−0.017	.57	−0.18	.054

Abbreviations: GRHL2, grainyhead-like 2; TAM, tumour-associated macrophage.
*$P < .01$; **$P < .001$; ***$P < .0001$.

actin filament polymerization, or depolymerization. This was consistent with the previous research,[35] which demonstrated that *GRHL2* could regulate EMT. Our results suggested that *GRHL2* might regulate actin filament status to determine the EMT phenotype of tumour cells. The third group included the cell cycle, DNA replication, nuclear division, mismatch repair, nucleotide excision repair, double-strand break repair, cell adhesion molecules, NF-kappa β signalling pathway, PI3K–Akt signalling pathway, and positive regulation of angiogenesis. This suggested that *GRHL2* could be involved in cell cycle control and have an effect on tumour cell proliferation. In addition, *GRHL2* might promote tumour invasiveness by co-operating with the NF-kappa β signalling pathway and PI3K–Akt signalling, affecting cell adhesion molecule expression and regulating angiogenesis.

Methylation could regulate GRHL2 expression

To further elucidate the mechanism by which *GRHL2* expression is regulated in BC, we explored the correlation between *GRHL2* expression levels and methylation. First, as shown in Figure 8A, GRHL2 was altered in 218 of 960 (23%) BC patients, including mutation in 8 cases (0.8%), amplification

(AMP) in 168 cases (17.5%), deep deletion in 2 cases (0.2%), high mRNA in 50 cases (5.2%), and low mRNA in 8 cases (0.8%). Thus, AMP is the most common type of GRHL2 copy number variation (CNV) in BC. Grainyhead-like 2 AMP led to high expression of GRHL2 (Figure 8B). However, GRHL2 AMP corresponds to a low methylation level (Figure 8C), and the *GRHL2* mRNA expression level was mainly related to GRHL2 AMP and promoter methylation. The analysis of *GRHL2* from the UALCAN database showed that the promoter methylation level in normal tissues was higher than that in cancer tissues (Figure 8D). The results of MEXPRESS analysis showed that in the DNA methylation sequences of *GRHL2*, there were 25 methylation sites that were negatively correlated with its expression level (Figure 8E). In addition, we analysed the relationship between *GRHL2* mRNA expression and methylation levels through the cBioPortal database, which showed a negative correlation (Figure 8F). One of the probes, cg15679829, was related to promoter methylation of GRHL2 in MethSurv. We analysed this methylation site and survival in this database, which showed no significant relationship (Figure 8G). However, the density and methylation level of *GRHL2* were different in different age groups of BC patients (Figure 8H and I). It can be seen from the density graph that the β-value is 0.844, which is significant (β-value > 0.6). These results demonstrate that the promoter methylation of GRHL2 could regulate GRHL2 expression.

Discussion

Breast cancer is a very common female disease. Although early detection and treatment have reduced the mortality rate of BC, patients with metastases have a poor prognosis.[36] Therefore, exploring new biomarkers for BC diagnosis and predicting recurrence, metastasis, and survival outcomes are valuable for BC patients.

In mammals, the structure and regeneration of various epithelial cells depend on the 3 members of the GRHL family of transcription factors – GRHL1, GRHL2, and GRHL3. A recent review found that all GRHLs are associated with various types of cancer.[6] GRHL2 has been shown to be a key determinant of keratinocyte differentiation and lung epithelial morphogenesis and is considered a lineage determinant of BC epithelial cells.[37] However, its prognostic effects in other aspects have not been fully studied. New evidence shows that GRHL2 is a novel oncogene,[38] but it has a tumour suppressor effect in gastric cancer, cervical cancer, clear cell renal cell carcinoma, and sarcoma.[39,40] Therefore, GRHL2 has different regulatory effects in different cancers, and it has not been studied in depth in BC.

