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ORIGINAL RESEARCH

Overexpression of *FAM234B* Predicts Poor Prognosis in Patients with Luminal Breast Cancer

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Background: Family with sequence similarity 234 member B (*FAM234B*), a protein-coding gene, is mainly expressed in brain tissues. Its clinical significance and biological function in tumors, especially in breast cancer (BC), have not been elucidated.

Methods: We firstly investigated the expression pattern of *FAM234B* at the mRNA and protein levels using Oncomine, TCGA portal, GEPIA, TIMER, HPA, and UALCAN databases, then applied bc-GenExMiner to assess the associations between expression level of *FAM234B* and clinicopathological features of BC. Besides, we also verified the expression of *FAM234B* expression in clinical BC samples using qRT-PCR. Subsequently, GEPIA, bc-GenExMiner, and TIMER databases were used to analyze the prognostic significance of *FAM234B* in all BC and different molecular subtypes. Finally, we conducted co-expression analysis and gene set enrichment analysis (GSEA). Additionally, we explored the regulatory mechanism of *FAM234B* in BC.

Results: Both bioinformatics analysis and experimental verification confirmed that the *FAM234B* expression was significantly higher at the mRNA and protein levels in luminal BC tissues than in adjacent normal tissues. High *FAM234B* expression was significantly correlated with older age, estrogen receptor-positive, progesterone receptor-positive, human epidermal growth factor receptor 2-negative, wild-type p53, low Nottingham prognostic index, low Scarff-Bloom-Richardson grade, lymph node metastasis positivity, and high tumor stage. Moreover, survival analysis indicated that high *FAM234B* expression was significantly related to a worse prognosis in patients with luminal BC. GSEA indicated that *FAM234B* was positively related to membrane transport process and negatively associated with immune response function. Besides, mechanism exploration indicated that pseudogene *HTR7P1* might act as endogenous RNA to compete with *has-miR-1271-5p* or *has-miR-381-3p* for binding to *FAM234B*, thereby upregulating the expression of *FAM234B* in luminal BC.

Conclusion: Our results suggest that *FAM234B* may be a candidate therapeutic target or prognostic marker for luminal breast cancer.

Keywords: *FAM234B*, luminal breast cancer, *HTR7P1*, prognosis

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Introduction

Breast cancer (BC) is one of the three most common cancers worldwide.[1] Among females, BC is the leading cause of cancer-related deaths in the vast majority of countries.[2,3] According to estrogen or progesterone receptor (ER or PR) expression and human epidermal growth factor receptor 2 (HER2) gene amplification, BC is classified into several molecular subtypes, including luminal, HER2-enriched, and basal-like.[4,5] Different molecular subtypes have different clinical outcomes and treatment regimens. The luminal subtype is the most common, accounting for

approximately 75% of all BC cases.[6] Although great advances in early detection, surgical procedures, adjuvant endocrine therapy, and chemotherapy have substantially improved clinical outcomes in luminal BC, relapses and resistance to endocrine therapy are still important issues, leading to a low overall survival (OS) of patients with luminal BC.[6,7] Since the disease remains a significant global health burden,[8,9] the identification of specific and sensitive molecular biomarkers that may serve as therapeutic targets or prognostic indicators for patients with luminal BC is an important research goal.

Family with sequence similarity 234 member B (*FAM234B*), also known as *KIAA1467*, is a protein-coding gene located on chromosome 12. *FAM234B* serves as integral component of the membrane and is expressed in many cell types and organs, especially in brain tissues.[10] Diseases associated with *FAM234B* include neurodevelopmental disorders and recurrent childhood high hyperdiploid acute lymphoblastic leukemia.[11,12] Nevertheless, the clinical significance and underlying roles of *FAM234B* in solid tumors are not well studied. Thus, we analyzed the expression profile, prognostic value, biological functions, and molecular mechanisms of *FAM234B* in BC through the combination of bioinformatics analysis and experimental verification.

Materials and Methods

Expression Analysis Using a Bioinformatics Approach

The *FAM234B* mRNA expression profile was evaluated in samples of 20 cancer types and matched non-tumor samples using Oncomine (https://www.oncomine.org).[13] *P*-values < 0.0001, fold change values of > 2.0, and genes ranking in the top 10% were set as thresholds. Then, *FAM234B* mRNA expression levels in BC were validated using data from TCGA portal (http://tumorsurvival.org/index.html),[14] GEPIA (http://gepia.cancer-pku.cn/),[15] TIMER (https://cistrome.shinyapps.io/timer/),[16] and bc-GenExMiner (http://bcgenex.centregauducheau.fr/BC-GEM/)[17] databases. Moreover, the protein expression of *FAM234B* in BC was explored using the Human Protein Atlas (HPA, https://www.proteinatlas.org/)[18] and CPTAC data from the UALCAN (http://ualcan.path.uab.edu/analysis.html)[19] database. In addition, mRNA expression level of *HTR7P1* in BC were evaluated using GEPIA.[15]

Associations Between *FAM234B* or *HTR7P1* Expression Levels and Clinicopathological Features of BC

The bc-GenExMiner (http://bcgenex.centregauducheau.fr/BC-GEM/)[17] tool was applied to analyze the relationships between *FAM234B* or *HTR7P1* mRNA expression levels and clinicopathological features of BC, including age, ER, PR, HER2, p53, Scarff-Bloom-Richardson (SBR) grades, Nottingham prognostic index (NPI), and molecular subtype. Additionally, we also analyzed the relationship between mRNA expression of *FAM234B* or *HTR7P1* and tumor stages by GEPIA.[15] The distribution of *FAM234B* or *HTR7P1* mRNA expression levels across molecular subtypes was evaluated using UCSC Xena (http://xena.ucsc.edu/)[20] and TCGA portal.[14] Finally, CPTAC data from UALCAN[19] was used to explore the protein expression of *FAM234B* in different subtypes.

Survival Analysis of *FAM234B* and *HTR7P1*

GEPIA,[15] TIMER,[16] and bc-GenExMiner[21] databases were used to evaluate the effects of *FAM234B* mRNA expression levels on survival of patients with all BC and different molecular subtypes. The prognostic values of *HTR7P1* in different subtypes were also evaluated using bc-GenExMiner[21] database.

Co-Expressed Genes of *FAM234B* and Gene Set Enrichment Analysis

LinkedOmics (http://www.linkedomics.org/)[22] was used for obtaining co-expression genes of *FAM234B* via the LinkFinder module and for performing gene set enrichment analysis (GSEA) via the LinkInterpreter module. A heat map of the top 50 positively or negatively correlated genes was generated. The correlation was evaluated by Spearman test.

Correlation Analysis of *FAM234B* with *HTR7P1*

Pearson's pairwise correlation analysis of *FAM234B* with *HTR7P1* in BC and different molecular subtypes was performed using bc-GenExMiner.[17] Then, TIMER[16] was used to verify the correlations based on Spearman correlation coefficients.

