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ORIGINAL ARTICLE

Identification of key tumor stroma-associated transcriptional signatures correlated with survival prognosis and tumor progression in breast cancer

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Abstract

Background The aberrant expression of stromal gene signatures in breast cancer has been widely studied. However, the association of stromal gene signatures with tumor immunity, progression, and clinical outcomes remains lacking.

Methods Based on eight breast tumor stroma (BTS) transcriptomics datasets, we identified differentially expressed genes (DEGs) between BTS and normal breast stroma. Based on the DEGs, we identified dysregulated pathways and prognostic hub genes, hub oncogenes, hub protein kinases, and other key marker genes associated with breast cancer. Moreover, we compared the enrichment levels of stromal and immune signatures between breast cancer patients with bad and good clinical outcomes. We also investigated the association between tumor stroma-related genes and breast cancer progression.

Results The DEGs included 782 upregulated and 276 downregulated genes in BTS versus normal breast stroma. The pathways significantly associated with the DEGs included cytokine–cytokine receptor interaction, chemokine signaling, T cell receptor signaling, cell adhesion molecules, focal adhesion, and extracellular matrix–receptor interaction. Protein–protein interaction network analysis identified the stromal hub genes with prognostic value in breast cancer, including two oncogenes (*COL1A1* and *IL21R*), two protein kinases encoding genes (*PRKACA* and *CSK*), and a growth factor encoding gene (*PLAU*). Moreover, we observed that the patients with bad clinical outcomes were less enriched in stromal and antitumor immune signatures (CD8 + T cells and tumor-infiltrating lymphocytes) but more enriched in tumor cells and immunosuppressive signatures (MDSCs and CD4 + regulatory T cells) compared with the patients with good clinical outcomes. The ratios of CD8 + /CD4 + regulatory T cells were lower in the patients with bad clinical outcomes. Furthermore, we identified the tumor stroma-related genes, including *MCM4, SPECC1, IMPA2,* and *AGO2*, which were gradually upregulated through grade I, II, and III breast cancers. In contrast, *COL14A1, ESR1, SLIT2, IGF1, CH25H, PRR5L, ABCA6, CEP126, IGDCC4, LHFP, MFAP3, PCSK5, RAB37, RBMS3, SETBP1,* and *TSPAN11* were gradually downregulated through grade I, II, and III breast cancers. It suggests that the expression of these stromal genes has an association with the progression of breast cancers. These progression-associated genes also displayed an expression association with recurrence-free survival in breast cancer patients.

Conclusions This study identified tumor stroma-associated biomarkers correlated with deregulated pathways, tumor immunity, tumor progression, and clinical outcomes in breast cancer. Our findings provide new insights into the pathogenesis of breast cancer.

Keywords Breast tumor stroma · Stromal hub genes · Tumor immunity · Tumor progression · Clinical outcomes

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Background

The tumor microenvironment (TME) consists of heterogeneous components including borders, blood vessels, lymph vessels, extracellular matrix (ECM), immune and/or inflammatory cells, secretomes, coding or non-coding RNAs, small organelles, tumor cells, and surrounding associated or regulatory cells [1]. Among the tumor-associated regulatory cells, stromal cells playing various crucial oncogenic roles in the TME. In the TME, heterogeneous stromal cells are associated with tumor growth, invasion, progression, and metastasis [2, 3]. These tumor-associated stromal cells promote various dysregulated biological functions including extracellular matrix remodeling, cellular migration, neoangiogenesis, and evasion of immunosurveillance through the production of several types of onco-regulators including cytokines, chemokines, matrix metalloproteinases (MMPs), extracellular matrix (ECM), and growth factors [4]. It is recently demonstrated that the tumor-associated stromal cells playing pivotal roles in the resistance of cancer therapy [5]. The oncogenic intrinsic properties of stromal cells substantially regulated the genotype and phenotype of surrounding cancer cells in the TME [6].

In the breast cancer TME, tumor-associated stromal cells are associated with cancer initiation, development, progression, angiogenesis, metastasis, recurrence, and therapeutic resistance [7]. Survival of breast cancer patients is correlated with stromal biology including the reorganization of the extracellular matrix (ECM) to promote cancer invasion and migration, changes in the phenotypes of stromal cell, variability in the stromal gene expression profiles, and changes in cellular signaling cascades to aid surrounding cancer cells [8]. Various parts of stromal compartments have crucial effects on breast cancer TME. The tumor-promoting intrinsic properties of the stroma are associated with the tumorigenesis of breast cancer [7]. Finak et al. reported that the expression of stromal gene signatures is correlated with the clinical outcomes of breast cancer patients [9]. Winslow et al. identified stromal gene signatures that are associated with clinical features in different types of molecular subtypes of breast cancer [10]. Altogether, these studies provide the clue that the stromal cells have substantial onco-regulatory roles in the TME of breast cancer.

Herein, we performed comprehensive bioinformatic analyses to identify molecular alterations in breast tumor stroma versus normal stroma. We identified differentially expressed genes (DEGs), hub genes from the interactions of DEGs, and regulatory transcription factors (TFs), and kinases and pathways associated with the DEGs. We also identified stromal genes having a significant link with recurrence-free survival in breast cancer patients. Moreover, we found certain tumor stromal genes, which were gradually dysregulated through the three different grades of breast cancer, and their dysregulation was associated with poor prognosis in patients.

Materials and methods

Data selection and pre-processing

We systematically searched for the gene expression omnibus (GEO) database using keywords "breast cancer," "stroma," and "tumor stroma". Ultimately, we identified eight datasets: GSE9014 (sample size $n=123$) [9], GSE83591 ($n=53$) [11], GSE31192 ($n=17$) [12], GSE26910 ($n=12$) [13], GSE10797 ($n=33$) [14], GSE8977 ($n=22$) [15], GSE33692 ($n=22$) [16], and GSE14548 ($n=34$) [17] (Supplementary Table S1). We combined the eight datasets into a single dataset (including stromal data and excluding other data) using the NetworkAnalyst software [18]. The ComBat method was utilized to remove batch effects from the eight datasets [19] (effects of batch removal shown in Supplementary Fig. S1). Each dataset was normalized by base-2 log transformation or quantile normalization. The combined dataset included 240 tumor stroma and 76 normal stroma samples. We also downloaded the dataset of gene expression profiles (RSEM normalized) in TCGA breast cancer cohort ($n=1212$) from the genomic data commons (GDC) data portal (https://portal.gdc.cancer.gov/). We further normalized the RSEM gene expression values by base-2 log transformation [20]. In addition, we used the clinical data of a breast tumor stroma cohort (GSE9014) to evaluate the survival time differences in breast cancer patients [9].

Identification of DEGs between breast tumor stroma and normal stroma by a meta-analysis

We employed the R package "limma" to identify the DEGs between BTS and normal stroma [21]. A meta-analysis of the eight datasets was performed using Cochran's combination test [22]. The false discovery rate (FDR), calculated by the Benjamini–Hochberg method [23], was used to adjust for multiple tests. We selected the all DEGs with a threshold of absolute value with combined effect size (ES) >0.41 and FDR <0.05.

Pathway and functional enrichment analysis

We performed pathway enrichment analysis of the set of genes that were differentially expressed between BTS and normal stroma using the GSEA software [24]. The KEGG pathways [25] significantly associated with the set of genes upregulated and downregulated in BTS versus normal stroma were identified, respectively, using a threshold of FDR <0.05.

Identification of TFs, protein kinases, and master transcriptional regulators (MTRs) that are significantly associated with the DEGs

To link gene expression signatures to upstream cell signaling networks, we used the eXpression2Kinases algorithm [26] to identify the upstream TFs and kinases that regulate the DEGs. In the eXpression2Kinases algorithm, we used a threshold of hypergeometric P value ≤ 0.05 for identifying upstream TFs and kinases. Besides, we utilized the Cytoscape plug-in iRegulon [27] to identify the MTRs for the upregulated and downregulated DEGs, with a threshold of the minimum normalized enrichment score (NES) > 3.0 which corresponds to an approximate FDR between 3 and 9%.

Protein–protein interactions (PPIs)

We constructed PPI networks of the DEGs using STRING (version v11 [28]). We input all DEGs into STRING. The rank of genes was identified by the Cytoscape plugin cytoHubba [29]. Hub nodes were identified using a threshold of medium interaction score ≥ 0.40 and we selected the degree of interaction ≥ 25 for identifying most interacted genes in the PPI. We identified hub genes, protein kinases, oncogenes, and tumor suppressor genes (TSGs) by comparing the hub nodes with TFs, protein kinases, oncogenes, and TSGs obtained from GSEA (https://www.gsea-msigdb.org/gsea/index.jsp). The online tool "Calculate and draw custom Venn diagrams" (http://bioinformatics.psb.ugent.be/webtools/Venn/) was used to identify common TF encoding genes, protein kinase encoding genes, oncogenes, and TSGs between different groups. We visualized the PPI networks using Cytoscape (version 3.6.1) [30].

Evaluation of immune scores, stromal scores, and tumor purity in stromal breast cancer subtypes

We utilized the "ESTIMATE" R package to calculate immune score representing the enrichment levels of immune cells, stromal score representing content of stromal cells, and tumor purity for each breast tumor sample [31] in (GSE9014 [9]). We compared immune scores, stromal scores, and tumor purity between the patients without disease recurrence and the patients with disease recurrence. We consider the Wilcoxon sum rank test P value ≤ 0.05 for identifying significant difference between both groups.

Quantification of the enrichment levels of immune and stromal signatures

We used the single-sample gene-set enrichment analysis (ssGSEA) to quantify the enrichment scores of immune and stromal signatures in tumors based on the expression levels of their marker genes [32]. We defined the ratio of immune signatures in a tumor sample as the ratio of the average expression levels of their marker genes. The immune and stromal signatures analyzed included B cells, CD8 + T cells, CD4 + regulatory T cells, macrophages, neutrophil, natural killer (NK) cells, tumor-infiltrating lymphocytes (TILs), regulatory T cells (Tregs), cytolytic activity, T cell activation, T cell exhaustion, T follicular helper cells (Tfh), M2 macrophages, tumor-associated macrophage (TAM), myeloid-derived suppressor cell (MDSC), endothelial cell, and cancer-associated fibroblasts (CAFs). Their marker genes are shown in Supplementary Table S2.

Survival analysis

We used the clinical data of a BTS cohort (GSE9014) which involved 53 breast cancer patients with clinical information available [9] (Supplementary Table S3) for survival analysis. We compared the recurrence-free survival (RFS) between breast cancer patients classified based on gene expression levels (expression levels > median versus expression levels < median). Kaplan–Meier survival curves were used to show the survival time differences, and the log-rank test was utilized to evaluate the significance of survival time differences between both groups. We used the function "survfit" in the R package "survival" to perform survival analysis and the function "coxph" in the R package "survival" for the univariate and multivariable Cox regression analyses [33].

Identification of DEGs between breast cancer patients with different tumor grades, clinical outcomes, and survival prognosis

In the GSE9014 database [9], we identified the DEGs between the breast cancer patients without and with disease recurrence and the DEGs among the breast cancer patients with different grades (grade I, grade II, and grade III) (Student's t test, $P < 0.05$). We then identified the common genes between both groups of DEGs. We further analyzed the association of the expression of these common genes with the RFS of breast cancer patients. To identify the DEGs among the breast cancer patients with different grades, we utilized the R package "multcomp" [34].