In this study, the Oncomine and TIMER databases were used to assess the correlation between GRHL2 expression and the prognosis of 33 different types of cancer, demonstrating that there are significant differences between normal tissues and
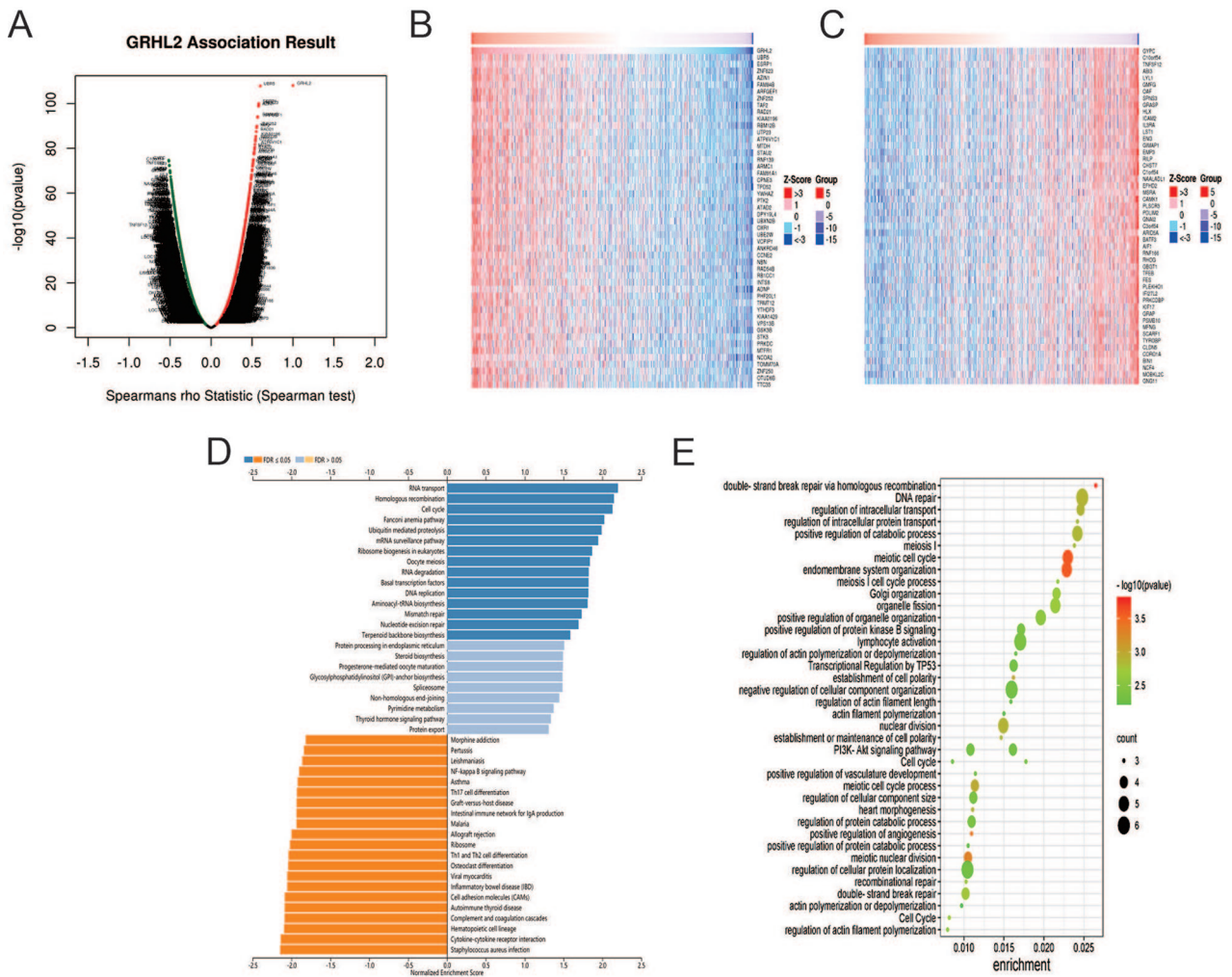
Figure 7. Function enrichment analysis. (A) GRHL2 upstream and downstream genetic volcano map. (B) Heat map of GRHL2 co-expression upstream genes. (C) Heat map of GRHL2 co-expression downstream genes. (D) KEGG signalling pathway enrichment analysis. (E) Gene Ontology enrichment analysis.
GRHL2 indicates grainyhead-like 2; KEGG, Kyoto Encyclopedia of Genes and Genomes.

