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Original Article

Gamma-Aminobutyric Acid Type A Receptor Subunit Delta (*GABRD*) Inhibits Breast Cancer Progression by Regulating the Cell Cycle

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Abstract

Background: The role of γ-aminobutyric acid receptor (*GABR*) in breast cancer (BC) is unknown.

Methods: The expression of different *GABR* subunits between BC and adjacent normal tissues was compared using transcriptome data set. The clinical and prognostic importance of the various *GABR* subunit genes in BC was determined using clinical and survival data (Data downloaded from TCGA, May 2022). Only *GABRD* was discovered to be substantially expressed and strongly related to the prognosis of BC cases.

Results: Compared with normal tissues, *GABRD* expression was increased in all subgroups of breast cancer tissues. Knockdown of *GABRD* inhibited the growth of BC cells. Mechanistically, the function of *GABRD* may be attributed to its effect on major pathways such as oxidative phosphorylation, Parkinson disease, and cell cycle. *GABRD* deletion significantly blocked the G2/M phase in BC cells.

Conclusion: Overall, *GABRD* might be a novel prognostic predictor of BC, providing clues for further studies on *GABRD*.

Keywords: γ-aminobutyric acid receptor; Breast cancer; Prognosis; Growth; Cell cycle

Introduction

Breast cancer (BC) is currently one of the most common cancers in women and the fifth leading cause of cancer-related deaths (1). Only in 2020, there was an estimated 2.26 million [95% UI, 2.24–2.79 million] new cases worldwide (2). Despite advances in early screening and treatment in recent years (3), there will be 5-year overall survival rates of up to 90% for early-stage breast cancer (3). However, long-term survival of patients with advanced cancer remains unsatisfactory, is mainly due to high recurrence rates and distant metastases (5,6). It is important to investigate certain sensitive molecular indicators related with breast cancer prognosis.

The gamma- aminobutyric acid (GABA) is the main inhibitory neurotransmitter for central nervous system. Originally, three groups of *GABA* receptors, namely $GABA_A$, $GABA_B$, and $GABA_C$, were identified (7). $GABA_A$ receptors ($GABA_AR$) are ligand-gated chloride channels, mading up of pentameric subunit combinations. In humans, there are 19 $GABA_A$ receptor subunit genes that code for six α (alpha1-6), three β (beta1-3), three ρ (rho1-3), three γ (gamma1-3), and one each of the π (pi), δ (delta), ε (epsilon), together with θ (theta) (8). These different subunit isoforms, including *GABRE*, *GABRD*, *GABRP*, *GABRQ*, *GABRB1*, *GABRB2*, *GABRB3*, *GABRA6*, *GABRG1*, *GABRG2*, *GABRG3*, *GABRR1*, *GABRR2* and *GABRR3* (9).

$GABA_AR$ is one of the most important pharmacological targets in the treatment of neuropsychiatric diseases including insomnia, epilepsy, and anxiety, as well as in surgical anesthesia (10). Epilepsy (11), eating disorder (**Error! Reference source not found.**), autism (13), and bipolar illness (14) have all been linked to $GABA_AR$ subunit gene variants in genetic research. However, it is unknown about the functional role of $GABA_AR$ in breast cancer.

In this paper, we explored the role of *GABRD* in BC based on its effect on cell cycle related pathways.

Materials and Methods

Data collection

In May 2022, we downloaded RNA sequencing (RNA-seq) data of 1,217 TCGA-BC patients (including 113 normal breast tissue samples and 1,104 BC samples) from TCGA (https://portal.gdc.cancer.gov) (15). As the FPKM values span a wide range of values, the gene expression for each gene is presented as log2 (FPKM+1). Clinical and prognostic information were also obtained.

Identification of differential gamma-aminobutyric acid receptor (GABR) expression

To compare the differential expression of *GABRs* in normal and tumour tissues, *P*-values for each *GABR* gene were generated using the edgeR (16) R package. *GABR* genes with P-values less than 0.05 were defined as differentially expressed *GABRs*.

Identification of candidate biomarkers for BC survival and clinicopathological features.

We used the Kaplan Meier method (17,18) and log-rank test for overall and progression-free survival analysis. The log-rank test *P*-values < 0.05 was applied to select candidate *GABR* genes that were associated with BC prognosis.

The association between *GABRs* and clinicopathological features was evaluated by the "survival" (19) package of R software.

