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CANCER INNOVATION

ORIGINAL ARTICLE

Integrative analyses identified gap junction beta-2 as a prognostic biomarker and therapeutic target for breast cancer

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Abstract

Background: Increasing evidence has shown that connexins are involved in the regulation of tumor development, immune escape, and drug resistance. This study investigated the gene expression patterns, prognostic values, and potential mechanisms of connexins in breast cancer.

Methods: We conducted a comprehensive analysis of connexins using public gene and protein expression databases and clinical samples from our institution. Connexin mRNA expressions in breast cancer and matched normal tissues were compared, and multiomics studies were performed.

Results: Gap junction beta-2 mRNA was overexpressed in breast cancers of different pathological types and molecular subtypes, and its high expression was associated with poor prognosis. The tumor membrane of the gap junction beta-2 mutated group was positive, and the corresponding protein was expressed. Somatic mutation and copy number variation of gap junction beta-2 are rare in breast cancer. The gap junction beta-2 transcription level in the p110α subunit of the phosphoinositide 3-kinase mutant subgroup was higher than that in the wild-type subgroup. Gap junction beta-2 was associated with the phosphoinositide 3-kinase-Akt signaling pathway, extracellular matrix–receptor interaction, focal adhesion, and proteoglycans in cancer. Furthermore, gap junction beta-2 overexpression may be associated with phosphoinositide 3-kinase and histone deacetylase inhibitor resistance, and its expression level correlated with infiltrating CD8+ T cells, macrophages, neutrophils, and dendritic cells.

Abbreviations: BC, breast cancer; CDK, cyclin-dependent kinase; CNV, copy number variant; CSC, cancer stem cell; CTRP, Cancer Therapeutics Response Portal; Cx, connexin; DAVID, The Database for Annotation, Visualization and Integrated Discovery; DMFS, distant metastasis-free survival; EGFR-TKI, epidermal growth factor receptor–tyrosine kinase inhibitor; ER, estrogen receptor; FDR, false discovery rate; GJ, gap junction; GJB2, gap junction beta-2; GJIC, gap junction intercellular communication; GO, Gene Ontology; GSCA, Gene Set Cancer Analysis; HDAC, histone deacetylase; HER2, human epidermal growth factor receptor 2; HPA, Human Protein Atlas; IC50, half-maximal inhibitory concentration; KEGG, Kyoto Encyclopedia of Genes and Genomes; MEK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; OS, overall survival; PPS, postprogression survival; RFS, relapse-free survival; TCGA, The Cancer Genome Atlas; TIMER, The Tumor Immune Estimation Resource; TNBC, triple-negative breast cancer.

Conclusions: Gap junction beta-2 may be a promising therapeutic target for targeted therapy and immunotherapy and may be used to predict breast cancer prognosis.

KEYWORDS
breast cancer, connexin, gap junction beta-2, phosphoinositide 3-kinase-Akt-mTOR pathway, prognosis

1 | INTRODUCTION

Breast cancer (BC) is one of the most common types of malignancy in women, with approximately 1.7 million new cases diagnosed worldwide annually [1, 2]. Despite advancements in screening, testing methods, and treatment modalities over the past 10 years, BC is still one of the leading causes of cancer-related deaths worldwide [3]. Tumor relapse and metastasis are the primary factors contributing to BC-specific deaths. Moreover, the frequency of drug resistance increases with prolonged survival time [4, 5]. Thus, identifying specific prognostic markers and therapeutic targets is key to improving the overall survival (OS) of patients with BC.

Gap junctions (GJs), also known as connexins (Cxs), consist of 21 Cx genes in humans [6]. GJ channels are formed by the docking of two hexameric hemichannels from different cells, and they maintain intercellular communication between cells through the exchange of ions, small metabolites, and electrical signals [7, 8]. Gap junction intercellular communication (GJIC) plays a crucial role in the maintenance of cell homeostasis, regulation of cell differentiation, and occurrence and development of tumors [9, 10]. Functional defects of Cxs are associated with numerous functional and pathophysiological processes. Cx disorders can cause various genetic diseases, including nonsyndromic deafness and skin disease [11].

Cxs, such as Cx26 and Cx43, are downregulated in primary tumors and are candidate tumor suppressors [12]. Structural variations in Cx genes can result in abnormal GJIC, thereby compromising the body's monitoring and regulatory mechanisms and facilitating excessive proliferation of tumor cells. GJs also enhance apoptosis caused by a variety of chemotherapeutic agents [13]. However, the evidence for Cx genes as tumor suppressor genes is insufficient, and contradictory results have been reported. Overexpression of Cxs was shown to enhance the activity of cancer stem cells (CSCs) and promote tumor cell growth and metastasis [14–18]. Moreover, higher mRNA expression of Cxs predicts poor prognosis in several tumors [19–21]. Additionally, intercellular communication between CSCs and the cellular niche is involved in long-term dormancy. GJIC between dormant cancer cells and the cellular niche facilitates the exchange of molecules to partially induce drug resistance and immune evasion of cancer cells [22].

The expression patterns, prognostic values, and potential mechanisms of Cxs in BC have not been fully elucidated. Therefore, we performed a comprehensive analysis of Cxs in BC using public databases and clinical samples from our hospital. By comparing the mRNA expression of Cxs in BC and matched normal tissues, we identified differentially expressed Cxs and conducted multiomics studies, including analysis of transcription, protein, and methylation levels and somatic variations. We also analyzed the potential function and mechanism of Cxs and the relationship between Cxs and drug sensitivity.

2 | MATERIALS AND METHODS

2.1 | Data sources

We used ONCOMINE gene expression array data sets from https://www.oncomine.org/resource [23, 24] to analyze the transcription levels of Cxs in different cancers. The mRNA expression of Cxs was compared in clinical cancer specimens and normal controls, and Student's *t*-test was performed to generate *p*-values. The cutoff and fold change were set at 0.01 and 2, respectively.

2.2 | Protein expression comparison

Gap junction beta-2 (GJB2) protein expression in human BC and normal tissues was analyzed using the Human Protein Atlas (HPA) (https://www.proteinatlas.org) [25]. GJB2 protein expression was evaluated in human BC and normal tissues by immunohistochemistry (IHC) using anti-GJB2 rabbit polyclonal antibody (Sangon Biotech; Order NO.D160410). Tissue sample preparation, antigen retrieval, primary antibody incubation, secondary antibody application, and visualization were performed following standard IHC procedures. Moreover, to assess

the protein expression levels between the GJB2 non-mutated group and the GJB2 mutated group, clinical samples were obtained from our hospital. IHC analysis was performed on these samples using specific antibodies against the GJB2 protein. The staining patterns of the tumor membranes were then examined under a microscope. To ensure accuracy, each sample was evaluated by a trained pathologist who was blinded to the sample groups. Positive staining on the tumor membrane was indicative of GJB2 protein expression. The comparison between the two groups was conducted based on these staining results.

2.3 | Survival analysis

Kaplan–Meier Plotter (http://kmplot.com/analysis) was used for survival analysis [26]. Patient samples were divided into two groups using the best cutoff value of mRNA expression and assessed using a Kaplan–Meier survival plot, with hazard ratio, 95% confidence interval, and log rank *p*-value.

2.4 | Gene expression analysis

The cBioPortal (http://cbioportal.org) [27], which contains data from 225 cancer studies, was used to examine *GJB2* expression. The data set of The Cancer Genome Atlas (TCGA)-Firehose Legacy was used to analyze the expression of *GJB2*. The co-expression interface and acquired genes correlated with *GJB2* were downloaded.

2.5 | Epigenetic analysis

UALCAN (http://ualcan.path.uab.edu) [28], a comprehensive and interactive web resource, allows exploration of epigenetic regulation through promoter methylation. The beta value indicates the level of DNA methylation, ranging from 0 (unmethylated) to 1 (fully methylated). Beta cutoff values 0.7–0.5 and 0.3–0.25 were considered to indicate hypermethylation and hypomethylation, respectively.

2.6 | Functional annotation

Functional annotations were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) database (http://david.abcc.ncifcrf.gov/) [29]. DAVID allows for comprehensive functional annotations to examine the biological meaning of genes. Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed using the DAVID database. A false discovery rate (FDR) < 0.01 and $p < 0.05$ were set as the cutoff criteria.

2.7 | Immune response analysis

The Tumor IMmune Estimation Resource (TIMER) algorithm database (https://cistrome.shinyapps.io/timer) [30] was used to analyze the expression of *GJB2* and its association with infiltrating immune cells in patients with BC. Tumor purity, which is an important factor affecting the analysis of immune infiltration in tumor samples by genomic methods, was adjusted.

2.8 | Drug sensitivity analysis

Gene Set Cancer Analysis (GSCA; http://bioinfo.life.hust.edu.cn/GSCA) integrates genomic and immunogenomic gene sets for drug sensitivity analysis. Over 750 small molecule drugs from the Genomics of Drug Sensitivity in Cancer (GDSC) and Cancer Therapeutics Response Portal (CTRP) databases were incorporated. Results with FDR < 0.05 were considered statistically correlated.

