GABARAP suppresses EMT and breast cancer progression via the AKT/mTOR signaling pathway

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Abstract
Background
Recent studies document that γ-aminobutyric acid type A (GABAA) receptor-associated protein (GABARAP) plays an important role in cancer autophagy. However, little is known about its role in tumor invasion, migration and metastasis. Here, the authors investigated the expression and significance of GABARAP in breast cancer.

Method:
A large group of clinical samples was assessed to detect GABARAP expression and its associations with clinicopathological features and prognosis. Gain- and loss-of-function experiments in cell lines and mouse xenograft models were performed to elucidate the function and underlying mechanisms of GABARAP-regulated tumor progression.

Results
We analyzed GABARAP levels in clinical breast cancer samples and cell lines and confirmed that GABARAP was negatively correlated with advanced clinicopathologic features, such as tumor size (P = 0.025) and TNM stage (P = 0.001). Importantly, patients with low GABARAP levels had a poor prognosis (p = 0.0047). Functionally, our data revealed that GABARAP can inhibit proliferation, migration and invasion in vitro and in vivo. Importantly, low levels of GABARAP induced epithelial-mesenchymal transition (EMT), one of the most important mechanisms for the promotion of tumor metastasis, in breast cancer cells. Mechanistically, low levels of GABARAP increased the levels of p-AKT (S473) and p-mTOR (S2448), and a specific AKT pathway inhibitor reversed the downregulation of GABARAP-induced tumor progression. In clinical breast cancer specimens, immunohistochemistry (IHC) revealed that the distribution and intensity of GABARAP expression were negatively correlated with those of matrix metalloproteinase (MMP) 2 (P = 0.0013) and MMP14 (P = 0.019).

Conclusions
Collectively, these data indicated that GABARAP suppressed the malignant behaviors of breast cancer cells, illuminating that the possible mechanism acts via the AkT/mTOR pathway. Targeting GABARAP may provide a potential diagnosis and treatment strategy for breast cancer.

Background
Breast cancer is the most common cancer in women and is the second leading cause of cancer-


related death among females worldwide [1]. In China, the number of new breast cancer cases has continually risen in recent years, and the age of onset tends to be younger [2]. For patients with aggressive tumors that are refractory to multiple treatments, a dismal prognosis is expected, and a vast majority of these patients die of complications relating to metastatic diseases [3, 4]. Metastasis and chemoresistance lead to treatment failure, and the mechanisms responsible are poorly understood. Thus, it is essential to further characterize the molecular mechanism of breast cancer progression and to develop novel mechanism-based therapeutic strategies to block this process [5, 6]. The purpose of this study was to evaluate the potential value of γ-aminobutyric acid type A (GABAA) receptor-associated protein (GABARAP) as a therapeutic target for breast cancer metastasis. GABARAP, located at 17p13.1, is a 14 kDa cytoplasmic protein that is a member of the autophagy-related protein 8 (Atg8) family and shows a high degree of sequence homology with the autophagy marker light chain 3 (LC3) [7]. Most studies have reported that GABARAP plays a crucial role in modulating the level of autophagy [8-10]. For example, Kabeya et al. stated that GABARAP binds to autophagic vesicles in a manner similar to that of LC3 and may also be involved in the formation of autophagosomes [11]. By modulating the level of autophagy, it has been reported to play a key role in inhibiting inflammation progression [12] and regulating angiogenic activity [13]. These processes are all associated with vesicle transport, apoptotic cell death, and reactive oxygen species generation, and the molecular mechanisms involve tubulin, the cytoskeleton, angiotensin II type 1 (AT1) receptor, transient receptor potential vanilloid, mTOR and PI3K/AKT [11–16]. However, the expression and underlying mechanism of GABARAP in breast cancer are poorly understood. These observations have prompted us to speculate the role of GABARAP in the initiation and development of breast cancer.

