

Systematic Analyses of the Expression, Function, and Prognostic Value of CCNBs in Breast Cancer

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Abstract

Background Cyclin B (CCNB) family plays key roles in the cell cycle, cell division and proliferation. Three members of CCNB family have been identified, including CCNB1, CCNB2 and CCNB3. Many studies have explored the roles of CCNBs in the tumorigenesis and pathogenesis of different types of cancer. However, the expression level, function, and prognostic value of CCNBs in breast cancer (BC) are still unclear.

Methods We explored the specific alterations of CCNBs in BC and predicted their prognostic value for BC patients. Oncomine, Gene Expression Profiling Interactive Analysis (GEPIA), Kaplan-Meier plotter, cBioPortal, STRING, Database for Annotation, Visualization and Integrated Discovery (DAVID) databases were used for above analyses.

Results We found that CCNB1 and CCNB2 were significantly overexpressed in BC compared with normal samples, but not CCNB3. Survival analysis showed that upregulated CCNB1 and CCNB2 expression levels were associated with poor prognosis of BC patients, while high CCNB3 expression was related to good prognosis for BC patients. Furthermore, gene ontology (GO) enrichment analysis was performed to reveal the functions of CCNBs and the interacted genes related to CCNBs. In addition, hsa-miR-139-5p and hsa-miR-944 were identified to be potentially involved in the regulation of CCNB1.

Conclusion Our study suggests that CCNB1, CCNB2 are potential targets of precise therapy for BC patients and that CCNB3 is a novel biomarker for the good prognosis of BC patients.

Introduction

Breast cancer (BC) is the most common cancer and the most common cause of cancer-related death among women [1–4]. Efficient tools for preventive intervention, therapeutic evaluation, and prognostic prediction are lacking [5]. Traditional histopathological classification does not provide sufficient predictive value, and therefore, in recent studies, researchers have gradually identified biomarkers indicating intrinsic features of tumors at the molecular level [6–9]. Elucidation of the genes associated with breast cancer play a key role in the development of BC treatment and prognostic prediction.

The Cyclin B (CCNB) family is a specific group that mainly functions in the cell cycle pathway, regulating cyclin-dependent kinase 1 (CDK1) enzyme activity through binding and degradation. CCNB synthesis occurs in the late S phase and G2 phase [10–12]. CDKs are Ser/Thr kinase systems involved in the cell cycle. Active CDK1-CCNB-Cks phosphorylate critical substrates during mitosis, leading to dramatic recombination and eventual separation of sister chromatids on mitotic spindles. Once separated, expression related to chromatids must return to a low level so that nuclear membrane recombination, spindle disassembly, and cytokinesis can occur [13].

At present, three members of the CCNB family have been identified: CCNB1, CCNB2 and CCNB3. CCNBs were reported to play key roles in human BC. High CCNB1 expression has been reported in many tumors, including colorectal cancer[14], lung cancer[15], ovarian cancer[16], liver cancer[17]. High CCNB2

expression is related to bladder cancer[18] and non-small cell lung cancer [19]. BCOR-CCNB3 gene fusion was also shown to be closely related to round cell sarcomas [20, 21]. Moreover, many drugs and upstream genes block the G2/M transition by inhibiting the action of CCNB1 and CCNB2, thus affecting cancer-related processes [15, 22, 23].

Although it has been reported that CCNB family play key roles in many cancers, its bioinformatics analysis in BC has not been systematically explored. In our study, the expression level, functions, and prognostic value of CCNBs in BC were analyzed by online databases and demonstrated based on a series of cell experiments. Our study suggests that CCNB1 and CCNB2 are potential biomarkers and precise therapy targets for BC patients and CCNB3 is a novel biomarker for good prognosis of breast cancer. Our study contributes to enriching the acknowledge of CCNBs in human BC.

Materials And Methods

Ethics Statement

All analyses and protocols in this study strictly comply with the requirements of the Ethics Committee and Institutional Review Board of Qingdao University. Datasets were sourced from publicly available databases on the web and were consistent with the appropriate requirements.

Oncomine analysis

Oncomine[24] (<http://www.oncomine.org>), which integrates RNA and DNA data from open databases such as GEO and TCGA, was used to explore the mRNA expression of CCNB family genes between BC and normal samples. Student's t-test was applied to generate a P value for comparison, and the criteria were as follows: $P = 0.01$ and fold change = 2.