Prediction of Candidate MicroRNAs of *FAM234B* and *HTR7P1*

To better understand the molecular mechanism of *HTR7P1* regulating *FAM234B*, we first explored the interaction molecules of *HTR7P1* and *FAM234B* using RNAInter (http://www.rna-society.org/rnainter/home.html)[23] database. Then, the subcellular localization of *HTR7P1* was explored by its sequence extracted from the National Center for Biotechnology Information using IncLocator (http://www.csbio.sjtu.edu.cn/bioinf/lncLocator/).[24] We applied miRanda (http://www.microrna.org/)[25] and starBase (http://starbase.sysu.edu.cn/index.php)[26] to determine potential microRNAs (miRNAs) binding to *HTR7P1* 3′-UTR. Subsequently, potential binding miRNAs of *FAM234B* 3′-UTR were predicted using TargetScan Human7.2 (http://www.targetscan.org/),[27] starBase,[26] and miRWalk (http://mirwalk.umm.uni-heidelberg.de/).[28] Then, we analyzed the potential miRNAs using a Venn diagram. The expression level of the candidate miRNA in BC was detected using dbDEMC2 (https://www.picb.ac.cn/dbDEMC/).[29] The correlations of miRNAs with *HTR7P1* and *FAM234B* were analyzed using starBase.[26]

Gathering of Clinical Samples and Ethics Statement

A total of 120 patients suffering from primary BC were recruited from the Second Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China). All the patients were pathologically diagnosed, did not exhibit distant metastasis, and did not receive any other treatment before surgery. Samples were immersed in RNAlater reagent and further stored at −80°C. The clinicopathological parameters of patients were obtained from their medical records. The study was approved by the Ethics Committee of Xi'an Jiaotong University, and each patient gave an informed consent.

RNA Extraction and qRT-PCR Analysis

Total RNA was isolated from the tissue samples using Trizol reagent (Invitrogen, USA). Then, the RNA was reverse-transcribed into cDNA using PrimeScript TM RT reagent Kit (TAKARA, Japan) according to the manufacturer's protocol. The levels of *FAM234B* and *HTR7P1* were detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using the SYBR® Premix Ex Taq TM II kit (Takara, Japan) on ABI StepOne Real-Time PCR system. The primers for *FAM234B, HTR7P1*, and the internal control *GAPDH* are listed in Supplementary Table 1. The relative expression of *FAM234B* and *HTR7P1* was quantified using $2^{-\Delta Ct}$ values.

Statistical Analysis

The difference in the expression of *FAM234B* or *HTR7P1* between two groups for our clinical samples was examined by Mann–Whitney test. The association of *FAM234B* or *HTR7P1* expression with BC clinical characteristics was assessed by chi-square test. Spearman correlation coefficients were calculated to evaluate the association between *FAM234B* and *HTR7P1*. All the statistical analyses were conducted with IBM SPSS Statistics 18.0 software. P-value < 0.05 was considered statistically significant.

Results

High *FAM234B* Expression Indicated Poor Prognosis in Luminal BC Patients

The expression profile of *FAM234B* in human cancer was first determined using microarray data by ONCOMINE database (Supplementary Figure 1A). *FAM234B* mRNA expression levels were highest in BC tissues in the Bittner multi-cancer dataset (Supplementary Figure 1B). Thus, we next focused on the role of *FAM234B* in BC. In the Curtis BC dataset, the mRNA level of *FAM234B* in BC tissues was highest in mucinous breast carcinoma, lowest in medullary breast carcinoma, and obviously higher than that in normal breast tissues (Supplementary Figure 1C and D, $p < 0.001$). Interestingly, compared with basal-like BC tissues, the *FAM234B* mRNA expression was significantly upregulated in luminal-like BC in the Farmer Breast 4 dataset (Supplementary Figure 1E, $p < 0.001$). We further examined *FAM234B* mRNA levels using RNA-seq data from TCGA via TCGA portal, GEPIA, TIMER, and bc-GenExMiner databases. As demonstrated in Figures 1A–D and 2A–C, consistent with the results of the ONCOMINE analysis, the mRNA levels of *FAM234B* were higher in luminal BC tissues and lower in basal-like BC tissues than that in normal breast tissues ($p < 0.001$). For further validation, we also detected the expression of *FAM234B* in clinical BC samples. As shown in Figure 1E, *FAM234B* expression in BC tissues was markedly higher than that in normal tissues ($p = 0.027$). Moreover, compared with adjacent-normal tissues, the mRNA levels of *FAM234B* were obviously upregulated in luminal BC tissues (Figure 2D, $p = 0.0006$) and downregulated in triple-negative BC (TNBC) tissues (Figure 2D, $p = 0.0409$). Using combination bioinformatics analysis and experimental validation, we demonstrated that *FAM234B*

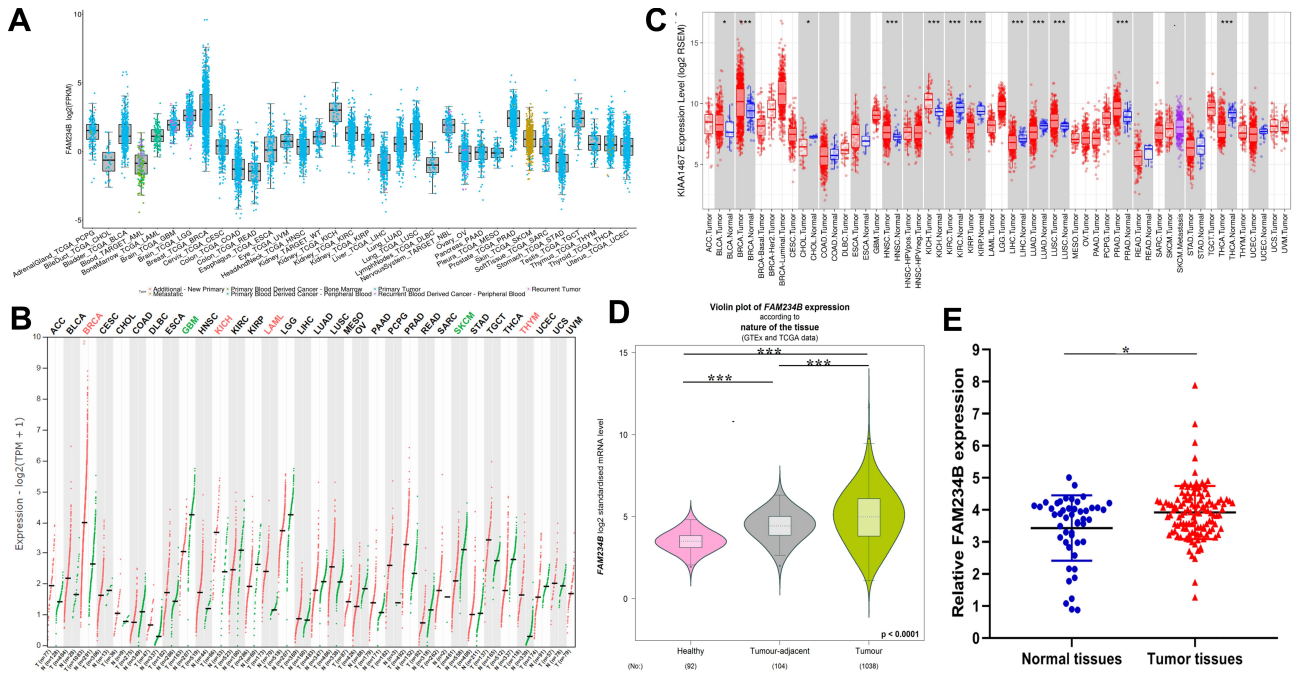

Figure 1 Overexpression of *FAM234B* mRNA level in breast cancer. (**A**) The mRNA expression of *FAM234B* was highest in breast cancer tissues (TCGA portal); (**B**) Transcriptional expression of *FAM234B* in breast cancer was higher than that in normal breast tissues (GEPIA); (**C**) The mRNA expression of *FAM234B* was upregulated in breast cancer tissues, especially in luminal subtype (TIMER); (**D**) The mRNA expression of *FAM234B* was higher in breast cancer tissues than that in healthy and tumor-adjacent breast tissues (bc-GenExMiner); (**E**) qRT-RCR results of *FAM234B* in BC tissues and normal breast tissues. Error bars represent SD. **P* < 0.05, ****P* < 0.001.

