FAM49B Promotes Breast Cancer Proliferation, Metastasis , and Chemoresistance by Stabilizing ELAVL1 Protein and Regulating Downstream Rab10/TLR4 Pathway

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Primary research

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Abstract

Background: Breast cancer (BC) is one of the most common cancers and the leading cause of death in women. Analysis of online databases revealed that FAM49B is highly expressed in BC and is associated with reduced disease-free survival (DFS) and overall survival (OS). However, the role of FAM49B in BC has not been elucidated yet. Therefore, in this study, we aimed to systematically study the role of FAM49B in the proliferation, metastasis, and chemoresistance of BC, as well as the corresponding mechanisms, and to provide new predictors and targets for BC treatment.

Methods: We used western blotting and immunohistochemistry to quantify FAM49B expression levels in human BC samples. A combination of shRNA, co-immunoprecipitation, MTT, migration/invasion, and apoptosis assays, in addition to microarray detection and data analysis and a nude mouse xenograft tumor model, was used for further mechanistic studies.

Results: In BC, the results showed that the expression level of FAM49B was significantly higher than that in normal breast tissue, and highly expression of FAM49B was significantly positively correlated with tumor volume, histological grade, lymph node metastasis rate, and poor prognosis. Knockdown or overexpression of FAM49B inhibited or promoted, respectively, the proliferation and migration of BC cells *in vitro* and *in vivo*. Microarray analysis revealed that the Toll-like receptor signaling pathway was inhibited upon FAM49B knockdown. In addition, the gene interaction network and downstream protein validation of FAM49B revealed that FAM49B positively regulates BC cell proliferation and migration by promoting the Rab10/TLR4 pathway. Furthermore, endogenous FAM49B interacted with ELAVL1 and positively regulated Rab10 and TLR4 expression by stabilizing ELAVL1. Moreover, mechanistic studies indicated that the lack of FAM49B expression in BC cells conferred more sensitivity to anthracycline and increased cell apoptosis by downregulating the ELAVL1/Rab10/TLR4/NF- κ B signaling pathway.

Conclusion: FAM49B may be a novel prognostic marker and therapeutic target for BC.

Background

Breast cancer (BC) is currently the most common cancer among women, with approximately 522,000 deaths worldwide in 2012 [1]. Comprehensive treatment can significantly improve the prognosis of patients with early-stage BC. However, due to the biological complexity of BC, it is difficult to fully evaluate the treatment response, metastasis pattern, and clinical outcomes using the most common clinicopathological markers [estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and Ki-67] [2]. Therefore, identifying new and effective biomarkers could improve BC clinical behavior and provide new therapeutic strategies.

The family with sequence similarity 49 member B (FAM49B) gene is a member of FAM49 in vertebrates. FAM49A and FAM49C are the other two members of the FAM49 gene family. They all have a conservative domain of DUF 1394. FAM49A and FAM49B only exist in the human genome, whereas FAM49C only exists in fishes, amphibians, reptiles, birds, and some lower mammals [3]. Currently, there are few reported

studies on the FAM49B gene in tumors. Some studies suggest that FAM49B is a regulator of actin kinetics, and T cell activation is related [4], which participates in mitochondrial function and inhibits proliferation and metastasis of pancreatic cancer [5]. In addition, FAM49B is a downstream target of ZFR and a potential tumor suppressor in colorectal and liver cancers [6]. However, a recent study revealed that FAM49B promotes gallbladder carcinoma cell proliferation and migration. Inhibition of PI3K/AKT pathway via FAM49B expression abrogated Myc-TASP1/Lv-shTASP1-induced gallbladder carcinoma cell proliferation and motility [7]. The above studies suggest that FAM49B may have various important tissue-specific functions, and its deregulation could promote or inhibit the emergence and evolution of some diseases, including cancer. However, the mechanism by which FAM49B plays its role in the genesis, development, and outcome of BC remains unclear. We analyzed differentially expressed genes in cancer and adjacent tissues using the ONCOMINE database, which contains data from clinical BC samples. This indicated that the expression of FAM49B was significantly higher than that in normal breast tissue in many BC samples. Therefore, in this study, we aimed to further analyze the expression pattern of FAM49B in BC, investigate its biological functions, and correlate these findings with BC patient prognosis.

Methods

Human specimens

We enrolled 180 patients with invasive BC who underwent a breast surgery at the Affiliated Hospital of the Hebei University of Engineering between January 2012 and December 2014; moreover, eight more patients were enrolled between January and February 2018. The selection criteria were as described previously [8, 9]. Written informed consent was obtained from all participants, based on the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the Affiliated Hospital of Hebei University of Engineering.

Human BC cell lines

MCF-7, MDA-MB-231, MDA-MB-361, HCC1937, and SKBR-3 cell lines were obtained from the American Type Culture Collection (USA). The cancer cell lines were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS) and incubated at 37°C in an atmosphere containing 5% CO₂. Recombinant retroviruses expressing either pLNCX2-vector or pLNCX2 with the FAM49B gene were generated according to the manufacturer's instructions (Clontech). MDA-MB-231 or MCF-7 cells were infected with these retroviruses using polybrene (8 µg/mL; Sigma-Aldrich), and expressing cells were then selectively isolated with G418 (750 µg/mL; Calbiochem).