Statistical and computational analysis

We used the two-tailed Student's t test to compare two classes of normally distributed data, including gene expression levels and the ratios of immune signatures, and the one-tailed Mann–Whitney U test to compare two classes of data that were not normally distributed, including immune scores,

stromal scores, tumor purity, and ssGSEA scores. The FDR evaluated by the Benjamini–Hochberg method [23] was used to adjust for multiple tests. We used the R package "ggplot2" to visualize the plots. For multiple probes of a single gene, we averaged the expression values of all probes into a single value by NetworkAnalyst [18]. The online tool "Calculate and draw custom Venn diagrams" (http://bioinformatics.psb.ugent.be/webtools/Venn/) was used to identify common genes between the different groups.

Results

Identification of DEGs between BTS and normal stroma

We identified 1058 DEGs between BTS and normal stroma. Among these DEGs, 782 were upregulated (Supplementary Table S4) and 276 were downregulated (Supplementary Table S5) in BTS. The top 25 upregulated (the highest ES) genes included *COL10A1, SULF1, INHBA, NOX4, COMP, COL11A1, RAB31, IFI30, COL8A1, CTSB, LRRC15, SDC1, WISP1, LAMP5, LEF1, ASPN, MSR1, MNDA, SLAMF8, UNC5B, SLA, TYROBP, C3AR1, ITGAX,* and *COL8A2* (Table 1). Among them, the upregulation of *COL11A1* and *IFI30* was associated with a worse prognosis in breast cancer patients (Fig. 1A–C). In addition, the top 25 downregulated (the lowest ES) genes included *FIGF, SPRY2, DLK1, SFRP1, TGFBR3, HLF, CD36, GPC3, LIFR, CAPN6, RELN, AKR1C3, CAV1, PLP1, MATN2, SDPR, SOCS2, ITM2A, LDB2, SYNM, EGFR, NACA, NOVA1, SPTBN1,* and *SEMA3G* in the BTS (Table 1). Among these genes, the downregulation of *SPRY2, CAV1, SOCS2, ITM2A, LDB2,* and *NACA* in BTS was associated with worse prognosis of breast cancer patients (Fig. 1A, D–I).

Identifications of pathways significantly associated with the breast tumor stromal DEGs

GSEA [24] identified 82 KEGG pathways [25] significantly associated with the DEGs upregulated in BTS (Fig. 2A and Supplementary Table S6). Among them, the top 20 (the lowest FDR) pathways are displayed in Fig. 2A. These pathways were mainly involved in immune, stromal signatures, including cytokine–cytokine

Table 1 Top 25 upregulated and top 25 downregulated genes in breast tumor stroma

Top 25 upregulated stromal genes				Top 25 downregulated stromal genes			
Entrez ID	Gene symbol	Combined ES	Adjusted *P* value	Entrez ID	Gene symbol	Combined ES	Adjusted *P* value
1300	*COL10A1*	4.07	2.35E–09	2277	*FIGF*	− 2.61	2.74E–02
23213	*SULF1*	3.41	0.00E+00	10253	*SPRY2*	− 2.41	0.00E+00
3624	*INHBA*	3.09	1.37E–06	8788	*DLK1*	− 2.40	1.41E-11
50507	*NOX4*	2.61	4.21E–07	6422	*SFRP1*	− 2.35	0.00E+00
1311	*COMP*	2.52	3.36E–08	7049	*TGFBR3*	− 2.32	1.73E–11
1301	*COL11A1*	2.44	1.04E–05	3131	*HLF*	− 2.03	7.16E–05
11031	*RAB31*	2.38	5.18E–08	948	*CD36*	− 1.94	2.83E–02
10437	*IFI30*	2.37	4.35E–09	2719	*GPC3*	− 1.86	3.29E–02
1295	*COL8A1*	2.36	6.03E–10	3977	*LIFR*	− 1.82	1.38E–09
1508	*CTSB*	2.22	1.48E–05	827	*CAPN6*	− 1.78	4.72E–02
131578	*LRRC15*	2.21	1.67E–09	5649	*RELN*	− 1.75	1.42E–05
6382	*SDC1*	2.16	1.69E–04	8644	*AKR1C3*	− 1.71	5.79E–04
8840	*WISP1*	2.14	1.15E–04	857	*CAV1*	− 1.71	1.24E–02
24141	*LAMP5*	2.11	2.46E–10	5354	*PLP1*	− 1.69	1.75E–03
51176	*LEF1*	2.10	9.45E–08	4147	*MATN2*	− 1.66	4.19E–03
54829	*ASPN*	2.02	2.30E–13	8436	*SDPR*	− 1.64	3.71E–05
4481	*MSR1*	2.01	3.18E–11	8835	*SOCS2*	− 1.63	9.21E–04
4332	*MNDA*	2.00	5.89E–06	9452	*ITM2A*	− 1.62	1.83E–04
56833	*SLAMF8*	1.97	4.34E–07	9079	*LDB2*	− 1.60	3.85E–02
219699	*UNC5B*	1.97	1.43E–06	23336	*SYNM*	− 1.60	8.76E–07
6503	*SLA*	1.92	2.27E–07	1956	*EGFR*	− 1.56	1.88E–04
7305	*TYROBP*	1.90	8.43E–12	4666	*NACA*	− 1.54	2.35E–09
719	*C3AR1*	1.90	3.77E–10	4857	*NOVA1*	− 1.53	4.56E–02
3687	*ITGAX*	1.89	5.01E–09	6711	*SPTBN1*	− 1.52	2.28E–05

A

Regulatory status	Entrez ID	Gene Symbol	Combined ES	Adjusted P-value
Upregulated	1301	*COL11A1*	2.4425	1.04E-05
	10437	*IFI30*	2.3713	4.35E-09
Downregulated	10253	*SPRY2*	-2.4098	0
	857	*CAV1*	-1.7089	1.24E-02
	8835	*SOCS2*	-1.633	9.21E-04
	9452	*ITM2A*	-1.622	1.83E-04
	9079	*LDB2*	-1.6014	3.85E-02
	4666	*NACA*	-1.5352	2.35E-09

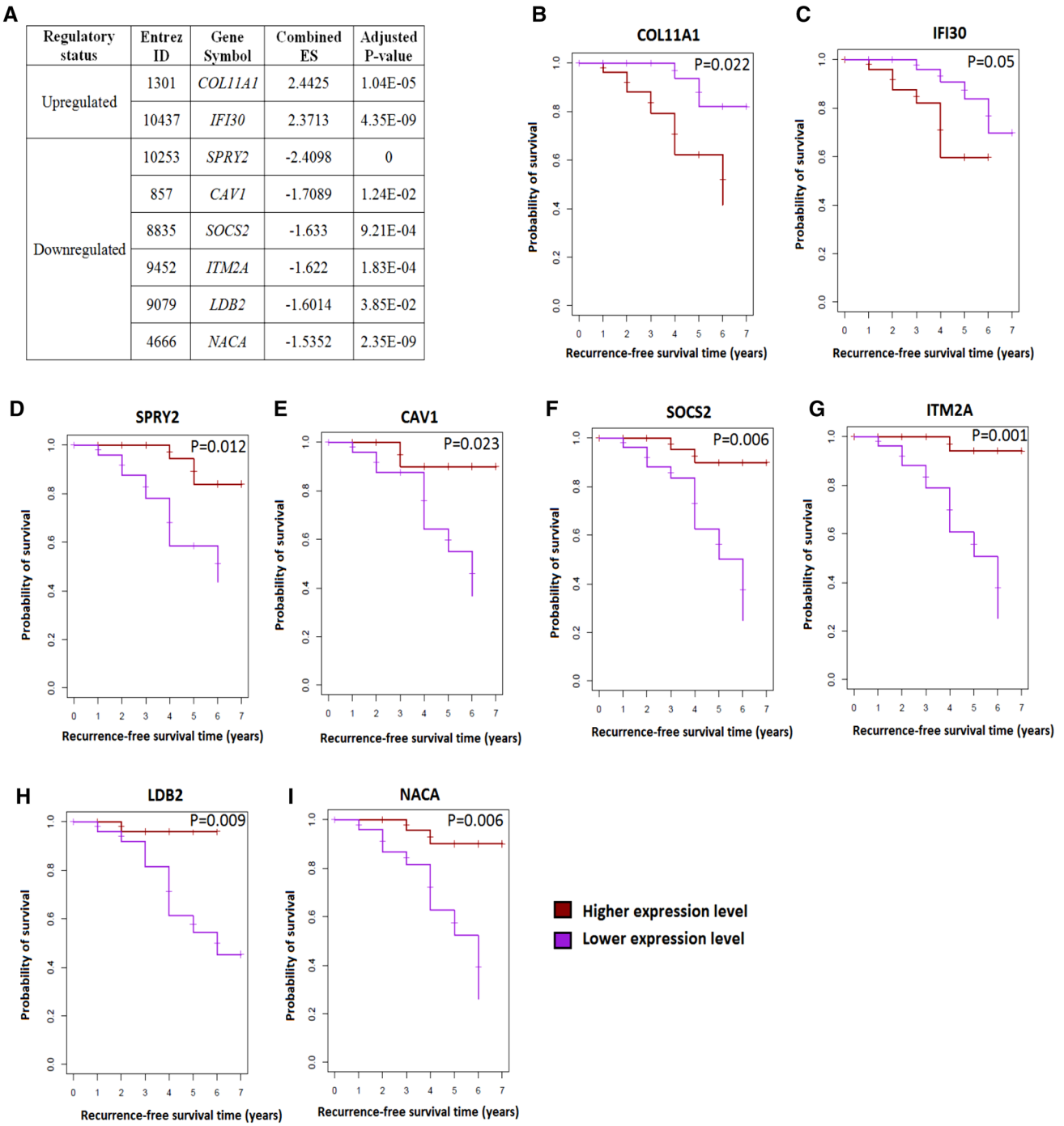


Fig. 1 Expression levels of eight prognostic genes in BTS and their associations with survival prognosis in breast cancer. **A** Expression of eight prognostic genes in BTS versus normal stroma. We investigated the survival of top 25 upregulated and top 25 downregulated genes. The upregulation of *COL11A1* and *IFI30* (upregulated in BTS) is associated with a worse prognosis (**B, C**). The downregulation of *SPRY2, CAV1, SOCS2, ITM2A, LDB2,* and *NACA* (downregulated in BTS) is associated with a worse prognosis (**D**–**I**)

receptor interaction, Toll-like receptor signaling, antigen processing and presentation, chemokine signaling, T cell receptor signaling, B cell receptor signaling, natural killer cell-mediated cytotoxicity, leukocyte transendothelial migration, hematopoietic cell lineage, complement and coagulation cascades, Fc gamma R-mediated phagocytosis, Fc epsilon RI signaling pathway, NOD-like receptor signaling, Jak-STAT signaling pathway, cytosolic DNA-sensing, RIG-I-like receptor signaling, cell adhesion molecules (CAMs), focal adhesion, ECM–receptor