The PI3K/AKT signaling pathway plays an important role in many cellular functions, including proliferation, adhesion, migration, invasion, metabolism, and survival. AKT has been reported to be overactivated in > 60% of cancer types [17–19], is involved in tumor development and is a potential target for tumor therapy. Epithelial-mesenchymal transition (EMT) is known to be a central mechanism responsible for the invasiveness and metastasis of various cancers [20, 21]. The
mechanism of EMT is complex and involves different signals from the tumor microenvironment, tumor
cells and the interactions between these two components [22–24]. In this study, we explored the
expression and biological functions of GABARAP using a comprehensive investigative approach that
included clinical breast cancer samples along with cellular and animal models. Furthermore, we
elucidated the mechanisms by which GABARAP suppressed EMT breast cancer cells, which partly
occurs through the Akt/mTOR signaling pathway.

Materials And Methods
Tissue specimens and patients
This study was approved by the Ethics Committee of Harbin Medical University. Samples from 87
cases of histopathologically confirmed invasive ductal breast cancer (IDC) and 48 cases of ductal
carcinoma in situ (DCIS) as well as 24 samples of normal breast tissue were included in this study.
The IDC patients were female, hospitalized at the Affiliated Tumor Hospital of Harbin Medical
University from March 2010 to November 2010 and followed-up until March 2015. The median follow-
up was 58.9 months (range 16.8 to 63.3 months). Formalin-fixed paraffin-embedded (FFPE) tissues
and complete clinical records of the patients were collected. None of the patients had undergone
preoperative chemotherapy or radiotherapy.

Cell Culture
The human breast cancer cell lines MDA-MB-231, MCF7, MDA-MB-468, T47D, UACC-812, MDA-MB-453,
SKBR-3, and HCC70 and nontransformed breast cell line MCF-10A were obtained from the Cancer
Research Institute of Heilongjiang Province, China. MCF7, T47D, UACC-812 and HCC70 cells were
cultured in DMEM (Gibco, Carlsbad, CA, USA), whereas MDA-MB-453 and SKBR-3 cells were cultured in
RPMI 1640 (Gibco, Carlsbad, CA, USA). All media for the abovementioned cancer cell lines were
supplemented with 10% fetal bovine serum (FBS) (PAN) (Gibco, Grand Island, NY, USA) and 1%
penicillin-streptomycin (Gibco, Grand Island, NY, USA). MCF-10A cells were maintained in DMEM-F12
(Gibco, Carlsbad, CA, USA) medium supplemented with hydrocortisone (0.5 µg/ml), insulin (10 µg/ml),
hEGF (20 ng/ml) and 10% FBS. All cells were cultured in a humidified incubator at 37 °C with 5% CO2.
MDA-MB-231 and MDA-MB-468 cells were cultured in RPMI 1640 (Gibco, Carlsbad, CA, USA)
supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C in a humidified incubator
without CO2.

Cell Transfection

UACC-812 and T47D cells were infected with lentiviruses expressing specific shRNA to knock down GABARAP (GABARAP-shRNA). The GABARAP sequence-specific shRNA and vector control shRNA (scrambled shRNA) were designed and synthesized by GeneChem Co. (Shanghai, China), and knockdown of GABARAP in T47D cells was achieved by lentivirus-mediated infection with three different shRNAs. shRNA2 (GABARAP-shRNA2; sequence: GCCUACAGUGACGAAAGUGTT) was most efficient at knocking down GABARAP and was therefore used in subsequent experiments. For overexpression of GABARAP, full-length human GABARAP cDNA was cloned into the pcDNA3.1 vector between the EcoRI and KpnI sites, and the resulting plasmids were transfected into MDA-MB-453 cells with Lipofectamine 2000 in accordance with the approved guidelines. Transfection efficiency was determined by western blot.

Cell Viability Assay

The proliferation of T47D, UACC-812 and MDA-MB-453 cells proliferation assay was assessed with a Cell Counting Kit-8 (CCK-8) (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer’s instructions. Briefly, 1000–3000 cells per well (depending on the cell type) were plated in each well of a 96-well plate and cultured overnight. CCK-8 reagent (10 µL per well) was added at 24, 48 or 72 h after seeding and incubated at 37 °C for 2 h, at which point the absorbance of optical density (OD) value at 450 nm was measured. Three parallel experiments were performed for each assay with five replicate wells per condition.