Immunohistochemistry (IHC)

Immunohistochemical staining was performed as previously described using antibodies against FAM49B, ER, PR, and HER2. A dual scoring system was used for staining range and intensity for immunohistochemical analysis. Based on the previous [8, 9] criteria, semi-quantitative evaluation was

conducted on the expression of FAM49B, ER, and HER2. Two independent pathologists performed the analysis separately.

RNA interference

Recombinant lentiviruses encoding short-hairpin RNAs (shRNAs) specific for human FAM49B, Rab10, and ELAVL1 were designed and prepared by GeneChem (Shanghai, China). The FAM49B target sequence (FAM49B-shRNA#1) used here was 5'-GCAGGCTCTTGCTAAACAGTT-3'; FAM49B-shRNA#2 used was 5'-GCAGCTAATTATGCATTGCAT-3'; FAM49B-shRNA#3 used was 5'-ATCCTGCCATACAGAATGATT-3'; Rab10-shRNA used was 5'-GCCTTCAATACTACCTTTATT-3'; ELAVL1-shRNA used was 5'-GACGATCAAATTCGTTCTCTT-3'; scrambled (scr)-shRNA was used as a negative control, and the target sequence was 5'-TTCTCCGAACGTGTCACGTTT-3'. Lentivirus was added to the cells according to the manufacturer's recommended protocol. Quantitative real-time polymerase chain reaction (PCR) and western blotting were used to quantify the FAM49B gene knockout rate. Stable cell lines were defined as those with a knockout rate > 80%.

RNA extraction and quantitative real-time PCR

TRIzol (Invitrogen) was used to extract total RNA and reverse transcribed according to the manufacturer's instructions (Invitrogen). The expression level of FAM49B was detected by real-time fluorescence quantitative PCR. The primers used were as follows. FAM49B: forward, GGCAACTCCAATGCTGAAA; reverse, CACCCACCATTACCCTCAA. GAPDH (used as a control): forward, TGACTTCAACAGCGACACCCA; reverse, CACCCTGTTGCTGTAGCCAAA.

Co-immunoprecipitation and immunoblotting

The collected cells were lysed with lysis buffer containing 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton x-100, and protease inhibitors. Total protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce). Cell extracts (2–3 mg) were combined with rabbit anti-FAM49B antibodies for 2 h at 4°C followed by a second incubation with protein G Plus-agarose for 2 h and subjected to 10% SDS-PAGE followed by transfer to PVDF membranes for immunoblotting. Membranes were blocked in 5% milk/TBS-Tween for 1 h at room temperature. The membranes were incubated overnight with the primary antibody (FAM49B, 1:300, BIOS; Rab10, 1:1000, Abcam; TLR4, 1:1000, Abcam; ELAVL1, 1:500, Abcam) at 4°C, followed by incubation with the secondary antibody for 2 h at room temperature. Proteins were visualized using enhanced chemiluminescence (Amersham), and the results were quantified using ImageJ (NIH).

MTT assay

Approximately 1.5×10^4 cells/mL were seeded in a 96-well plate for 2–5 days, and the cell proliferation rate was determined by the MTT assay. At each time point, 10 mol/L MTT (5 mg/mL) was added to each

well, and the incubation time was 4 h. Thereafter, 100 mol/L DMSO was added to each well, and the absorbance was measured at 490 nm using an enzyme marker. Each set uses three duplicate holes.

Wound healing assay

The experiment was performed as recommended by the manufacturer, using 2.0×10^5 cells per well. Wound field images were obtained at 0, 8, and 24 h after removal of the insertion. Mobility was calculated as the migration distance/initial width of the wound.

Transwell assay

Serum-free cell suspensions were prepared and counted at a density of 5×10^4 cells/well (24-well plate). The upper culture medium was carefully removed, and 100 μ l cell suspension was added. In the lower cavity, 600 μ l of 30% FBS medium was added. The mixture was incubated at 37°C for 24 h. The cells were fixed with 4% paraformaldehyde for 30 min. Then, 1–2 drops of stain were added and the cells were transferred to the submembrane surface for 1–3 min. Photographs were taken under a microscope.

Tumor growth and metastasis in nude mice

Female BALB/c nude mice (4–6 weeks old) were purchased from Shanghai Lingchang Biological Technology Ltd. (Shanghai, China). MDA-MB-231 cells (1×10^7) transfected with FAM49B-shRNA or scr-shRNA were implanted subcutaneously into the flanks of BALB/c female nude mice according to previously described criteria [8, 10].

For the *in vivo* metastasis experiment, shFAM49B- or scr-shRNA-expressing MDA-MB-231 cells were injected into the caudal vein of nude mice under anesthesia by inhalation of a 1:1 mixture of isoflurane gas and oxygen. The mice were sacrificed 10 weeks later, and their metastatic lung nodules were quantified.

For the *in vivo* chemoresistance experiment, shFAM49B- MDA-MB-231 cells were injected into the flanks of nude mice (10 mice/group). After two weeks, each group was divided randomly into two subgroups that were either left untreated or received intraperitoneal injections of doxorubicin (4 mg kg^{-1}) every 5 days (three cycles), as previously described by Ghebeh et al. [11]. Animal handling and research protocols were approved by the Ethics Committee of the Affiliated Hospital of Hebei Engineering University.

Cell apoptosis analysis

After washing with PBS, the cells were stained with annexin V and propidium iodide (PI) using the annexin V-PI detection kit (Roche, Mannheim, Germany). Apoptosis was measured using flow cytometry (BD Biosciences, San Jose, CA, USA).