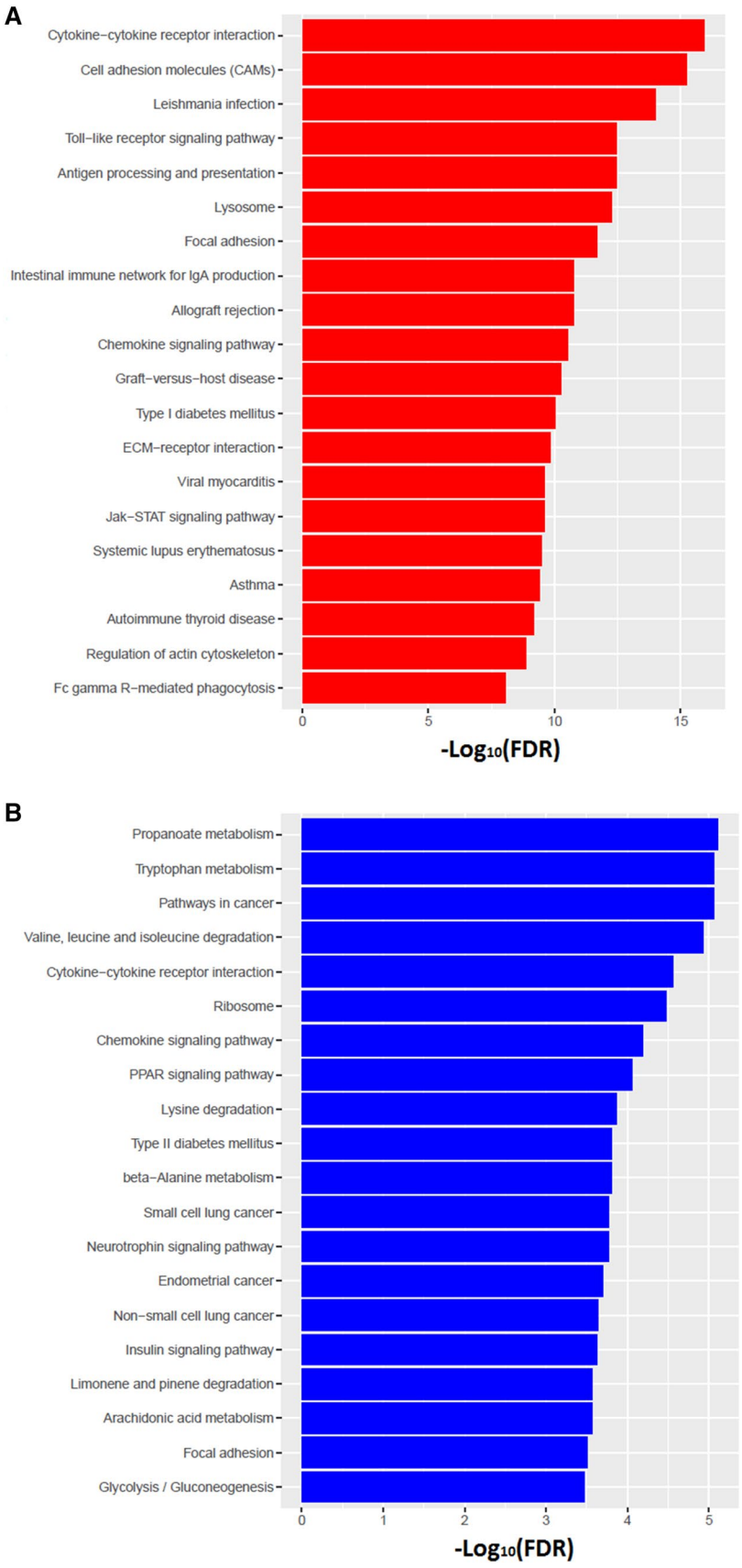


Fig. 2 KEGG pathways are significantly associated with the upregulated and downregulated genes in BTS versus normal stroma identified by GSEA [24]. **A** Top 20 pathways significantly associated with the DEGs upregulated in BTS. **B** Top 20 pathways significantly associated with the DEGs downregulated in BTS. FDR: false discovery rate

interaction, regulation of actin cytoskeleton, adherens junction, tight junction, and gap junction. Moreover, many cancer-associated pathways were included in the 82 pathways, including MAPK signaling, TGF-beta, VEGF signaling, calcium signaling, mTOR signaling, and apoptosis. Besides, we identified 67 KEGG pathways [25] associated with the DEGs downregulated in BTS. The top 20 (the lowest FDR) pathways are displayed in Fig. 2B and Supplementary Table S7. The downregulated pathways are mainly associated with metabolism (propanoate metabolism, tryptophan metabolism, valine, leucine and isoleucine degradation, lysine degradation, beta-alanine metabolism, limonene and pinene degradation, arachidonic acid metabolism, butanoate metabolism, fatty acid metabolism, histidine metabolism, metabolism of xenobiotics by cytochrome P450, drug metabolism-cytochrome P450, pyruvate metabolism, glycerolipid metabolism, arginine and proline metabolism, steroid hormone biosynthesis, retinol metabolism, ascorbate and aldarate metabolism, linoleic acid metabolism, glycine, serine and threonine metabolism, alanine, aspartate and glutamate metabolism, ether lipid metabolism, glycolysis/gluconeogenesis, etc.), cancers (pathways in cancer, small cell lung cancer, endometrial cancer, non-small cell lung cancer, bladder cancer, thyroid cancer, pancreatic cancer, renal cell carcinoma, prostate cancer, acute myeloid leukemia, colorectal cancer, etc.), and cellular signaling and development (ribosome, PPAR signaling pathway, neurotrophin signaling pathway, insulin signaling pathway, spliceosome, p53 signaling pathway, Wnt signaling pathway, etc.). Altogether, our pathway analysis underlines that the stromal gene signatures are associated with the alteration of pathways that regulating tumor immunity, cellular signaling, metabolism, and cancers.

Upstream TFs, kinases, and MTRs regulating the DEGs

The eXpression2Kinases algorithm identified 20 upstream TFs playing a significant regulatory role toward the DEGs (Supplementary Table S8). These TFs included IRF8, NFE2L2, TP63, RUNX1, SPI1, SMAD4, TRIM28, GATA2, AR, SUZ12, EGR1, KLF4, GATA1, RELA, TCF3, PPARD, RCOR1, TP53, SALL4, and NANOG. Interestingly, among the 20 upstream TFs, the genes encoding *IRF8, PPARD*, and *RUNX1* were significantly upregulated in BTS and the gene encoding *KLF4* was significantly downregulated in BTS (Supplementary Fig. S2A). *IRF8* is a tumor suppressor involved in the regulation of the signaling of breast cancer cells [35]. Dysregulation of *NFE2L2* is correlated with poor outcomes in breast cancer patients [36]. In the harsh metabolic conditions of the TME, *PPARD* promotes the survival of breast cancer cells [37]. In triple-negative breast cancer (TNBC), the expression of *RUNX1* is correlated with the poor survival prognosis [38]. One of the isoforms of *KLF4* is associated with the carcinogenesis of breast cancer [39]. Besides, we identified 116 upstream protein kinases, including CDK1, LYN, CSNK2A1, MAPK3, RPS6KA1, PRKACA, and TGFBR2 (Supplementary Table S9). Among these kinases, the genes encoding *DYRK2, LYN, ERBB2, RPS6KA1,* and *PRKACA* were significantly upregulated in BTS and the gene encoding *PKD1, RPS6KA5, EGFR,* and *FOXO3* were significantly downregulated in BTS (Supplementary Fig. S2B). *CSNK2A1* expression levels are significantly higher in a basal subtype of breast cancer [40].

MAPKs are associated with the downstream oncogenic signaling pathways in breast tumorigenesis [41]. Furthermore, we identified 13 MTRs, including IRF8, ETV7, STAT2, SPI1, IRF1, RELA, STAT1, IKZF1, RUNX1, IRF7, NFKB1, NKX3-2, and BATF, which were involved in the regulation of the genes upregulated in BTS (Fig. 3A and Supplementary Table S10). We also identified 11 MTRs targeting the genes downregulated in BTS, including HLF, FOXP2, JUND, NANOS1, RBBP9, FOS, TAF1, FOSL1, JUN, HAND1, and FOXJ3 (Fig. 3B and Supplementary Table S10). Interestingly, the genes encoding the MTRs *RUNX1, IRF7, ETV7, STAT2,* and *IRF8* were upregulated in BTS (Fig. 3A), and the genes encoding the MTRs *HLF* and *FOXJ3* were downregulated in BTS (Fig. 3B). Altogether, these results indicate that a number of TFs and protein kinases play significant roles in regulating the breast cancer stromal gene signatures and are associated with the pathogenesis of breast cancer.

Identification of prognostic hub genes in breast tumor stroma

To identify the hub genes of the DEGs in BTS, we input all the DEGs into the STRING tool [28]. We identified 233 hub genes (degree ≥ 25), including 194 upregulated and 39 downregulated genes in BTS (Supplementary Table S11 and Supplementary Fig. S3). Finally, we displayed the top 50 hub genes (*EGFR, TLR4, ITGAM, IL10, TLR2, CD86, IL1B, MMP9, ITGB2, TLR8, TLR7, ITGAX, MYC, CXCL10, TYROBP, CXCR4, IRF8, TLR3, CASP3, CTLA4, CSF1R, PLEK, LCP2, CD80, C3AR1, MYD88, IL10RA, PIK3R1, CYBB, SYK, SELL, FCGR2A, CXCL9, CCR7, CCR1, LYN, IRF7, CXCL1, PTGS2, RAC2, ERBB2, FCER1G, ISG15, HCK, CXCR3, CD4, IL7, FCGR2B, COL1A1,* and *OASL*) in Fig. 4A. We found that the upregulation of hub genes *MMP9, FCER1G, CD86, ITGAM, TLR2,* and *COL1A1* (upregulated DEGs in BTS) was significantly associated with poor RFS (Fig. 5A–F). These data indicate that the dysregulation of breast cancer stromal hub genes is likely