Cell Invasion Assays

For the invasion assays, cells in serum-free medium were seeded into the upper chamber of a transwell insert (8-µm pore size, Millipore) coated with Matrigel (Sigma-Aldrich, USA). Medium containing 10% FBS was added to the lower chamber. After the cells were incubated at 37 °C for 48 h, noninvading cells that remained in the top chambers were removed with a cotton swab, and the cells that had migrated to the underside of the membrane were fixed in 100% methanol for 30 min, air-dried, stained with 0.5% crystal violet (Sigma, St. Louis, MO, USA), imaged, and counted under a light microscope.
Colonization Formation Assay
Cells were seeded into a 6-well plate and cultured for 14 days in medium containing 10% FBS. Colonies were fixed in methanol for 30 min, and 500 µL of 0.5% crystal violet was added to each well for 30 min for visualization and counting.

Wound Healing Assay
For wound healing assays, cells cultured to 95% confluence in a 6-well plate were scratched with a 10-µL pipette tip. Then, images of the wound were taken at 0 h and 24 h. Mobility was calculated as follows: (mean area at 0 h−mean area at × h)/(mean area at 0 h) × 100%.

Immunohistochemistry (IHC) And Western Blot
IHC and western blotting were performed as previously described [25]. The antibodies targeting the following proteins were used: GABARAP (Abcam, ab109364), E-cadherin (Abcam, ab40772), N-cadherin (Abcam, ab76011), Vimentin (Proteintech, 10366-1-AP), matrix metalloproteinase (MMP) 2 (Abcam, ab110186), MMP14 (Abcam, ab3644), PI3K (Cell Signaling Technology, 4292), p-PI3K (Cell Signaling Technology, 4228), AKT (Abcam, ab179463), p-AKT (Ser473; Bioworld Technology, BS4007), mTOR (Abcam, ab2732), p-mTOR (Ser2448; Cell Signaling Technology, 5536), p-70S6K (Proteintech, 14485-1-AP), and p-P70S6K (Thr389; Cell Signaling Technology, 9234). Equal protein loading was assessed using a mouse anti-β-actin antibody (ZSGB-BIO, TA-09).

Nude Mouse Tumor Xenograft Model
BALB/c athymic nude mice (female, 4-5 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and bred in pathogen-free conditions at the Animal Center of the Affiliated Tumor Hospital of Harbin Medical University. UACC-812/vector control or UACC-812/GABARAP-shRNA cells (5 × 10⁶ cells in 100 µL of PBS) were subcutaneously injected into the left flanks of mice in each group (n = 10 per group). When palpable tumors formed, the mice bearing UACC-812 cells with or without stable knockdown of GABARAP were randomized into two subgroups (n = 5 per subgroup) and injected with LY294002 (75 mg/kg) or sterile water into the peritoneal cavity twice weekly for 3 weeks. Subsequently, the tumor volume was monitored with Vernier calipers every five days for 3 weeks and calculated using the equation (L × W²)/2, where L is the length and W is the width. Tumor weight was measured after excision on the final day of the experiment. After all the
mice were sacrificed, a portion of the tumor tissue was fixed in formalin and embedded in paraffin for immunohistochemical analysis. All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of Harbin Medical University in China and the NIH Guidelines for the Care and Use of Laboratory Animals.

**Statistical analysis**

Statistical analyses were performed with SPSS 22.0 and GraphPad Prism software. The data are expressed as the means ± standard deviations (SDs). All experiments were performed at least three times. The differences between two groups were analyzed with Student’s tests and the χ² test. The survival analysis was performed using Kaplan-Meier analysis and log-rank tests. Data from The Cancer Genome Atlas Breast Invasive Carcinoma (TCGA-BRCA) cohort were downloaded from the UALCAN Database (http://ualcan.path.uab.edu/index.html) and Oncomine Database (https://www.oncomine.org/resource/login.html); these data sets were used to detect GABARAP mRNA expression and survival analysis in breast cancer. A two-tailed p value < 0.05 was considered significant.