Microarray detection and data analysis

Total RNA was extracted from MDA-MB-231 cells treated with the TRIzol reagent. MDA-MB-231 cells were treated with lentiviral vectors of SCR-shRNA or Fam49B-shRNA. RNA was evaluated using NanoDrop 2000 and Agilent 2100. Subsequent experiments were conducted using these samples. Both cDNA strands were synthesized via reverse transcription. Next, labeled cRNA was synthesized by *in vitro* transcription using the GeneChip 3IVT Expression Kit. GeneChip Hybridization Wash and Stain Kit was used for hybridization, washing, and staining. GeneChip Scanner 3000 was used to scan the arrays for evaluation of data. Gene expression profiling was performed using the Affymetrix Human Gene 1.0 ST platform. The criteria used to determine the genes differentially expressed between MDA-MB-231 cells infected with FAM49B-shRNAs and scr-shRNAs were $p < 0.05$ and $|FC| > 1.5$. The Innovative Pathway Analysis (IPA) database was used for pathway analysis, disease analysis, functional analysis, network analysis, and downstream gene analysis.

ONCOMINE analysis

The mRNA levels of FAM49B in BC samples (and normal control samples) were determined using the ONCOMINE database (www.oncomine.org), a publicly accessible online cancer microarray database designed to facilitate discovery from genome-wide expression analyses. In this study, Student's *t*-test was used to generate a p-value for comparing cancer specimens and normal control datasets. The fold change was defined as 2, and the p-value was set at 0.01.

Kaplan–Meier plotter survival analysis

Prognostic values of FAM49B expression in BC samples were assessed by displaying the disease-free survival (DFS) and overall survival (OS) using the Kaplan–Meier plotter (<http://kmplot.com/analysis/>) [12]. Kaplan–Meier survival curve, log-rank p-value, and hazard ratio (HR) with a 95% confidence interval (CI) were calculated and plotted in R using Bioconductor packages.

Statistical analysis

Student's *t*-test was used to analyze the real-time PCR, western blotting, MTT, and migration assays. χ^2 analysis was used to assess whether and to what extent FAM49B expression was correlated with tumor size, histological grade, and lymph node metastasis rate. Statistical significance of differences was considered to be at a p-value less than 0.05.

Results

FAM49B mRNA levels are elevated in BC and correlate with poor prognosis

FAM49B expression has also been observed in several human cancers (Fig. 1A). ONCOMINE analysis revealed that FAM49B mRNA expression was significantly higher in BC samples than in normal samples across a wide variety of datasets covering different types of BC. FAM49B transcripts were elevated 1.664-fold in BC samples compared to normal tissues, in a dataset containing 450 samples derived from The

Cancer Genome Atlas (TCGA) database (Fig. 1B). The pooled results of the eight clinical cohorts showed a significant increase in FAM49B expression in BC ($p = 0.003$, Fig. 1C). Thereafter, we assessed the prognostic effect of FAM49B in BC using the Kaplan–Meier survival analysis plot. This revealed that high FAM49B mRNA expression was correlated with reduced DFS and OS in all BC patients (HR = 1.29, $p = 0.016$; HR = 1.26, $p = 0.034$, respectively, Fig. 1D, E).

FAM49B protein expression is elevated in BC tissues and correlates with poor prognosis

The expression of FAM49B protein in eight BC tissues and eight adjacent non-tumor tissues was assessed using western blotting. As shown in Fig. 2A, FAM49B protein expression levels were significantly higher in BC tissues than in normal breast tissues ($p < 0.01$).

Next, we performed IHC to visualize FAM49B protein expression in BC tissues. This revealed that FAM49B staining occurred mainly in the cytoplasm of BC samples. ER staining was observed in the nucleus of BC cells, whereas HER2 staining was localized in the cellular membrane. Examples of positive protein expression of FAM49B, ER, and HER2 are shown in Fig. 2B–D. IHC analysis showed that the positive rate of FAM49B expression in cancer tissue samples was 52.8% (95/180 cases). The positive rate was significantly higher than that of the adjacent normal breast tissue (28.9%; 26/90 samples) ($p = 0.000$, Table 1). In addition, positive FAM49B protein expression was positively correlated with tumor size, histological grade, and lymph node metastasis of BC ($p = 0.006$, $p = 0.013$, and $p = 0.023$, respectively, Table 1).

Table 1
The relationship between FAM49B expression and the
clinicopathological factors (n = 180)

Variable	n	FAM49B ⁻	FAM49B ⁺	<i>p</i> variable
Tissue				0.000
Cancer tissue	180	85	95	
Adjacent tissue	90	64	26	
Age				0.927
≥ 40	152	72	80	
< 40	28	13	15	
Tumor size				0.006
T1	38	25	13	
T2	112	52	60	
T3	30	8	22	
Histological grades				0.013
I	20	12	8	
II	65	38	27	
III	95	35	60	
Lymph node metastasis				0.023
Negative	67	39	28	
Positive	113	46	67	
“+”, positive; “-”, negative.				

Correlation analysis showed that the FAM49B-positive expression rate was significantly higher in ER-negative (ER-) cases than in ER-positive (ER+) cases ($p = 0.007$, Table 2). Conversely, FAM49B-positivity was significantly higher in HER2 + cases than in HER2- cases ($p = 0.011$, Table 2). However, there was no significant difference in FAM49B expression with regard to PR positivity ($p = 0.071$, Table 2).