3 | RESULTS

3.1 | Transcription level of Cxs in BC

The transcription levels of Cxs in BC were analyzed in this study. Cxs are a family of proteins that form GJ channels, which enable direct intercellular communication. In humans, twenty-one Cx genes have been identified. The transcription levels of Cxs in cancer tissues and the paired normal tissues were compared using the ONCOMINE database (Figure 1a). Specifically, Cxs, such as *GJA1, GJA4, GJB1*, and *GJB2*, which encode GJ proteins, were found to be highly expressed in a variety of solid tumors.

Table 1 lists the Cx genes that are differentially expressed in breast tumors compared with normal tissues. The transcription levels of *GJA3, GJA4, GJA5, GJA8, GJA9, GJB1, GJB2, GJB3, GJB7, and GJC2* differed between BC and normal tissues in at least one data set. The mRNA expression of GJA1, GJA10, GJB4, GJB5, GJB6, GJC1, GJC3, GJD2, GJD3, GJD4, and GJE1 was not significantly different between BC and matched normal tissues. Notably, *GJB2* was upregulated in different pathological types of BC, whereas the mRNA expression of other Cxs correlated with the pathological type and sex. The TIMER1.0 online tool was used to

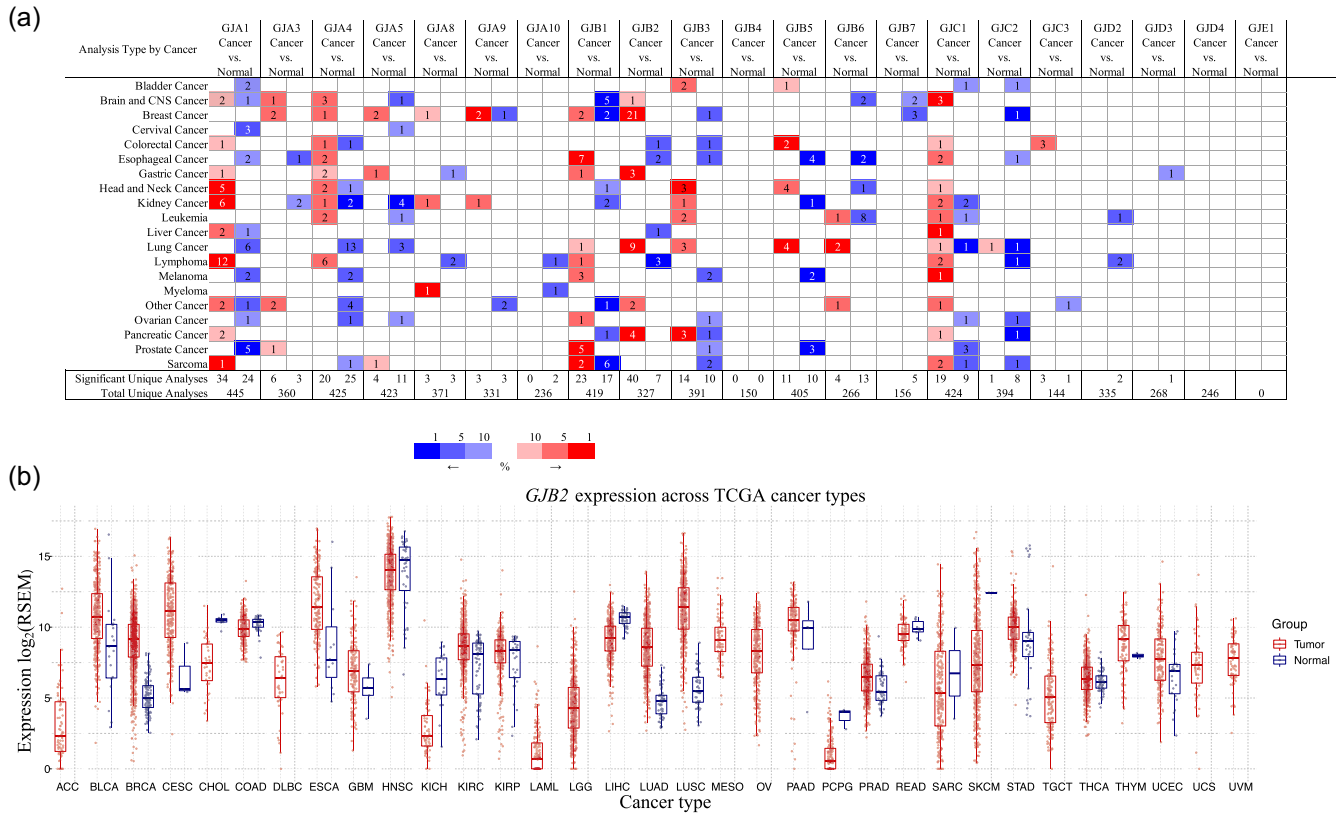

FIGURE 1 Transcriptional levels of connexins in different cancer types. (a) The transcription levels of different Cxs in cancer and paired normal tissues were compared using the ONCOMINE database; (b) GJB2 expression level in different cancer types. Cx, connexin; *GJB2*, gap junction beta-2.

further verify that *GJB2* is overexpressed in BC tissues. *GJB2* was found to be highly expressed in different molecular subtypes of BC (Figure 1b). Therefore, we hypothesized that *GJB2* may be a potential biomarker for BC and further explored the relationship between *GJB2* and BC.

3.2 | The close association between GJB2 transcription levels and BC prognosis

We next analyzed the relationship between *GJB2* transcription level and BC prognosis in public data sets using the Kaplan–Meier Plotter. The results showed that BC patients with high *GJB2* expression had worse OS and distant metastasis-free survival (DMFS) compared with patients with low GJB2 expression (Figure 2a,b). However, *GJB2* level was not associated with relapse-free survival (RFS) and postprogression survival (PPS). Subgroup analysis indicated that luminal type B BC patients with high *GJB2* expression had worse OS than those with low *GJB2* expression (Figure 2e). In BC with estrogen receptor (ER) negativity, human epidermal growth factor receptor 2 (HER2) positivity, luminal B subtype, HER2-positive subtype, and lymph node status negativity, patients with high *GJB2* expression had worse DMFS compared with those with low *GJB2* expression (Figure 2f). In BC with ER negativity, triple-negative breast cancer (TNBC), and negative lymph node status, patients with low *GJB2* expression had better RFS compared with those with high expression (Figure 2g). However, in ER-positive and luminal type A BC, patients with high expression of *GJB2* had better RFS (Figure 2g). Similarly, in ER-negative BC, patients with high *GJB2* expression had better PPS (Figure 2h).

3.3 | GJB2 protein expression and genomic alteration

We next examined the expression of GJB2 by IHC in our public database and clinical samples. The immunohistochemical results for GJB2 in BC and normal breast tissues were obtained using the HPA database. GJB2 was significantly overexpressed in breast tumor tissues compared with normal tissues (Figure 3a,b). We further conducted a comparison of the protein expression

TABLE 1 Differential expression of connexins in breast cancer and paired normal tissues.