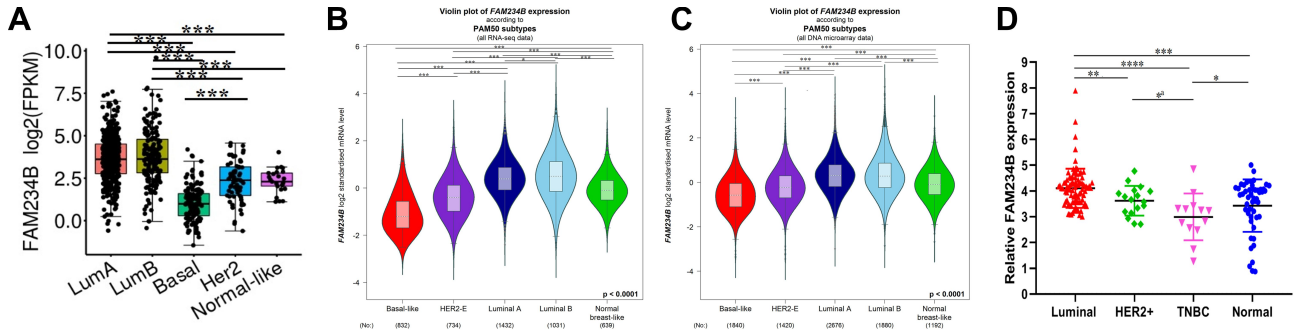

Figure 2 The distribution of *FAM234B* mRNA expression across molecular subtypes of breast cancer. (**A**–**C**) *FAM234B* mRNA level was highest in luminal subtypes of breast cancer (TCGA portal and bc-GenExMiner). (**D**) qRT-RCR results of *FAM234B* in different subtypes of BC samples and normal breast tissues. Error bars represent SD, [a]Represent unpaired *t*-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.
Abbreviation: TNBC, triple-negative breast cancer.

mRNA was significantly overexpressed in luminal BC. Next, we probed the protein expression pattern of *FAM234B* in BC using HPA and UALCAN. As depicted in Figure 3A, *FAM234B* was not expressed in normal breast tissues and was expressed at weak and moderate intensity in BC tissues, and located in cytoplasm. Similarly, a CPTAC analysis showed that the protein expression levels of *FAM234B* differed significantly between BC tissues and normal breast tissues (Figure 3B, $p < 0.001$). Moreover, CPTAC analysis also indicated that the protein expression of *FAM234B* was obviously higher in luminal BC than in TNBC (Figure 3C, $p = 0.015$). All evidence taken together, our results showed that *FAM234B* was upregulated at the mRNA and protein levels in luminal BC.

To better understand the clinical significance and function of *FAM234B* in BC, we assessed the relationship between *FAM234B* expression and clinical outcomes using GEPIA, TIMER, and bc-GenExMiner databases. First, the effect of *FAM234B* mRNA expression on survival in all BC patients was evaluated using GEPIA and TIMER databases. High *FAM234B* mRNA expression was significantly related to a worse OS (Figure 4A1 and B1, $p < 0.05$) in all BC patients.

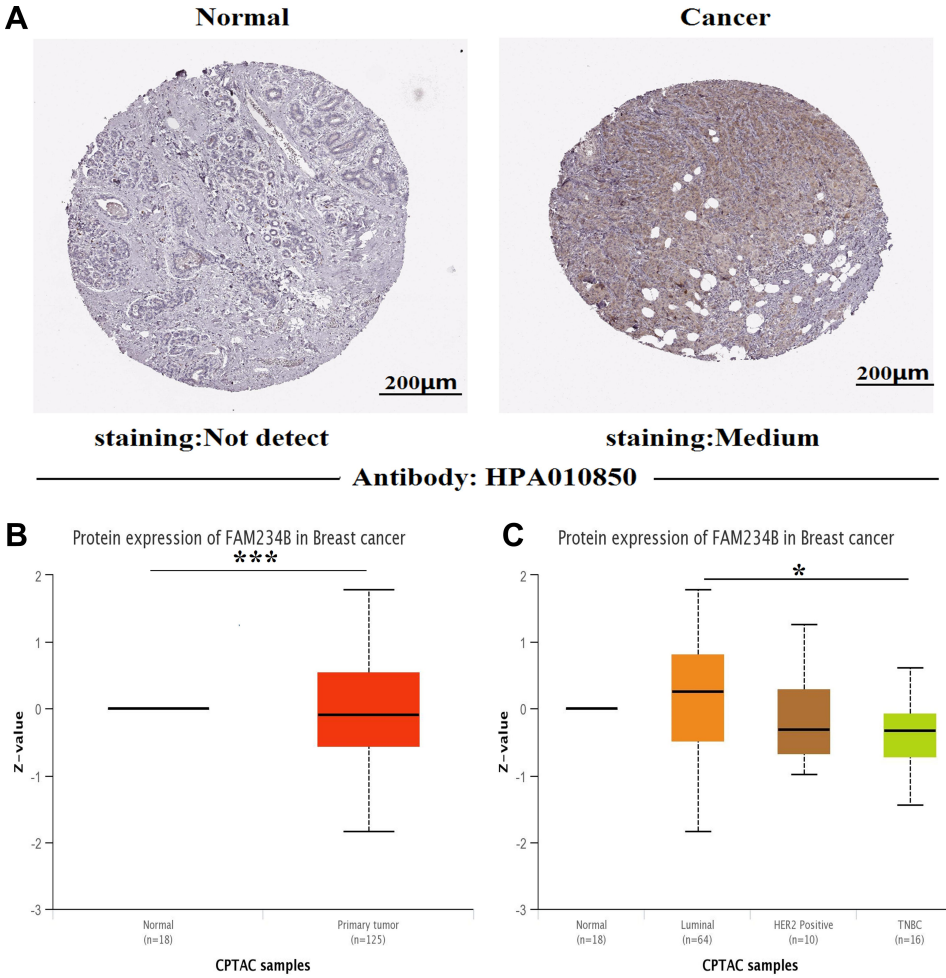

Figure 3 Upregulation of *FAM234B* protein level in breast cancer. (**A**) FAM234B protein was not expressed in normal breast tissues, whereas weak and moderate expressions were observed in breast cancer tissues (Human Protein Atlas). (**B, C**) CPTAC analysis showed FAM234B protein was significantly increased in breast cancer tissues, especially in luminal subtype (UALCAN). *$P < 0.05$, ***$P < 0.001$.
Abbreviation: TNBC, triple-negative breast cancer.

Subgroup analyses were subsequently performed to assess the prognostic value of *FAM234B* for different molecular subtypes. An analysis of TCGA data of GEPIA and TIMER revealed that increased *FAM234B* mRNA levels predicted worse survival in BC patients with luminal subtype (Figure 4A2 and B3, $p < 0.05$), but there was no significant correlation between *FAM234B* expression and prognosis in BC patients with HER2-enriched and basal-like BC (Figure 4A3, A4 and B2, B4). As determined using the bc-GenExMiner database, high *FAM234B* mRNA levels were obviously associated with a shorter OS in BC patients with luminal A and luminal B subtypes (Figure 4C1 and C2) but with a longer OS in BC patients with the HER2-enriched subtype (Figure 4C3). However, no significant correlation was found between *FAM234B* expression and prognosis in patients with basal-like BC (Figure 4C4).