**Results**

GABARAP is downregulated in primary breast cancer specimens as well as in breast cancer cell lines

To investigate the potential role of GABARAP in breast cancer, we first analyzed GABARAP mRNA expression via breast cancer RNA-seq data from the TCGA database. We found that GABARAP expression was significantly downregulated in breast cancers compared with normal tissues (P < 0.0001, Fig. 1a). GABARAP expression was lower in all subtypes of breast cancer tissues than in normal tissues (Fig. 1b) (P < 0.0001). Moreover, the results of the TCGA data analysis were validated in 87 IDC samples, 48 DCIS samples and 24 noncancerous tissue samples via IHC (Fig. 1e). Positive staining (brown) was detected in the majority of noncancerous tissues (16/24) and in some DCIS tissues (17/48) but was detected less frequently in IDC tissues (27/87) (P < 0.01, Fig. 1f, Additional file 1: Table S1). This suggests that the low expression of GABARAP is related to the occurrence and development of breast cancer.

To test whether GABARAP was also decreased in cultured breast cancer cell lines, we conducted western blot analysis on eight breast cancer cell lines and nontransformed MCF-10A cells. As shown in
Fig. 1g and h, GABARAP displayed high expression levels in the nontransformed MCF-10A cells but only weak expression in T47D, UACC-812, and MCF-7 cells; furthermore, GABARAP had low expression in MDA-MB-453, SKBR3, HCC70, MDA-MB-231 and MDA-MB-468 cells (P < 0.05). These results indicated that GABARAP is downregulated in breast cancer cells.

The clinical significance of GABARAP in patients with breast cancer

To further investigate whether low GABARAP expression is involved in breast cancer progression, we analyzed GABARAP mRNA expression based on RNA-seq data of individual cancer stages in breast cancer from the TCGA database (Fig. 1c). GABARAP downregulation was strongly associated with pTNM stage (P < 0.01). Then, we analyzed the correlation between GABARAP levels and the clinicopathological features of 87 patients with histopathologically confirmed IDC at Harbin Medical University Cancer Center (HMUCC). As shown in Additional file 1: Table S2, statistical analyses using the IHC results revealed that low levels of GABARAP in patients correlated with advanced pT classification (p = 0.025), axillary lymph node metastasis (P = 0.023), advanced pTNM stage (p = 0.001), histological grade (p = 0.019) and ER status (p = 0.039). However, no significant association was found between GABARAP and age, Her-2 status, Ki-67 levels or P53 status. Therefore, we hypothesize that low expression of GABARAP is involved in tumor cell proliferation and plays an important role in breast cancer development. Additionally, survival analysis showed that patients with low levels of GABARAP expression had lower overall survival (p = 0.0047) than those with high GABARAP expression levels (TCGA, Fig. 1d). These results demonstrate that GABARAP is expressed at low levels in breast cancer tissue and that low expression is associated with a poor prognosis for these patients.

Low GABARAP levels enhance the malignant behavior of breast cancer cells

We performed western blotting analysis to compare the expression levels of GABARAP in breast cancer cell lines and nontransformed MCF-10A cells. As shown in Fig. 1g, T47D and UACC-812 cells were used as GABARAP “loss-of-function” models, while MDA-MB-453 cells served as a GABARAP “gain-of-function” model. We detected the protein expression level of GABARAP in these target cells by western blot. As shown in Fig. 2a and b, compared with control cells, T47D and UACC-812 cells
exhibited significant knockdown of GABARAP while MDA-MB-453 cells presented increased GABARAP expression was increased in (Fig. 3a).

To test whether GABARAP affects the proliferation of breast cancer cell lines, we conducted CCK8 assays and measured cell viability. Knockdown of GABARAP significantly accelerated cell growth in T47D and UACC-812 cells compared to the respective control cells (Fig. 2c and d), whereas overexpression of GABARAP in MDA-MB-453 cells attenuated cell growth (Fig. 3b). Accordingly, we observed similar patterns in the colony forming efficiency of GABARAP-overexpressing MDA-MB-453 cells (Fig. 3c) and GABARAP-shRNA-transfected T47D and UACC-812 cells (Fig. 2e and f), suggesting that GABARAP negatively regulates cell proliferation in vitro.