Gene	Type of breast cancer versus normal breast tissue	Fold change	*p* value	*t* test	Source and/or reference
GJA1	NA	NA	NA	NA	NA
GJA3	Invasive breast carcinoma	2.468	1.16×10^{-14}	8.603	TCGA
	Mucinous breast carcinoma	2.027	7.96×10^{-4}	5.168	TCGA
GJA4	NA	NA	NA	NA	NA
GJA5	NA	NA	NA	NA	NA
GJA8	Invasive breast carcinoma	2.217	9.79×10^{-14}	11.282	Finak Breast statistics
GJA9	Invasive ductal breast carcinoma epithelia	2.57	8.69×10^{-8}	7.778	Ma Breast 4 statistics
	Ductal breast carcinoma in situ	2.311	2.25×10^{-6}	6.139	Ma Breast 4 statistics
	Intraductal cribriform breast adenocarcinoma	6.81×10^{-4}	−6.575	−2.249	TCGA Breast Statistics
GJA10	NA	NA	NA	NA	NA
GJB1	Lobular breast carcinoma	2.502	2.50×10^{-5}	5.521	Zhao Breast statistics
	Invasive ductal breast carcinoma	2.377	3.77×10^{-5}	4.543	Zhao Breast statistics
	Male breast carcinoma	−3.566	5.21×10^{-17}	−12.042	TCGA Breast Statistics
	Mucinous breast carcinoma	−2.491	5.34×10^{-5}	−6.199	TCGA Breast Statistics
GJB2	Invasive ductal breast carcinoma	4.342	2.89×10^{-292}	57.299	Curtis Breast statistics
	Ductal breast carcinoma in situ	2.662	2.51×10^{-5}	7.091	Curtis Breast statistics
	Tubular breast carcinoma	6.67	5.87×10^{-29}	18.628	Curtis Breast statistics
	Invasive ductal and invasive lobular breast carcinoma	4.037	6.07×10^{-25}	13.987	Curtis Breast statistics
	Medullary breast carcinoma	3.9	2.73×10^{-10}	8.78	Curtis Breast statistics
	Invasive lobular breast carcinoma	2.539	7.70×10^{-29}	13.569	Curtis Breast statistics
	Invasive breast carcinoma	3.363	2.61×10^{-05}	5.109	Curtis Breast statistics
	Invasive ductal breast carcinoma	18.849	8.99×10^{-69}	30.789	TCGA
	Invasive breast carcinoma	20.153	2.55×10^{-47}	23.111	TCGA
	Invasive lobular breast carcinoma	15.99	2.39×10^{-16}	12.499	TCGA
	Intraductal cribriform breast adenocarcinoma	34.833	1.80×10^{-5}	24.55	TCGA
	Ductal breast carcinoma	10.13	7.93×10^{-12}	10.650	Richardson breast
	Ductal breast carcinoma in situ stroma	11.642	1.32×10^{-5}	6.163	Ma Breast 4 statistics
	Invasive breast carcinoma stroma	15.549	2.97×10^{-15}	16.263	Finak Breast statistics
GJB3	NA	NA	NA	NA	NA
GJB4	NA	NA	NA	NA	NA
GJB5	NA	NA	NA	NA	NA
GJB6	NA	NA	NA	NA	NA
GJB7	Invasive lobular breast carcinoma	−2.558	1.56×10^{-15}	−9.769	TCGA Breast Statistics
	Invasive breast carcinoma	−2.371	2.74×10^{-14}	−8.424	TCGA Breast Statistics
	Intraductal cribriform breast adenocarcinoma	−2.936	0.008	−5.805	TCGA Breast Statistics
GJC1	NA	NA	NA	NA	NA
GJC2	Breast carcinoma	−2.188	3.52×10^{-14}	−13.722	Curtis Breast statistics
GJC3	NA	NA	NA	NA	NA

(Continues)

TABLE 1 (Continued)

Gene	Type of breast cancer versus normal breast tissue	Fold change	*p* value	*t* test	Source and/or reference
GJD2	NA	NA	NA	NA	NA
GJD3	NA	NA	NA	NA	NA
GJD4	NA	NA	NA	NA	N
GJE1	NA	NA	NA	NA	NA

Abbreviations: NA, not available; TCGA, The Cancer Genome Atlas.

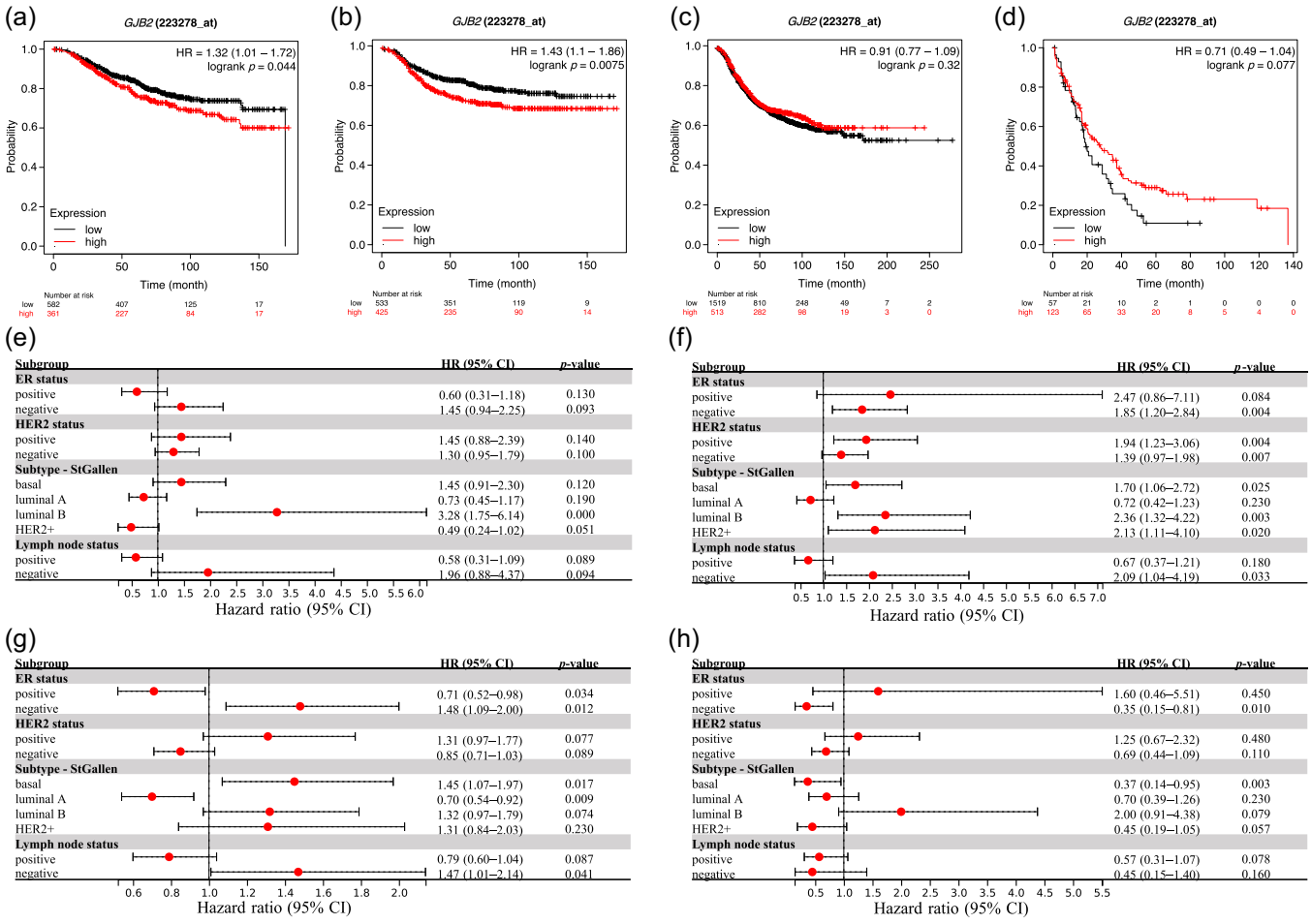

FIGURE 2 Relationship between *GJB2* expression and prognosis. OS (a), DMFS (b), RFS (c), and PPF (d) in patients with high and low expression of *GJB2*. Subgroup analysis of OS (e), DMFS (f), RFS (g), and PPF (h). DMFS, distant metastasis-free survival; *GJB2*, gap junction beta-2; OS, overall survival; PPS, postprogression survival; RFS, relapse-free survival.

between the *GJB2* nonmutated group and the *GJB2* mutated group using clinical samples obtained from our hospital. Our analysis revealed positive tumor membrane staining in the *GJB2* mutated group, indicating expression of the corresponding protein (Figure 3c–f).

We further analyzed *GJB2* somatic mutations and copy number variants (CNVs) and co-expressing genes for invasive BC using the cBioPortal online tool (TCGA, Firehose Legacy). Somatic mutation of *GJB2* in BC was rare. Among the 960 patients, only one missense mutation was identified, and the frequency of somatic mutation was 0.1% (Figure 4a). CNV was detected in 1.4% ($n = 13$) of TCGA BC samples, and amplification was the common variant type (Figure 4b). In the p110α subunit of phosphoinositide 3-kinase altered group, *GJB2* was overexpressed (Figure 4c).