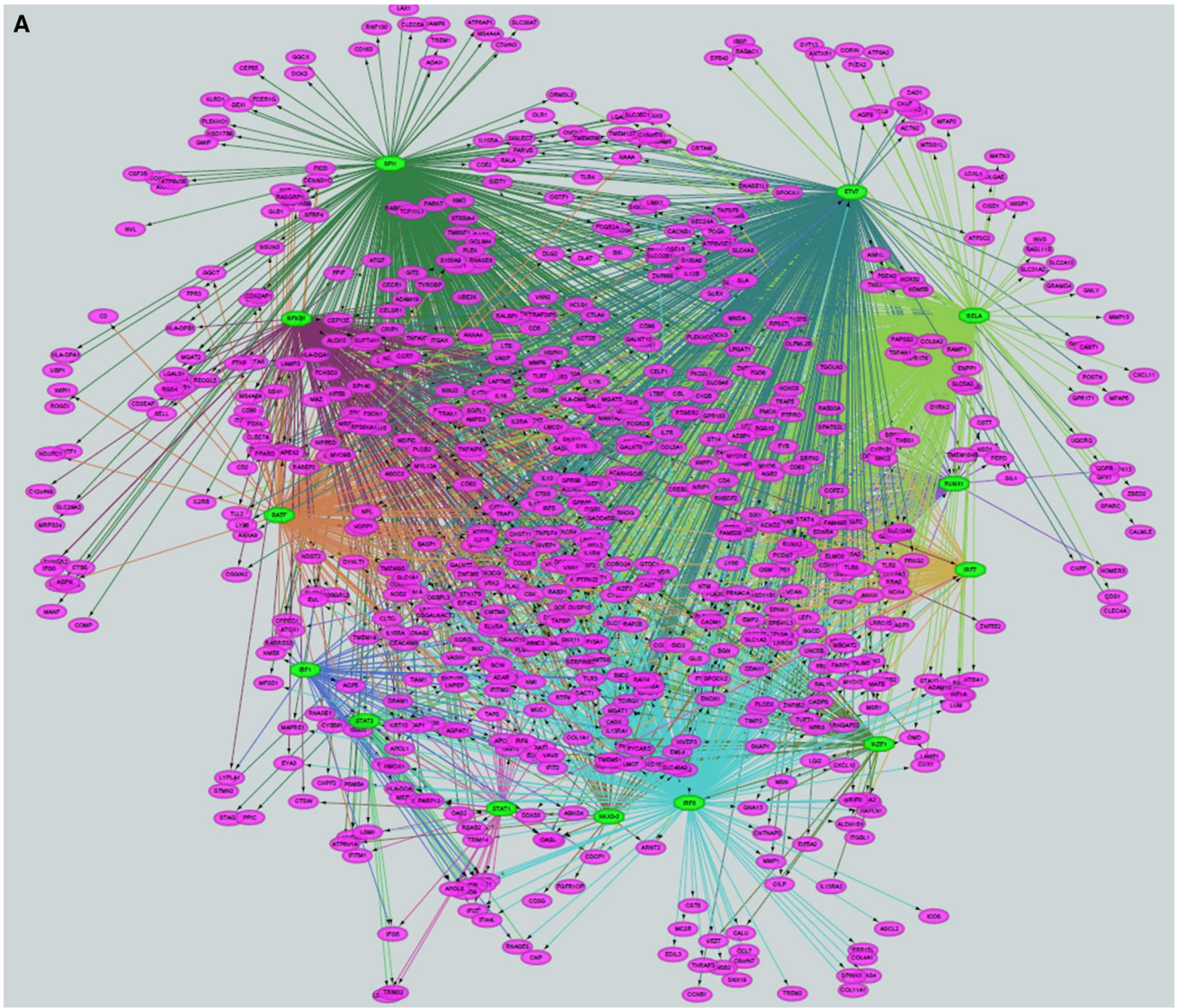


Fig. 3 Regulatory networks of the master transcriptional regulators (MTRs) and their targeted differentially expressed genes (DEGs) between BTS and normal stroma. **A** Regulatory network of the MTRs and their targeted upregulated genes in BTS. **B** Regulatory network of the MTRs and their targeted downregulated genes in BTS. In the center, green color octagon indicates MTRs, and purple color oval indicates DEGs

to be associated with poor prognosis in breast cancer patients.

Hub oncogenes, protein kinases encoding genes, and cytokines and growth factor-encoding genes are associated with poor survival prognosis in BTS

We identified the hub genes belonging to four gene families, including oncogenes, genes encoding protein kinases, genes encoding cytokines and growth factors, and tumor suppressor genes (Supplementary Fig. S3). We found 17 oncogenic hub genes, including *CD74, CIITA, CLTC, COL1A1, ERBB2, FCGR2B, IL21R, MUC1, SYK* (upregulated in BTS), and *EGFR, EPS15, FOXO3, MET, MYC, PPARG, RPL22, ZBTB16* (downregulated in BTS). Also, we found 11 protein kinases encoding genes (*CSF1R, CSK, EIF2AK2, HCK, LYN, PRKACA, RNASEL, SYK,* and *ERBB2* (upregulated in BTS) and *EGFR* and *MET* (downregulated in BTS), 20 cytokines and growth factors encoding genes (*CCL11, CCL7, CMTM6, CXCL10, CXCL11, CXCL9, IL10, IL16, IL1B, IL1RN, IL7, OSM, PLAU, PMCH,* and *TNFSF4* (upregulated in BTS) and *CAT, CCL27, CXCL1, CXCL2,* and *CXCL3*

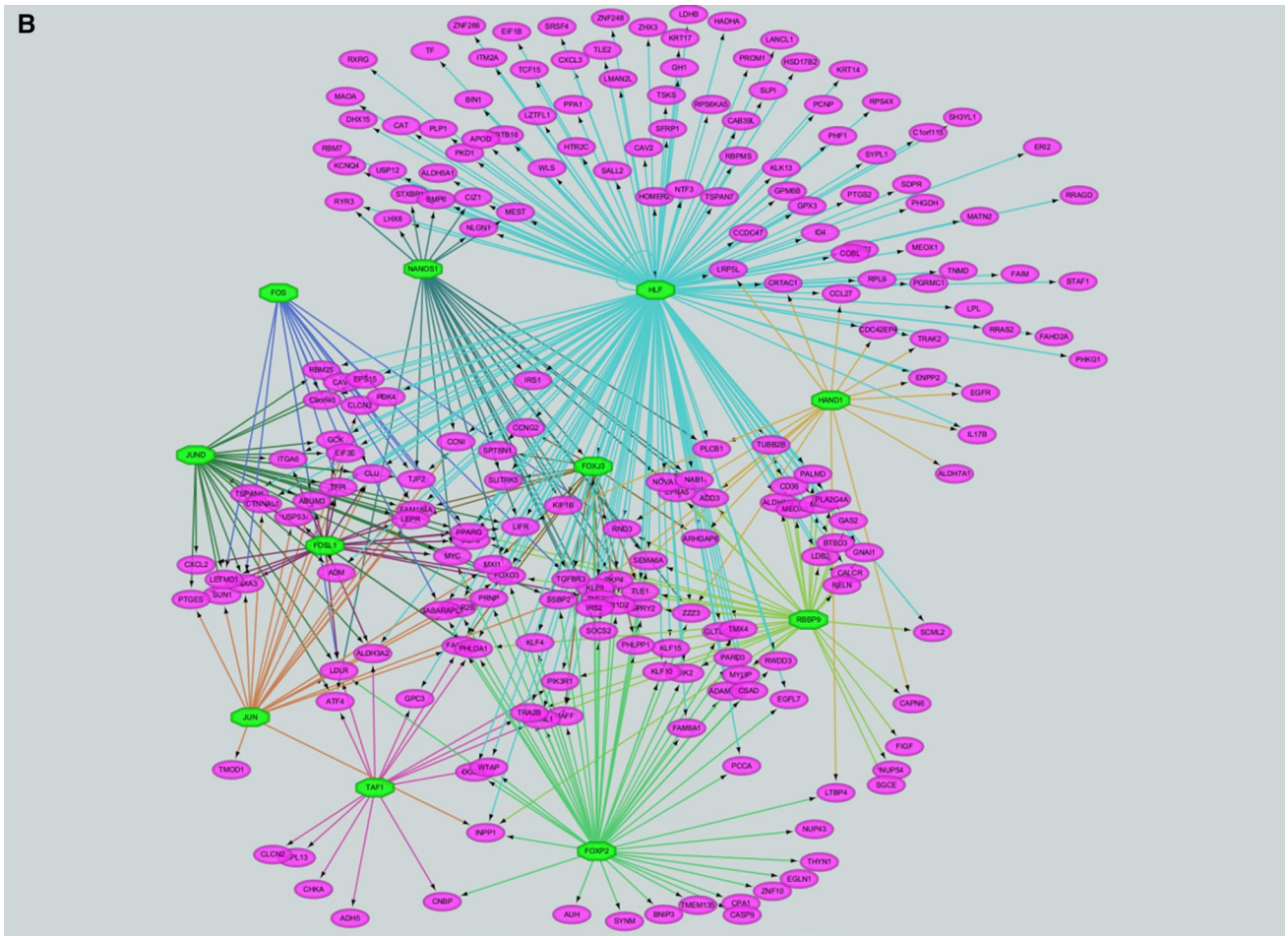


Fig. 3 (continued)

(downregulated in BTS), and 2 tumor suppressors genes (upregulated *TNFAIP3* and downregulated *PIK3R1*).

We investigated the association of these hub genes with survival prognosis. Besides, we investigated the specific gene-family-centric PPI of prognostic hub oncogenes (*IL21R* and *COL1A1*) (Fig. 4B), hub protein kinase genes (*PRKACA* and *CSK*) (Fig. 4C), and hub cytokines-and-growth-factor genes (*PLAU*) (Fig. 4D) with other stromal hub genes. We revealed that these families of genes were interacted with other stromal hub genes (Fig. 4B–D), indicating their regulatory roles in the TME of breast cancer. Survival analysis revealed that the upregulation of two oncogenes (*IL21R* and *COL1A1*), two protein kinase genes (*PRKACA* and *CSK*), and a cytokine and growth factor gene (*PLAU)* is associated with shorter RFS in breast cancer patients (Fig. 5F–J). Altogether, these results indicate that the dysregulation of many tumor stroma-derived gene signatures is associated with unfavorable clinical outcomes in breast cancer patients.

Comparisons of immune and stromal signatures between breast cancer patients with good and bad clinical outcomes

We found that stromal scores were lower in the breast cancer patients with bad clinical outcomes (Wilcoxon sum rank test, $P \leq 0.05$) (Fig. 6A). In contrast, tumor purity was higher in breast cancer patients with bad clinical outcomes (Fig. 6A). Interestingly, the enrichment scores (ssGSEA scores) of CD8 + T cells ($P = 0.007$), TILs ($P = 0.03$), and endothelial cells ($P = 0.05$) were lower in breast cancer patients with bad clinical outcomes than in those with good clinical outcomes (Fig. 6B). In contrast, MDSCs ($P = 0.05$) is more highly enriched in the breast cancer patients with bad clinical outcomes (Fig. 6B). The ratios of CD8 + /CD4 + regulatory T cells were lower in the breast cancer patients with bad clinical outcomes (Student's *t* test, $P = 4.5 \times 10^{-05}$) (Fig. 6C).