Association of *FAM234B* Expression Levels with Clinicopathological Features of BC Patients

We further investigated the correlations between the expression level of *FAM234B* and BC clinicopathological parameters using bc-GenExMiner. *FAM234B* mRNA expression levels were positively related to age, with upregulated expression in the older group (Figure 5A and B, $p < 0.0001$). *FAM234B* mRNA expression levels were significantly higher in the ER-positive group and PR-positive group than in the corresponding negative groups (Figure 5C and D, $p < 0.0001$), and the opposite trend was observed for HER2 status (Figure 5E, $p < 0.0001$). Patients with BC harboring wild-type p53 displayed higher *FAM234B* levels than those of patients harboring mutant p53 (Figure 5F, $p < 0.0001$). Not surprisingly, compared

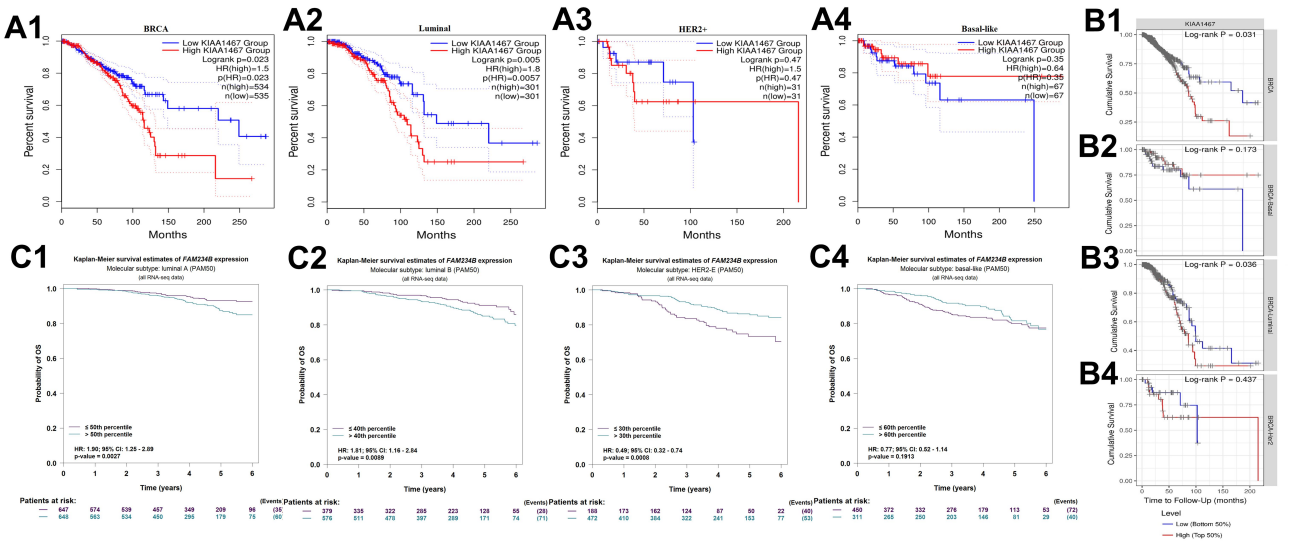

Figure 4 Prognostic significance of *FAM234B* mRNA expression in all BC patients and distinct molecular subtypes. (**A1, A2**) high expression of *FAM234B* predicted worse prognosis in all breast cancer and luminal subtypes, (**A3, A4**) *FAM234B* expression was not related to prognosis of breast cancer patients with HER2-enriched and basal-like subtypes (GEPIA); (**B1, B3**) high expression of *FAM234B* predicted worse prognosis in all breast cancer and luminal subtypes, (**B2, B4**) *FAM234B* expression was not related to prognosis of breast cancer patients with basal-like and HER2-enriched subtypes (TIMER); (**C1, C2**) high expression of *FAM34B* indicated poor prognosis of breast cancer patients with luminal A and B subtypes, (**C3**) high expression of *FAM34B* predicted better survival rate of breast cancer patients with HER2-enriched subtype, (**C4**) *FAM234B* expression showed no correlation with prognosis of breast cancer patients with basal-like subtype (bc-GenExMiner).
Abbreviations: HR, hazard ratio; CI, confidence interval.

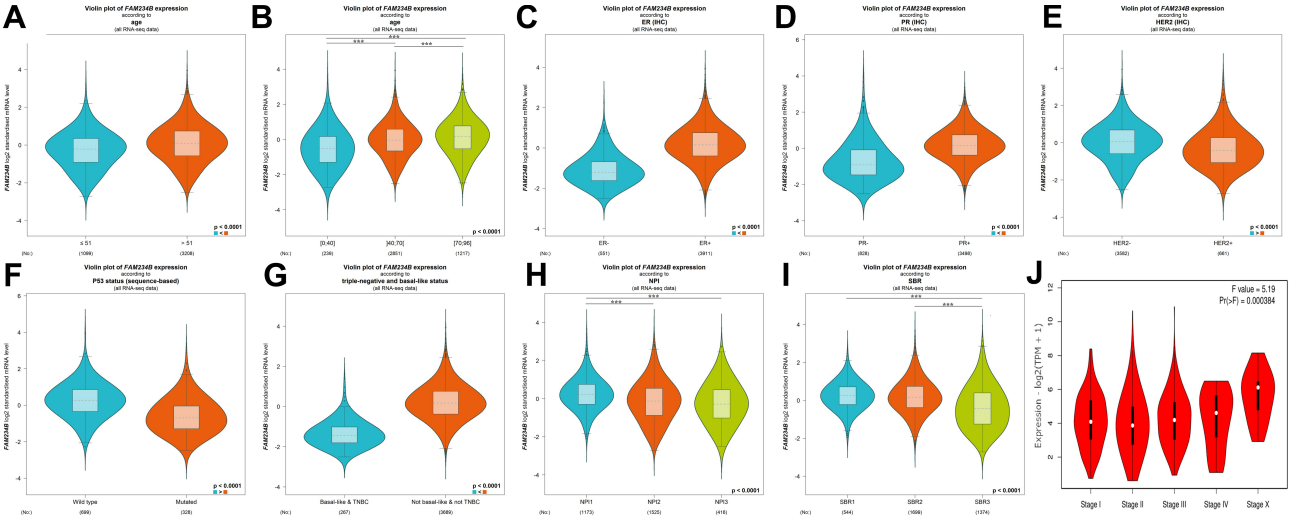

Figure 5 Relationship between expression of *FAM234B* and various clinicopathological parameters of breast cancer using bc-GenExMiner and GEPIA databases. (**A, B**) Violin plot of *FAM234B* expression according to age; (**C**) Violin plot of *FAM234B* expression according to ER status; (**D**) Violin plot of *FAM234B* expression according to PR status; (**E**) Violin plot of *FAM234B* expression according to HER2 status; (**F**) Violin plot of *FAM234B* expression according to p53 status; (**G**) Violin plot of *FAM234B* expression according to triple-negative and basal-like status; (**H**) Violin plot of *FAM234B* expression according to NPI; (**I**) Violin plot of *FAM234B* expression according to SBR grade status; and (**J**) Violin plot of *FAM234B* expression according to tumor stage (GEPIA). Difference of mRNA expression was compared by Welch's tests and Dunnett–Tukey–Kramer's test. ***$P < 0.001$.
Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; SBR, Scarff-Bloom-Richardson; NPI, Nottingham prognostic index; TNBC, triple-negative breast cancer; IHC, immunohistochemistry.

with levels in TNBC and basal-like BC, the mRNA levels of *FAM234B* were clearly higher in the not-basal-like BC and non-TNBC group (Figure 5G, $p < 0.0001$). However, the SBR grade and NPI were negatively related to mRNA levels of *FAM234B* (Figure 5H and I, $p < 0.0001$). *FAM234B* mRNA expression was remarkably correlated with tumor stages, and the highest *FAM234B* mRNA expression was found in stage X (Figure 5J, $p < 0.001$).

