GABRQ is Overexpressed and Correlated with Tumor Progression in Breast Cancer

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Research Article

Keywords: GABRQ, Breast cancer, tumor progression

DOI: https://doi.org/10.21203/rs.3.rs-503791/v1

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Abstract

Background: Breast cancer (BRCA) is the most common type of women's cancer with a high incidence. The function of gamma-aminobutyric acid A receptor θ subunit (GABRQ) has been studied in other cancers. The results demonstrated that the expression levels of GABRQ were closely associated with tumor prognosis. However, the functions and mechanisms of GABRQ in BRCA remain unclear.

Materials and methods: We used the public genome datasets and a tissue microarray (TMA) cohort to analyze the GABRQ expression levels. We performed Immunohistochemistry (IHC) and Western blot to determine GABRQ expression in BRCA cell lines and tissues. Cell proliferation was assessed by EDU assay and colony formation assay. Transwell assay was carried out to investigate the cell invasion ability \textit{in vitro} and Xenograft nude mouse model was constructed to test the function of GABRQ on tumor growth \textit{in vivo}. Moreover, we utilized bioinformatic analysis to identify the potential molecular mechanisms mediated by GABRQ modification in BRCA.

Results: GABRQ was markedly up-regulated in BRCA tissues, and the expression levels of GABRQ were closely associated with BRCA prognosis. Functional analysis elucidated that knockdown of GABRQ could suppress BRCA cell growth and invasion \textit{in vitro}, and inhibit tumor development \textit{in vivo}. Moreover, we found that GABRQ overexpression activated the EMT signaling pathway.

Conclusions: These results demonstrated that the function of GABRQ in BRCA progression provided potential prognostic predictors for BRCA patients.

1. Introduction

Breast cancer affects over 2.26 million women all over the globe and leads to the highest number of deaths in women (IARC, International Agency for Research on Cancer, 2020). Though the death rates have steadily declined over the past two decades due to advancements in detection and treatment, the etiology of the majority of breast cancer cases remains unknown\cite{1-3}. Thus, it is essential to identify underlying molecular mechanisms of BRCA and develop new therapeutic targets.

As best known, γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain. GABAA receptor subunit θ (GABRQ) can combine with other receptors to form a functional chloride channel that mediates inhibitory synaptic transmission in the mature central nervous system \cite{4, 5}. However, the precise function of GABRQ in peripheral tissues is poorly understood. In the previous study, Y. H. Li \textit{et al.} \cite{6} reported that GABRQ is over-expressed in hepatocellular carcinoma (HCC) and that GABA promotes the proliferation of cancer cells through GABRQ. On the contrary, D. Lee \textit{et al.} \cite{7} demonstrated that lower GABRQ expression was associated with a worse clear cell renal cell carcinoma (ccRCC) prognosis. Therefore, it is controversial whether GABRQ expression could act positively or negatively regulating cancer cell behavior.
In the study, we found that GABRQ expression was up-regulated in BRCA tissues and patients with high GABRQ expression had a shorter survival time. In addition, we corroborated that over-expression of GABRQ promoted cell's invasive ability and activated epithelial-to-mesenchymal transition (EMT) signaling pathway, which was deemed to be associated with migration and invasion of cancer cells[8, 9]. Thus, our results showed that GABRQ might be a pivotal target for BRCA therapy.

2. Materials And Methods

2.1 TCGA data sets

The TCGA-BRCA cohort data of 104 normal patients and 1079 BRCA patients and all relevant clinical date were downloaded from The Cancer Genome Atlas (TCGA, https://tcga-data.nci.nih.gov/tcga/). The expression profiles of GABRQ were analyzed by these datasets. The downloaded original data preprocessing and bioinformatics analysis were conducted by the R studio software (3.51).

2.2 Patients and specimens

The specimens containing 16 paired paraffin embedding BRCA tissues and adjacent non-tumor tissues were obtained from the First Affiliated Hospital of Zhengzhou University (ZZU cohort). The study was approved by the Institutional Review Board of the First Affiliated Hospital of Zhengzhou University.

2.3 TMA cohorts

We analyze another BRCA TMA (the tissue microarray) cohort, which contains 44 GABRQ high expression BRCA tissue specimens and 42 GABRQ low expression BRCA tissue specimens. The TMA cohort was purchased from Outdo Biotech (Shanghai, China).

2.4 Cells and culture

The BRCA cell lines (MCF-7, MDA-MB-231, T47D, BT-474, BT-549) and normal breast cell lines used in this study were purchased from the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin (Corning, NY, United States) in a humid incubator (5% carbon dioxide, 95% air) at 37°C.