Biofunctional and pathway enrichment analysis

Gene Ontology (GO) and KEGG enrichment analysis (20) of *GABRD*-related genes was carried out using Gene Set enrichment analysis (GSEA) (21). Significantly differentially expressed genes were uploaded to the Molecular Signatures Database of GSEA for gene set studies. A *P*-value less than 0.05 as well as q-value less than 0.05 were used as the screening criteria for significant pathways.

BC cell lines screening

We first downloaded the *GABRD* expression of 56 breast cancer cell lines, CRISPR-treated *GABRD* gene dependent index of 34 cell lines and RNAi-treated GABRD gene dependent index of 77 cell lines From the Cancer the Dependency Map (Depmap, https://depmap.org/portal/) (22). The bubble map was made by the mean expression of *GABRD*, CRISPR and RNAi of 34 BC cell lines, and 5 BC cell lines were screened out according to the results for follow-up experiments.

Cell lines and cell culture

From the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), five human BC cell lines together with a normal human breast epithelial cell line were collected. MCF10A cells were cultivated in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) containing 1% penicillin/streptomycin (Invitrogen) and 5 percent horse serum (Invitrogen). Cells were grown in DMEM containing 100 g/mL penicillin, 10 percent FBS, as well as 100 mg/mL streptomycin, all at 100 g/mL each. All cells were kept at 37 degrees Celsius in a humidified incubator containing 5 percent CO2 (Thermo Fisher Scientific, Waltham, MA).

Cell transfection

GABRD (using GV248 vector) and the corresponding controls' short hairpin RNA (shRNA) oligonucleotides were constructed by Shanghai Genetic Chemical Co (SGC). There were three sets of target sequences: TGTTCTCGGAGGACAT (shGABRD-#1), CTCATTTCAACGCCGACTA (shGABRD-#2), and TTCCTCGAACGTGTCACGT (sh-NC). Lipofectamine 3000 from Invitrogen (L3000015, San Diego, CA, USA) was used for transient transfection of cells in accordance with the manufacturer's instructions.

RNA extraction and the quantitative polymerase chain reaction (q-PCR)

Cellular RNA was extracted and q-PCR was carried out according to previously described. q-PCR primer sequences were: *GABRD* forward primer 5' -GCATCCGAATCACCTCCACTG-3'; *GABRD* reverse primer 5'- GATGAGTAACCGTAGCTCTCCA-3'. Electrophoresis and sequencing were used to confirm the specificity of the primers. We utilized *GAPDH* as an internal standard for comparisons and measurements. The trials were carried out three times to ensure accuracy.

Western blotting

Western blotting and total cellular protein extraction were carried out (23). The antibodies including: a rabbit anti-GABRD polyclonal antibody (abs141150; 1:1,000 dilution;), which was from Absin Bioscience Inc., Shanghai, China, and a rabbit anti-GAPDH polyclonal antibody (1:2,000 dilution), which was from Beyotime Biotechnology, Shanghai, China, as the loading control.

Cell Cycle Analysis

It took 3 mL of very cold 70 percent ethanol overnight at 20 degrees Celsius to fix cells. Fixed cells were centrifuged for 5 minutes at 1000 g. The cells were then washed in PBS and stained for 30 minutes using a staining solution containing 20 g/mL propidium iodide and 0.2 mg/mL RNase A, followed by flow cytometric analysis. At least three separate trials were used to calculate the average value of G0/G1, S, and G2/M phases (23).

Statistical analysis

We used not only SPSS software version 22 for Windows (IBM Corp., Armonk, NY, USA) but also Microsoft Excel 2010 (Microsoft, WA, USA), as well as GraphPad Prism 7 (GraphPad, CA, USA) to statistical analyses. Continuous variables were subjected to paired or unpaired Student's *t* tests. Categorical comparisons were made by the Fisher exact test and the chi-square test. A minimum of three replications of each experiment were carried out, after that the mean together with standard deviation of all data collected was calculated (SD). According to the statistical significance definition, n.s, not significant; $*P \leq 0.05$; $**P \leq 0.01$

Results

Identification of differentially expressed DABR genes in BC

As mentioned in the introduction, there are 19 known different subunit isoforms of the $GABA_A$ receptor. We obtained genome-wide fpkm matrices from TCGA-BC for 1104 breast cancer patients and 113 paraneoplastic tissues, and analyzed the expression of the $GABA_A$ receptor in breast cancer after excluding genes with an aver-

age fpkm < 0.1. Eight $GABA_A$ receptors were found to be expressed in breast cancer (Fig. 1), with *GABRA3*, *GABRQ* and *GABRD* significantly highly expressed in breast cancer (Fig. 1A-C), *GABRE*, *GABRP* and *GABRR2* significantly low expressed in breast cancer (Fig. 1D-F), and *GABRB2* and *GABRB3* not significantly different (Fig. 1G-H).