We further studied the potential effects of GABARAP on cell migration and invasion using wound healing and transwell assays, respectively. As shown in Fig. 2g - j, both migration and invasion were significantly enhanced in T47D and UACC-812 cells with knockdown of GABARAP. However, overexpression of GABARAP in MDA-MB-453 cells markedly inhibited cell migration and invasion compared to MDA-MB-453 cells transfected with vector controls (Fig. 3d and e). These results suggest that GABARAP inhibits cell migration and invasion in vitro.

**GABARAP Regulates Breast Cancer Progression Via EMT**

EMT plays an important role in tumor metastasis, and we hypothesized that GABARAP affects the EMT process and inhibits breast cancer progression. Then, we analyzed the expression profile data of breast cancer patients clinical cohort from TCGA database. Pearson correlation was calculated among genes related to GABARAP, CDH1, MMP2, MMP14 and Akt/ mTOR signaling pathway. In result, the expression correlation of GABARAP and Akt/mTOR signaling pathway was identified in the clinical cohort of TCGA breast cancer patients, revealed the potential influence of GABARAP and Akt/mTOR signaling pathway. Furthermore, Akt/mTOR signaling pathway mediates genes related to epithelial mesenchymal transformation, such as CDH1, MMP2 and MMP14 (Fig. 4a). Here, we show that downregulation of GABARAP was associated with high levels of Vimentin, N-cadherin, MMP2 and MMP14 and low levels of E-cadherin (Fig. 4b). Conversely, GABARAP upregulation inhibited Vimentin, N-cadherin, MMP2 and MMP14 expression and upregulated E-cadherin expression (Fig. 4c). Taken
together, these results indicate that GABARAP inhibits EMT and thereby suppresses breast cancer progression.

Low levels of GABARAP induce EMT by activating the AKT/mTOR pathway

Multiple pathways, including mTOR, PI3K/AKT and NF-κB signaling, are involved in GABARAP-regulated autophagy, inflammation and angiogenic activity [8–13]. To further verify how GABARAP regulates EMT, we measured AKT/mTOR, NF-κB and ERK/MAPK signaling in breast cancer cells. Knockdown of GABARAP in T47D and UACC-812 cells increased the levels of phosphorylated AKT, mTOR and p70s6k (Fig. 4b) but had no effect on p-ERK, p-MEK, p-IKK-β and p-IκBα levels (Additional file 3: Fig. S1). By contrast, overexpression of GABARAP suppressed the phosphorylation of AKT, mTOR and p70s6k (Fig. 4c), and showed no effect on p-ERK, p-MEK, p-IKK- β and p-IκBα levels (Additional file 3: Fig. S1). These results indicate that GABARAP levels are negatively correlated with the activation of AKT/mTOR pathways. Using pathway inhibitors, we found that GABARAP-mediated inhibition of invasion, migration, and EMT was reversed by the AKT pathway inhibitor LY-294002 (50 µM, 24 h; Fig. 5a, b, c, d and e). Taken together, these data indicate that downregulation of GABARAP activates the AKT/mTOR pathway, which in turn promotes EMT of breast cancer cells.

GABARAP Suppresses Breast Cancer Progression In Vivo

To evaluate the role GABARAP plays in regulating breast cancer progression in vivo, we established a xenograft tumor model in nude mice using UACC-812 cell lines stably transfected with vector control or GABARAP-shRNA and treated the animals with LY294002 (75 mg/kg) or sterile water. Mice were randomly assigned to the following experimental groups: vector control group, GABARAP-shRNA group, vector control + LY294002 group and GABARAP-shRNA + LY294002 group (n = 5 per subgroup). (Fig. 6a). As expected, silencing GABARAP significantly increased tumor volumes and weight in mice compared with those in the control group. However, GABARAP-mediated inhibition of tumor formation was reversed by LY-294002 (Fig. 6b and c). Furthermore, tumors with GABARAP knockdown had a higher metastasis capacity, as evidenced by the increased MMP2 and p-mTOR staining and reduced E-cadherin staining (Fig. 6d). These results indicate that knocking down GABARAP suppresses breast cancer progression in vivo.
GABARAP Suppresses Tumor Progression In Clinical Breast Cancer Specimens

To further examine the relationship between GABARAP and metastasis in human breast cancer, we performed IHC staining of GABARAP, MMP2 and MMP14 in 87 IDC specimens (Fig. 7a and c).