To examine the mechanism regulating *GJB2* expression, we analyzed the methylation level of the *GJB2* promoter

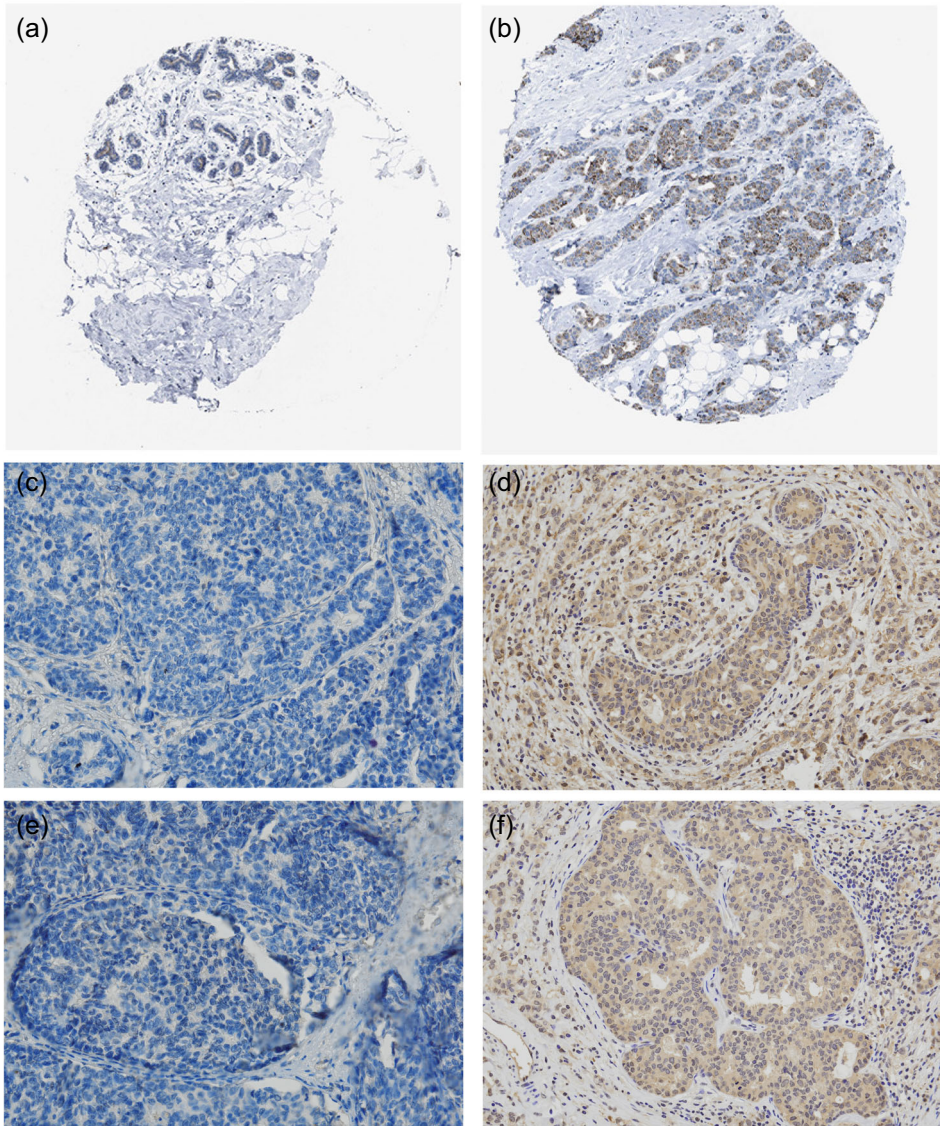

FIGURE 3 Immunohistochemical results. Compared with *GJB2* expression in normal tissues (a), *GJB2* was significantly overexpressed in breast tumor tissues (b). Compared with the tumor membrane in the *GJB2* nonmutated group (c and e), the tumor membrane of the *GJB2* mutated group was positive and the corresponding protein was expressed (d and f). *GJB2*, gap junction beta-2.

in BC tissues and paired normal tissues. The *GJB2* promoter region in BC tissues showed hypermethylation ($p = 1.62 \times 10^{-12}$, Figure 4d), and the corresponding *GJB2* mRNA was overexpressed ($p < 1 \times 10^{-12}$, Figure 4e).

3.4 | Functions and pathway analysis of GJB2

We screened out the top 100 genes positively and negatively co-expressed with *GJB2* through correlation coefficients and predicted the functions and related pathways of *GJB2* co-expressed genes through GO term and KEGG pathway analyses using DAVID database. In the GO term enrichment analysis, the top three biological functions in the enrichment analysis of GJB2 co-expressed genes were external encapsulating structure organization, collagen fibril organization, and endodermal cell differentiation (Figure 5a). The top three cellular components were the external encapsulating structure, collagen-containing extracellular matrix, and collagen trimer (Figure 5b). The top three molecular functions were collagen binding, extracellular matrix structural constituent, and platelet-derived growth factor binding (Figure 5c). KEGG pathway enrichment analysis showed that the cancer-related pathways enriched by *GJB2* co-expression genes included the phosphoinositide 3-kinase-protein kinase B (PI3K-Akt) signaling, focal adhesion, proteoglycans in cancer, extracellular matrix–receptor interaction, and human papillomavirus infection pathways (Figure 5d).

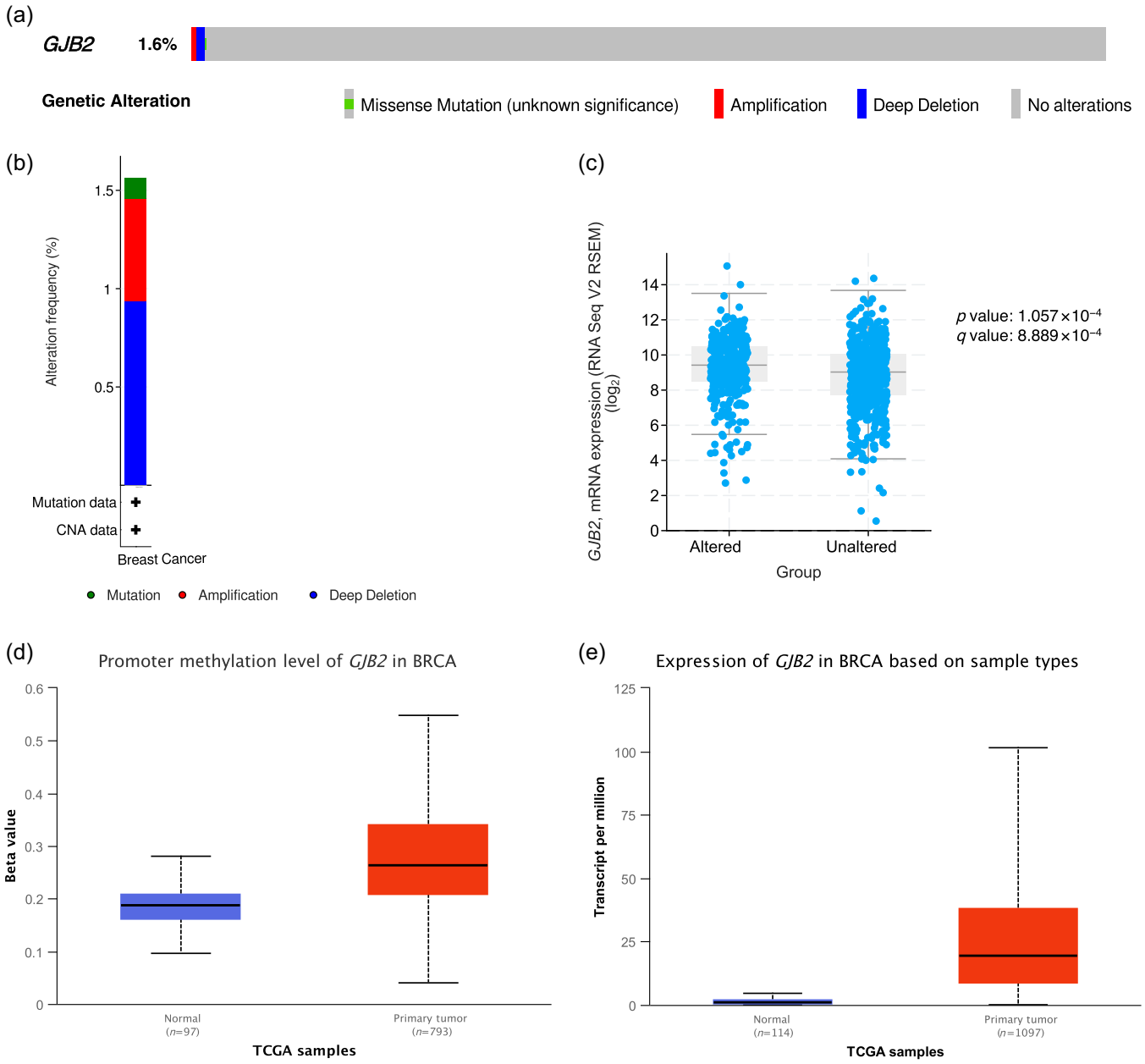

FIGURE 4 Genomic alteration and methylation of *GJB2*. (a) Genomic alteration of *GJB2* in BRCA; (b) Frequency of *GJB2* alteration in BRCA; (c) *GJB2* mRNA expression in altered group and unaltered group; (d) Level of *GJB2* promoter methylation in breast cancer; (e) *GJB2* expression in BRCA tumor and normal tissue. BRCA, breast cancer; GJB2, gap junction beta-2.

3.5 | Correlation analysis between GJB2 expression and infiltrating immune cells

To explore the relationship between *GJB2* and immunotherapy, we investigated whether *GJB2* expression was correlated with the six main infiltrating immune cells (B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells) in BC using the TIMER database. The results showed that *GJB2* expression level correlated with CD8+ T cells ($r = 0.141$, $p = 9.88 \times 10^{-6}$), macrophages ($r = 0.288$, $p = 3.40 \times 10^{-20}$), and neutrophils ($r = 0.197$, $p = 8.93 \times 10^{-10}$) (Figure 6).