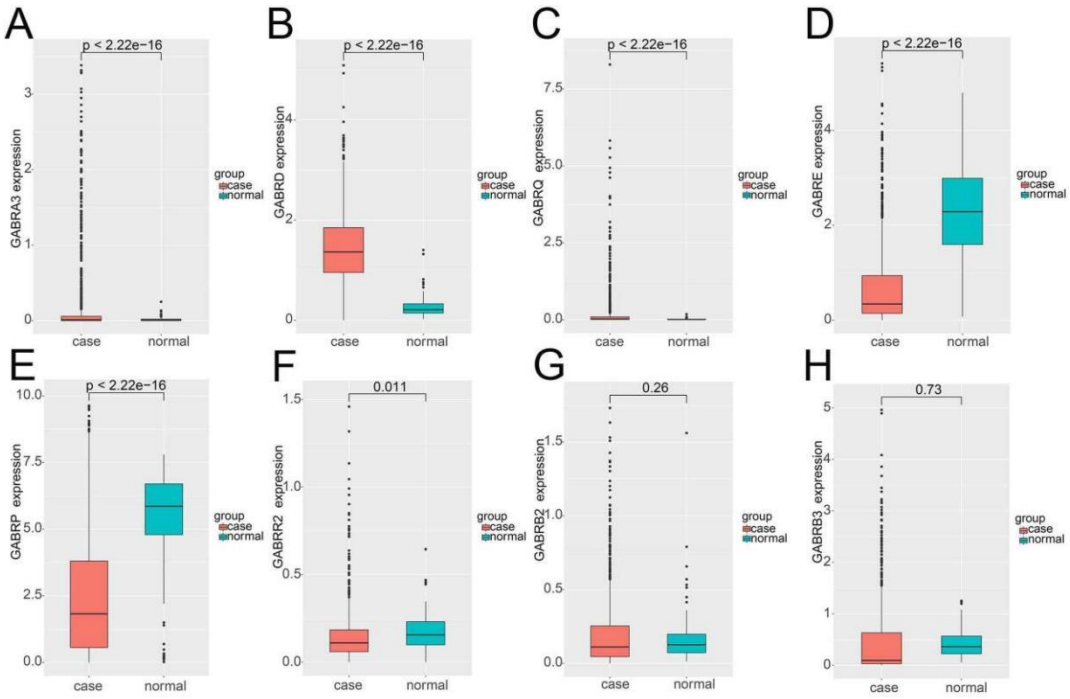

Fig. 1: Expression of $GABA_A$ receptor subunits in breast cancer
(A) The expression of *GABRA3* was increased in cancer tissues; (B) the expression of *GABRD* was high; (C) the expression of *GABRQ* was increased; (D) the expression of *GABRE* was significantly low; (E) the expression of *GABRP* was significantly low; (F) the expression of *GABRR2* was significantly low; (G-H) the expression of *GABRB2* and *GABRB3* were not significantly differential

Survival analysis of differentially expressed GABAA receptors

To investigate whether the six differentially expressed *$GABA_A$* receptor genes have an impact on the prognosis of BC cases, we combined the survival data of BC patients and the expression of the six *$GABA_A$* receptor genes. Fig. 2 shows the Kaplan-Meier overall and progression-free survival curves in breast cancer. The results showed that BC cases in the low expression group of *GABRA3*, *GABRD, GABRQ*, and *GABRR2* had significantly higher OS rates than the high expression group (Fig. 2A-D, P<0.05). Conversely, BC cases of high expression of *GABRE*, and *GABRP* had a better prognosis compared with those of low expression (Fig. 2E-F, P<0.05). *GABRD*'s low expression group had a considerably greater PFS rate than the high expression group. (Fig. 2H, *P*<0.05). However, BC cases of high *GABRA3* expression had a better prognosis for progression-free survival compared with those of low *GABRA3* expression. (Fig. 2G, *P*<0.05). The PFS rates of BC cases in the low expression group for the remaining four genes were not significantly different from those in the high expression group (Fig. 2 I-L).As mentioned above only in the high *GABRD* expression group, both the OS and PFS rates were lower than those in the low *GABRD* expression group, suggesting that the high *GABRD* expression is

associated with the poor prognosis of BC patients.