Subsequently, we assessed the associations between *FAM234B* expression and BC clinicopathological

Table 1 Correlations of *FAM234B* Expression with Breast Cancer Clinical Features

Clinicopathological Parameter	Total (*N*=120)	*FAM234B* Expression		OR	χ^2	*P*-value
		High (*N*=60)	Low (*N*=60)			
Age (years)				1.072	0.035	0.852
>50	73	37	36			
≦50	47	23	24			
Menopause status				1.070	0.034	0.854
Postmenopausal	67	34	33			
Premenopausal	53	26	27			
Tumor size				0.714	0.839	0.360
>2 cm	65	30	35			
≦2 cm	55	30	25			
Lymph node metastasis				2.418	5.647	**0.017**
Positive	57	35	22			
Negative	63	25	38			
TNM stage				1.567	0.891	0.345
III–IV	22	13	9			
I–II	98	47	51			
ER status				6.000	14.400	**0.000**
Positive	90	54	36			
Negative	30	6	24			
PR status				6.682	21.860	**0.000**
Positive	73	49	24			
Negative	47	11	36			
HER2 status				0.300	8.044	**0.005**
Positive	34	10	24			
Negative	86	50	36			
Ki67 status				1.453	0.370	0.543
>14%	108	55	53			
≦14%	12	5	7			
Molecular subtype				6.000	14.400	**0.000**
Luminal	90	54	36			
Not-luminal	30	6	24			

Note: The bold values indicate that the results are statistically significant.
Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

parameters in clinical samples, including age, menopause status, tumor size, lymph node metastasis, TNM stage, ER status, PR status, HER2 status, and Ki67 expression level. BC patients were divided into two groups (high expression vs. low expression) using median value as the cut-off. As shown in Table 1 and Supplementary Figure 2, the increased expression of *FAM234B* was correlated with lymph node metastasis-positive, ER-positive, PR-positive, HER2-negative, and luminal subtype, whereas *FAM234B* did not show correlation with age, menopause status, tumor size, TNM stage, and Ki67 level.

Co-Expressed Genes of *FAM234B* and Functional Enrichment Analysis

The identification of related genes will contribute to our understanding of the underlying roles of *FAM234B* in the development and progression of BC. We thus obtained the correlated genes of *FAM234B* by LinkFinder module using LinkedOmics database (Figure 6A). The heatmap of the top 50 positively and negatively correlated genes of *FAM234B* are presented in Figure 6B and C. Next, the gene set enrichment analysis (GSEA) of all significantly correlated genes

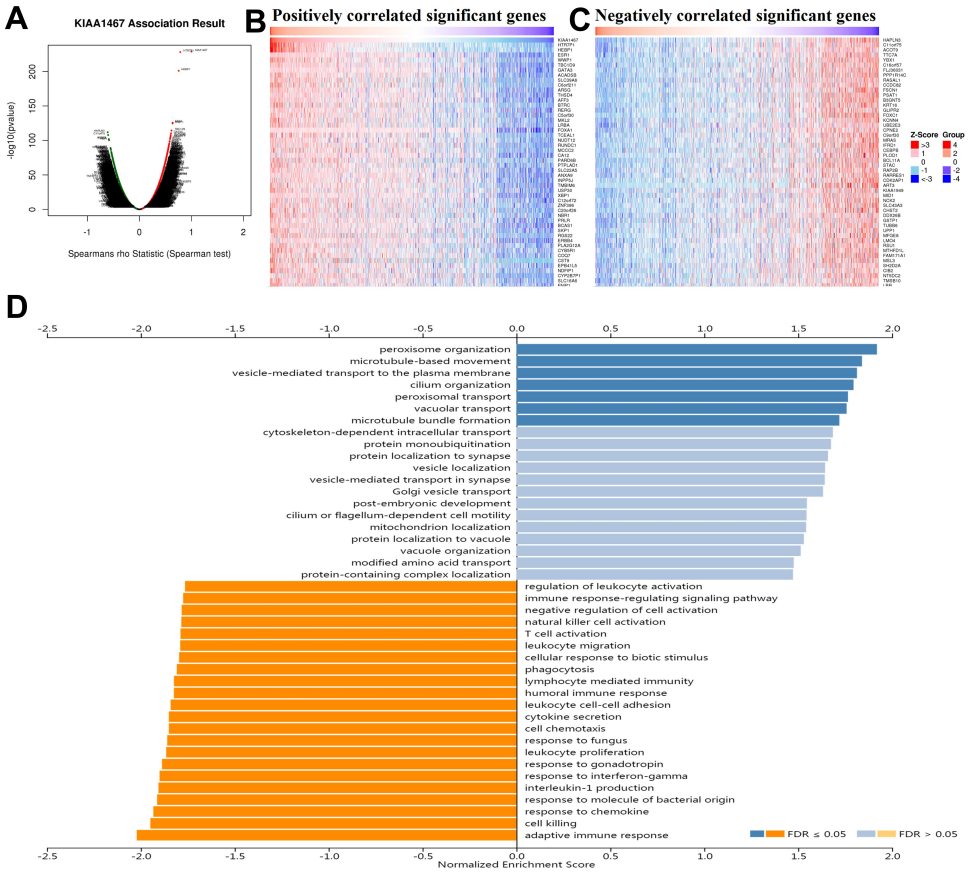

Figure 6 Co-expression analysis and gene set enrichment analysis in breast cancer using Linkedomics database. (**A**) The volcano plot of Spearman positive and negative association result of *FAM234B* in breast cancer; (**B, C**) The heatmap of the top 50 positively and negatively correlated genes of *FAM234B* in breast cancer; (**D**) Gene set enrichment analysis for all positively and negatively correlated significant genes of *FAM234B*.

was conducted via LinkInterpreter module. As shown in Figure 6D, we observed that *FAM234B* was positively related to the membrane transport and cytoskeleton formation process, such as peroxisome organization, microtubule-based movement, vesicle-mediated transport to the plasma membrane, cilium organization, peroxisomal transport, vacuolar transport, and so on, and negatively associated with immunity-related biological function, such as adaptive immune response, response to chemokine, interleukin-1 production, response to interferon-gamma, humoral immune response, lymphocyte-mediated immunity, natural killer cell and T cell activation, and so on.

HTR7P1 Was the Most Significantly Correlated with *FAM234B*, and High *HTR7P1* Expression Predicted Poor Survival Rate in Luminal BC Patients

As depicted in Figure 6A, *HTR7P1* (5-hydroxytryptamine receptor 7 pseudogene 1) had the most correlation with *FAM234B*. They were located on chr12p13.1, and *HTR7P1* was located upstream of *FAM234B* (Figure 7A). Specially, a correlation analysis using bc-GenExMiner database revealed that correlation of *FAM234B* with *HTR7P1* varied among various molecular subtypes, with higher correlation coefficient in luminal A and luminal B subtypes (Figure 7B and Supplementary Figure 3, $p < 0.001$). Similarly, we observed that the correlation between *FAM234B* and *HTR7P1* was the most significant in luminal BC by TIMER database (Figure 7C, $p < 0.001$). We also found that *HTR7P1* and *FAM234B* have a significant correlation in luminal subtype ($p < 0.05$), whereas no significant correlation was found in not-luminal subtype using clinical BC samples (Figure 7D, $p > 0.05$). Based on the above findings, we speculated that *HTR7P1* expression in BC varied among molecular subtypes.