3.6 | Relationship between GJB2 expression and drug sensitivity

The relationship between *GJB2* expression and drug sensitivity was analyzed using the Genomics of Drug Sensitivity in Cancer and CTRP databases. There were several consistent results regarding drug sensitivity from

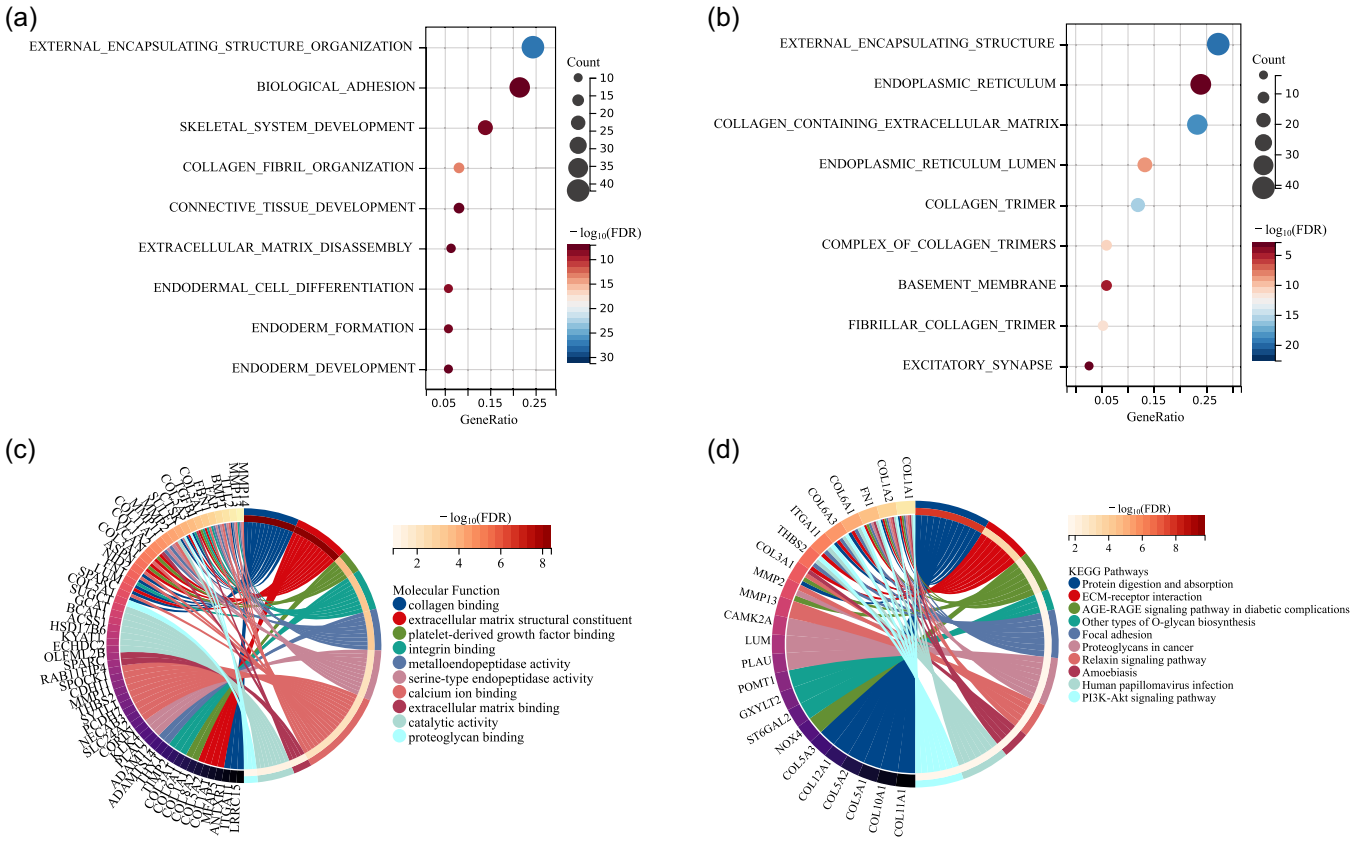

FIGURE 5 Functions and pathway analysis of *GJB2*. (a) Biological function, (b) cellular component, and (c) molecular function in Gene Ontology analysis; (d) Kyoto Encyclopedia of Genes and Genomes analysis. *GJB2*, gap junction beta-2.

different databases (Table 2). The expression of *GJB2* mRNA was negatively correlated with the half-maximal inhibitory concentration (IC50) of epidermal growth factor receptor–tyrosine kinase inhibitors (EGFR-TKIs) and mitogen-activated protein kinase (MEK) inhibitors. *GJB2* mRNA expression positively correlated with the IC50 of other types of agents, such as inhibitors of histone deacetylase (HDAC), PI3K, mammalian target of rapamycin (mTOR), cyclin-dependent kinase (CDK), and c-kit. Our findings indicate that BC patients with high *GJB2* expression may be sensitive to EGFR-TKIs and MEK inhibitors and resistant to HDAC, PI3K, mTOR, and CDK inhibitors and other BC-related targeted agents (Figure 7).

4 | DISCUSSION

Cxs are considered tumor suppressors in several solid cancer models [31]. However, conflicting evidence suggests that Cxs are also upregulated in some tumors, suggesting they play a dual role as tumor suppressors and facilitators of disease progression [14, 32]. In this study, we performed a comprehensive bioinformatic analysis of the relationship between Cxs and BC using public data sets. To the best of our knowledge, this is the first study to investigate *GJB2* expression patterns, genetic changes, prognostic value, and functional enrichment in BC using multiomics analysis.

GJB2 encodes Cx26, a member of the Cx family, and is located on the long arm of chromosome 13. Cx26 is involved in the regulation of mammary gland development [33], and the blockage of intercellular communication caused by Cx26 downregulation is one of the mechanisms of tumor pathogenesis [34]. *Cx26* promotes the progression of ductal carcinoma in situ to invasive ductal carcinoma by regulating the activity of CSCs [14]. Additionally, animal models with high expression of Cx43 and Cx26 are more prone to tumor brain metastasis compared with control groups [15]. By cross-validation of different public data sets, we found that Cx26 was highly expressed in BC of different pathological types and molecular subtypes. Notably, Kaplan–Meier survival analysis indicated that patients with high *GJB2* expression had a shorter OS and DMFS compared with those with low expression ($p < 0.05$), suggesting that the upregulation of *GJB2* expression is a poor prognostic indicator in patients with BC. *GJB2* has also been shown to be overexpressed in other malignant tumors, such as lung and pancreatic cancers, and associated with a poor prognosis [35, 36].