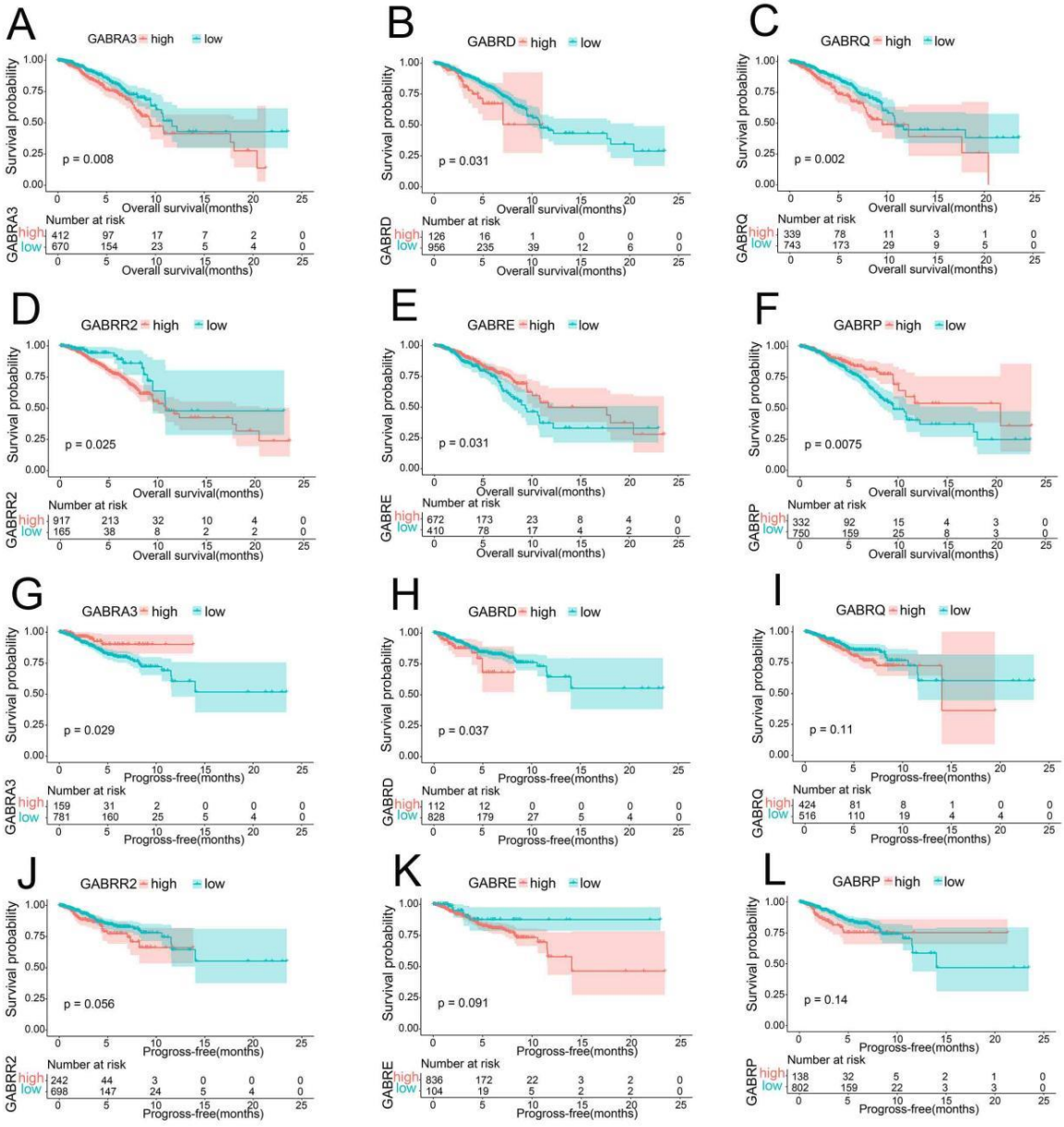

Fig. 2: Overall and progression-free survival analysis of the six differentially expressed $GABA_A$ receptors
(A-F) Overall survival of the six differentially expressed *$GABA_A$* receptors tested by KM analysis. (G-L) The KM analysis was used to detect the progression-free survival of the six differentially expressed $GABA_A$ receptors

The GABRD gene was proved to be overexpressed in a variety of BC cases subgroups

GABRD expression in TCGA BC cases was analyzed by the R software package "ggplot2" to explore the relationship between *GABRD* expression and the development of the disease. *GABRD* expression was proved considerably higher in each subgroup than the normal group in breast cancer patients split by age, pathology, and stage of cancer (Fig. 3A-E). *GABRD* expression in all subgroups of BC patients increased as the disease progressed, regardless of TMN stage or cancer stage (Fig. 3B-E). As a result, *GABRD* may be linked to breast cancer development.