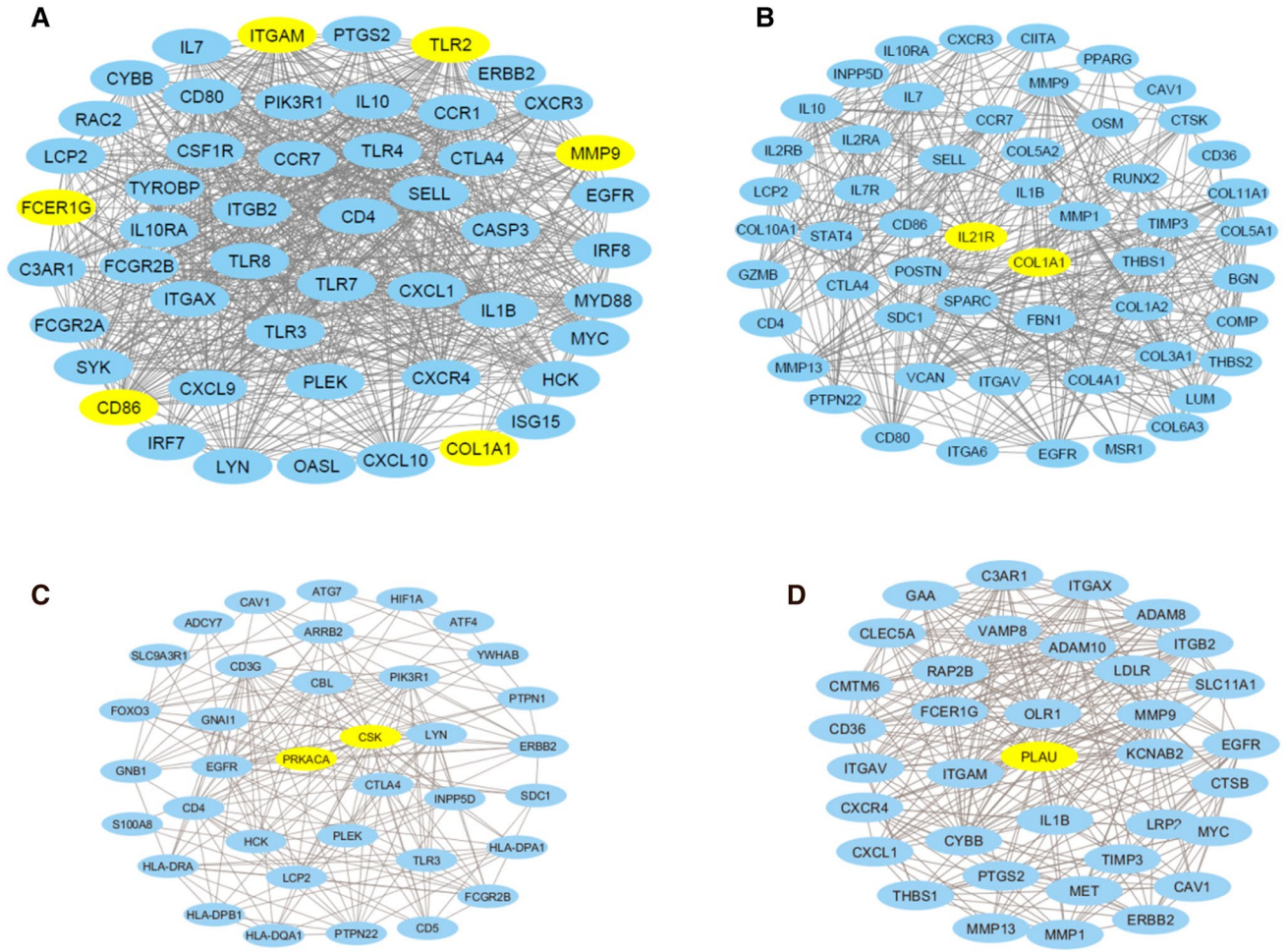


Fig. 4 Protein–protein interactions of prognostic hub genes in BTS. **A** Protein–protein interaction network of top 50 hub genes. The hub genes in yellow are associated with poor prognosis in breast cancer. **B** Two prognostic hub oncogenes (*IL21R* and *COL1A1*) interact with other hub genes. **C** Interactions of two prognostic protein kinases encoding genes (*PRKACA* and *CSK*) with other hub genes. **D** Prognostic growth factor encoding gene *PLAU* is a hub gene that interacts with other stromal hub genes

These results indicate that increased immune-promoting signatures are associated with better prognosis in breast cancer, while increased immunosuppressive signatures are associated with worse prognosis. This is consistent with the findings of previous studies [42–46].

Stromal gene signatures significantly altered with the grades, clinical outcomes, and survival prognosis

We found 1955 DEGs among three grades (grade I, II, and III) of breast cancers (F-test, $P < 0.05$) (Fig. 7 and Supplementary Table S12). Besides, we found 1471 DEGs between the good clinical outcome (patients without disease recurrence) and bad clinical outcome (patients with disease recurrence) groups (Student's t test, $P < 0.05$) (Fig. 7 and Supplementary Table S13). There were 124 common genes between both groups of DEGs (Fig. 7 and Supplementary Table S14). Furthermore, we found 20 of the 124 genes (*MCM4, SPECC1, IMPA2, AGO2, COL14A1, ESR1, SLIT2, IGF1, CH25H, PRR5L, ABCA6, CEP126, IGDCC4, LHFP, MFAP3, PCSK5, RAB37, RBMS3, SETBP1,* and *TSPAN11*) whose expression had a significant association with RFS prognosis (Fig. 7 and Supplementary Table S14).

MCM4, SPECC1, IMPA2, and *AGO2* were gradually upregulated through grade I, II, and III of breast cancers, and their elevated expression was associated with the worse clinical outcomes RFS (Fig. 8A). In contrast, *COL14A1, ESR1, SLIT2, IGF1, CH25H, PRR5L, ABCA6, CEP126, IGDCC4, LHFP, MFAP3, PCSK5, RAB37, RBMS3, SETBP1,* and *TSPAN11* were gradually downregulated through grade I, II, and III of breast cancers and their reduced expression were associated with worse clinical outcomes and RFS (4 genes are shown in Fig. 8B and 12 genes in Supplementary Fig. S4).

The univariate Cox regression analyses identified 23 genes (out of the 38 prognostic genes) were significant

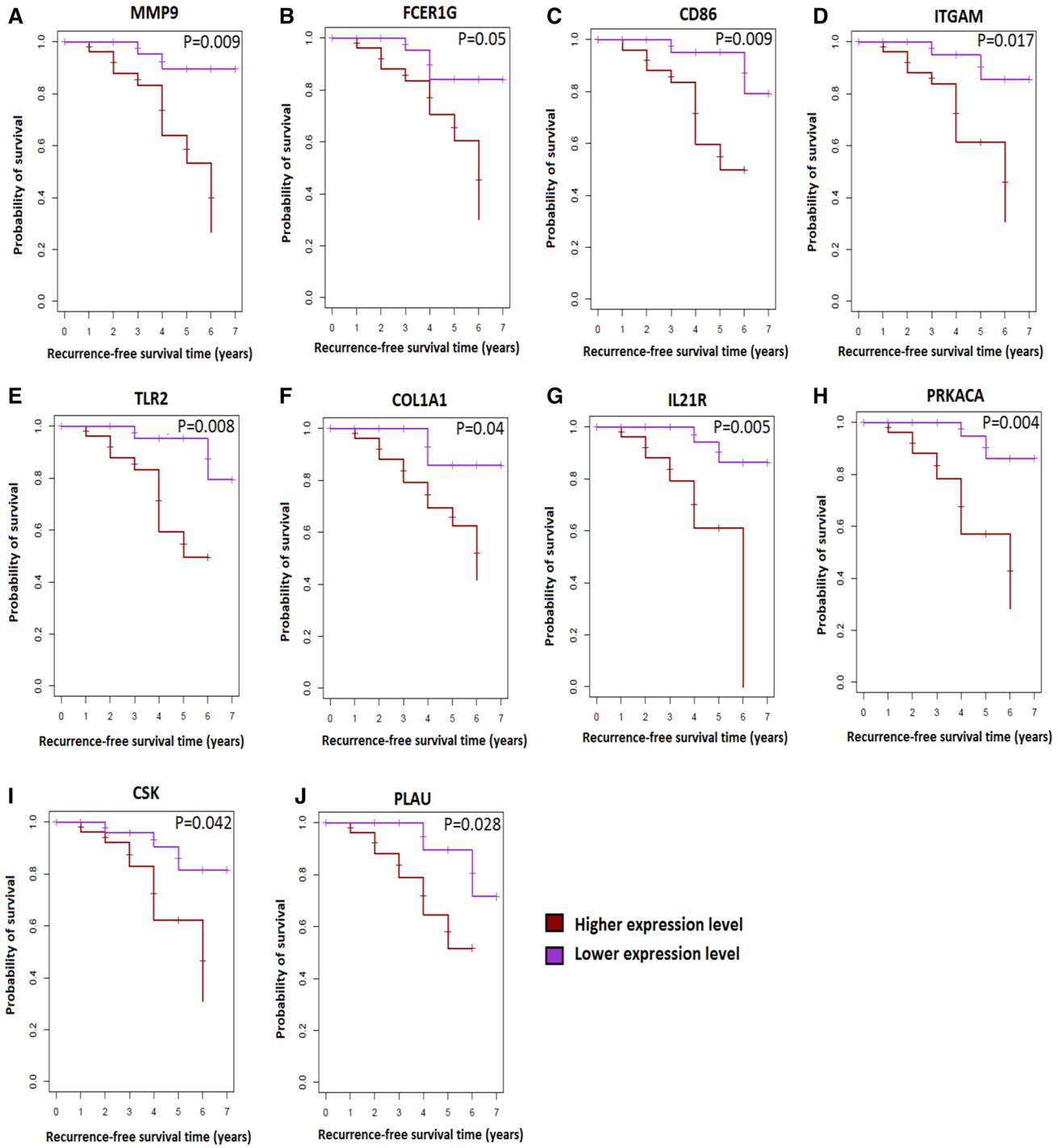


Fig. 5 Protein–protein interaction network analysis identifies prognostic hub genes in BTS. **A**–**F** High expression of the top hub genes is associated with poor prognosis in breast cancer. **F**–**G** The elevated expression of oncogenic hub genes *COL1A1* and *IL21R* is associated with poor prognosis in breast cancer. **H**–**I** The elevated expression of protein kinases encoding genes (*PRKACA* and *CSK*) is correlated with poor prognosis in breast cancer. **J** The elevated expression of growth factor encoding gene *PLAU* is linked with poor prognosis in breast cancer. The survival analysis is performed in the breast cancer dataset GSE9014

prognostic factors. These genes included *COL11A1, CAV1, ITM2A, LDB2, CD86, TLR2, COL1A1, SPECC1, IMPA2, AGO2, COL14A1, SLIT2, IGF1, CH25H, PRR5L, ABCA6, CEP126, IGDCC4, LHFP, MFAP3, RAB37, SETBP1,* and *TSPAN11* (Supplementary Fig. S5). We further performed the multivariate Cox regression analysis with the expression