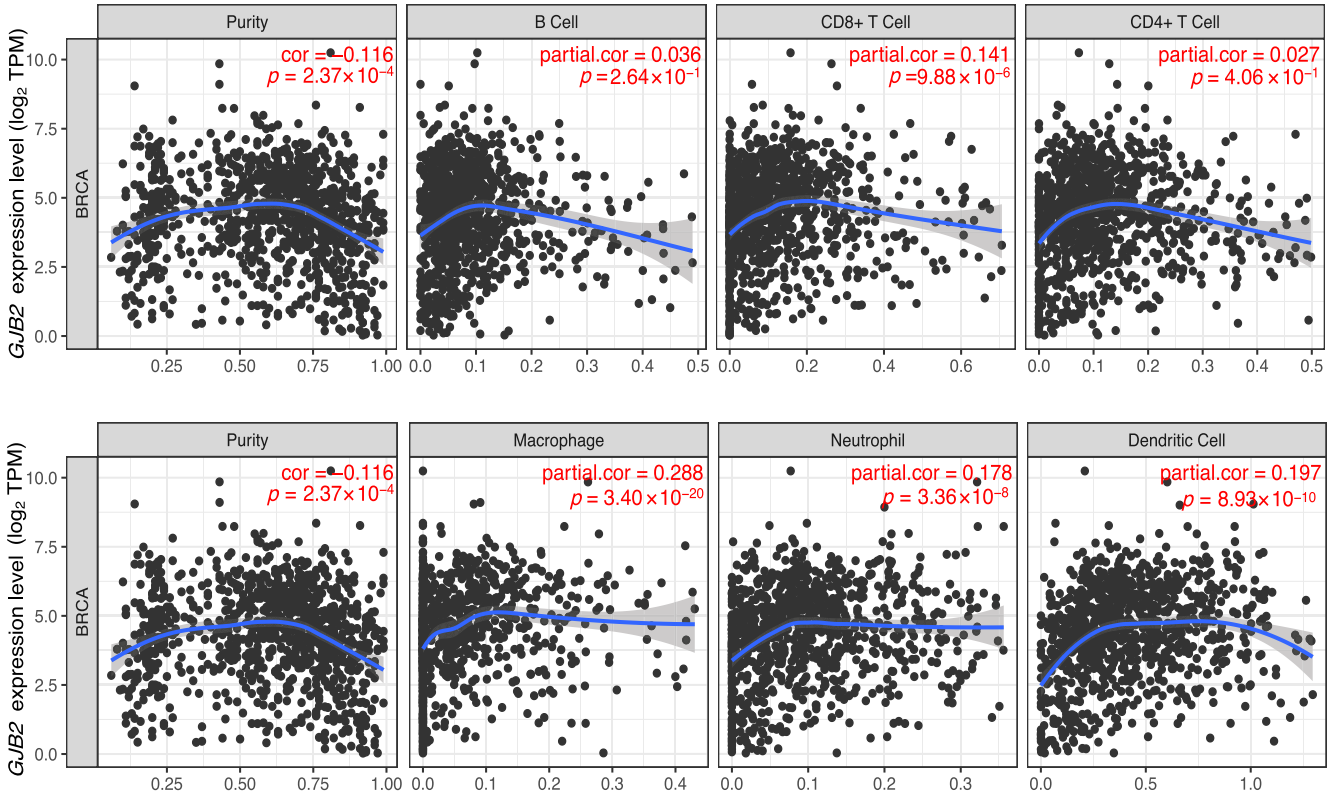

FIGURE 6 Correlation analysis between *GJB2* expression and infiltrating immune cells. BRCA, breast cancer; GJB2, gap junction beta-2.

The relationship between Cx gene overexpression and BC prognosis is controversial. For instance, differential expression of Cx43 and Cx30 may serve as potential positive and negative prognostic markers for BC, respectively. Elevated levels of Cx43 protein were correlated with favorable RFS, whereas heightened levels of Cx30 were linked to poor OS [37]. However, increasing studies have reported that Cx26 overexpression is associated with aggressive clinical features and decreased survival [38, 39], which is consistent with the results obtained in our study. We also observed a significant association between high *GJB2* expression and distant metastasis in different molecular subtypes of BC, excluding the luminal A subtype. However, the relationship between *GJB2* and other clinical prognostic indicators, including recurrence-free survival and PPS, was not as pronounced. Mechanistically, elevated *GJB2* expression may be linked to the invasion and migration of tumor cells. By influencing intercellular communication, Cx26 may modulate the infiltrative capacity of tumor cells, facilitating their passage through the basement membrane and subsequent metastasis to other tissues and distant organs. Furthermore, the aberrant expression of *GJB2* may alter the tumor microenvironment, including the cells and matrix surrounding the tumor. These microenvironmental changes could provide more favorable conditions for tumor cells to invade neighboring tissues and enter the vascular system, thereby promoting metastasis.

To explore the potential mechanism underlying *GJB2* upregulation in BC, we analyzed the frequency of *GJB2* changes in BC in cBioPortal and the structural mutations and CNVs. Somatic mutations and CNVs of *GJB2* were rare, whereas the methylation level of the *GJB2* promoter in breast tumors was significantly higher than that in the normal tissues. We further found that *GJB2* promoter hypermethylation corresponded to upregulation at the transcriptional level. Hypermethylation of CpG islands in the promoter regions is associated with transcriptional repression, especially in tumor suppressor genes. Recent studies have shown that promoter hypermethylation can also activate target genes by blocking the binding of transcription inhibitors and interacting with enhancers [40]. Abnormal methylation of the *GJB2* promoter has been detected in BC, colon and lung cancers, and other tumors [41–43]. The relationship between *GJB2* methylation and mRNA expression has been controversial. Singal et al. [43] reported that hypermethylation may not be the primary mechanism of regulating Cx26 gene repression in human mammary cancer cell lines. Loncarek et al. [42] reported that methylation was not involved in the regulation of Cx26 in human esophageal

TABLE 2 Results of GDSC and CTRP analysis.

	GDSC		CTRP		
Drug name	**Correlation**	**FDR**	**Correlation**	**FDR**	**Mechanism**
ZSTK474	0.09	0.01	0.106593	0.0324666	Pan-class I PI3K inhibitor
PI-103	0.17	2.22×10^{-6}	0.261965	9.014×10^{-12}	Multitargeted PI3K inhibitor
PIK-93	0.15	1.58×10^{-5}	0.12463	0.002066	PI4K inhibitor
OSI-027	0.14	5.56×10^{-5}	0.183637	4.267×10^{-6}	mTORC1/2inhibitor
Vorinostat	0.08	0.04	0.189241	4.712×10^{-7}	HDAC inhibitor
Belinostat	0.12	1.20×10^{-3}	0.187723	0.0005925	HDAC inhibitor
Tubastatin A	0.18	1.33×10^{-7}	0.203388	0.0003867	HDAC inhibitor
Erlotinib	−0.23	2.00×10^{-4}	−0.240653	5.314×10^{-10}	EGFR-TKI
Lapatinib	−0.22	1.16×10^{-4}	−0.222042	2.891×10^{-8}	EGFR and HER2 inhibitor
Afatinib	−0.33	4.00×10^{-23}	−0.265411	6.103×10^{-11}	EGFR-TKI
Gefitinib	−0.3	4.63×10^{-17}	−0.182615	1.438×10^{-5}	EGFR-TKI
Trametinib	−0.21	3.61×10^{-9}	−0.24121	0.0001204	MEK inhibitor
selumetinib	−0.14	1.29×10^{-4}	−0.19417	3.383×10^{-6}	MEK 1/2 inhibitor
Masitinib	0.12	1.02×10^{-3}	0.150473	0.0001684	c-kit inhibitor
OSI-930	0.08	0.04	0.103624	0.0184498	c-kit inhibitor
Navitoclax	0.16	6.27×10^{-6}	0.105161	0.0107637	BCL-2 inhibitor
PAC-1	0.11	5.22×10^{-3}	0.134127	0.0006554	Procaspase-3 activator
PHA-793887	0.09	9.37×10^{-3}	0.130132	0.0005292	Inhibitor of CDK2, CDK5 and CDK7
UNC0638	0.17	3.59×10^{-7}	0.217577	3.926×10^{-8}	HMTase inhibitor

Abbreviations: CDK, cyclin-dependent kinase; CTRP, Cancer Therapeutics Response Portal; EGFR-TKI, epidermal growth factor receptor–tyrosine kinase inhibitor; FDR, false discovery rate; GDSC, Genomics of Drug Sensitivity in Cancer; HDAC, histone deacetylase; HER2, human epidermal growth factor receptor 2; MEK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase.

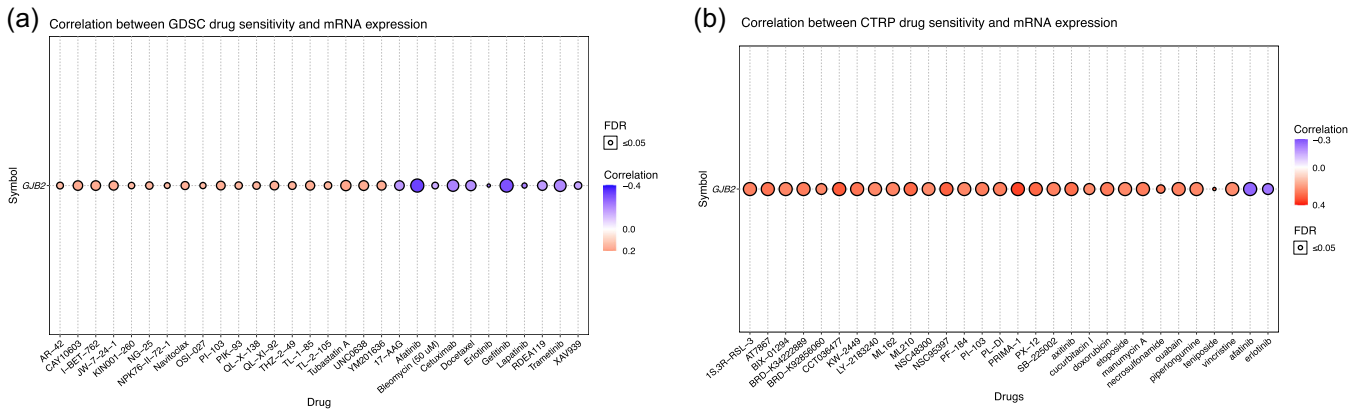

FIGURE 7 *GJB2* expression and drug sensitivity analysis. (a) GDSC analysis of drug sensitivity and *GJB2* mRNA expression; (b) CTRP analysis of the correlation between drug sensitivity and *GJB2* mRNA expression. CTRP, Cancer Therapeutics Response Portal; GDSC, Genomics of Drug Sensitivity in Cancer; *GJB2*, gap junction beta-2.

cancer cell lines. Additionally, a few studies have reported that promoter methylation is related to the downregulation of *GJB2* expression [44, 45]. Therefore, alterations in the methylation status of *GJB2* may be associated with a feedback regulatory mechanism, indicating that high DNA methylation levels may facilitate the activation and overexpression of *GJB2*. This suggests that other cancer-specific elements may be

involved in the regulation of GJB2 gene expression, reflecting the complexity of regulatory mechanisms across different cancer types. Furthermore, methylation exhibits spatiotemporal heterogeneity, indicating that methylation levels may vary across different tissues, time points, and individuals, which is crucial for understanding the complexity of GJB2 regulation mechanisms.