Consistent with our observations in the tumor cell lines and xenograft models, the distribution and intensity of GABARAP were negatively correlated with MMP2 (P = 0.0013, Fig. 7b) and MMP14 (P = 0.019, Fig. 7d) in breast cancer tissue specimens. Which clearly indicated that low GABARAP expression was associated with elevated metastasis in breast cancer patients.

Discussion

Metastasis and chemoresistance lead to treatment failure in breast cancer patients. Metastasis is a highly inefficient process, and the mechanisms involved are poorly understood [26, 27]. Thus, it is essential to further characterize the molecular mechanism(s) of breast cancer progression and to develop novel therapeutic strategies based on mechanisms to block breast cancer progression. Our study demonstrates that GABARAP is significantly downregulated in breast cancer tissues and that a low level of GABARAP is associated with a malignant breast cancer phenotype. Our findings indicate that, in terms of the mechanism, GABARAP inactivates the AKT/mTOR pathway and suppresses cell EMT. Clinically, we found that low levels of GABARAP were negatively associated with upregulation of the expression of MMPs, which we believe will serve as a promising prognostic indicator in breast cancer patients.

Several studies have reported that GABARAP plays a crucial role in modulating the level of autophagy. For example, Sasai M et al. demonstrated that GABARAP, as an autophagy protein, plays an essential role in interferon-inducible GTPase-mediated host defense [28]. Nath S et al. found that lipidation of the LC3/GABARAP family of autophagy proteins relies on the membrane-curvature-sensing domain in Atg3[29]. However, very little research on the role of GABARAP in tumor progression is available.

Based on our assessment of a large number of clinical samples, the present study showed that GABARAP is significantly downregulated in breast cancer tissues, a result that is consistent with that of the public database. Moreover, we found that decreased GABARAP levels promoted cell proliferation, invasion and migration in vitro and promoted breast cancer growth and metastasis in
vivo. Importantly, low levels of GABARAP induced EMT in breast cancer cells, which is one of the most important mechanisms for the progression of tumor metastasis.

A retrospective review of the literature indicates that GABARAP regulates multiple classical pathways, including the AKT/mTOR pathway. For example, Wu et al. showed that GABARAP promotes bone marrow mesenchymal stem cell-based osteoarthritis cartilage regeneration through the inhibition of the PI3K/AKT/mTOR signaling pathway [16]. Rho et al. reported that GABARBP inactivates cisplatin-induced dephosphorylation of the PI3K/Akt signaling pathway and promotes the sensitivity of ovarian cancer to apoptosis [30]. In line with these studies, we found that downregulation of GABARAP expression increased p-AKT and p-mTOR levels and induced EMT. Furthermore, EMT induced by low levels of GABARAP can be reversed by an AKT pathway inhibitor. We therefore conclude that low levels of GABARAP results in the activation of the AKT/mTOR signaling pathway.

Conclusions
Collectively, to the best of our knowledge, this is the first study to reveal a fundamental role of GABARAP in suppressing invasion, aggressiveness and metastasis, thus establishing GABARAP as a potential therapeutic target for the multifaceted progression of breast cancer.

Abbreviations
GABARAP
γ-aminobutyric acid type A (GABAA) receptor-associated protein (GABARAP);
EMT
epithelial-mesenchymal transition (EMT);
IHC
immunohistochemistry (IHC);
MMP
matrix metalloproteinase (MMP);
LC3
light chain 3 (LC3);
AT1
angiotensin II type 1 (AT1);
IDC
invasive ductal breast cancer (IDC);
DCIS
ductal carcinoma in situ (DCIS);
FFPE
Formalin-fixed paraffin-embedded (FFPE);
TCGA
The Cancer Genome Atlas;
LNM
lymph node metastasis (LNM)
OS
overall survival;
OD
optical density.