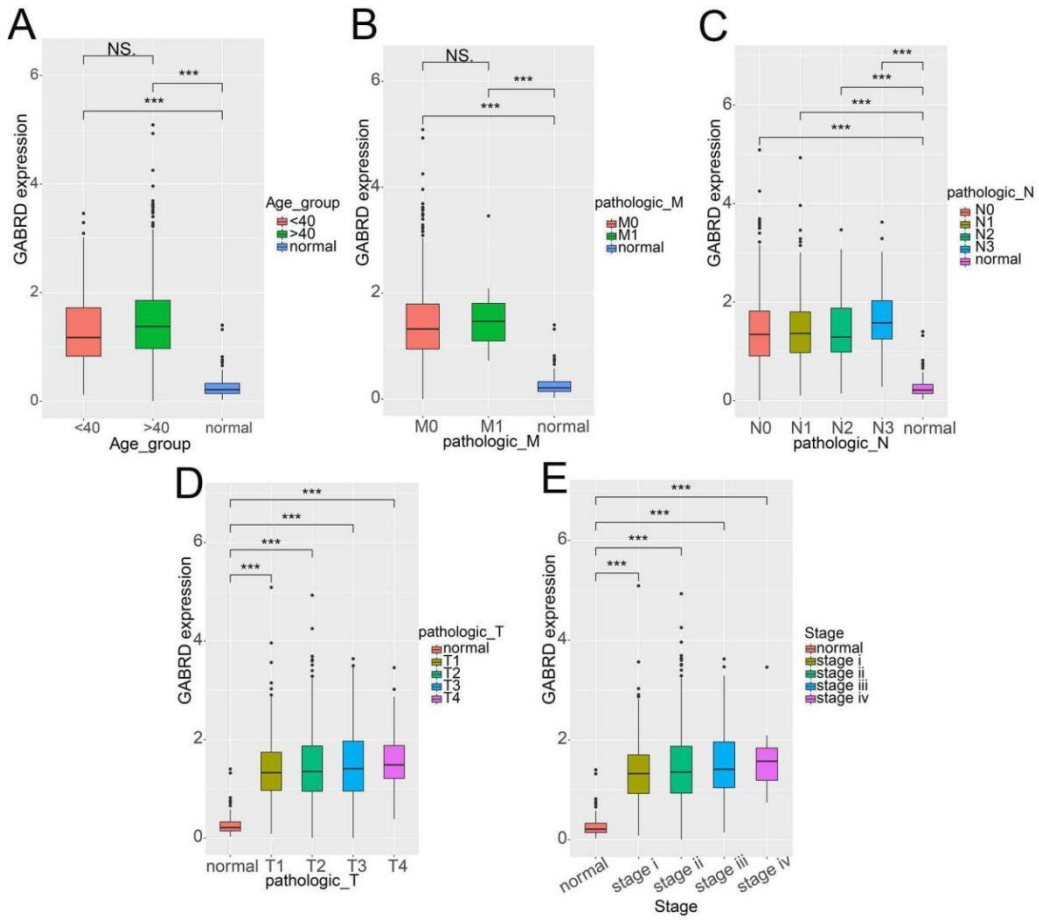

Fig. 3: Breast cancer patients of age and clinical stages have elevated *GABRD* expression
(A) *GABRD* is expressed in breast cancer and normal tissues in two age groups.(<40 and >40); (B) *GABRD* expression in healthy and cancerous breast tissues from two distinct pathogenic M subpopulations (M0 and M1); (C) *GABRD* expression in normal and BC tissues of four subgroups of pathological N(N0, N1, N2, and N3); (D) Four subtypes of pathogenic T cells express *GABRD* in normal and cancerous tissues.(T1, T2, T3 and T4); (E) Normal and breast cancer tissues of four different stages of malignancy showed *GABRD* expression (stages i, ii, iii and iv). ** represents *P* less than 0.01; *** represents p less than 0.001

BC cell proliferation is inhibited by GABRD deletion.