CCLC analysis

The Cancer Cell Line Encyclopedia [25] (CCLE, <https://portals.broadinstitute.org/ccle>) is an open database containing data from nearly a thousand cancer patients' cell lines, which can be used to study genomic data. The CCNBs expression levels in various cancer cell lines were analysed by CCLE.

Kaplan-Meier Plotter

Kaplan-Meier plotter[26] (<http://kmplot.com/analysis/>) is an online tool to generate survival plots that can be used to assess the correlation between the expression levels of genes and the clinical outcomes of BC patients. Survival curves, which included HR and log-rank P value, were used to show the prognostic value of CCNB1, CCNB2 and CCNB3 based on the Kaplan-Meier plotter.

STRING analysis

The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) [27]. (<https://string-db.org>) is a database that collects and integrates protein-protein interaction information

and predicts further interactions. The protein-protein interaction network of CCNBs was established by STRING.

GEPIA Dataset

Gene Expression Profiling Interactive Analysis (GEPIA) [28](<http://gepia.cancer-pku.cn/>) is a newly developed interactive web server for analyzing the RNA sequencing expression data from TCGA and GTEx projects using a standard processing pipeline. We used the GEPIA database to analyse the expression levels of CCNBs in BC tissues compared with normal samples.

cBioPortal analysis

The Breast Invasive Carcinoma database (TCGA, PanCancer Atlas) was selected to establish the Breast Cancer Genome Atlas of CCNBs by the cBioPortal [29] (<http://www.cBioPortal.org/>) database. The selected genomic profiles contained the following: mutations, putative copy-number alterations from DNA and mRNA expression data (microarray) and z-scores relative to diploid samples.

GO enrichment analysis

Database for Annotation, Visualization and Integrates Discovery (DAVID) [30] (<https://david.ncifcrf.gov/>), an integration and analysis tool, was used to analyze the CCNBs and interacted genes to identify Gene Ontology (GO) terms and visualize genes on KEGG pathway maps.

ENCORI analysis

The Encyclopedia of RNA Interactomes (ENCORI) (<http://starbase.sysu.edu.cn/index.php>) [31] database can be used to explore the potential relationships, such as miRNA-mRNA, miRNA-ncRNA. In our study, ENCORI was used to predict miRNAs that regulated CCNBs and showed the miRNA expression level in breast cancer. The options used in our study were as follows: CLIP data: medium stringency (≥ 3); Degradome data: with or without data; Pan-Cancer: 1 cancer type.

Cell culture and qPCR detection

BC cell line MDA-MB-231 was stored in the laboratory and MCF-10A (human normal breast epithelial tissue cell) was acquired from Biotechnology Co., Ltd. Shanghai enzyme research. MDA-MB-231 cells were cultured in culture medium comprising DMEM supplemented with 10% fetal bovine serum (Tianhang Biotechnology Co., Ltd., China) while MCF-10A cells were cultured in MCF-10 cultural solution (CC-Y1607M from EK-Bioscience) and both were maintained at 37 °C in a 5% CO₂-incubator.

Total RNA of TNBC and MCF-10A was isolated using RNA extraction kit (R0032, Beyotime Biotechnology, China). BeyoFast™ SYBR Green qPCR Mix(2x) kit (Beyotime Biotechnology, China) was utilized for all gene qRT-PCR assays according to the manufacturer's protocol. Quantitative RT-PCR was performed on a real-time PCR system using QuantStudio3 by Thermo Fisher. PCR cycles were run as follows: predenaturation for 2 min at 95°C followed by 40 cycles of 15 s at 95°C, 30 s at 56°C and 30 s at 72°C.

The primers used in qPCR for human CCNB1 were forward 5'-AATAAGGCGAAGATCAACATGGC-3' and reverse 5'-TTTGTTACCAATGTCCCCAAGAG-3', CCNB2 were forward 5'-CCGACGGTGTCCAGTGATTT-3' and reverse 5'-TGTTGTTTTGGTGGGTTGAACT-3' and CCNB3 were forward 5'-ATGAAGGCAGTATGCAAGAAGG-3' and reverse 5'-CATCCACACGAGGTGAGTTGT-3'(all synthesized by Sangon Tech).

Statistical Analysis

Student's t-test was used to analyse the statistical difference of the CCNBs expression levels in BC and normal samples. The analysis of qRT-PCR was used to show the relative expression level of CCNBs in human BC and normal breast cells by using $2^{-\Delta\Delta C(T)}$ method. Using Kaplan-Meier plotter, the survival curves, including Log-rank test and hazard ratio (HR), of different subtypes of BC patients with different expression levels of CCNBs were drawn. $P < 0.05$ was considered to be statistically significant.