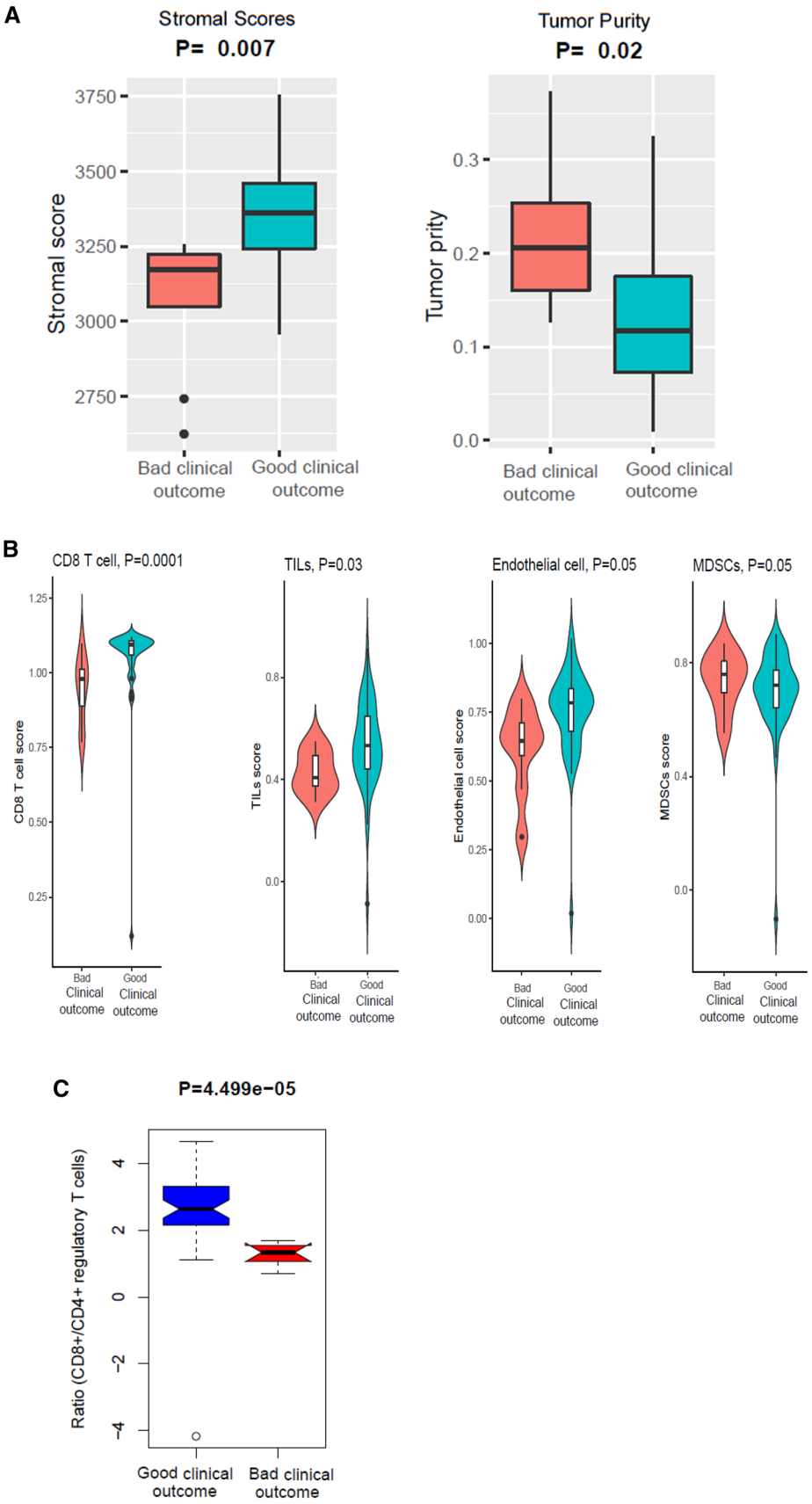


Fig. 6 Comparisons of immune and stromal signatures between breast cancer patients with good and bad clinical outcomes. **A** Comparisons of stromal scores and tumor purity between the bad and good clinical outcome groups of breast cancer patients. **B** The enrichment scores (ssGSEA scores) of CD8 + T cells, TILs, and endothelial cells are lower in the bad clinical outcome group. The enrichment scores of MDSCs is higher in the bad clinical outcome. **C** The ratios of CD8 + /CD4 + regulatory T cells are lower in the bad clinical outcome group

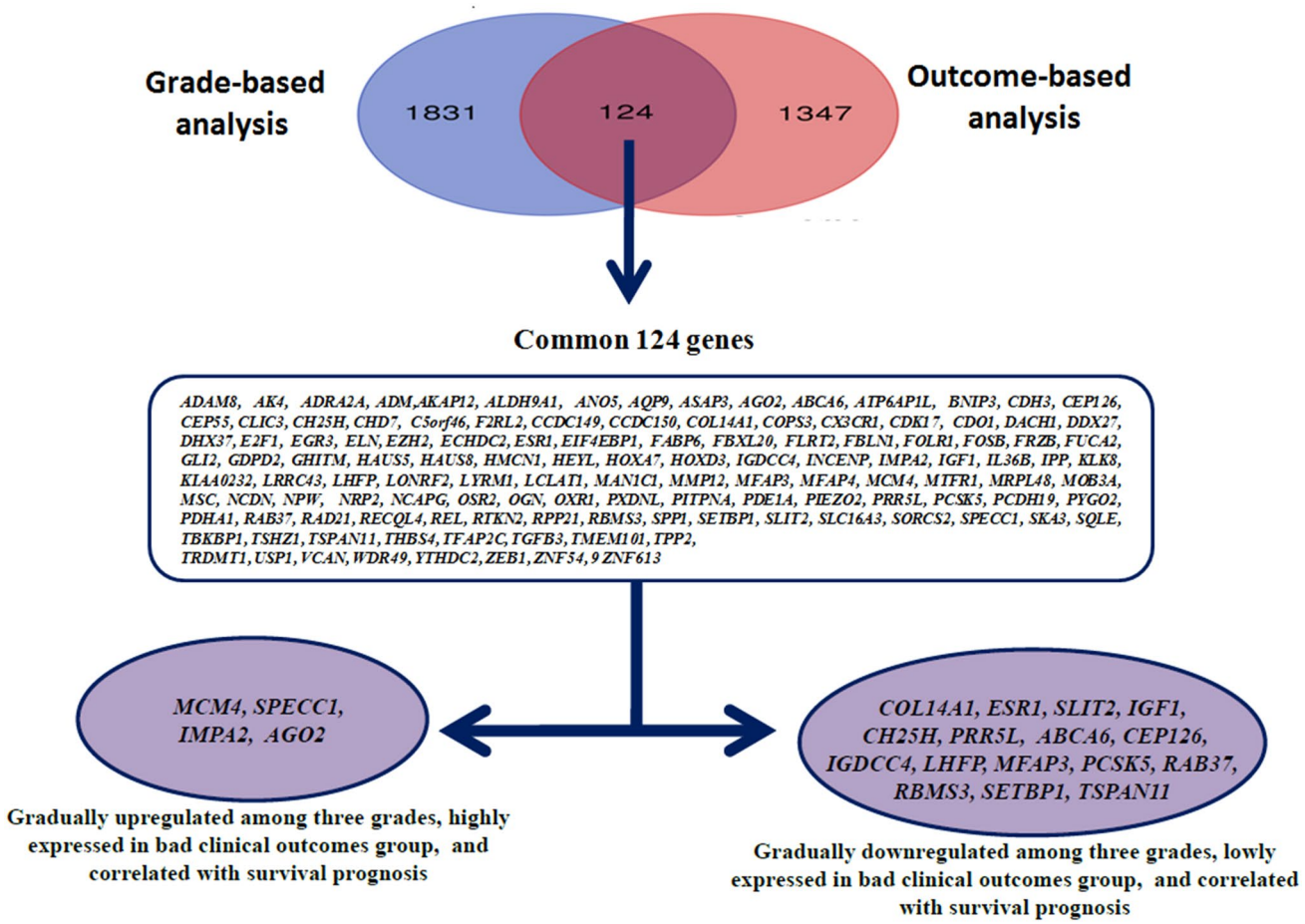


Fig. 7 20 genes which are significantly and gradually upregulated (*MCM4, SPECC1, IMPA2,* and *AGO2*) or downregulated (*COL14A1, ESR1, SLIT2, IGF1, CH25H, PRR5L, ABCA6, CEP126, IGDCC4, LHFP, MFAP3, PCSK5, RAB37, RBMS3, SETBP1,* and *TSPAN11*) through grade I, II, and III of breast cancers, deregulated in the bad clinical outcome group, and associated with poorer survival prognosis

levels of the 23 genes, breast cancer subtypes (including Her2 status, ER status, and PR status), and age being the predictor variables. We found that 19 prognostic genes (*COL11A1, ITM2A, LDB2, CD86, TLR2, COL1A1, IMPA2, AGO2, COL14A1, SLIT2, CH25H, PRR5L, CEP126, IGDCC4, LHFP, MFAP3, RAB37, SETBP1,* and *TSPAN11*), the three subtypes, and age were significant prognostic factors (Supplementary Fig. S6).

Analysis of the dysregulated stromal genes in TCGA BRCA cohort

We further analyzed the expression of the stromal prognostic upregulated and downregulated genes in the TCGA breast invasive carcinoma (BRCA) cohort. Interestingly, we found that the stromal upregulated prognostic genes *COL11A1* and *IFI30* were significantly upregulated in BRCA than in healthy tissue (Fig. 9A). Also, the downregulated stromal prognostic genes *SPRY2, CAV1, SOCS2, ITM2A, LDB2,* and *NACA* were significantly downregulated in BRCA (Fig. 9A). Moreover, we compared the expression levels of stromal prognostic hub genes (*COL1A1, MMP9, CD86, FCER1G, ITGAM,* and *TLR2*), oncogenes (*COL1A1* and *IL21R*), protein kinases encoding genes (*PRKACA* and *CSK*), and chemokines and growth factors encoding gene (*PLAU*) between TCGA BRCA and healthy tissue. We found that the expression levels of *COL1A1, MMP9, CD86, FCER1G, ITGAM, IL21R, CSK,* and *PLAU* genes were consistently upregulated in BRCA (Fig. 9B). Only *PRKACA* and *TLR2* were slightly downregulated in BRCA. Finally, we investigated the expression levels of prognostic stromal genes whose expression levels gradually altered among the three grades of tumor stroma and also altered between breast cancer patients with bad and good clinical outcomes. Interestingly, we found that *MCM4, SPECC1,* and *AGO2* genes were upregulated, and *ABCA6, CEP126, CH25H, COL14A1, IGDCC4, IGF1, MFAP3, PCSK5, RBMS3, SLIT2, TSPAN11, LHFP,* and