To further investigate the role of *GABRD* in BC, we first downloaded the *GABRD* expression of 56 breast cancer cell lines, CRISPR-treated *GABRD* gene dependent index of 34 cell lines and RNAi-treated *GABRD* gene dependent index of 77 cell lines from the Cancer Dependency Map (Depmap, https://depmap.org/portal/) (Fig. 4A). Finally, we made a bubble plot by *GABRD* mean expression, CRISPR and RNAi combined score from 34 breast cancer cell lines (Fig. 4B). According to the results, we selected 5 BC cell lines HCC1428, McF-7, T47D, ZR751 and CAL51 from 34 BC cell lines for further screening. Western blotting and q-PCR were used to evaluate the endogenous expression of *GABRD* mRNA and protein in MCF-10A normal breast cells and five BC cell lines (Fig. 4C and D; mode pattern). As shown in Fig. 4B-D, in the above BC cell lines, HCC1428 and CAL51 showed high *GABRD* expression levels, and CRISPR and RNAi had strong effects on them, so they were selected for subsequent experiments. Western blotting together with q-PCR was used to look for *GABRD* expression in two

BC cell lines that had been transduced with vector, sh-NC, or *GABRD* shRNAs. (Fig. 4E and F; mode pattern). *GABRD's* in vitro activity was tested utilizing the CCK-8 cell proliferation assay. As shown in Fig. 4G and H mode pattern, knock-down of *GABRD* significantly hindered the proliferative capacity of two BC cells. The above use indicated that *GABRD* was essential for the proliferation of BC cell lines.