Table 2
Correlations between FAM49B expression and immunohistochemical markers

Variable	n	FAM49B ⁻	FAM49B ⁺	<i>p</i> variable
ER				0.007
-	58	19	39	
+	122	66	56	
PR				0.071
-	64	29	45	
+	116	56	50	
HER2				0.011
-	137	72	65	
+	43	13	30	
“+”, positive; “-”, negative. ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor				

Furthermore, our patient follow-up analysis showed that 45 of 180 patients died, and the 6-year overall survival rate was 85.0%. FAM49B expression was positive in the BC samples of 29 out of the 45 patients that died, while only 16 cases of death occurred in the group with negative FAM49B expression. Kaplan–Meier analysis showed that compared with FAM49B-negative BC patients, the survival rate of FAM49B-positive BC patients was significantly reduced (log-rank test, $p < 0.05$, HR = 1.874, 95% CI = 1.045–3.362, Fig. 2E).

FAM49B promotes the proliferation and migration of BC cells *in vitro*

First, FAM49B mRNA expression levels in BC cell lines were evaluated using real-time PCR, which showed that FAM49B was highly expressed in the five BC cell lines (Fig. 3A). MDA-MB-231 and MCF-7 cell lines were selected for subsequent knockdown or overexpression studies.

We used shRNA to knock out FAM49B in MCF-7 and MDA-MB-231 BC cells and confirmed that the infection efficiency of FAM49B-shRNA and scr-shRNA exceeded 80% at 3 days after infection (Fig. 3B). The results of western blotting and real-time PCR showed that the levels of FAM49B protein and mRNA in FAM49B knockout cells were lower than those in scr-shRNA control cells ($p < 0.01$, Fig. 3C). FAM49B-shRNA#1 was selected for subsequent analyses. The MTT analysis method was used to analyze the proliferation rate of MCF-7 and MDA-MB-231 cells. MCF-7 and MDA-MB-231 cells were infected with FAM49B-shRNA or scr-shRNA. Within 5 days of FAM49B downregulation, the number of cells decreased and the cell proliferation rate was significantly reduced, as assessed by MTT analysis ($p < 0.01$, Fig. 3D).

However, re-expression of FAM49B in the two FAM49B-shRNA BC cell lines completely restored cell proliferation ($p < 0.01$, Fig. 3D).

Furthermore, wound healing and transwell assays were performed to evaluate the effect of FAM49B on BC cell migration. MDA-MB-231 cells with downregulated FAM49B migrated much slower than shRNA control cells, indicating that the inhibitory effect of FAM49B effectively inhibited cell migration ($p < 0.01$, Fig. 3E, F). However, reducing FAM49B back to the FAM49B-shRNA BC cell line completely restored cell migration ($p < 0.01$, Fig. 3E, F). Therefore, FAM49B plays a significant role in cancer growth and metastasis *in vitro*. FAM49B knockdown inhibits tumor growth and metastasis *in vivo*.

We investigated whether FAM49B could regulate the growth capacity of BC cells *in vivo*. MDA-MB-231 cells expressing scr-shRNA or FAM49B-shRNA were implanted into nude mice ($n = 10$), and tumor progression was monitored for 7 weeks, after which the mice were sacrificed. Compared with the scr-shRNA control group, the volume of MDA-MB-231 tumors expressing FAM49B-shRNA was significantly reduced ($p < 0.05$, Fig. 4A, B). At the end of the observation period, the tumors of FAM49B-shRNA MDA-MB-231 were significantly reduced in weight compared with the control group ($p < 0.05$, Fig. 4C).

To test whether FAM49B regulates metastatic potential *in vivo*, this study quantified lung metastatic nodules following injection of MDA-MB-231-shFAM49B and their corresponding control cells into the caudal vein of nude mice. Compared with control mice, the lung metastasis nodules of mice injected with MDA-MB-231-shFAM49B cells were significantly reduced ($p < 0.05$, Fig. 4D, E). These results indicate that FAM49B also plays a role in cancer growth and metastasis *in vivo*.

FAM49B regulates expression of BC genes

To clarify the mechanism by which FAM49B plays a role in BC, we performed a genome-wide expression microarray on MDA-MB-231 cells expressing scr-shRNA or FAM49B-shRNA. Consequently, we detected 1063 genes that showed differential expression ($|\text{fold change}| \geq 1.5$ and $p < 0.05$), including 393 upregulated genes and 670 downregulated genes (Fig. 5A). Using on the IPA database, FAM49B knockdown was found to affect the expression of related genes, such as cancer, cell movement, and cell death and survival (Fig. 5B). Knockdown of FAM49B significantly inhibited tumor cell migration and invasion of tumor cells (Fig. 5C). In addition, FAM49B knockdown had a significant inhibitory effect on several key cancer pathways, such as TWEAK, PPAR, and Toll-like receptor signaling pathways (Fig. 5D), indicating that FAM49B can regulate the malignant phenotype of BC.

FAM49B promoted BC cell proliferation and migration by upregulating Rab10/TLR4 pathway

According to the IPA database, the expression of Rab10 and Toll-like receptor 4 (TLR4) mRNA was inhibited when FAM49B was silenced in Toll-like receptor signaling and TLR4 may be the downstream target of Rab10 (Fig. 5A, E). Rab10 can accelerate the transport of TLR4 to the plasma membrane. Rab10 knockout reduced the expression of membrane TLR4 and reduced the production of inflammatory factors induced by LPS [13]. To further study the regulatory mechanism between FAM49B, Rab10, and TLR4 in

BC, FAM49B knockdown in the BC cell lines MCF-7 and MDA-MB-231 was performed using shRNA, and we found that FAM49B knockdown significantly inhibited the protein expression of Rab10 and TLR4 in BC cell lines. However, re-expression of FAM49B back into the two FAM49B-shRNA BC cell lines completely restored Rab10 and TLR4 expression ($p < 0.01$, Fig. 6A), indicating that FAM49B positively regulates Rab10 and TLR4 expression in BC cells.