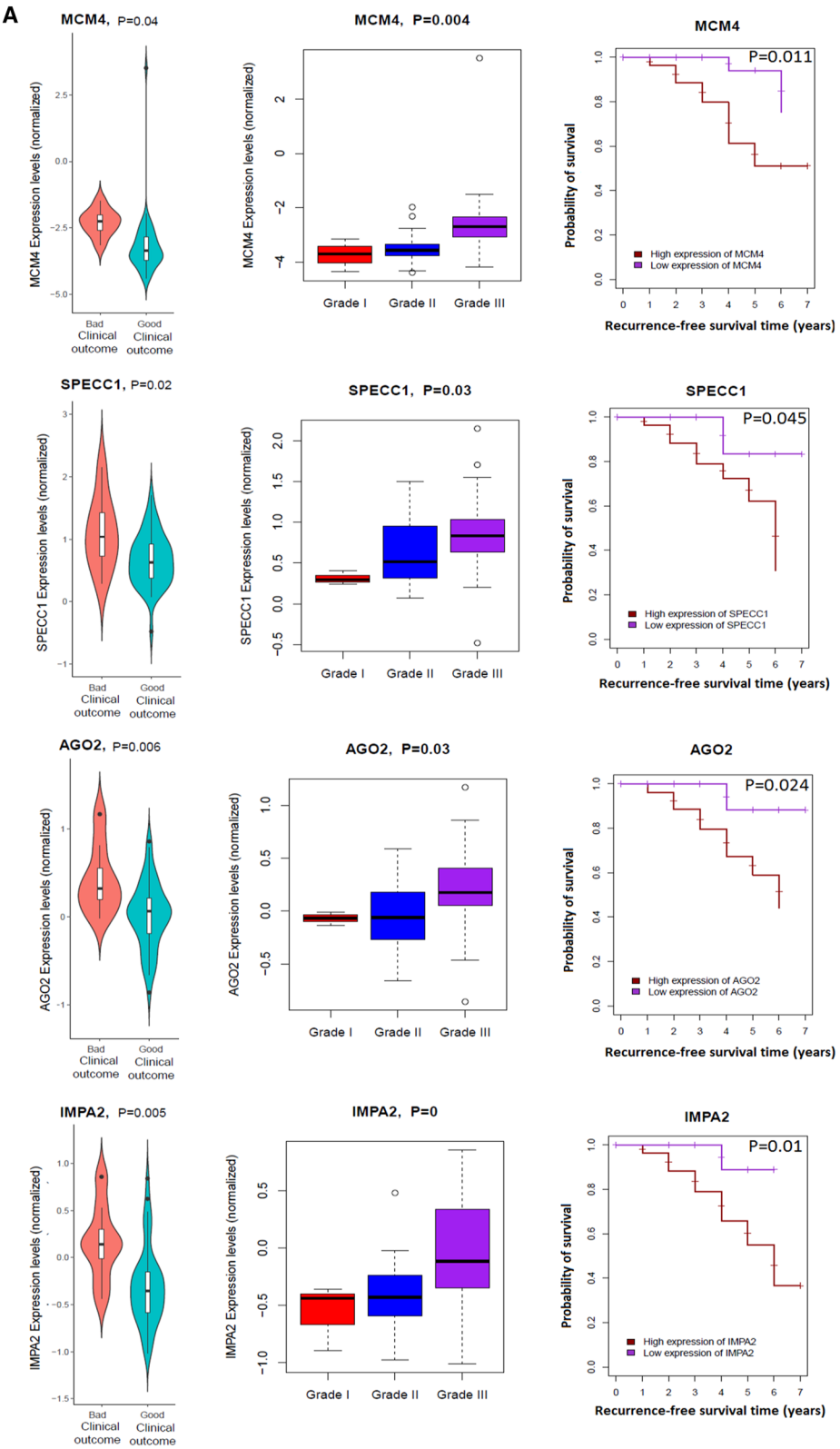
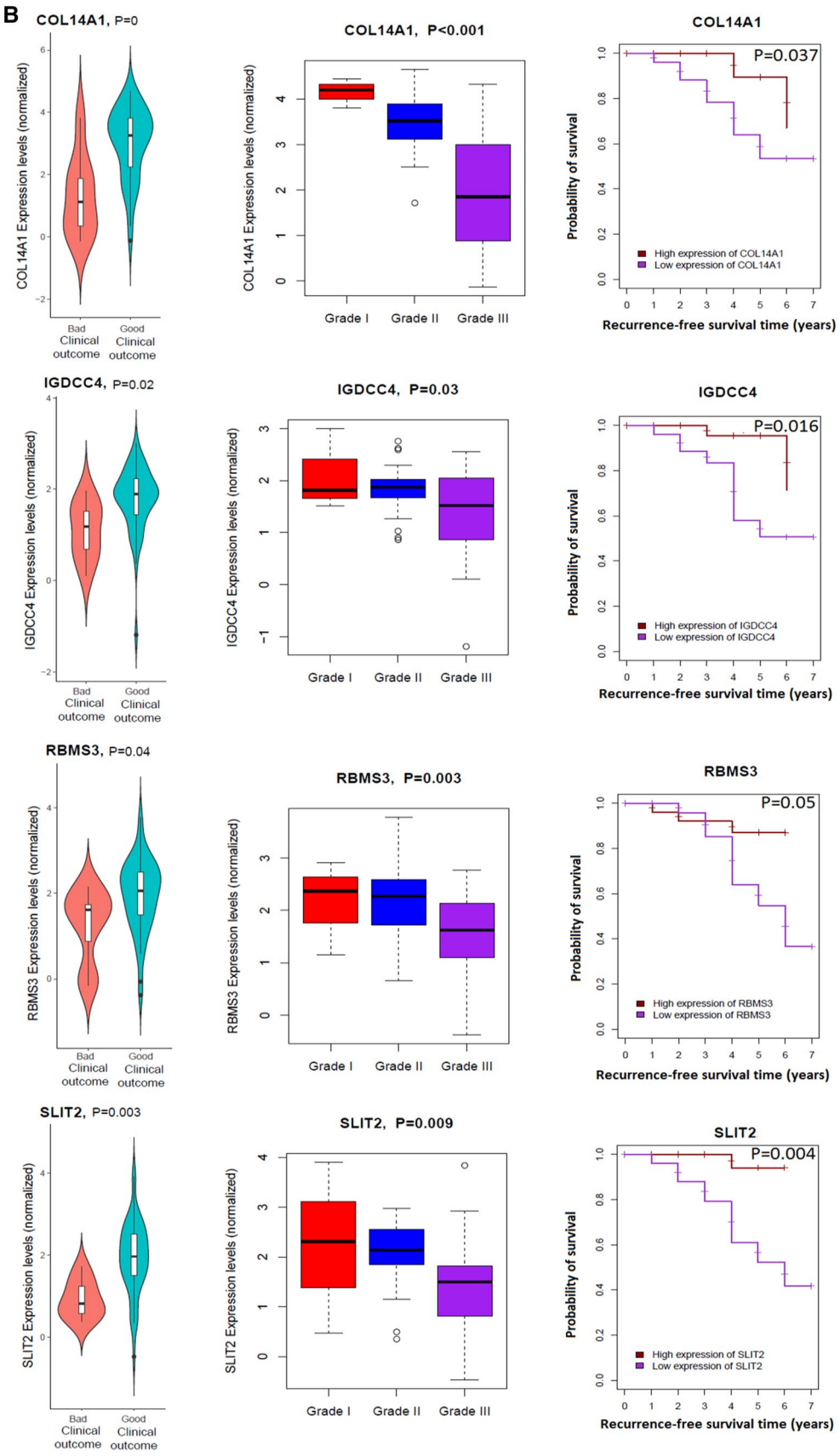
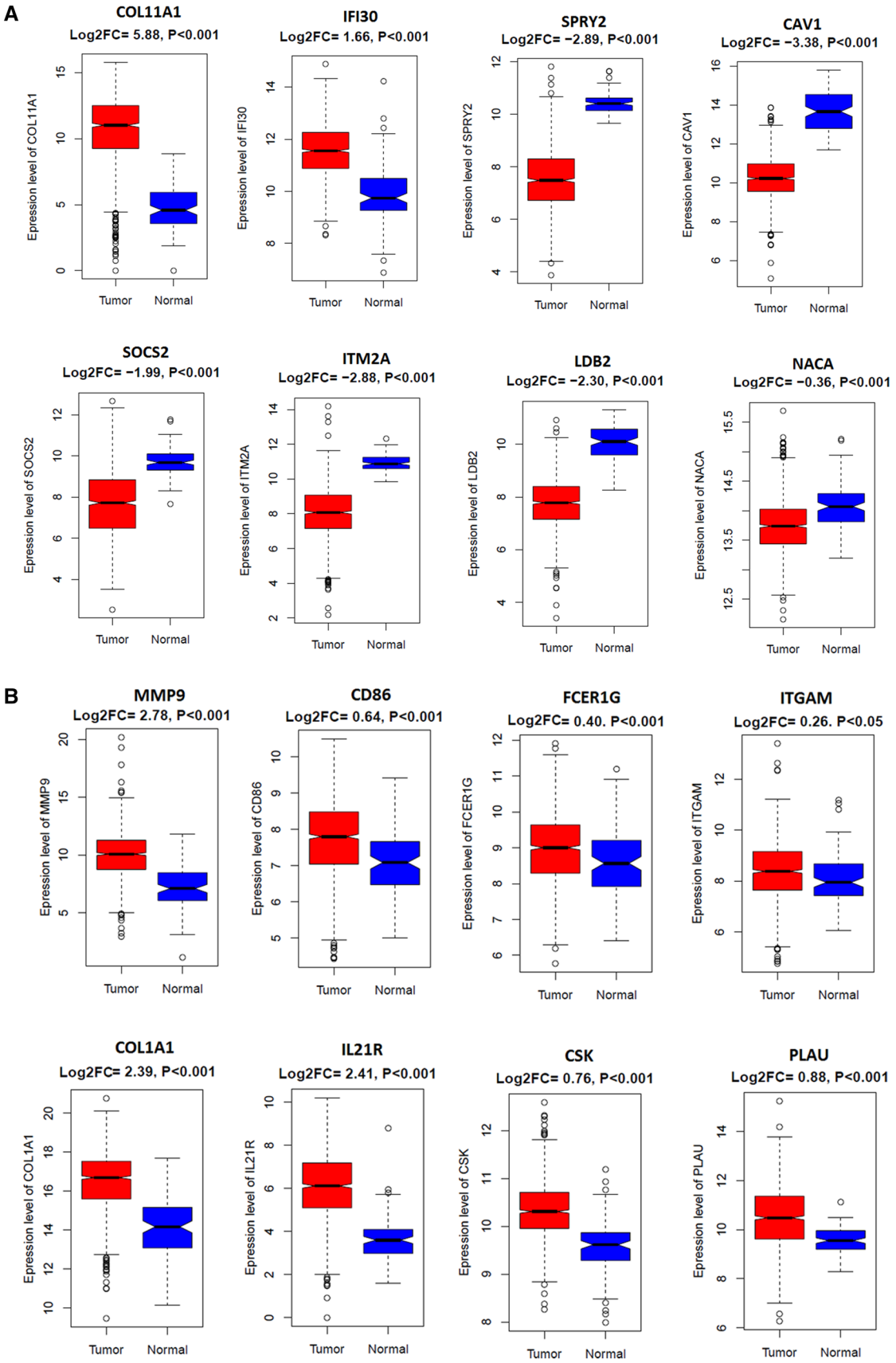


Fig. 8 Stromal genes significantly altered among the grades (grade I, II, and III), clinical outcomes, and their association with survival prognosis. **A** Gradually upregulated genes among three grades and their association with bad clinical outcome. **B** Gradually downregulated genes among three grades and their association with bad clinical outcome (Only 4 genes are shown)

Fig. 8 (continued)




A
COL11A1
Log2FC= 5.88, P<0.001
Epression level of COL11A1
IFI30
Log2FC= 1.66, P<0.001
Epression level of IFI30
SPRY2
Log2FC= −2.89, P<0.001
Epression level of SPRY2
CAV1
Log2FC= −3.38, P<0.001
Epression level of CAV1
SOCS2
Log2FC= −1.99, P<0.001
Epression level of SOCS2
ITM2A
Log2FC= −2.88, P<0.001
Epression level of ITM2A
LDB2
Log2FC= −2.30, P<0.001
Epression level of LDB2
NACA
Log2FC= −0.36, P<0.001
Epression level of NACA
Tumor
Normal
B
MMP9
Log2FC= 2.78, P<0.001
Epression level of MMP9
CD86
Log2FC= 0.64, P<0.001
Epression level of CD86
FCER1G
Log2FC= 0.40. P<0.001
Epression level of FCER1G
ITGAM
Log2FC= 0.26. P<0.05
Epression level of ITGAM
COL1A1
Log2FC= 2.39, P<0.001
Epression level of COL1A1
IL21R
Log2FC= 2.41, P<0.001
Epression level of IL21R
CSK
Log2FC= 0.76, P<0.001
Epression level of CSK
PLAU
Log2FC= 0.88, P<0.001
Epression level of PLAU

◂**Fig. 9** Comparisons of the expression levels of stromal dysregulated prognostic genes between TCGA BRCA and healthy tissue. **A** Stromal upregulated prognostic genes (*COL11A1* and *IFI30)* are upregulated in BRCA, and downregulated prognostic genes (*SPRY2, CAV1, SOCS2, ITM2A, LDB2,* and *NACA*) are downregulated in BRCA. **B** The stromal hub genes (*MMP9, COL1A1, CD86, FCER1G, ITGAM,* and *TLR2*), oncogenes (*COL1A1* and *IL21R*), protein kinases encoding gene (*CSK*), and chemokines and growth factors encoding gene (*PLAU*) are significantly upregulated in BRCA. The Student's *t* test *P* values and fold change are shown

SETBP1 were downregulated in BRCA (Table 2). These results confirmed that the stromal transcriptomes contribute to breast tissue carcinogenesis.