Not surprisingly, although *HTR7P1* expression was not statistically different between BC and normal tissues (Supplementary Figure 4A and B, $p > 0.05$), its expression in luminal subtype was obviously higher than that in normal

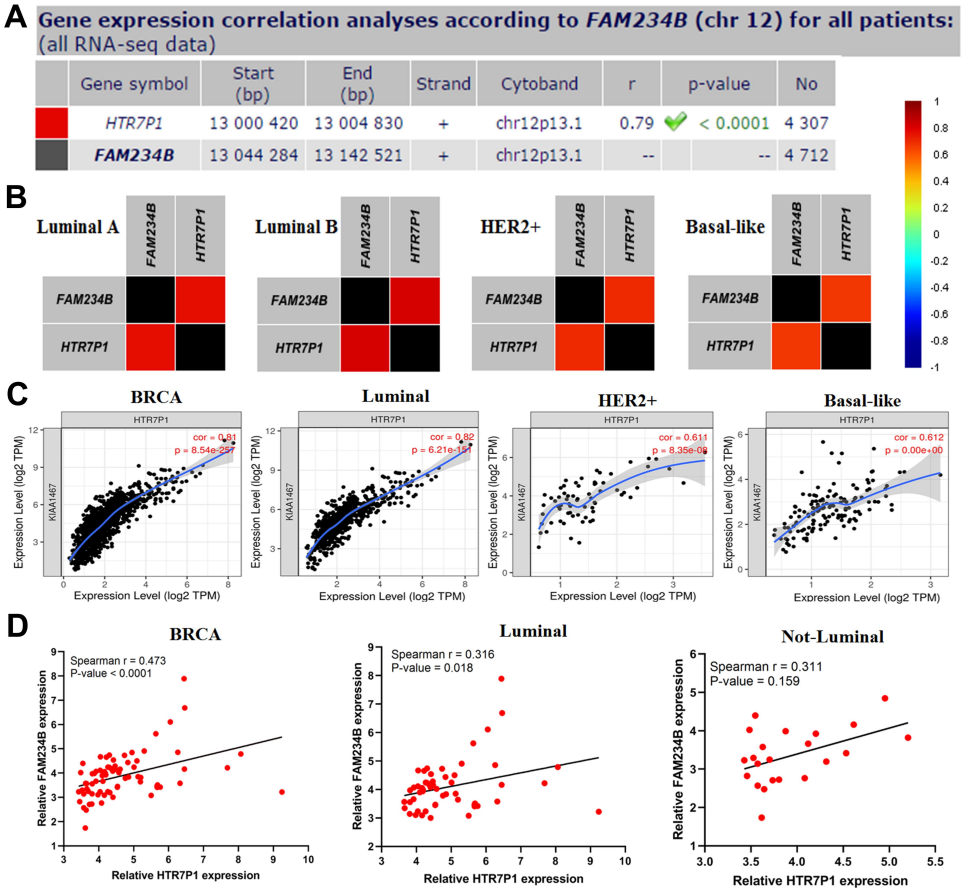

Figure 7 Correlation analysis of *FAM234B* with *HTR7P1* in breast cancer and its molecular subtypes. (**A**) Gene correlation analysis by chromosomal location of *FAM234B* with *HTR7P1* (bc-GenExMiner); (**B**) Pearson correlation of *FAM234B* with *HTR7P1* varied in different molecular subtypes (bc-GenExMiner); (**C**) Spearman correlation of *FAM234B* with *HTR7P1* in breast cancer and molecular subtypes (TIMER); (**D**) Spearman correlation of qT-PCR results of *FAM234B* with *HTR7P1* in breast cancer and molecular subtypes.

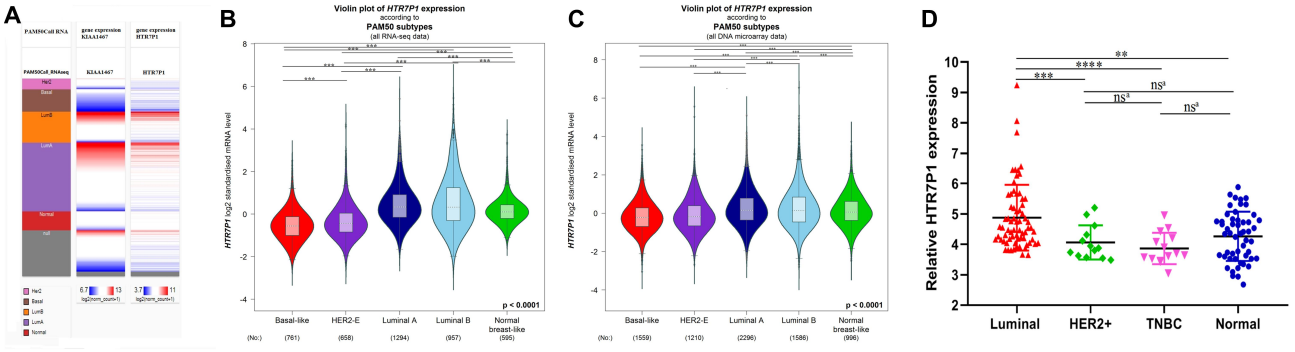

Figure 8 The distribution of *HTR7P1* mRNA expression across molecular subtypes of breast cancer. (**A**) The heatmap of *FAM234B* and *HTR7P1* gene expression in PAM50 subtypes (UCSC Xena); (**B, C**) *HTR7P1* mRNA level was highest in luminal subtype of breast cancer, too (bc-GenExMiner). (**D**) qRT-RCR results of *HTR7P1* in different subtypes of BC samples versus normal breast tissues. Error bars represent SD; ns, no significance, [a]Represent unpaired *t*-test. ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.
Abbreviation: TNBC, triple-negative breast cancer.

tissues (Figure 8A–D, $p < 0.001$). What is more, increased *HTR7P1* mRNA level indicated worse prognosis in luminal A and luminal B subtypes (Figure 9A and B, $p < 0.05$), whereas it predicted better survival rate in HER2-enriched subtype (Figure 9C, $p < 0.001$). However, no significant correlation was found between *HTR7P1* expression and prognosis in patients with basal-like BC (Figure 9D, $p > 0.05$). Subsequently, we analyzed associations of *HTR7P1* mRNA expression with clinicopathological features of BC. The results from bc-GenExMiner database