To identify whether Rab10 is a key factor in this pathway, endogenous Rab10 was silenced in FAM49B-transfected MCF-7 and MDA-MB-231 cells. BC cells transfected with a non-functional vector were used as controls. It was found that Rab10 could inhibit protein expression of TLR4 in the control group and FAM49B upregulated protein expression of TLR4 in the FAM49B overexpression group ($p < 0.05$, Fig. 6B). However, TLR4 expression was significantly decreased by silencing Rab10 in the FAM49B overexpression group ($p < 0.05$, Fig. 6B). These results suggest that Rab10 positively regulates TLR4 expression in BC cells and is required in the FAM49B/TLR4 pathway.

To verify whether FAM49B promotes the proliferation and migration of BC cells through Rab10 regulation, Rab10 was knocked down in FAM49B-transfected MDA-MB-231 and MCF-7 cells. We found that proliferation and migration were promoted in FAM49B overexpressing cells, as assessed by MTT assay, wound healing assay, and transwell assay ($p < 0.01$, Fig. 7A–C). However, the promotion of proliferation and migration mediated by FAM49B overexpression was reversed by Rab10 knockdown ($p < 0.01$, Fig. 7A–C). These observations demonstrate that Rab10 is required for the FAM49B pathway-mediated migration and proliferation of BC cells.

FAM49B positively regulates Rab10/TLR4 pathway by stabilizing ELAVL1 protein

According to the IPA database, ELAVL1 may be the downstream target of FAM49B and plays a central role in regulating the Rab10/TLR4 pathway (Fig. 5E). ELAV-like RNA binding protein 1 (ELAVL1) is a member of the ELAVL family of RNA-binding proteins that contain several RNA recognition motifs, and it selectively binds AU-rich elements (AREs) found in the 3' untranslated regions of mRNAs. It is highly expressed in many cancers and could be potentially useful in cancer diagnosis, prognosis, and therapy [14–19]. To identify target proteins downstream of FAM49B, the Pathway Commons Protein-Protein Interactions dataset (<http://amp.pharm.mssm.edu/Harmonizome/>) was used. This dataset identified ELAVL1 as a potential interactor of FAM49B [20]. Thereafter, we performed a co-immunoprecipitation assay and found that exogenous FAM49B interacted with ELAVL1 in 293 cells (Fig. 8A). Moreover, FAM49B knockdown reduced protein expression of ELAVL1 in MCF-7 and MDA-MB-231 BC cells ($p < 0.01$, Fig. 8B). Co-transfection of FAM49B into FAM49B-shRNA BC cells completely restored ELAVL1 expression ($p < 0.01$, Fig. 8B). In addition, FAM49B knockdown did not alter ELAVL1 mRNA expression (Fig. 8C). Therefore, FAM49B may regulate ELAVL1 protein expression at the posttranslational level. To test this hypothesis, we determined whether FAM49B maintained ELAVL1 stability by treating MDA-MB-231 cells with cycloheximide (CHX) to inhibit protein synthesis. The downregulation of FAM49B induced ELAVL1 degradation in MDA-MB-231 cells (Fig. 8D), suggesting that FAM49B stabilizes ELAVL1 in BC cells. Moreover, FAM49B knockdown promoted ELAVL1 ubiquitination (Fig. 8E).

To further understand the role of ELAVL1 in this pathway, endogenous ELAVL1 was silenced in FAM49B-transfected MCF-7 and MDA-MB-231 cells. BC cells transfected with a non-functional vector were used as controls. We found that ELAVL1 could inhibit protein expression of Rab10 and TLR4 in the control group and FAM49B upregulated protein expression of Rab10 and TLR4 in the FAM49B overexpression group ($p < 0.01$, Fig. 8F, G). However, Rab10 and TLR4 expression was significantly decreased by silencing ELAVL1 in the FAM49B overexpression group ($p < 0.01$, Fig. 8F, G). These results suggest that ELAVL1 positively regulates Rab10 and TLR4 expression in BC cells and is required in the FAM49B pathway.

FAM49B promotes anthracycline resistance to chemotherapy in triple-negative BC (TNBC) cells by targeting the ELAVL1/Rab10/TLR4/NF- κ B signaling pathway

ROC Plotter showed that the level of FAM49B mRNA in BC samples of anthracycline responders was significantly lower than that in BC samples of anthracycline non-responders ($p = 5.3e-07$, Fig. 9A) [21]. Furthermore, Kaplan–Meier survival analysis showed that high FAM49B mRNA expression was correlated with reduced RFS in BC patients who received chemotherapy (HR = 1.39, $p = 0.019$, Fig. 9B). These results suggest that high FAM49B expression may inhibit the chemosensitivity of BC. TNBC is generally malignant, and there are no effective targeted drugs. Therefore, chemotherapy is the main treatment method for TNBC [22]. To evaluate whether FAM49B can directly promote anthracycline resistance to chemotherapy in TNBC cells, MDA-MB-231-shFAM49B cells (or control MDA-MB-231 cells) were treated with doxorubicin. The levels of apoptosis were significantly higher in FAM49B-shRNA MDA-MB-231 cells than in control cells, following treatment with 200 ng/mL doxorubicin ($p < 0.01$, Fig. 9C). We used FAM49B-shRNA MDA-MB-231 cells (or control MDA-MB-231 cells) in a xenograft tumor model (Fig. 9D). The size of tumors formed by the control group cells was slightly reduced by doxorubicin treatment ($p > 0.05$, Fig. 9E), whereas the size of the tumors formed by FAM49B-shRNA cells was significantly reduced by doxorubicin treatment ($p < 0.05$, Fig. 9E). These results show that the expression of FAM49B is directly related to an increase in anthracycline resistance via inhibition of apoptosis.