Discussion

Since stromal cells control various types of tumor phenotypes, including tumor growth, invasion, progression, metastasis, and angiogenesis [2, 3], identification of novel molecular features in BTS is significant. In this study, by analyzing a combined dataset composed of eight breast tumor stromal transcriptomic datasets, we identified the deregulated stromal gene signatures and their associated cellular signaling pathways and PPIs, as well as their associations with antitumor immunosuppression, poor clinical outcomes, and tumor progression in breast cancer (Fig. 10). Our meta-analysis identified 782 upregulated and 276 downregulated stromal genes in BTS versus normal stroma. The previous studies have shown that *COL10A1, COL11A1, NOX4,* and *COL8A1* were upregulated in the TME of breast cancer, and their upregulation was associated with the progression of aggressive breast cancers [17]. Overexpression of *COL11A1* is associated with worse clinical outcomes, including overall survival and disease-free survival [47]. *SULF1*, elevated with the second-highest ES in the BTS, is associated with the remodeling of extracellular matrix during the progression of breast cancer [48]. Elevated expression of *COMP* was found in the epithelial and stromal cells of invasive breast carcinomas [49]. Another bioinformatics study revealed that *IFI30, INHBA,* and *CTSB* were upregulated in breast cancer [50]. A previous study showed that *FIGF, SFRP1,* and *SPRY2* were consistently downregulated in multifocal invasive lobular breast tumors [51]. In breast cancer reactive stroma, *ITM2A* downregulation is associated with shorter survival of patients [13]. Matrix-producing stromal *LDB2* has prognostic value in breast cancer [52]. Among the downregulated genes in BTS, many have been associated with breast cancer onset, invasion, progression, and metastasis [53–58]. It was also reported that the expression levels of *DLK1* and *CD36* were downregulated in breast cancer [53]. The type III TGF-beta receptor (*TGFBR3*), a tumor suppressor gene, is associated with breast cancer progression and metastasis [54]. Silencing of *GPC3* expression is associated with the growth, invasion, and metastasis of MCF-7 human breast cancer cells [55]. Altogether, many of the aberrantly expressed genes in BTS have been associated with breast cancer pathogenesis and carcinogenesis.

Based on these dysregulated genes, we identified pathways upregulated and downregulated in BTS. The upregulated pathways were mainly involved in immune signatures, stromal signatures, oncogenic signatures, and metabolism. Many of these pathways were involved in cancer initiation, progression, angiogenesis, and metastasis in breast cancer [59–61]. For example, the group of cytokines and their receptors are associated with breast cancer growth and progression [59]. The ECM–receptor interaction pathway, a major stromal signaling pathway, is involved in breast cancer development [62]. The downregulated pathways were mainly associated with the alteration of metabolisms. In fact, deregulated cellular metabolisms in glucose, amino acid, and other nutrients, are major hallmarks of cancers [63]. The ribosome pathway is one of the major targeting pathways in cancer therapeutic [64]. As a tumor-suppressive signaling, the p53 signaling pathway is a crucial target in cancer therapy [65]. These results indicate that the transcriptional signatures of BTS are associated with the alteration of numerous cancer-associated pathways.

We identified 13 and 11 MTRs which regulate the upregulated stromal DEGs and downregulated stromal DEGs, respectively**.** Previous studies have shown that these MTRs are associated with breast carcinogenesis [13, 66–69]. For

Table 2 Comparisons of the expression levels of the genes between TCGA BRCA and healthy tissue, whose expression levels gradually altered among breast cancers with three different grades and between breast cancers with bad and good clinical outcomes

SL. number	Gene symbol	$\log_2$FC	*P* value
1	*MCM4*	1.47	4.06E–48
2	*SPECC1*	0.32	0.000388
3	*AGO2*	0.38	3.84E–05
4	*ABCA6*	− 3.33	4.75E–80
5	*CEP126*	− 1.64	1.16E–27
6	*CH25H*	− 1.30	1.13E–19
7	*COL14A1*	− 2.35	4.32E–38
8	*IGDCC4*	− 0.64	1.28E–07
9	*IGF1*	− 2.87	5.9E–55
10	*MFAP3*	− 0.19	0.00179
11	*PCSK5*	− 1.87	5.61E–38
12	*RBMS3*	− 2.66	7.32E–81
13	*SLIT2*	− 1.93	5.82E–31
14	*TSPAN11*	− 1.57	7.59E–33
15	*LHFP*	− 2.24	3.9E–99
16	*SETBP1*	− 1.17	6.19E–22

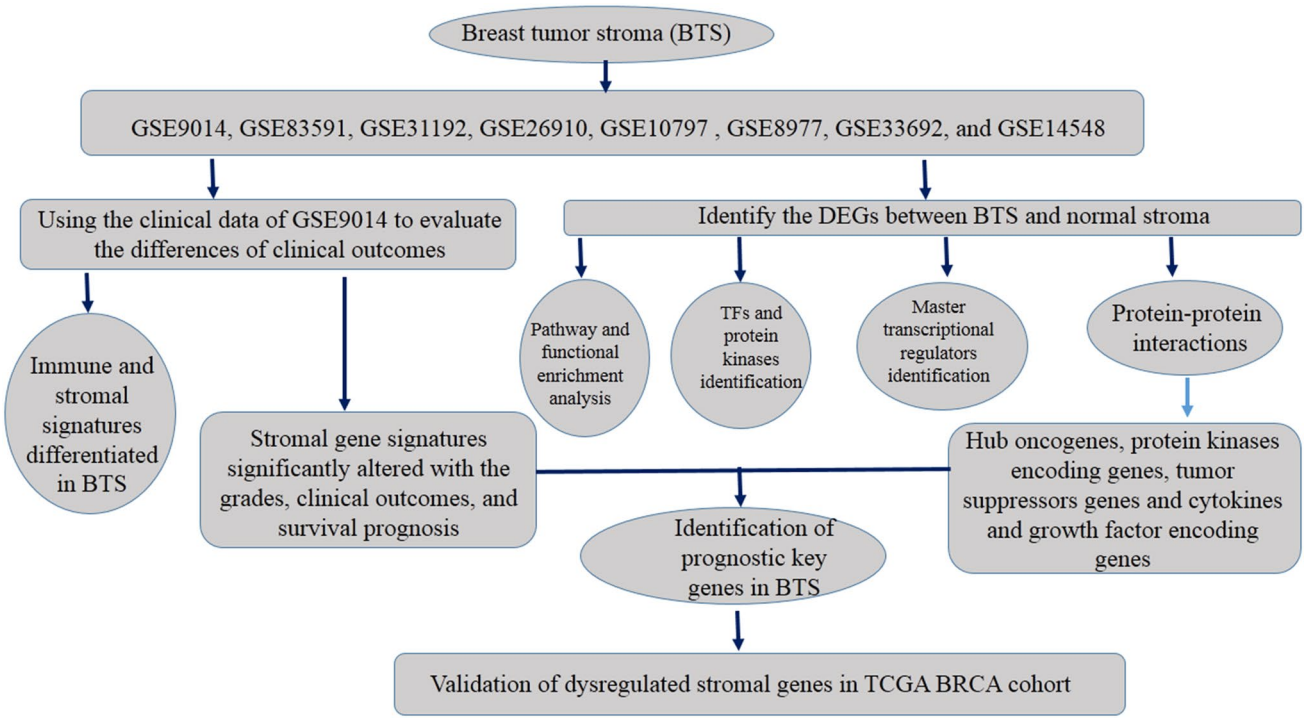


Fig. 10 The overall flow of the study that identifying key genes and pathways in the BTS

example, *ETV7* plays a substantial role in the oncogenesis of breast tissue [67]. In nodal positive breast cancer patients, another MTR, *STAT2* is associated with RFS [66]. *RELA* is elevated in the CAFs derived from Her2 + breast cancer tissue [68]. Another study confirmed the altered expression of *JUND, FOS,* and *JUN* in breast cancer tissue [69]. These results indicate that these TFs may control the BTS-associated transcriptions in the TME of breast cancer.

We also identified key hub genes, especially oncogenes, protein kinases encoding genes, cytokines and growth factors encoding genes in BTS based on the PPI network analysis. PPI networks indicate cellular signaling, communication, and crosstalk between the cells [70]. These hub genes included adverse prognostic factors, such as *MMP9*, *FCER1G*, *CD86*, *ITGAM*, *TLR2*, *COL1A1*, *IL21R*, *PRKACA* , *CSK*, *PLAU*, *MYC*, and *RNASEL*. In luminal A breast cancers, the matrix metalloproteinase gene *MMP9* is associated with poor clinical outcomes [10]. In addition, a previous study have shown that *ITGAM* and *TLR2* act as hub genes in breast cancer [71]. *IL21R* is highly expressed in breast cancer cells and is associated with proliferation, invasion, and migration of breast cancer cells [72]. The elevated levels of *COL1A1* are linked with shorter survival and chemotherapy resistance [73]. Moody et al. reported that *PRKACA* mediated the therapy resistance in breast cancer [74]. Another prognostic stromal protein kinase, *CSK*, is associated with the cellular growth of hormone-independent breast cancer tissue [75]. In metastatic breast cancer, elevated *PLAU* is associated with poor survival prognosis [76]. However, we found that the upregulation of *MYC* (an oncogene downregulated in BTS) and downregulation of *RNASEL* (a protein kinase gene downregulated in BTS) were associated with worse survival in breast cancer patients. These data suggest that the deregulation of key molecules in PPI networks associated with stromal signatures contributes to the aggressive TME compartment in breast cancer.

The BTS-specific molecules and their associated pathways and interaction networks are potential prognostic biomarkers and therapeutic targets for breast cancer. For example, the upregulation of *COL11A1* and *IFI30* could indicates a worse prognosis, while the downregulation of *SPRY2, CAV1, SOCS2, ITM2A, LDB2,* and *NACA* could indicates a better prognosis in breast cancer. The small molecule inhibitors targeting the kinases upregulated in BTS, such as *CSF1R, CSK, EIF2AK2, HCK, LYN, PRKACA, RNASEL, SYK,* and *ERBB2*, could be effective in controlling breast tumor progression.

This study has several limitations. First, the findings were obtained by the bioinformatics analysis but lack of experimental validation. Second, the number of cancer samples with clinical data, such as survival time, tumor grade, and cancer subtypes, is limited in this study. Finally, we analyzed mRNA expression profiles which are not necessarily the same as protein expression profiles, due to some factors affecting the translation from mRNA level to protein level, such as post-translational modification. Therefore,

to translate our findings into clinical applications, further experimental and clinical validation would be necessary.

Conclusions

Our data provide pivotal molecular insights into breast tumor stroma characterization, which may have substantial effects on the stroma-based treatment recommendations for breast cancer patients.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12282-022-01332-6.

Author contributions MNU performed data analyses, conceived the research, and prepare the manuscript. XW conceived the research, designed analysis strategies, and wrote the manuscript. Both authors read and approved the final manuscript.

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Data availability The datasets (GSE9014 [9], GSE83591 ($n=53$)[11], GSE31192 ($n=17$) [12], GSE26910 ($n=12$) [13], GSE10797 ($n=33$) [14], GSE8977 ($n=22$) [15], GSE33692 ($n=22$) [16], and GSE14548 ($n=34$) [17]) were downloaded from the NCBI GEO database (https://www.ncbi.nlm.nih.gov/geo/), the TCGA breast cancer dataset was downloaded from the website https://gdc-portal.nci.nih.gov/.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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