2.5 Immunohistochemistry

In brief, 5-µm-thick TMA sections were deparaffinized and then hydrated, blocking endogenous peroxidases and antigen retrieval. TMA sections were incubated with primary antibody overnight at 4°C after blocking for one hour at room temperature. Subsequently, TMA sections were probed with secondary biotinylated goat anti-rabbit antibody, and then detected by SignalStain® DAB (Cell Signaling Technology, Danvers, MA) and counterstained with Hematoxylin QS (Vector Laboratories). Two experienced pathologists, who were blinded to the clinicopathologic data, independently evaluated immunostaining. Sections were scored for the GABRQ staining patterns as follows: 1+, weak staining; 2+,
moderate staining; 3+, strong staining; and 4+, intense staining. Simultaneously, scores of 3+ and 4+ were definitized as high expression and the other scores were deemed as low expression for statistical analysis.

2.6 Real-time quantitative PCR (RT-qPCR)

Different BRCA cell lines tissue and normal tissues were selected for RT-qPCR assay. Total RNA was extracted utilizing TRIzol reagent (Life Technologies) according to the manufacturer instructions. TransScript First-Strand cDNA Synthesis SuperMix (TransGen) was used to reverse-transcribe cDNA. The RT-qPCR assay was performed using PowerUp SYBR Green Kit (ABI) and QuantStudio 6 System (ABI). Data were analyzed by using the comparative Ct method (2^−ΔΔCt). β-Actin was served as the internal control.

2.7 Western blot

RIPA buffer was utilized to extract total protein from cultured cells. Following extraction, BCA assays (Beyotime) were performed to quantify all proteins. An equal amount of protein samples was separated by 12% SDS-PAGE and then transferred to the nitrocellulose membranes (Millipore). The membranes were blocked with 5% non-fat milk/PBS for 1 hour. Then, the membranes were incubated by primary antibodies at 4°C overnight. After washing the membranes with PBST three times, the membranes were further incubated with secondary antibodies for 2 hours. The membranes were developed using enhanced chemiluminescence solution (Beyotime) and exposed to the photographic film for visualization.

2.8 Cell proliferation assay

For colony formation assays, MCF-7 or T47D cells treated with si-GABRQ-2 were placed in 6-well plates and incubated for 2 weeks, then, all cells were fixed, stained, photographed and analyzed. Furthermore, an EDU kit (RiboBio, Guangzhou, China) was used to further assess the cell proliferation ability after GABRQ knockdown. Images were taken with a microscope at 100× (Olympus, Tokyo, Japan) for further analysis. The ratio of EDU-stained cells (red fluorescence) and DAPI-stained cells (blue fluorescence) were used to evaluate the cell proliferation activity.

2.9 Cell invasion assay

Cell invasion assay was performed with Matrigel Invasion Chambers (BD Biosciences, San Jose, CA, USA). Here, 1×10^4 transfected cells were cultured on the upper chamber of the transwell insert in serum-free medium, and the bottom chamber is filled with DMEM with 10% FBS. After 24 hours, the invasive cells were stained with 0.5% crystal violet, imaged and counted.

2.10 Tumor Xenografts

Mice assays were approved by the Animal Health Committee of Zhengzhou University. The male nude mice (4–6 weeks) were purchased from Beijing Vital River Laboratory (Beijing, China). Cells transfected with GABRQ knockdown lentivirus (Sh-GABRQ) and empty lentivirus control (Sh-NC) were subcutaneously implanted into the lower flank of nude mice. Tumor growth was examined every week. Mice were
euthanized at 5 weeks post-implantation. The tumor tissues were weighed and extracted for further IHC staining.

2.11 Statistical analysis

GraphPad Prism software (version 7.0, United States) and SPSS (Version 23.0, IBM, Seattle, WA, United States) were carried out for statistical analyses. Differences between two groups were analyzed by Student's t-test or the Mann–Whitney U test. Overall survival (OS) and disease-free survival (DFS) was calculated with Kaplan-Meier curves and log-rank tests. The correlation was performed by Spearman rank analysis. A p-value < 0.05 was considered to be statistically significant. All data were presented as the mean ± standard deviation (SD).

3. Results

3.1 Expression of GABRQ in TCGA BRCA cohort

We performed TCGA data analysis to verify the expression level of GABRQ in normal patients and BRCA patients. As shown in the figure, GABRQ expression was increased in BRCA tissues (N = 1079) compared to the non-tumor tissues (N = 104) (P < 0.001) (Fig. 1a). The predictive value of GABRQ expression in BRCA patients was further evaluated by Kaplan-Meier survival analysis. BRCA patients with high GABRQ expression (n = 465) had observably worse OS and DFS than those with low GABRQ expression (n = 569) in the TCGA BRCA cohort (Fig. 1b 1c). As shown in Fig. 1D and 1E, by Kaplan-Meier analysis, TNM34 patients with high GABRQ expression (n = 111) had more inferior OS and DFS than TNM34 patients with low GABRQ expression (n = 139) (p < 0.0001).