Next, we determined whether FAM49B expression exerts anthracycline resistance in TNBC cells through ELAVL1 and the Rab10/TLR4 signaling pathway. After FAM49B knockout MDA-MB-231 cells were treated with doxorubicin, the protein levels of ELAVL1, Rab10, TLR4, phosphorylate-p65 (p-p65), XIAP, and survivin decreased in a dose-dependent manner (Fig. 9F). In addition, ELAVL1, Rab10, TLR4, p-p65, XIAP, and survivin protein levels were significantly lower in FAM49B-knockdown cells than in control cells following treatment with the corresponding doses of doxorubicin ($p < 0.01$, Fig. 9F). However, the protein levels of cleaved caspase 3 (c-caspase 3) and cleaved PARP1 (c-PARP1) were significantly higher in FAM49B-knockdown cells than in control cells following treatment with the corresponding doses of doxorubicin ($p < 0.05$, Fig. 9F). These results suggest that FAM49B may inhibit the apoptosis and pro-apoptotic protein activation in BC cells through the ELAVL1/Rab10/TLR4/NF- κ B signaling pathway, resulting in anthracycline resistance.

Discussion

To our knowledge, this is the first study to explore the role and mechanism of FAM49B in BC. ONCOMINE analysis revealed that FAM49B mRNA expression was significantly higher in BC samples than in normal samples, which corroborated with the observed elevation in its protein expression in BC samples, as assessed via western blotting and IHC. Many scholars believe that BC is a heterogeneous disease because of its clinical and pathological characteristics and differences in molecular characteristics observed in multiple BC subtypes [23]. The prognosis of luminal type BC is often better than that of other subtypes because HER2-positive and TNBC subtypes are prone to recurrence and metastasis after treatment [23–25]. Our results revealed that FAM49B expression was negatively correlated with ER expression and positively correlated with HER2 expression. These results revealed that FAM49B expression correlated with the molecular subtype of BC.

Few studies have revealed that FAM49B is a potential tumor suppressor in pancreatic cancer, colorectal cancer, and liver cancer [5, 6]. Another study revealed that FAM49B promotes gallbladder carcinoma cell proliferation and migration [7]. According to our results, high FAM49B mRNA expression is correlated with reduced OS and DFS in BC patients using Kaplan–Meier plotter survival analysis. Consistent with this, we found that the survival rate of patients positive for FAM49B expression was significantly lower than that of patients negative for FAM49B expression, in our BC samples. In addition, our results revealed that FAM49B can significantly promote the proliferation and migration of BC cells. These results suggest that FAM49B may play different roles in different cancer types; however, it has clear potential as a novel biomarker with pivotal prognostic value in BC.

TLR4 is a member of the Toll-like protein family, mainly located in the cell membrane and cytoplasm, and it was initially studied in immune cells [26]. TLR4 protein expression strongly correlates with the expression of pro-inflammatory mediators and correlates with a decreased survival rate in patients with BC [27]. In addition, TLR4 plays an important role in increasing the efficiency of conventional anticancer treatments. Moreover, TLR4 has also been shown to promote metastasis in non-small cell lung cancer, hepatocellular carcinoma, oral squamous cell carcinoma, BC, and colon cancer [28–32]. According to our results, FAM49B knockdown significantly repressed the Toll-like receptor signaling pathway. The expression of Rab10 and TLR4 mRNA was inhibited when FAM49B was silenced in Toll-like receptor signaling, and TLR4 may be the downstream target of Rab10.

Rab10 is a protein coding gene with GTP- and GDP-binding domains and belongs to the RAS superfamily of small GTPases [33, 34]. A recent study demonstrated that Rab10 was highly expressed in liver cancer tissue samples [35, 36]. Furthermore, inhibition of Rab10 represses osteosarcoma cell proliferation and metastasis [37]. A previous study demonstrated that Rab10 regulated the transport of TLR4, which was vital for innate immune responses [38]. Our results showed that FAM49B could positively regulate Rab10 and TLR4 expression, and Rab10 expression was necessary for FAM49B to regulate this pathway in BC cell lines. Rab10 has been studied as an oncogene in BC for the first time. However, based on the mechanism of regulation of Rab10 by FAM49B, ELAVL1 may be the downstream target of FAM49B and plays a central role in regulating Rab10, according to the IPA database.