Results

Transcriptional Levels of CCNBs in BC

At present, three members have been identified in the CCNB family, including CCNB1, CCNB2 and CCNB3. However, it is not clearly known that the roles of CCNBs in BC. The Oncomine database was used to analyse the mRNA expression levels of CCNBs in BC patients and those in normal samples. As shown in Fig. 1a, 445 CCNB1 analyses, 415 CCNB2 analyses and 249 CCNB3 analyses were performed in the Oncomine database. Our results showed that CCNB1 (upregulated in 14 datasets) and CCNB2 (upregulated in 25 datasets) were significantly overexpressed in BC, but not CCNB3.

As shown in Fig. 1b, Curtis et al. [32] and TCGA data showed that the CCNB1 expression level was significantly upregulated in BC compared with normal samples (Curtis breast, P value = $1.28E-117$, Fold change = 2.088; TCGA breast, P value = $5.84E-44$, Fold change = 4.531). Moreover, Curtis et al. [32] and TCGA data also showed that the CCNB2 expression level was significantly overexpressed in BC compared with normal samples (Curtis breast, P value = $4.42E-113$, Fold change = 4.835; TCGA breast, P value = $8.53E-38$, Fold change = 5.289) (Fig. 1c). However, we did not find that upregulated expression of CCNB3 in BC compared with normal samples (Fig. 1d).

Overexpression of CCNB1 and CCNB2 were closely related to BC

Furthermore, a meta-analysis was performed to show that the expression levels of CCNB1 and CCNB2 were significantly increased in BC (supplement material 1). The results demonstrated that the CCNB1 expression level was significantly upregulated in BC (P value = $4.79E-8$) and the mRNA expression level of CCNB2 was also prominently increased in BC (P value = $6.39E-5$), indicating that upregulation of CCNB1 and CCNB2 were closely associated with BC.

In addition, qRT-PCR analysis confirmed that CCNBs mRNA was up-regulated at the cellular level. Compared with MCF-10A cell line, the average expression of CCNB1 mRNA in MDA-MB-231 cell line was 9.97 (Fig. 2a), and CCNB2 expression was 8.35 (Fig. 2b), while CCNB3 expression has no difference between 2 cell lines (Fig. 2c). These results indicate that CCNBs may be a special factor of breast cancer.

In order to verify that CCNB1 and CCNB2 were overexpressed in BC compared with normal samples, but not CCNB3, a series of analyses were performed. We used the Cancer Cell Line Encyclopedia (CCLE) database to show the mRNA transcription levels of CCNB1, CCNB2 and CCNB3 in BC. As shown in supplement material 2, the mRNA transcription levels of CCNB1 and CCNB2 in BC cell line and BC tissue were significantly up-regulated. However, we did not observe that the increased CCNB3 mRNA transcription level in BC cell line and BC tissue.

High expression of CCNB1 and CCNB2 were associated with poor prognosis for BC patients, while high expression of CCNB3 was related to good prognosis for BC patients

Further, the prognostic value of CCNB1, CCNB2 and CCNB3 in BC patients was evaluated through the Kaplan-Meier plotter. High mRNA expression level of CCNB1 was significantly associated with shorter (relapse free survival) RFS in all BC patients (HR = 1.51, $p = 2e-07$). In particular, analysis of different BC subtypes showed that high CCNB1 mRNA expression was significantly related to PR-positive (HR = 2.05, $p = 0.00034$), ER-positive (HR = 1.82, $p = 7e-05$), Her2-negative (HR = 1.97, $p = 1.1e-05$), Luminal A (HR = 1.55, $p = 0.00055$), Luminal B (HR = 1.69, $p = 0.00079$), Lymphnode-positive (HR = 1.53) and Lymphnode-negative (HR = 1.64, $p = 0.013$) subtypes of BC (supplement material 3).

As shown in supplement material 4, high mRNA expression level of CCNB2 was significantly associated with shorter RFS in all BC patients (HR = 2, $p < 1e-16$). The analysis of different BC subtypes showed that high CCNB2 mRNA expression was significantly related to PR-positive (HR = 2.3, $p = 5.8e-06$), ER-positive (HR = 1.92, $p = 1e-14$), Her2-negative (HR = 1.97, $p = 5.6e-07$), Luminal A (HR = 2.28, $p < 1e-16$), Luminal B (HR = 1.57, $p = 3.7e-06$), Lymphnode-positive (HR = 1.93, $p = 7.5e-11$) and Lymphnode-negative (HR = 1.78, $p = 2.6e-11$) subtypes of BC.