Declarations

**Ethics approval and consent to participate**

Breast cancer tissues were taken from patients undergoing surgical resection at the Harbin Medical University Cancer Hospital (Harbin, China). All work was approved by the Ethics Committee of Harbin Medical University. All animal work was performed in compliance with the Institutional Animal Care and Use Committee (IACUC) of the second affiliated hospital of Harbin medical university in China and the NIH Guide for the Care and Use of Laboratory Animals.

**Consent for publication**

We have obtained consents to publish this paper from all the participants of this study.

**Availability of data and materials**

All data generated or analysed during this study are included in this published article [and its supplementary information files].

**Competing interests**

The authors declare that they have no competing interests.

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Not applicable.

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Authors' contributions

CL, CX and LY designed this research. LY carried out most of the experiments, analyzed the data, drew the figures and drafted this manuscript. LM, GJ, CY and JX helped with cell culture, western blot experiments and the CCK-8 assay. WD and JY helped with the IHC assay. YQ, GY and LY helped with the animal experiment. CX helped check the manuscript and figures. All authors read and approved the final manuscript.

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Figures
GABARAP is downregulated in breast cancer specimens and cell lines. a. Expression profile of GABARAP in primary breast cancer tissues (n = 1097) and normal breast tissues (n = 114) (TCGA). b. Expression of GABARAP in BRCA based on breast cancer subclasses (TCGA). c. Expression of GABARAP in BRCA based on individual cancer stages (TCGA). d. Overall survival of breast cancer patients according to GABARAP expression (TCGA). e. Representative images of GABARAP immunohistochemical staining. f. A statistically significant of ‘positive’ or ‘negative’ GABARAP staining in 87 cases of IDC, 48 cases of DCIS and 24 cases of normal breast tissue. g. Western blotting analysis of GABARAP expression in 8 human breast cancer cell lines and non-transformed MCF-10A. h. A statistically significant of GABARAP expression in 8 human breast cancer cell lines and non-transformed MCF-10A. (*P < 0.05, **P < 0.01, ***P < 0.001 ****P < 0.0001. TCGA = The Cancer Genome Atlas).
Low GABARAP level enhances the malignant behavior of breast cancer cells. a. Knockdown of GABARAP in T47D cells by several shRNAs, GABARAP expression was determined using Western blot. b. Knockdown of GABARAP in T47D and UACC-812 cells, GABARAP expression was determined using Western blot. c. Cell proliferation in T47D-vector, T47D-shRNA cells was detected by CCK-8 assays. d. Cell proliferation in 812-vector, 812-shRNA cells was...
detected by CCK-8 assays. e. Colony-forming efficiency was determined in T47D-vector, T47D-shRNA cells. f. Colony-forming efficiency was determined in 812-vector, 812-shRNA cells. g. Invasive abilities conveyed by T47D-vector, T47D-shRNA cells were measured by Matrigel invasion assays. h. Invasive abilities conveyed by 812-vector, 812-shRNA cells were measured by Matrigel invasion assays. i. Migration ability conveyed by T47D-vector, T47D-shRNA cells were assessed by wound-healing migration assays. j. Migration ability conveyed by 812-vector, 812-shRNA cells were assessed by wound-healing migration assays. ( *P < 0.05, **P < 0.01, ***P < 0.001 )
High GABARAP level suppresses the malignant behavior of breast cancer cells. a. Over-expression of GABARAP in MDA-MB-453 cells, GABARAP expression was determined using Western blot. b. Cell proliferation in 453-vector, 453-GABARAP cells was detected by CCK-8 assays. c. Colony-forming efficiency was determined in 453-vector, 453-GABARAP cells. d. Invasive abilities conveyed by 453-vector, 453-GABARAP cells were measured by Matrigel invasion assays. e. Migration ability conveyed by 453-vector, 453-GABARAP cells were assessed by wound-healing migration assays. ( *P < 0.05, **P < 0.01, ***P < 0.001 )
|       | b Vector | b shRNA | c Vector | c GABARAP |
|-------|----------|---------|----------|-----------|
| GABARAP | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| N-cadherin | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
| Vimentin | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) |
| E-cadherin | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| MMP-14 | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) |
| MMP-2 | ![Image](image16.png) | ![Image](image17.png) | ![Image](image18.png) |
| AKT | ![Image](image19.png) | ![Image](image20.png) | ![Image](image21.png) |
| p-AKT | ![Image](image22.png) | ![Image](image23.png) | ![Image](image24.png) |
| mTOR | ![Image](image25.png) | ![Image](image26.png) | ![Image](image27.png) |
| p-mTOR | ![Image](image28.png) | ![Image](image29.png) | ![Image](image30.png) |
| p70s6k | ![Image](image31.png) | ![Image](image32.png) | ![Image](image33.png) |
| p-p70s6k | ![Image](image34.png) | ![Image](image35.png) | ![Image](image36.png) |
| β-actin | ![Image](image37.png) | ![Image](image38.png) | ![Image](image39.png) |
Low GABARAP level promotes cellular EMT via AKT/mTOR signaling in breast cancer. a. Pearson correlation was calculated among genes related to GABARAP, CDH1, MMP2, MMP14 and Akt/ mTOR signaling pathway in breast cancer patients clinical cohort (TCGA). b. Western blot analyses were used to detect the expression levels of E-cadherin, N- cadherin, Vimentin, MMP2, MMP14 p-AKT, AKT, P-mTOR, mTOR, p-p70s6k and p70s6k in T47D-vector, T47D-shRNA, 812-vector, 812- shRNA cells. c. Western blot analyses were used to detect the expression levels of E-cadherin, N- cadherin, Vimentin, MMP2, MMP14 p-AKT, AKT, P-mTOR, mTOR, p-p70s6k and p70s6k in 453-vector, 453-GABARAP cells.
LY-294002 (AKT pathway inhibitor) reverses GABARAP-inhibited proliferation, invasion,
migration and EMT. a. Colony-forming efficiency were performed in T47D-Vector, T47D-shRNA, T47D-shRNA cells incubated with LY-294002, 812-vector, 812-shRNA, 812-shRNA cells incubated with LY-294002. b. Invasion assays were performed in the indicated cells. c. Migration assays were performed in the indicated cells. d. Western blot analyses were used to detect the expression levels of E-cadherin, N-cadherin, Vimentin, MMP2 and MMP14 in the indicated cells. e. Western blot analyses were used to detect the expression levels of p-AKT, AKT, P-mTOR, mTOR, p-pI3k and pI3k in the indicated cells.