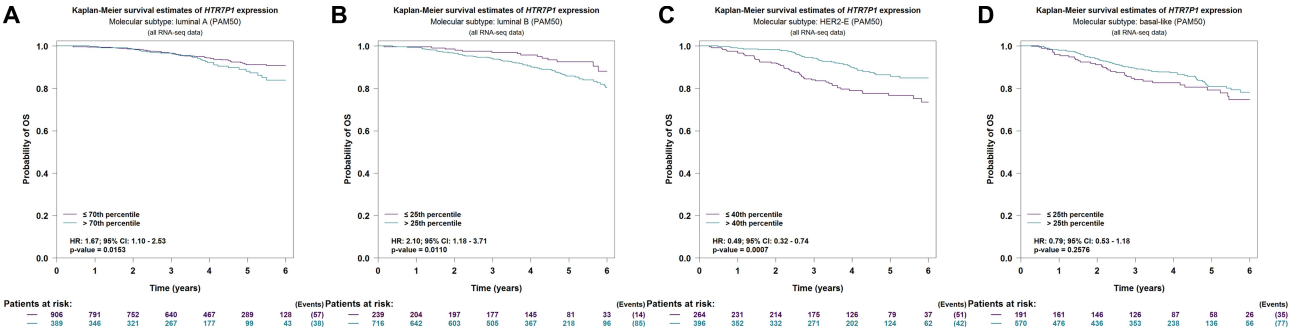

Figure 9 Prognostic value of *HTR7P1* in distinct molecular subtypes of breast cancer (bc-GenExMiner). (**A, B**) High expression of *HTR7P1* indicated poor prognosis of breast cancer patients with luminal A and B subtypes; (**C**) high expression of *HTR7P1* indicated better survival of breast cancer patients with HER2-enriched subtype; (**D**) *HTR7P1* expression showed no correlation with prognosis of breast cancer patients with basal-like subtype.
Abbreviations: HR, hazard ratio; CI, confidence interval.

showed that *HTR7P1* mRNA expression was obviously higher in older, ER-positive, PR-positive, HER2-negative, wild-type p53, not-basal-like and not-TNBC, low SBR grade, low NPI, and high-stage BC patients (Supplementary Figure 5, $p < 0.05$). Likewise, as shown in Table 2 and Supplementary Figure 6, the expression of *HTR7P1* was correlated with ER status, PR status, and molecular subtype using clinical samples ($p < 0.05$).

HTR7P1 Functions as an Competing Endogenous RNA of *has-miR-1271-5p* or *has-miR-381-3p* to Upregulate *FAM234B*

The pseudogene *HTR7P1* acts as a special long non-coding RNA (lncRNA) and is located upstream of *FAM234B*. According to the above results, high *HTR7P1* and *FAM234B* expression indicated poor prognosis in luminal BC. We thus assumed that *HTR7P1* may cis-regulate the expression of *FAM234B*. The interaction network of the top 100 interactions of *HTR7P1* and *FAM234B* are shown in Figure 10A and B. Subcellular localization of pseudogene *HTR7P1* determined the underlying mechanisms. lncLocator predicted that *HTR7P1* was mainly located in the cytosol but also distributed in the exosome (Figure 10C), which suggested that *HTR7P1* regulated *FAM234B* expression more likely in a competing endogenous RNA (ceRNA) way. As shown in Figure 10D, after taking the intersection of the prediction results from four databases, there were three candidate miRNAs (*has-miR-1271-5p, has-miR-381-3p*, and *has-miR-330*). We further analyzed their expression in BC using dbDEMC. Compared with normal tissues, *has-miR-1271-5p* and *has-miR-381-3p* were down-regulated (Figure 10E and Supplementary Table 2, $p < 0.01$), whereas *has-miR-330* was upregulated in BC tissues (Figure 10E). Correlation analysis using starBase also confirmed a significantly negative correlation of expression of *has-miR-1271-5p* or *has-miR-381-3p* with *HTR7P1* and *FAM234B* (Supplementary Figure 7A–D). These results demonstrated that *HTR7P1* might serve as ceRNA to cis-regulate *FAM234B* expression by sponging *has-miR-1271-5p* or *has-miR-381-3p* in luminal BC.

Discussion

BC is a heterogeneous disease with distinct histological, molecular, and clinical phenotypes.[30] Molecular stratification based on gene expression profiles has shown that BC could be divided into so-called intrinsic subtypes (luminal A and B, HER2-enriched, and basal-like).[31] These molecular subtypes differ clearly with respect to incidence, prognosis, and response to treatments. Over 70% of BC cases belong to the two luminal categories. Patients with luminal subtypes often respond to "endocrine" therapies targeting ER.[32] Unfortunately, many patients show intrinsic or acquired resistance to these therapies over time.[33] In fact, more than 30% of ER-positive cancers do not respond to endocrine therapy, exhibiting intrinsic resistance. Another 30–40% of tumors initially respond to endocrine therapy and eventually develop resistance.[34] Therefore, the current focus of research is the identification of new molecular targets or adjuvant endocrine therapies to overcome resistance in patients with luminal BC.

Several luminal subtype-specific genes have been identified, including *NUP43*,[35] *FANCM*,[36] *SYCP2*,[37] and *NCOA5*.[38] *FAM234B* is a recently identified gene in the *FAM234* family. Previous studies have reported that it is mainly expressed in the nervous system.[11] However, few studies have evaluated the role of *FAM234B* in human cancers. Results of bioinformatics analysis showed that *FAM234B* mRNA expression is highest in BC tissues

Table 2 Correlations of *HTR7P1* Expression with Breast Cancer Clinical Features

Clinicopathological Parameters	Total (*N*=96)	*HTR7P1* Expression		OR	χ^2	*P*-value
		High (*N*=48)	Low (*N*=48)			
Age (years)				0.771	0.389	0.533
>50	57	27	30			
≦50	39	21	18			
Menopause status				0.709	0.686	0.408
Postmenopausal	56	26	30			
Premenopausal	40	22	18			
Tumor size				1.543	1.080	0.299
>2 cm	57	31	26			
≦2 cm	39	17	22			
Lymph node metastasis				1.800	2.043	0.153
Positive	49	28	21			
Negative	47	20	27			
TNM stage				1.140	0.066	0.798
III–IV	19	10	9			
I–II	77	38	39			
ER status				5.444	11.594	**0.001**
Positive	69	42	27			
Negative	27	6	21			
PR status				4.200	10.971	**0.001**
Positive	56	36	20			
Negative	40	12	28			
HER2 status				0.462	2.650	0.104
Positive	25	9	16			
Negative	71	39	32			
Ki67 status				0.636	0.447	0.504
>14%	86	42	44			
≦14%	10	6	4			
Molecular subtype				5.444	11.595	**0.001**
Luminal	69	42	27			
Not-luminal	27	6	21			

Note: The bold values indicate that the results are statistically significant.
Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

among various tumor types, and its mRNA and protein levels are higher in BC tissues than in matched normal tissues, which was also verified by qRT-PCR for clinical samples. In particular, compared with normal breast tissues, the expression of *FAM234B* was increased in luminal BC but decreased in basal-like BC. qRT-PCR for clinical samples also demonstrated a high expression level of *FAM234B* in luminal BC tissues when compared with normal tissues. Subsequently, survival analysis indicated that high *FAM234B* levels are associated with a poor prognosis in patients with luminal BC. Positive associations between *FAM234B* expression and age, ER status, PR status, and tumor stage were found. Conversely, HER2 status, p53 status, NPI, SBR grade, basal-like status, and TNBC status were negatively related to *FAM234B* expression. Likewise, we also found that high mRNA expression of *FAM234B* was correlated with lymph node metastasis, ER-positive, PR-positive, HER2-negative, and luminal subtype by analyzing the qRT-PCR for clinical samples. Different from the results of bioinformatic analysis, no significant correlation between *FAM234B* expression and age and stage was found in our experimental verification