We also found that high mRNA expression level of CCNB3 was significantly associated with longer RFS in all BC patients (HR = 0.66, $p = 2.4e-07$). In particular, analysis of different BC subtypes showed that high CCNB3 mRNA expression was significantly related to ER-positive (HR = 0.74, $p = 0.046$), Luminal A (HR = 0.76, $p = 0.026$), Luminal B (HR = 0.64, $p = 0.0051$) and Lymphnode-negative (HR = 0.68, $p = 0.049$) (Fig. 3).

The predictive function of CCNBs and their frequently interacted genes in patients with BC

To detect the expression pattern and mutation of CCNBs, the cBioPortal database was used to analyze the alterations of CCNBs in BC patients. As shown in supplement material 5A, CCNBs mRNA expression levels were altered in 184 (17%) of queried 1108 BC patients. In addition, the correlations of the mRNA expressions of CCNBs in BC was analysed by the Gene Expression Profiling Interactive Analysis (GEPIA) database. We found that there were significant positive correlations between following CCNBs: CCNB1

with CCNB2 (R = 0.75), CCNB1 with CCNB3 (R = 0.086) and CCNB2 with CCNB3 (R = 0.13) (supplement material 5B).

We established a protein-protein interaction network of CCNBs, and 50 most frequently altered genes were also included in it (Fig. 4a). Furthermore, the functions of CCNBs and the interacted genes associated with CCNBs were predicted by gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses in the Database for Annotation, Visualization and Integrated Discovery (DAVID). Using GO enrichment analysis, we predicted the potential functions of target genes through the GO terms, including biological process (BP), cellular component (CC), and molecular function (MF) (Fig. 4b). We found that GO:0051301 (cell division), GO:0007067 (mitotic nuclear division), GO:0007062 (sister chromatid cohesion), GO:0051439 (regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle), GO:0031145 (anaphase-promoting complex-dependent catabolic process) and GO:0000086 (G2/M transition of mitotic cell cycle) were the BPs significantly regulated by CCNBs alterations in BC (). GO:0000776 (kinetochore), GO:0005680 (anaphase-promoting complex), GO:0005634 (nucleus) and GO:0000775 (chromosome, centromeric region) were the CCs affected by alterations of CCNBs expression; and GO:0004693 (cyclin-dependent protein serine/threonine kinase activity)

and GO:0030332 (cyclin binding) were the MFs that were significantly controlled by CCNBs alterations.

The pathways related to the altered functions of CCNBs and frequently altered interaction genes were defined by KEGG analysis. KEGG analysis revealed pathways associated with the altered functions of CCNBs in BC, such as cell cycle and p53 signaling pathway (supplement material 6), which involved in the tumorigenesis and pathogenesis of BC.

Identification of regulatory miRNA and their roles on prognostic value in breast cancer patients

As shown in Table 1–2(supplementary material), 42 miRNAs regulating CCNB1 and 5 miRNAs regulating CCNB2 were predicted by the ENCORI database. We did not find any miRNA that met the criteria to regulate CCNB3. Among them, 22 miRNA-CCNB1 pairs and 3 miRNA-CCNB2 pairs were negatively correlated. However, only 2 miRNA-CCNB1 pairs were decreased expression in BC compared with normal samples and predicted poor prognosis for patients with BC. As shown in Fig. 6a and 6b, the high expression of hsa-miR-139-5p and hsa-miR-944 in BC were related to longer overall survival in BC patients. Therefore, hsa-miR-139-5p and has-miR-944 may target CCNB1 to exert anti-tumor function in BC (Fig. 6c).

Discussion

It has been reported that BC is the most common type of cancer in women [33, 34]. Classic clinical prognostic biomarkers, including PR, HER2 and ER, have positive roles in precision therapy in BC patients [35]. However, the current limitations of tumor markers are sensitivity and specificity, which are determined by the unique heterogeneity of various tumors. Therefore, we need valuable novel biomarkers as prognostic predictors for cancer patients to effectively increase the prognosis and precise therapy

effects of cancer patients. Fortunately, some open bioinformatics databases have been established by biomedical researchers, such as Oncomine[36], CCLE[25], cBioPortal [29], and some potential novel biomarkers were identified as valuable prognostic predictors and therapeutic targets[37–39].