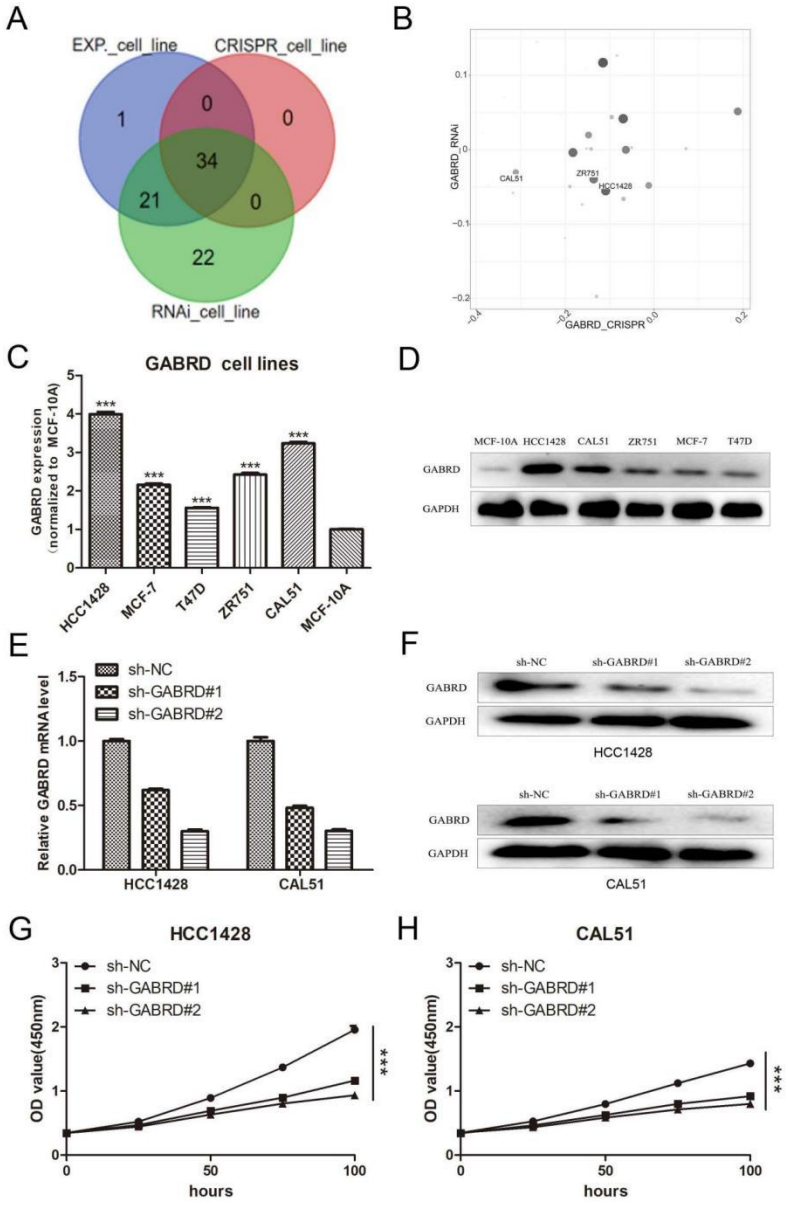

Fig. 4: In BC cell lines, *GABRD* expression was elevated, and *GABRD* knockdown reduced cell growth (A) The effect of *GABRD* on cell proliferation was related to 34 BC cell lines; (B) Bubble plot of *GRBRD* mean expression, CRISPR and RNAi combined score; (C-D) q-PCR as well as western blotting was used to examine *GABRD* mRNA and protein expression in MCF-10A and five BC cell lines. (E-F) Western blotting together with q-PCR was used to look for *GABRD* expression in two BC cell lines that had been transduced with sh-NC or *GABRD* shRNAs. (G-H) A CCK-8 cell proliferation experiment was performed on HCC1428 and CAL51 cells that had been transduced with sh-NC or shGABRDs

GABRD deficiency leads to BC cell cycle arrest

KEGG pathway enrichment and GO annotation analysis were performed on *GABRD* co-expressed genes in order to better understand *GABRD's* role in breast cancer. The coexpressed genes of *GABRD* were particularly engaged in BP, such as double-strand break repair by homologous recombination and mitotic cell cycle G1/S transition, according to the results of GO analysis (Fig. 5A), and in CC, such as chromatin and mitochondrial inner membrane (Fig. 5B), besides in MF like antigen binding and chromatin binding (Fig. 5C). The co-expressed genes of *GABRD* were strongly engaged in oxidative phosphorylation, parkinson disease, cell cycle, and other pathways, according to the results of KEGG enrichment analysis. *GABRD* coexpressed genes were related to oxidative phosphorylation, Parkinson's disease, the cell cycle, and other processes according to KEGG enrichment analysis (Fig. 5D). Cell cycle analysis showed a significant G2/M phase block in *GABRD* deficient cells In GABRD-deficient cells, cell cycle studies revealed a severe G2/M phase block (Fig. 5E-G).