The RNA-binding protein ELAVL1 is one of the best-studied regulators of cytoplasmic mRNA fate [39]. Mechanistically, ELAVL1 regulates mRNA cargos that typically contain AREs in the 3'-untranslated region, including numerous mRNAs involved in diverse biological processes of carcinogenesis [19, 40]. Elevated cytoplasmic ELAVL1 protein levels have been observed in many types of cancer [41]. The protein level of ELAVL1 was found to be modulated by the ubiquitin-proteasome pathway [42]. Our results revealed that endogenous FAM49B could interact with ELAVL1 in BC cells and stabilize ELAVL1 from being ubiquitinated. Furthermore, we found that ELAVL1 positively regulated the expression of Rab10 and TLR4 in BC cells. Therefore, we speculate that FAM49B binds to ELAVL1 and stabilizes it to prevent its degradation via ubiquitination. ELAVL1 then increases the expression of Rab10 by binding to Rab10 mRNA. Later, Rab10 enhances the transport of TLR4 to the cell membrane and activates downstream pathways, such as NF- κ B [43, 44] and Akt/GSK3 β / β -catenin pathways [45], and ultimately promotes the proliferation and metastasis of BC cells (Fig. 10).

Chemotherapy is currently an effective treatment for TNBC. St Gallen experts recommend anthracyclines and taxanes as the main adjuvant chemotherapy drugs for TNBC. However, more than 50% of TNBC patients are resistant to adjuvant chemotherapy [46]. This study demonstrated that FAM49B knockout could significantly increase doxorubicin-induced apoptosis of cancer cells and the sensitivity of the TNBC xenograft tumor model to doxorubicin. In BC cells, the increase in ELAVL1 in the cytoplasm is related to doxorubicin-induced apoptosis [47, 48]. Furthermore, chemotherapy may induce the expression of TLR4, leading to chemotherapy resistance. Therefore, inhibition of TLR4 with TAK-242 could reverse chemotherapy resistance [49, 50]. TLR4 activates the NF- κ B pathway [51, 52] and downstream target genes XIAP and survivin [53]. XIAP and survivin directly inhibit the activity of caspase 3, which plays an important role in cell apoptosis and activation of poly ADP ribose polymerase (PARP) [54]. The results showed that FAM49B knockout in TNBC cells resulted in decreased ELAVL1, Rab10, TLR4, p-p65, XIAP, and survivin levels and increased c-caspase 3 and c-PARP1 levels. FAM49B knockdown increased sensitivity to chemotherapy and increased cell apoptosis and apoptotic protein activation by downregulating the ELAVL1/Rab10/TLR4/NF- κ B signaling pathway in BC cells.

Conclusion

In summary, we are the first to systematically study the functional mechanism of FAM49B-mediated proliferation, metastasis, and chemoresistance in BC. This study showed that FAM49B can activate the proliferation and metastasis of BC cells via the ELAVL1/Rab10/TLR4 pathway; however, FAM49B increased resistance of BC to anthracycline, induced by the ELAVL1/Rab10/TLR4/NF- κ B pathway. Therefore, FAM49B may be a potential target for making BC treatment more effective.

Declarations

Availability of data and materials

Datasets supporting the conclusions of this article are included within the article.

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Author Contributions

WL conceived the study and provided the project direction. WL, YL, and YX guided and performed the experiments, analyzed the data, and wrote the manuscript until final submission. WL, YL, YX, WZ, JH, SS, JH, and NS completed the cell experiments. YL, YX, WZ, WW, RW, WLv, and YD assisted in performing the animal experiments.

Ethics approval and consent to participate

This study was approved by the Ethical Committee of Affiliated Hospital of Hebei University of Engineering. The written informed consents were obtained from all the patients.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Analysis of FAM49B mRNA expression and prognostic values in BC. (A) FAM49B mRNA expression (shown in red frame) in normal and BC tissues, as per ONCOMINE. (B) FAM49B mRNA expression, as per TCGA database. (C) Meta-analysis of gene expression profiling for FAM49B in BC using ONCOMINE, with $p < 0.05$ and fold change > 1.5 . The colored squares indicate the median rank for FAM49B across each

analysis comparing BC tissue with normal tissue. (D, E) High FAM49B mRNA levels were correlated with reductions in DFS (D) and OS (E).

Figure 2

High expression of FAM49B in human BC. (A) Expression status of FAM49B in matched normal (N) and cancerous (T) regions isolated from BC patients following western blotting. Expression levels of FAM49B, ER, and HER2, as per immunohistochemical staining (B–D). Blue arrow represents staining in the nucleus; black arrow represents staining in the cytoplasm; red arrow represents staining in the cytomembrane. (E)

Kaplan–Meier survival curves showing survival in 180 BC patients, with respect to FAM49B expression. Results are presented as the mean $\pm$ SD. The statistical significance was assessed by Student’s t-test; **p < 0.01.

Figure 3

Effects of FAM49B on proliferation and migration in vitro. (A) Levels of FAM49B mRNA were analyzed by real time-PCR in five BC cell lines (n = 3). (B) MCF-7 and MDA-MB-231 cells infected with FAM49B-shRNA

lentivirus were examined by fluorescence microscopy, 3 days after infection. (C) FAM49B mRNA and protein expression levels were examined by real time-PCR and western blotting in MCF-7 and MDA-MB-231 cells infected with FAM49B shRNA (n = 3). pLNCX2-FAM49B or pLNCX2-vector were transfected into FAM49B-shRNA-expressing MCF-7 or MDA-MB-231 cells. Cell growth rate (D) was monitored by MTT assay in MCF-7 and MDA-MB-231 cells; (E, F) Cell migration was examined by wound healing assay (E) and transwell assay (F) in MDA-MB-231 cells (n = 3). Results are presented as the mean $\pm$ SD. The statistical significance was assessed by Student's t-test; **p < 0.01.