Cxs play an inhibitory role in tumor formation during the early stage of the disease. However, as the tumor progresses, Cx26 may shift its role to promote tumor invasion and metastasis. The function of Cx26 may vary in different types of cancers and at different stages of the same cancer, and research in this area is ongoing. Categorizing *GJB2* as a tumor suppressor gene or oncogene requires further experimental investigation and a detailed molecular mechanism analysis.

The KEGG pathway enrichment analysis showed that *GJB2* co-expressed genes were significantly enriched in BC-related pathways, including PI3K-Akt and cell adhesion pathways. Moreover, *GJB2* was expressed at high levels in the PI3KCA variant group. Yang et al. [46] reported that *GJB2* overexpression may lead to PI3K-Akt pathway activation and thereby promote epithelial–mesenchymal transition and gefitinib resistance in nonsmall-cell lung cancer cells. Cx26 promotes the self-renewal of TNBC stem cells via its interaction with the pluripotency transcription factor NANOG and focal adhesion kinase [47]. In most BCs, the PI3K-Akt-mTOR pathway is activated. The PI3K-Akt-mTOR pathway is involved in the regulation of various cell processes, including cell proliferation, growth, motility, and metabolism [48]. Additionally, the PI3K-Akt-mTOR pathway is related to BC resistance. PI3K-Akt-mTOR axis inhibitors, including everolimus, sirolimus, and alpelisib, have demonstrated good efficacy in delaying drug resistance and improving survival of BC patients. Drug sensitivity analysis indicated that the upregulation of *GJB2* may be associated with resistance to a variety of PI3K and HDAC inhibitors. We speculate that Cx26 may indirectly lead to drug resistance and poor prognosis of BC by activating the PI3K-Akt-mTOR pathway.

We also found that *GJB2* expression significantly correlated with CD8+ T cells, macrophages, neutrophils, and dendritic cells. Cx26 may play an important role in recruiting infiltrating immune cells and regulating the immunity against BC, thereby affecting prognosis. Thus, *GJB2* may be a potential therapeutic target for specific therapy and immunotherapy. Further in vitro and in vivo experiments are needed to verify our results and explore the potential mechanisms.

Our study has several limitations. First, there may be selection bias in the database choice. The cohort included in this study was derived from public databases, and sample distribution in these cohorts may be inconsistent with the clinical population. Moreover, the absence of wet experimental data hampers our ability to provide mechanistic insights into the relationship between *GJB2* and BC. Further experimental validation is essential to confirm and expand upon the observations made in this study. Future research is crucial for a more comprehensive understanding of the role of Cx26 in BC progression.

5 | CONCLUSION

In this study, we demonstrated *GJB2* mRNA overexpression in various pathological types and molecular subtypes of BC, correlating with an unfavorable prognosis. Somatic mutation and copy number variation of *GJB2* were rare in BC. In the p110α subunit of the PIK3CA mutant subgroup, the GJB2 transcription level was higher than that in the PIK3CA wild-type subgroup. *GJB2* was associated with the PI3K-Akt signaling pathway, extracellular matrix–receptor interaction, focal adhesion, and proteoglycans in cancer. *GJB2* overexpression was associated with PI3K and HDAC inhibitor resistance. Furthermore, *GJB2* expression level correlated with infiltrating immune cells. Our study identified *GJB2* as a prognostic biomarker and therapeutic target in BC. High expression of GJB2 in BC patients may indicate poor prognosis and drug resistance.

AUTHOR CONTRIBUTIONS

Di Zhang: Conceptualization (lead); methodology (equal); resources (lead); software (equal); visualization (equal); writing—original draft (equal); writing—review and editing (equal). **Lixi Li**: Conceptualization (equal); data curation (equal); formal analysis (supporting); investigation (supporting); methodology; resources (equal); software (supporting); supervision (supporting); validation (supporting); writing—original draft (equal); writing—review and editing (supporting). **Fei Ma**: Funding acquisition (lead); methodology (lead); project administration (lead); resources (supporting); supervision (lead); writing—review and editing (lead). All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Most of these data came from public databases, and readers can find them in public databases through the method section.

ETHICS STATEMENT

Data, except for some immunohistochemical results, were extracted from public databases. This study was approved by the ethics committee of the Chinese Academy of Medical Sciences, Peking Union Medical College (ethical approval number: 23/442-4185).

INFORMED CONSENT

All patients provided written informed consent at the time of entering this study.

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REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021;71(3):209–49. https://doi.org/10.3322/caac.21660
2. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, et al. Cancer statistics in China, 2015. CA Cancer J Clin. 2016;66(2):115–32. https://doi.org/10.3322/caac.21338
3. Gaudet MM, Gierach GL, Carter BD, Luo J, Milne RL, Weiderpass E, et al. Pooled analysis of nine cohorts reveals breast cancer risk factors by tumor molecular subtype. Cancer Res. 2018;78(20):6011–21. https://doi.org/10.1158/0008-5472.CAN-18-0502
4. Nedeljković M, Damjanović A. Mechanisms of chemotherapy resistance in triple-negative breast cancer-how we can rise to the challenge. Cells. 2019;8(9):957. https://doi.org/10.3390/cells8090957
5. Hanker AB, Sudhan DR, Arteaga CL. Overcoming endocrine resistance in breast cancer. Cancer Cell. 2020;37(4):496–513. https://doi.org/10.1016/j.ccell.2020.03.009
6. Beyer EC, Berthoud VM. Gap junction gene and protein families: connexins, innexins, and pannexins. Biochim Biophys Acta Biomembr. 2018;1860(1):5–8. https://doi.org/10.1016/j.bbamem.2017.05.016
7. Sáez JC, Retamal MA, Basilio D, Bukauskas FF, Bennett MVL. Connexin-based gap junction hemichannels: gating mechanisms. Biochim Biophys Acta Biomembr. 2005;1711(2):215–24. https://doi.org/10.1016/j.bbamem.2005.01.014
8. Sáez JC, Berthoud VM, Brañes MC, Martínez AD, Beyer EC. Plasma membrane channels formed by connexins: their regulation and functions. Physiol Rev. 2003;83(4):1359–400. https://doi.org/10.1152/physrev.00007.2003
9. Zefferino R, Piccoli C, Gioia SD, Capitanio N, Conese M. Gap junction intercellular communication in the carcinogenesis hallmarks: is this a phenomenon or epiphenomenon? Cells. 2019;8(8):896. https://doi.org/10.3390/cells8080896
10. Hervé JC, Bourmeyster N, Sarrouilhe D, Duffy HS. Gap junctional complexes: from partners to functions. Prog Biophys Mol Biol. 2007;94(1–2):29–65. https://doi.org/10.1016/j.pbiomolbio.2007.03.010
11. Srinivas M, Verselis VK, White TW. Human diseases associated with connexin mutations. Biochim Biophys Acta Biomembr. 2018;1860(1):192–201. https://doi.org/10.1016/j.bbamem.2017.04.024
12. Lee SW, Tomasetto C, Sager R. Positive selection of candidate tumor-suppressor genes by subtractive hybridization. Proc Natl Acad Sci USA. 1991;88(7):2825–9. https://doi.org/10.1073/pnas.88.7.2825
13. Tanaka M, Grossman HB. Connexin 26 gene therapy of human bladder cancer: induction of growth suppression, apoptosis, and synergy with Cisplatin. Hum Gene Ther. 2001;12(18):2225–36. https://doi.org/10.1089/10430340152710568
14. Liu Y, Pandey PR, Sharma S, Xing F, Wu K, Chittiboyina A, et al. ID2 and GJB2 promote early-stage breast cancer progression by regulating cancer stemness. Breast Cancer Res Treat. 2019;175(1):77–90. https://doi.org/10.1007/s10549-018-05126-3
15. Stoletov K, Strnadel J, Zardouzian E, Momiyama M, Park FD, Kelber JA, et al. Role of connexins in metastatic breast cancer and melanoma brain colonization. J Cell Sci. 2013;126(Pt 4):904–13. https://doi.org/10.1242/jcs.112748
16. Lamiche C, Clarhaut J, Strale PO, Crespin S, Pedretti N, Bernard FX, et al. The gap junction protein Cx43 is involved in the bone-targeted metastatic behaviour of human prostate cancer cells. Clin Exp Metastasis. 2012;29(2):111–22. https://doi.org/10.1007/s10585-011-9434-4
17. Oliveira R, Christov C, Guillamo J, de Boüard S, Palfi S, Venance L, et al. Contribution of gap junctional communication between tumor cells and astroglia to the invasion of the brain parenchyma by human glioblastomas. BMC Cell Biol. 2005;6(1):7. https://doi.org/10.1186/1471-2121-6-7
18. Zhang W, Nwagwu C, Le DM, Yong VW, Song H, Couldwell WT. Increased invasive capacity of connexin 43-overexpressing malignant glioma cells. J Neurosurg. 2003;99(6):1039–46. https://doi.org/10.3171/jns.2003.99.6.1039
19. Chasampalioti M, Green AR, Ellis IO, Rakha EA, Jackson AM, Spendlove I, et al. Connexin 43 is an independent predictor of patient outcome in breast cancer patients. Breast Cancer Res Treat. 2019;174(1):93–102. https://doi.org/10.1007/s10549-018-5063-9
20. Kim EY, Jun KH, Yim K. The roles of connexin 26, 32, and 43 as prognostic factors for gastric cancer. Anticancer Res. 2020;40(8):4537–45. https://doi.org/10.21873/anticanres.14459
21. Zhang W, Li HG, Fan MJ, Lv ZQ, Shen XM, He XX. Expressions of connexin 32 and 26 and their correlation to prognosis of non-small cell lung cancer. Ai Zheng. 2009;28(2):173–6.
22. Sinha G, Ferrer AI, Moore CA, Naaldijk Y, Rameshwar P. Gap junctions and breast cancer dormancy. Trends Cancer. 2020;6(4):348–57. https://doi.org/10.1016/j.trecan.2020.01.013
23. Rhodes DR, Yu J, Shanker K, Deshpande N, Varambally R, Ghosh D, et al. ONCOMINE: a cancer microarray database and integrated data-mining platform. Neoplasia. 2004;6(1):1–6. https://doi.org/10.1016/s1476-5586(04)80047-2
24. Rhodes DR, Kalyana-Sundaram S, Mahavisno V, Varambally R, Yu J, Briggs BB, et al. Oncomine 3.0: genes, pathways, and networks in a collection of 18,000 cancer gene