It has been reported that the expression levels of CCNBs in many types of cancer are dysregulated, such as colorectal cancer, lung cancer, ovarian cancer, liver cancer, bladder cancer, round cell sarcomas and BC [14–21, 40]. Although key roles of CCNBs in tumor occurrence, progression, distant metastasis and predicting the prognosis of tumor patients have been partially reported, the detailed biological information of CCNBs in BC has not been systematically explored. Bioinformatics analyses of CCNBs in BC were performed based on online databases to systematically explore the expression levels, functions and prognostic value of CCNBs in BC to enrich the acknowledge of CCNBs in BC.

We found that CCNB1 and CCNB2 were significantly overexpressed in BC compared with normal samples (Fig. 1). It has been reported that the expression level of CCNB1 and CCNB2 are increased in BC and predicts poor prognosis of BC patients [40–42]. However, the prognostic value of CCNBs in BC patients, including the survival rate of all BC patients and BC subgroups, still needs to be further clarified. Using Kaplan-Meier plotter, we further clarified the correlation between the high CCNB1 and CCNB2 expression in BC and poor prognosis of BC patients, including the relapse free survival of all BC patients and BC subgroups (PR+, PR-, basal-like, ER+, ER-, Her2+, Her2-, Luminal A, Luminal B, Lymphnode+ and Lymphnode- subgroups) (supplement material 1). Alterations and mutations of CCNBs in BC and the correlation of CCNB family members were also been shown (supplement material 5). We established a protein-protein interaction network of CCNBs and frequently altered genes. Furthermore, the functions of CCNBs and the interacted genes associated with CCNBs were predicted (Fig. 4). Our results indicated that cell cycle was the main pathway which CCNBs involved in the tumorigenesis and pathogenesis of BC. Korgun et al. reported that CCNB1 had an important role in the G2/M transition [43]. Gustafsson N et al. confirmed that downregulation of CCNB1 expression affected the G2/M phase transition [44]. Liu et al. demonstrated that acetylated MORC2 reduced the H3T11P expression and repressed the expression of its downstream gene CCNB1, which sensitized BC cells to DNA-damaging chemotherapy by arresting BC cells at the G2/M transition [45]. CCNB2 also plays an important role in BC. Acid ceramidase (ASAH1) can upregulated the CCNB2 expression and promote BC cell proliferation [46]. Meng et al. reported that PTTG1 was overexpressed in BC and might target CCNB2, resulting in more BC cells distributed in S phase [47]. To date, there is no report revealing the expression level of CCNB3 in BC and predicting the prognostic value for BC patients. In our study, we found that high CCNB3 expression was related to good prognosis of BC patients. Our study suggests that CCNB3 is a novel biomarker for good prognosis of BC patients.

According to previous reports, the expression of miRNA is closely associated with the occurrence, progression and therapy of various tumors, indicating that these miRNAs may become potential diagnostic, prognostic biomarkers for cancer [48]. Using ENCORI database, miRNAs (including has-miR-139-5p and has-miR-944) that negatively regulated CCNB1 in BC were identified. It has been reported that has-miR-139-5p can inhibit the proliferation of BC [49]. Flores-Pérez et al. reported that has-miR-944 could

target SIAH1 and PTP4A1 to suppress BC cells migration[50]. We established the has-miR-139-5p-CCNB1 and has-miR-944-CCNB1 regulatory network that may be potentially prognostic biomarkers and therapeutic targets in BC.

Conclusion

In this study, the expression level, function, and prognostic value of CCNBs were systematically explored using online databases. We confirmed that CCNB1 and CCNB2 expression were significantly increased in BC compared with normal samples, but not CCNB3. Our result show that high expression of CCNB1 and CCNB2 were associated with poor prognosis of BC patients. High CCNB3 expression was related to good prognosis of BC patients. We suggest that CCNB1 and CCNB2 are potential biomarkers and precise therapy targets for BC patients and CCNB3 is a novel biomarker for good prognosis of BC patients.

Declarations

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Conflicts of interest

All authors declare that there are no conflicts of interest in this study.

Availability of data and material

The data shown in this study can be obtained through online databases or corresponding author.

Code availability

GraphPad Prism 8

Authors' contributions

Study design: LL; Data collection: XL, YT; Statistical analysis: XL, YT, CL; Data interpretation: XL, YT; Manuscript preparation: YT, XL, LL; Literature research: YQ, XL; Funds collection: CS, LL. All authors read and approved the final manuscript.