Figure 4

Effects of FAM49B on proliferation and migration in vivo. (A–C) scr-shRNA- and FAM49B-shRNA- MDA-MB-231 cells were injected into the flanks of nude mice. (A) Tumor growth and (B) tumor volume were measured on the indicated days. (C) Tumor weights were measured after mice were sacrificed. (D, E) shFAM49B- MDA-MB-231 cells and control cells were injected into the caudal vein of nude mice. The mice were sacrificed, and their metastatic lung nodules were quantified. Results are presented as the mean $\pm$ SD. The statistical significance was assessed by Student's t-test; *p < 0.05.

Figure 5

Changes in gene expressions in MDA-MB-231 cells with FAM49B knockdown, analyzed using microarray. (A) Heat-map analysis showing 1063 genes that were detected as being altered, by microarray profiling. $p < 0.05$ and $|\text{fold Change}| \geq 1.5$. (B, C) Disease and function enrichment of whole-genome expression microarray in FAM49B knockdown MDA-MB-231 cells was performed using IPA. (D) Canonical pathway enrichment of whole-genome expression microarray in FAM49B knockdown MDA-MB-231 cells was

performed using IPA. (E) Gene interaction network of FAM49B and related genes in Toll-like receptor signaling pathway. The results show that Rab10, TLR4, and ELAVL1 (shown in red frame) were located in the central regulatory position. Data are shown as mean $\pm$ SD.

Figure 6

FAM49B positively regulates Rab10 and TLR4 expression. (A) pLNCX2-FAM49B or pLNCX2-vector were transfected into FAM49B-shRNA-expressing MCF-7 or MDA-MB-231 cells. FAM49B, Rab10, and TLR4

expression was quantified via western blotting (n = 3). (B) scr- or Rab10-shRNA were transfected into FAM49B-overexpressing MCF-7 and MDA-MB-231 cells. Rab10 and TLR4 expression was quantified via western blotting (n = 3). Results are presented as the mean ± SD. The statistical significance was assessed by Student's t-test; *p < 0.05, **p < 0.01.

Figure 7

FAM49B regulates proliferation and migration of BC cells by upregulating Rab10. scr- or Rab10-shRNA were transfected into FAM49B-overexpressing MCF-7 and MDA-MB-231 cells. Cell growth rate (A) was monitored by MTT assay in MCF-7 and MDA-MB-231 cells (n = 3). (B, C) Cell migration was examined via wound healing (B) and transwell assays (C) in MDA-MB-231 cells (n = 3). Results are presented as the mean ± SD. The statistical significance was assessed by Student's t-test; **p < 0.01.

Figure 8

FAM49B positively regulates Rab10/TLR4 pathway by stabilizing ELAVL1. (A) Exogenous interaction between FAM49B and ELAVL1 in 293 cells, observed via co-immunoprecipitation. (B) pLNCX2-FAM49B or pLNCX2-vector were transfected into FAM49B-shRNA-expressing MCF-7 or MDA-MB-231 cells. ELAVL1 expression was quantified via western blotting (n = 3). (C) Level of ELAVL1 mRNA was determined using quantitative real-time PCR in FAM49B-shRNA-expressing MCF-7 or MDA-MB-231 cells. (D) FAM49B mediated ELAVL1 stabilization in BC cells. scr- or FAM49B-shRNA-expressing MDA-MB-231 cells were

treated with CHX (50 μ M) for the indicated time points and analyzed for endogenous ELAVL1 expression via western blotting. (E) FAM49B inhibition increased ELAVL1 ubiquitination in MDA-MB-231 cells. HA-Ubiquitin was electroporated into scr- or FAM49B-shRNA-expressing MDA-MB-231 cells. Cells were treated with MG132 (20 μ M) for 2 h. ELAVL1 complex in resulting lysates was examined using an antibody against Ubiquitin (Ub). (F, G) scr- or ELAVL1-shRNA were transfected into FAM49B-overexpressing MCF-7 and MDA-MB-231 cells. ELAVL1, Rab10, and TLR4 expression was quantified via western blotting (n = 3). Results are presented as the mean $\pm$ SD. The statistical significance was assessed by Student's t-test; *p < 0.05, **p < 0.01.

Figure 9

FAM49B-knockdown BC cells are more sensitive to doxorubicin. (A) FAM49B mRNA expression was lower in anthracycline-sensitive BC samples, as per ROC Plotter analysis. (B) High FAM49B mRNA level was associated with decreased RFS in BC patients who had received chemotherapy. (C) The dot matrix of Annexin V/PI double staining was generated after scr- and FAM49B-shRNA MDA-MB-231 cells were treated or not treated with 200 ng/mL of doxorubicin for 24 h ($n = 3$). scr- and FAM49B-shRNA MDA-MB-

231 cells were injected into mice, as described previously. Tumor growth (D) and tumor volume (E) were measured on the indicated days. The arrows showed the time of doxorubicin injection. (F) FAM49B-shRNA or scr-shRNA were transfected into MDA-MB-231 cells treated with various doses of doxorubicin for 24 h. Levels of ELAVL1, Rab10, TLR4, p-p65, XIAP, survivin, c-caspase 3, and c-PARP1 in MDA-MB-231 cells were quantified via western blotting (n = 3). Results are presented as the mean $\pm$ SD. The statistical significance was assessed by Student's t-test; *p < 0.05, **p < 0.01.

Figure 10

The signaling pathway proposed in this study.