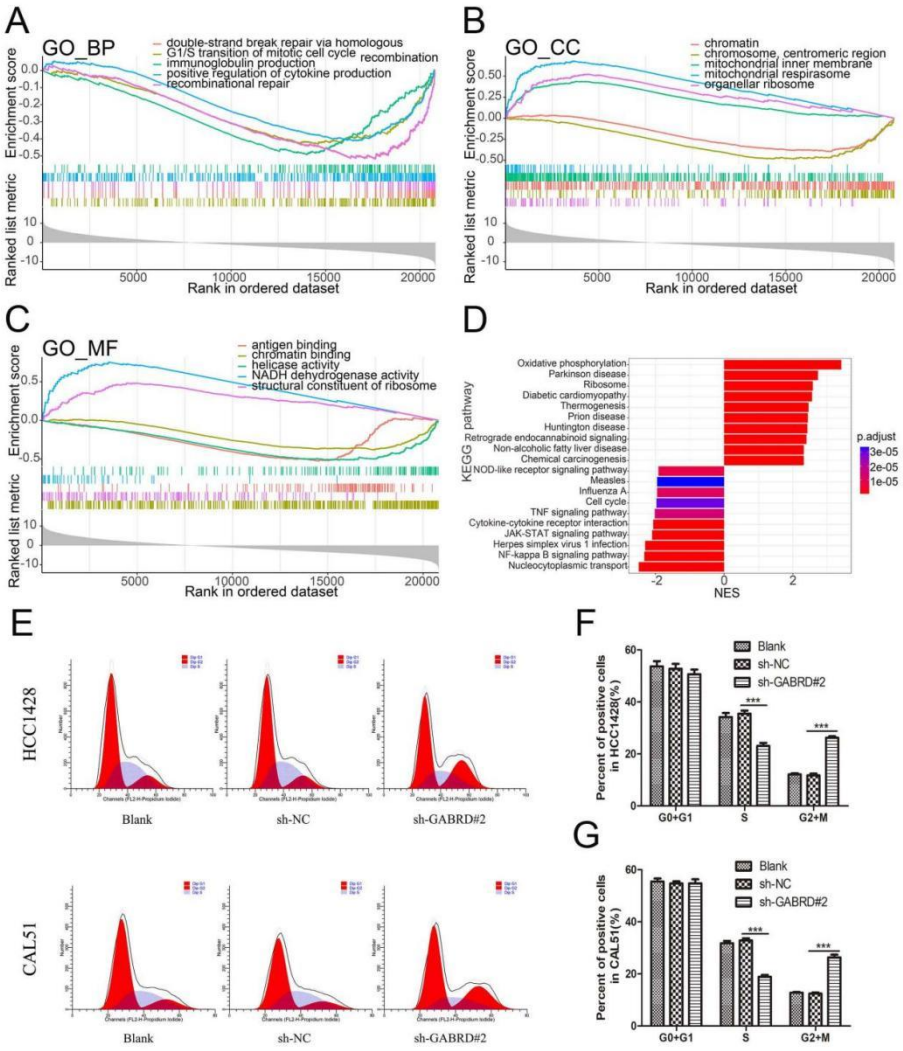

Fig. 5: The progression of the cell cycle was changed when *GABRD* was knocked out

(A-C) GO annotations were used to identify *GABRD* co-expressed genes in breast cancer, (D) Experiments were carried out on the *GABRD* co-expressed genes using KEGG pathway enrichment analysis; (E-G) G2/M phase block was significantly increased in the two cell lines after *GABRD* downregulation (negative control: parental breast cancer cells)

Discussion

We analyzed and screened the expression and survival of all *GABA$_A$R* subunits in BC, and found that only *GABRD* was overexpressed in breast cancer, and significantly related to overall and progression-free survival of breast cancer cases. The pathway analysis also revealed that *GABRD* was associated with the cell cycle, and further revealed that *GABRD* deletion inhibited the proliferation by affected the cell cycle progression. These findings reveal that overexpression of *GABRD* may serve as a prognostic marker for BC patients and an underlying object for BC therapy.

In this study, nineteen *GABA$_A$R* subunits were analyzed for expression and prognostic survival using the public database TCGA-BC data. Breast cancer patients with BC mutations who had high *GABRD* expression had better overall survival and progression-free survival compared to those with of *GABRD* expression.

Children with mental disorders and generalized epilepsies may be at risk for developing the *GABA$_A$* receptor subunit delta (*GABRD*), a gene that encodes the receptor's subunit (24,25). *GABRD* has been implicated with tumorigenesis in a number of recent investigations. *GABRD* was more prevalent in tumors of *USP8* mutations, which are the most common mutation factors in corticotrophinomas (26). A pan-cancer investigation based on the TCGA revealed that *GABRD* was overexpressed in almost all of the positive cases examined (nearly 90%) (27). Low-grade gliomas, on the other hand, have been linked to poor prognosis by *GABRD* expression, according to certain studies (28, 29). As can be observed, individual cancer types must be examined in order to better understand *GABRD*'s mechanism of action in cancer.

GABRD expression was considerably elevated in all categories, such as pathological grading and staging, in comparison to the normal group, when we looked at the link between its expression and breast cancer clinical characteristics. *GABRD* was discovered to be highly elevated in all stages of hepatocellular carcinoma (30). Colon cancer growth might be accelerated by a high expression of *GABRD* mRNA (31). A link exists between *GABRD* and the development of numerous tumors, as seen above.

The enrichment pathway of *GABRD* co-expressed genes was then analyzed using GO Annotation and KEGG Pathway Enrichment Analysis. KEGG enrichment analysis revealed a substantial enrichment in oxidative phosphorylation and Parkinson's disease for co-expressed genes related with *GABRD* in BC. In Parkinson's disease, *GABRD* may possibly have a regulatory function, which has not previously been examined. In addition, genes coexpressed with *GABRD* were shown to be substantially enriched in activities linked to the cell cycle, including negative control of cell cycle processes, mitotic cell cycle transition, also with regulation of cell cycle phase transition. Cancer therapy now relies heavily on the discovery of medications that target the cell cycle (32). *GABRD* may be able to control the cell cycle to help fight breast cancer, according to one study. *GABRD,* then, may be a promising target for cancer treatment.

GABRD's significance in BC carcinogenesis was discovered in this work, however it has inherent limitations that must be addressed in future research. Because we relied on public databases, we did not have enough clinical samples to validate our findings. It also failed to show that *GABRD* has an oncogenic function in vivo, which might be rather diverse in light of the complicated interactions of microenvironment and tumor that are present in this setting. Third, it only partially clarified the probable molecular mechanism potential the action of *GABRD*. There must be in vivo investigations and mechanistic research to verify this functional propensity.

Conclusion

The expression of *GABRD* in all breast cancer sub tissues is significantly higher than that in normal tissues. The down-regulation of *GABRD*

expression will inhibit the growth of BC cells, which may be a new prognostic marker of BC.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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