Figure 6
GABARAP suppresses breast cancer progression in vivo. a. Representative images of nude mice and tumors at day 28 after inoculation of UACC-812 cells with or without shRNA mediated silencing of GABARAP. Vector control group, mice inoculated with control UACC-812 cells; GABARAP-shRNA group, mice inoculated with GABARAP silenced UACC-812 cells; Vector control + LY294002 group, mice inoculated with control UACC-812 cells and treated
with LY294002; GABARAP -shRNA + LY294002 group, mice inoculated with GABARAP silenced UACC-812 cells and treated with LY294002. b. Tumor growth curves in 4 groups of nude mice. N=5 for 1), 2) and 3) groups, N=4 for 4)group. c. The tumor weights were measured, and the data are presented as the means±SDs. d. Immunostaining of proteins in tumors from the vector control group and GABARAP-shRNA group. First column, H&E staining; second column, immunostaining for GABARAP ; third column, immunostaining for E-cadherin; fourth column, immunostaining for MMP2; fifth column, immunostaining for p-mTOR. Magnification, 400×. (*p < 0.05; **p < 0.01; ***p < 0.001).
Correlation between MMPs and GABARAP expression in breast cancer tissues from patients.

a. Representative immunostaining profiles of MMP2 in GABARAP low expression and GABARAP high expression breast cancer tissues. Magnification, 400×. b. Correlation analysis of the expression of MMP2 and GABARAP using the pearson correlation coefficient. GABARAP negatively correlated with MMP2 at the protein level. c. Representative immunostaining profiles of MMP14 in GABARAP low expression and GABARAP high expression breast cancer tissues. Magnification, 400×. d. Correlation analysis of the expression of MMP14 and GABARAP using the pearson correlation coefficient. GABARAP negatively correlated with MMP14 at the protein level.

Supplementary Files
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