cancer tissues. In Oncomine, we found that GRHL2 was highly expressed in bladder cancer, BC, colorectal cancer, etc, compared with the expression level in normal tissues. Meanwhile, in the TIMER database, GRHL2 expression is higher in bladder urothelial carcinoma, breast invasive carcinoma, cervical squamous cell carcinoma, etc. In different databases, these different GRHL2 expression levels in cancer are due to different data collection methods and biological potential analysis methods. Interestingly, the results obtained for BC through these 2 databases are consistent. The expression of GRHL2 is high in BC tissues and low in normal tissues. Next, we used methylation databases and found that GRHL2 methylation levels are lower in BC tissues than in normal tissues. We found a significant negative correlation between *GRHL2* mRNA levels and promoter methylation levels through the cBioPortal database. Analysing the association between GRHL2 and genome-wide methylation in MEXPRESS showed that more methylation sites are closer to the open sea, suggesting that GRHL2 expression could be regulated by methylation. Then, we analysed

GRHL2 expression levels through the HPA, GEPIA, and UALCAN databases and conducted research on different ages, sex, and pathological data. Through the HPA database and immunohistochemical staining, it was found that GRHL2 protein expression is consistent with its mRNA expression and is also highly expressed in BC tissues. The ROC curve shows that the expression of GRHL2 has high diagnostic value in BC. Then, we used the Kaplan–Meier plotter, PrognoScan, and GEPIA and found that high expression of GRHL2 could induce shorter survival times in BC patients. The high expression level of GRHL2 can be used as an independent risk factor for poor prognosis of BC. Therefore, we infer that the high expression of GRHL2 may play a critical role in BC occurrence and development as a carcinogenic factor.