expression profiles. Neoplasia. 2007;9(2):166–80. https://doi.org/10.1593/neo.07112
25. Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Tissue-based map of the human proteome. Science. 2015;347(6220):1260419. https://doi.org/10.1126/science.1260419
26. Lánczky A, Nagy Á, Bottai G, Munkácsy G, Szabó A, Santarpia L, et al. miRpower: a web-tool to validate survival-associated miRNAs utilizing expression data from 2178 breast cancer patients. Breast Cancer Res Treat. 2016;160(3):439–46. https://doi.org/10.1007/s10549-016-4013-7
27. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal. 2013;6(269):pl1. https://doi.org/10.1126/scisignal.2004088
28. Chandrashekar DS, Bashel B, Balasubramanya SAH, Creighton CJ, Ponce-Rodriguez I, Chakravarthi BVSK, et al. UALCAN: a portal for facilitating tumor subgroup gene expression and survival analyses. Neoplasia. 2017;19(8):649–58. https://doi.org/10.1016/j.neo.2017.05.002
29. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009;4(1):44–57. https://doi.org/10.1038/nprot.2008.211
30. Li T, Fan J, Wang B, Traugh N, Chen Q, Liu JS, et al. TIMER: a web server for comprehensive analysis of tumor-infiltrating immune cells. Cancer Res. 2017;77(21):e108–10. https://doi.org/10.1158/0008-5472.CAN-17-0307
31. McLachlan E, Shao Q, Wang H, Langlois S, Laird DW. Connexins act as tumor suppressors in three-dimensional mammary cell organoids by regulating differentiation and angiogenesis. Cancer Res. 2006;66(20):9886–94. https://doi.org/10.1158/0008-5472.CAN-05-4302
32. Jamieson S, Going JJ, D'Arcy R, George WD. Expression of gap junction proteins connexin 26 and connexin 43 in normal human breast and in breast tumours. J Pathol. 1998;184(1):37–43. https://doi.org/10.1002/(SICI)1096-9896(199801)184:1<37:AID-PATH966>3.0.CO;2-D
33. McLachlan E, Shao Q, Laird DW. Connexins and gap junctions in mammary gland development and breast cancer progression. J Membr Biol. 2007;218(1–3):107–21. https://doi.org/10.1007/s00232-007-9052-x
34. Lee SW, Tomasetto C, Paul D, Keyomarsi K, Sager R. Transcriptional downregulation of gap-junction proteins blocks junctional communication in human mammary tumor cell lines. J Cell Biol. 1992;118(5):1213–21. https://doi.org/10.1083/jcb.118.5.1213
35. Tang Y, Zhang YJ, Wu ZH. High GJB2 mRNA expression and its prognostic significance in lung adenocarcinoma: a study based on the TCGA database. Medicine. 2020;99(14):e19054. https://doi.org/10.1097/MD.0000000000019054
36. Kyo N, Yamamoto H, Takeda Y, Ezumi K, Ngan C, Terayama M, et al. Overexpression of connexin 26 in carcinoma of the pancreas. Oncol Rep. 2008;19(3):627–31. https://doi.org/10.3892/or.19.3.627
37. Teleki I, Szasz AM, Maros ME, Gyorffy B, Kulka J, Meggyeshazi N, et al. Correlations of differentially expressed gap junction connexins Cx26, Cx30, Cx32, Cx43 and Cx46 with breast cancer progression and prognosis. PLoS One. 2014;9(11):e112541. https://doi.org/10.1371/journal.pone.0112541
38. Naoi Y, Miyoshi Y, Taguchi T, Kim SJ, Arai T, Tamaki Y, et al. Connexin 26 expression is associated with lymphatic vessel invasion and poor prognosis in human breast cancer. Breast Cancer Res Treat. 2007;106(1):11–7. https://doi.org/10.1007/s10549-006-9465-8
39. Kanczuga-Koda L, Sulkowski S, Lenczewski A, Koda M, Wincewicz A, Baltaziak M, et al. Increased expression of connexins 26 and 43 in lymph node metastases of breast cancer. J Clin Pathol. 2006;59(4):429–33. https://doi.org/10.1136/jcp.2005.029272
40. Smith J, Sen S, Weeks RJ, Eccles MR, Chatterjee A. Promoter DNA hypermethylation and paradoxical gene activation. Trends Cancer. 2020;6(5):392–406. https://doi.org/10.1016/j.trecan.2020.02.007
41. Chen Y, Hühn D, Knösel T, Pacyna-Gengelbach M, Deutschmann N, Petersen I. Downregulation of connexin 26 in human lung cancer is related to promoter methylation. Int J Cancer. 2005;113(1):14–21. https://doi.org/10.1002/ijc.20498
42. Loncarek J, Yamasaki H, Levillain P, Milinkevitch S, Mesnil M. The expression of the tumor suppressor gene connexin 26 is not mediated by methylation in human esophageal cancer cells. Mol Carcinog. 2003;36(2):74–81. https://doi.org/10.1002/mc.10102
43. Singal R, Tu ZJ, Vanwert JM, Ginder GD, Kiang DT. Modulation of the connexin26 tumor suppressor gene expression through methylation in human mammary epithelial cell lines. Anticancer Res. 2000;20(1A):59–64.
44. Tan L, Bianco T, Dobrovic A. Variable promoter region CpG island methylation of the putative tumor suppressor gene Connexin 26 in breast cancer. Carcinogenesis. 2002;23(2):231–6. https://doi.org/10.1093/carcin/23.2.231
45. Tsujiuchi T, Shimizu K, Itsuzaki Y, Onishi M, Sugata E, Fujii H, et al. CpG site hypermethylation of E-cadherin and Connexin26 genes in hepatocellular carcinomas induced by a choline-deficient L-amino acid-defined diet in rats. Mol Carcinog. 2007;46(4):269–74. https://doi.org/10.1002/mc.20268
46. Yang J, Qin G, Luo M, Chen J, Zhang Q, Li L, et al. Reciprocal positive regulation between Cx26 and PI3K/Akt pathway confers acquired gefitinib resistance in NSCLC cells via GJIC-independent induction of EMT. Cell Death Dis. 2015;6(7):e1829. https://doi.org/10.1038/cddis.2015.197
47. Thiagarajan PS, Sinyuk M, Turaga SM, Mulkearns-Hubert EE, Hale JS, Rao V, et al. Cx26 drives self-renewal in triple-negative breast cancer via interaction with NANOG and focal adhesion kinase. Nat Commun. 2018;9(1):578. https://doi.org/10.1038/s41467-018-02938-1
48. Guerrero-Zotano A, Mayer IA, Arteaga CL. PI3K/AKT/mTOR: role in breast cancer progression, drug resistance, and treatment. Cancer Metastasis Rev. 2016;35(4):515–24. https://doi.org/10.1007/s10555-016-9637-x

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