3.2 Expression of GABRQ in TMA cohort

IHC staining was carried out within the TMA cohort containing 86 BRCA samples, and the staining specimens of all tissues were shown in Fig. 2a. Besides, based on the staining intensity, the expression levels of GABRQ were scored between 1 to 4 (Fig. 2b). By Kaplan-Meier analysis, high GABRQ expression samples (n = 44) had the significantly poorer OS and DFS than low GABRQ expression samples (n = 42) (OS: P = 0.014, DFS: P = 0.029, Fig. 2c 2D).

3.3 Expression of GABRQ in BRCA cell lines and tissues

To evaluate the expression profile of GABRQ in BRCA cell lines and tissues, we analyzed GABRQ expression in human BRCA cell lines and tissues by Western blot and RT-qPCR. As shown in Fig. 3a, GABRQ is dramatically high in expression in BRCA cells. Consistently, RT-qPCR results showed that the expression levels of GABRQ were much higher in BRCA cells than in normal cells (Fig. 3b). As shown in Fig. 3c, GABRQ expression levels were significantly enhanced in BRCA tissues compared to the surrounding non-tumor tissues (n = 16).
3.4 Cell proliferation and invasion after knockdown of GABRQ

To analyze the biological function of GABRQ in BRCA cells, we transfected si-GABRQ into MCF-7 or T47D cells three times. The efficiency of si-GABRQ was assessed by Western blot 48 hours later (Fig. 3a). The most efficient si-GABRQ-2 was used for the following experiments. Colony assays showed that cell proliferation was significantly suppressed in GABRQ-downregulated MCF-7 or T47D cells (Fig. 3b). And EDU assays showed that GABRQ knockdown markedly decreased the DNA synthesis rate in BRCA cells (Fig. 3c). Meanwhile, transwell assay revealed that knockdown of GABRQ suppressed BRCA cell invasion (Fig. 3d 3e). Collectively, these findings suggested that GABRQ is a factor that affects the proliferation of BRCA cells.

3.5 The effect of GABRQ on tumor growth in vivo

Xenograft tumor model was established to confirm the oncogenic role of GABRQ further. sh-NC or sh-GABRQ were subcutaneously implanted into nude mice and the sh-GABRQ group showed significantly dampened tumor growth (Fig. 5a). Compared with the tumors from the corresponding control group, tumors from the experimental group had dramatically smaller tumor size and lower tumor weight (Fig. 5b 5c). In addition, IHC staining found that the tumors developed from the sh-GABRQ cells displayed reduced expression of GABRQ and Ki-67 compared to those from the sh-NC group (Fig. 5d). These results promoted that GABRQ might promote BRCA tumor development in vivo.

3.6 The activated signaling pathway by GABRQ

Gene Set Variation Analysis (GSVA) was adopted to predict the potential signaling pathway. As shown in Fig. 6a, GSVA results indicated that GABRQ might be associated with the regulation of epithelial-mesenchymal transition (EMT) signaling pathway. Besides, GABRQ expression was positively associated with the expression of proliferation marker Ki67 and PCNA, indicating that GABRQ levels were positively correlated to the cell proliferation (Fig. 6b). Furthermore, Gene Set Enrichment Analysis (GSEA) exhibited that EMT signatures were enriched in GABRQ high expression group (Fig. 6c). Altogether, these results further corroborated that GABRQ played an essential role in BRCA progression.

4. Discussion

In this study, we comprehensively analyzed the GABRQ expression levels between BRCA tissues and normal tissues based on three different methods. All the results showed that GABRQ was notably upregulated in BRCA tissues, and we corroborated that OS and DFS of patients with GABRQ high-expression were significantly poorer than that of patients with GABRQ low-expression by the Kaplan–Meier survival analysis. All the results above indicated that GABRQ expression might contribute to the progression of breast cancer by functioning as an oncogene. In order to detect the biological function of GABRQ on BRCA progression, we respectively performed sh-GABRQ to disrupt GABRQ expression in BRCA
cells *in vitro* and *in vivo*. Our data showed that the downregulation of GABRQ led to significant suppression of cell proliferation. Meanwhile, the invasion abilities of cells were dramatically decreased after treatment with sh-GABRQ. In agreement with our results, Y. H. Li *et al.* [6] reported that GABA and GABRQ promote HCC cell proliferation *in vivo* and *in vitro*. But on the contrary, D. Lee *et al.* [7] reported that low GABRQ expression was significantly associated with a poor prognosis of ccRCC by the analysis of TCGA and ICGA cohorts. (ICGA: International Cancer Genome Consortium). Furthermore, Joseph *et al.* [10] reported that GABA could inhibit colon carcinoma cell migration associated with the norepinephrine-induced pathway. Some previous studies also reported that GABA activates some other receptors to inhibit the proliferation without GABRQ[11–13].