More than 90% of cancer-related deaths are caused by metastasis. Epithelial–mesenchymal transition causes tumour cells to spread, whereas the opposite process, MET, allows cancer cells to grow and create potentially fatal metastatic lesions. But recently, partial EMT or hybrid E/M phenotypes have
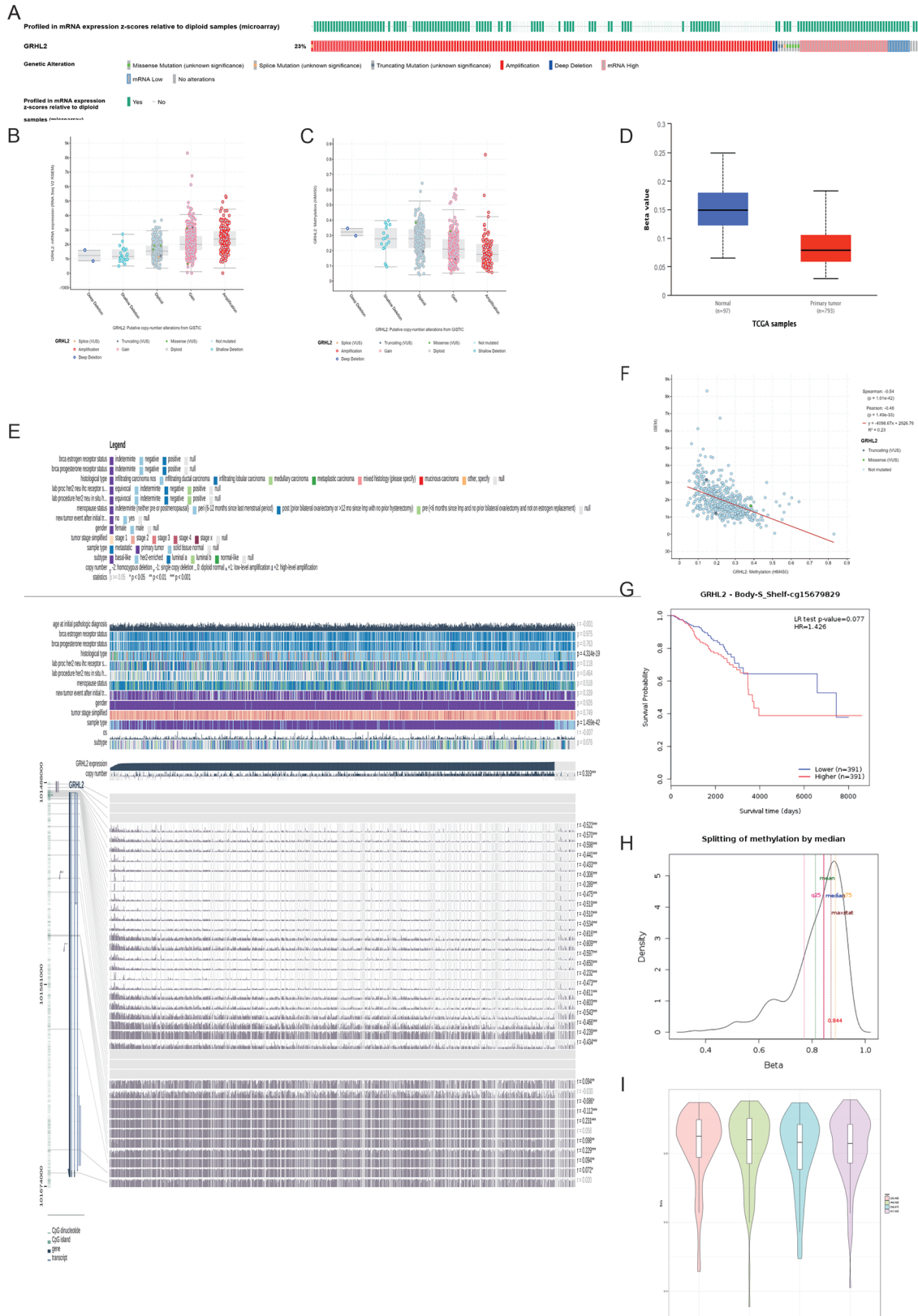
Figure 8. GRHL2 methylation analysis. (A) OncoPrint of GRHL2 alterations in breast cancer cohort. The different types of genetic alterations are highlighted in different colors. (B) GRHL2 expression in different GRHL2 CNV groups. (C) GRHL2 methylation in different GRHL2 CNV groups. (D) Using UALCAN analysed methylation. (E) The methylation site of GRHL2 DNA sequence association with gene expression was visualized using MEXPRESS. (F) GRHL2 and methylation expressions were shown on cBioPortal. (G) Survival analysis of cg15679829. (H) Density of cg15679829. (I) The violin chart shows the methylation levels between different age groups.
GRHL2 indicates grainyhead-like 2.

been increasingly recognized. In ovarian cancer and BC metastasis, tumour growth in vivo is mainly driven by hybrid E/M cells.[41,42] Our in vitro experimental results demonstrate that GRHL2 overexpression in the mesenchymal cell line MDA-MB-231 could induce epithelial characteristics in a portion of cells and then promote the hybrid E/M phenotype. It has been reported that the hybrid E/M phenotype is strongly correlated with aggressiveness and can pose a higher metastatic risk in patients compared with the pure and complete EMT phenotype.[15,43]