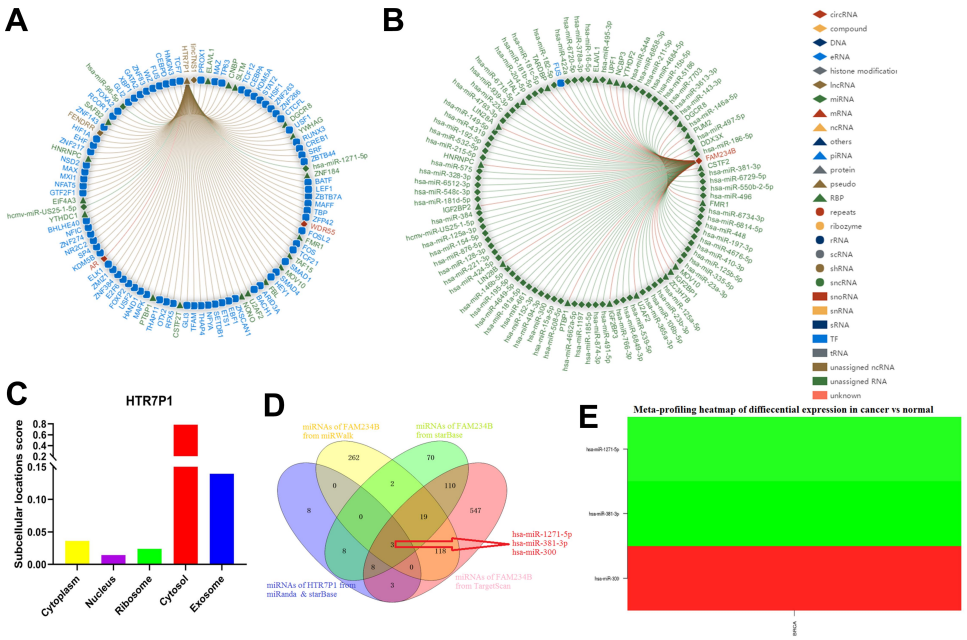

Figure 10 *HTR7P1* might cis-regulate the expression of *FAM234B* via competing with *has-miR-1271-5p* or *has-miR-381-3p* for binding to *FAM234B* in luminal breast cancer. (**A, B**) The interaction network of the top 100 interactions of *HTR7P1* and *FAM234B*; (**C**) Prediction of subcellular localization of *HTR7P1* using lncLocator; (**D**) Venn analysis of candidate miRNAs for *HTR7P1* and *FAM234B*; (**E**) Meta-profiling of differential expression of three candidate miRNAs in breast cancer tissues and normal tissues determined by dbDEMC 2.

using clinical samples. This may be due to the small sample size and the selection bias of the cohort of the present study. Additionally, *FAM234B* did not show correlation with menopause status, tumor size, and Ki67 level. These findings suggested that *FAM234B* functions as an oncogene and overexpression of *FAM234B* may be a promising prognostic biomarker in luminal BC.

Co-expression analysis facilitates understanding of the biological functions and molecular mechanisms of *FAM234B*; we obtained the positively and negatively correlated genes of *FAM234B* using Linkedomics database. Among them, genes in the ER-alpha pathway (*ESR1*, *GATA3*, *FOXA1*, and *XBP1*) were significantly positively correlated with *FAM234B* expression,[39–41] which can explain the high expression of *FAM234B* in luminal subtypes. Estrogen is a key regulator of mammary gland development, breast carcinogenesis, and progression.[42,43] The ER signaling pathway is the most promising target to date for the clinical treatment of ER-positive BC, and ERs are ligand-dependent transcription factors that regulate genes involved in cell proliferation, differentiation, apoptosis, and cell migration.[44] The dysregulation of these signaling pathways can lead to the initiation, progression, and invasion of breast tumors.[45] Therefore, we hypothesized that *FAM234B* is directly related to ER-mediated pathways and functions in luminal BC via these genes. Besides, GSEA showed that these positively correlated genes were mainly enriched in the membrane transport process, such as peroxisome organization, microtubule-based movement, vesicle-mediated transport to the plasma membrane, cilium organization, peroxisomal transport, vacuolar transport, and so on. We found that *FAM234B* was a single-pass membrane protein and was located in golgi apparatus, cytoplasm, cytoskeleton, microtubule organizing center, and membrane using UniProt (https://www.uniprot.org/uniprot/) database, which can explain that *FAM234B* was positively correlated with membrane transport process. Specially, GSEA showed that these negatively associated genes with *FAM234B* were mainly enriched in immunity-related biological functions, such as adaptive immune response, response to chemokine, interleukin-1 production, response to interferon-gamma, humoral immune response, lymphocyte-mediated immunity, natural killer cell and T cell activation, and so on. A large-scale quantitative phosphoproteomic analysis of TCR signaling identified that *FAM234B* protein phosphorylation was associated with T cell activation in human T cell leukemia.[46] At present, there are limited research reports on the correlation of *FAM234B* with immune response. Therefore, the relationship between *FAM234B* expression and immunity needs further research.

What makes *FAM234B* upregulated in luminal BC? Co-expression analysis also provided critical clues for investigating the regulatory mechanisms of *FAM234B* and showed

that *HTR7P1* was most significantly correlated with *FAM234B*. Moreover, correlation analysis suggested that, among various BC subtypes, *FAM234B* expression was the most significantly correlated with *HTR7P1* in luminal BC, which was also verified by qT-PCR using clinical samples. Simultaneously, both bioinformatics analysis and experimental verification have confirmed that *HTR7P1* expression was higher in luminal BC tissues than that in normal breast tissues. What is more, overexpression of *HTR7P1* indicated shorter survival in luminal BC patients. *HTR7P1* is a pseudogene that has not been reported in human tumors. Pseudogenes have long been considered junk genes. However, in recent years, more and more evidence has shown that pseudogenes play a key role in physiological and pathological processes in human diseases.[47] The dysregulation of pseudogenes also have a lot to do with tumor.[48,49] Pseudogenes, as a kind of lncRNA, serve as ceRNA for miRNAs or interacting with RNA-binding proteins to cis-regulate or trans-regulate the expression of their parent genes or other protein-coding genes, which depend on the subcellular localization of pseudogenes.[50–52] The prediction of subcellular localization of *HTR7P1* showed that it was mainly distributed in the cytosol, using IncLocator database. Therefore, we speculated that *HTR7P1* might be likely to upregulate the *FAM234B* expression through the ceRNA mechanism. Further exploration found that *has-miR-1271-5p* or *has-miR-381-3p* might be candidate miRNAs competing with *HTR7P1* for binding to *FAM234B*. We also observed a significantly negative correlation between the *has-miR-1271-5p* or *has-miR-381-3p* and *HTR7P1* or *FAM234B* in BC samples.

In summary, the overexpressions of *FAM234B* and *HTR7P1* predict a poor prognosis in patients with luminal BC. Pseudogene *HTR7P1* might cis-regulate the expression of *FAM234B* via competing with *has-miR-1271-5p* or *has-miR-381-3p* for binding to *FAM234B* in luminal BC. Besides, GSEA indicated that *FAM234B* was positively related to the membrane transport and cytoskeleton formation process and negatively associated with immune response biological function. However, the biological functions and molecular mechanisms of *FAM234B* in luminal BC still require laboratory research support.

Abbreviations

BC, breast cancer; TNBC, triple-negative breast cancer; ER, estrogen receptor; PR, progesterone receptor; OS, overall survival; HER2, human epidermal growth factor receptor 2; SBR, Scarff-Bloom-Richardson; NPI, Nottingham prognostic index; GSEA, gene set enrichment analysis; *FAM234B*, family with sequence similarity 234 member B; *HTR7P1*, 5-hydroxytryptamine receptor 7 pseudogene 1.

Data Sharing Statement

The data that support the findings of this study are available and derived from the available resource in the public domain.

Original Publication Statement

We declare that there are no prior or duplicate publication or submission elsewhere of any part of the work.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that there are no competing interests associated with the manuscript.

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