In our study, bioinformatics analysis indicated that GABRQ might act as an oncogene in BRCA patients by activating EMT signaling pathway. A. R. Lourenco *et al.* [14] confirmed that the significant contribution of the EMT pathway in BRCA metastasis. J. Chen *et al.* [15] reported that arachidonate lipoxygenase12 could suppress growth and migration of lung cancer cells by suppressing EMT pathway activity. Besides, some other studies have also noted that the activation of the EMT pathway was positively correlated with cancer metastasis, such as colorectal cancer [16], cervical cancer [17, 18] and prostate cancer [19].

In summary, our results show that GABRQ is overexpressed in BRCA tissues and cells, and overexpression of GABRQ is an adverse prognostic factor for BRCA patients. Functional experiments reveal that GABRQ knockdown suppresses BRCA tumorigenesis both *in vitro* and *in vivo*. Furthermore, we predict that GABRQ may acted as an oncogene in BRCA patients by activating EMT signaling pathway. However, the underlying mechanism based on bioinformatic prediction remains a subject for further study.

**Declarations**

**Conflict of Interest:**
None

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Figures
Figure 1

Expression of GABRQ in TCGA BRCA cohort. a GABRQ expression level in normal and tumor patients in TCGA BRCA cohort. b Kaplan–Meier analysis of OS of BRCA patients with low- or high-expression of GABRQ in TCGA BRCA cohort. (*P=0.00091). c Kaplan-Meier analysis of BFS of BRCA patients with low- or high-expression of GABRQ in TCGA BRCA cohort. (*P=0.043). d Kaplan–Meier analysis of the correlation between GABRQ expression and OS of BRCA patients at different TMN stages in TCGA BRCA cohort. (*P<0.0001). e Kaplan–Meier analysis of the correlation between GABRQ expression and BFS of BRCA patients at different TMN stages in TCGA BRCA cohort. (*P<0.0001)
Expression of GABRQ in TMA cohort. 

a Immunohistochemical staining specimens of all tissues. 
b Representative immunohistochemical staining patterns with different staining scores in BRCA tissues. 
c Kaplan–Meier analysis of OS with low- or high-expression of GABRQ in TCGA TMA cohort. (*P=0.014). 
d Kaplan–Meier analysis of BFS with low- or high-expression of GABRQ in TCGA BRCA cohort. (*P=0.029)
Expression of GABRQ in BRCA cell lines and tissues. 

a The expression level of GABRQ in different cell lines were analyzed by Western blot 48 hours later. 

b Relative expression levels of GABRQ in different cell lines. 

c GABRQ expression in 16 paired BRCA tissues (T) and adjacent non-tumor tissues (N) was analyzed by Western blot.

Figure 3
Cell proliferation and invasion after knockdown of GABRQ. a BRCA cells MCF-7 or T47D were transfected with negative control (NC) or different shRNAs targeting GABRQ. The knockdown efficiency was evaluated by Western blot 48 hours later. b Effects of GABRQ knockdown on cell proliferation were determined by colony formation assay. c DNA synthesis in BRCA cells MCF-7 or T47D was measured using the EDU incorporation assay. d-f Transwell assays were performed to evaluate the effect of GABRQ
knockdown on cell invasion capability. Cells were counted under a microscope in five randomly selected fields.

**Figure 5**

The effect of GABRQ on tumor growth in vivo. a Representative image of tumor growth. b The tumor volume was detected every week. c Images of the tumors were shown and the tumors weight were measured and represented as means of tumor weights ± S.D. d Representative images of H&E staining.
and Ki-67, GABRQ IHC staining of tumor sections from the sh-NC or sh-GABRQ group. Quantification of relative Ki-67 and GABRQ staining intensity in tumor sections from the sh-NC or sh-GABRQ group.

Figure 6

The activated signaling pathway by GABRQ. a Gene set variation analysis (GSVA) comparison of signaling pathways with differently expressed genes. b The relationship between Ki-67 and PCNA and
GABRQ. c The Gene Set Enrichment Analysis (GSEA) analysis the enrichment of Epithelial-mesenchymal transition (EMT) signaling pathways between GABRQ high expression and low expression groups.