The cellular environment in which tumour or cancer stem cells live is referred to as the TME. Immune cells, blood arteries, extracellular matrix, fibroblasts, bone marrow-derived inflammatory cells, and signalling molecules are components of the TME.[44,45] Immunity infiltration in the TME has been shown in the previous research to impact immune treatment responses and patient prognosis.[46-48] Some studies have shown that the density of CD8+ T cells is strongly linked to immune escape in BC, and the infiltration of CD8+ T and CD4+ T cells is also linked to BC prognosis.[49] In this study, the expression of GRHL2 was significantly positively correlated with tumour purity in BC tissue, indicating that its expression is different in tumour cells and the TME. We found that GRHL2 is associated with multiple types of immune cell infiltration of the TME in BC. First, CD8+ T cells were identified as related to GRHL2 expression in this study. A common type of T lymphocyte in the TME is CD8+ T cells, which can kill tumour cells by their immune killing effect. However, tumours progress despite the presence of CD8+ T cells in the TME, which suggests that CD8+ T-cell differentiation to dysfunctional states fails to achieve responses to immunotherapy.[50] Our results indicate that GRHL2 expression has a close relationship with CD8+ T cells and that the functional status of CD8+ T cells might be involved in BC aggressiveness. Second, in this study, both GRHL2 expression level and gene copy number were positively related to macrophages. Macrophages are the most prominent immune cell type of the TME.[51,52] Macrophages in the TME can promote tumour reoccurrence and metastases. They can facilitate the escape of tumour cells into the circulatory system and can inhibit the antitumor immune mechanism and response.[52] It has been reported that the macrophage M2 subgroup is endowed with a repertoire of tumour-promoting capabilities involving immunosuppression, angiogenesis, and neovascularization, as well as stromal activation and remodelling, thereby accelerating the pace of tumour aggressiveness and metastasis.[53] After adjusting tumour purity, GRHL2 expression is positively correlated with M2 macrophages, which suggests that GRHL2-expressing tumour cells may recruit M2 macrophages into tumour tissue to promote BC development. Third, our results also indicate that GRHL2 may be related to *Treg* gene markers. Tregs highly enriched in the TME are

widely known for their immunosuppressive effects in tumours.[54] Fourth, in this study, the B-cell markers CD19, CD38, and MS4A1 were negatively related to GRHL2 in BC but not in normal tissue, suggesting that a GRHL2-related B lymphocytes decrease also impacts BC progression. Recent research[55] supported a favourable prognostic value of tumour-infiltrating CD20+ B lymphocytes in colorectal cancer. In addition, KEGG and GO analyses also showed that GRHL2 and its related genes are involved in lymphocyte activation and T helper cell differentiation, demonstrating that GRHL2 expression in tumour cells is associated with immune cell infiltration in the TME. According to this study, GRHL2 may have a major impact on the immune response generated in the TME through signalling pathways and crosstalk between immune cells, thereby affecting the aggressiveness of BC. This phenomenon not only brings important clues for the prognosis of BC but also helps to explore new therapeutic targets. To further explore the biological functions of GRHL2, we performed KEGG and GO analyses of GRHL2. The enrichment analysis showed that GRHL2 and its related factors are involved in multiple tumour-related signalling pathways, which may be related to BC cell proliferation, invasion, and metastasis.

In summary, based on the results of bioinformatics analysis, GRHL2 plays a major role in BC progression. Overexpression of GRHL2 is present in BC tissue and is related to poor survival of patients. The expression of GRHL2 correlates with immune cell infiltration. Further in vitro experiments demonstrate an important role of GRHL2 in the regulation of the hybrid E/M phenotype of BC cells and promotion of BC invasion. Therefore, GRHL2 may be a valuable biomarker for evaluation of BC prognosis.

Author Contributions
XB, TL, and XZ contributed to the conceptualization. XB, YuL, YaL, and FL contributed to the software, data curation, and resources. XB, CN, and NC contributed to the formal analysis and resources. XB, YZ and NZ contributed to the methodology. XB contributed to the writing – original draft preparation. TL contributed to the writing – review and editing. XZ contributed to the supervision and project administration. TL contributed to the funding acquisition. All authors contributed to the article and approved the submitted version.

Data Availability Statement
The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

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Supplemental Material

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