Ethics approval

This research has been approved by the Ethics Committee and Institutional Review Board of Qingdao University, China. All procedures comply with the Declaration of Helsinki. In addition, all the data we obtained was from public database.

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Figures

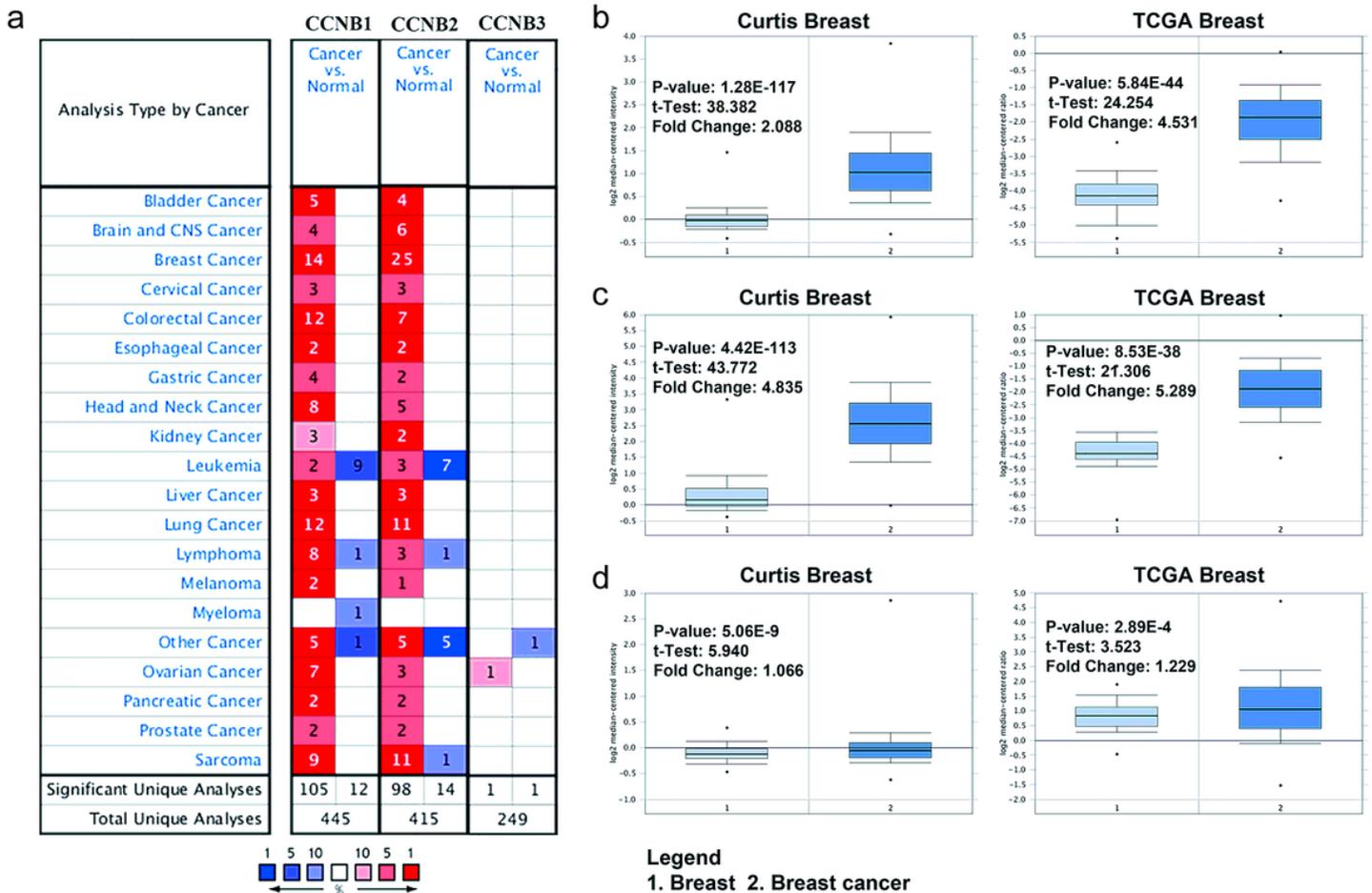


Figure 1

The transcriptional level, meta-analysis of CCNBs in different types of cancer. a This Fig. shows the different transcriptional levels of CCNB1, CCNB2 and CCNB3 between various cancers and normal samples based on the Oncomine database. The overexpression of CCNBs in cancer are marked in red, while the decreased expression levels of CCNBs in normal samples are marked in blue. The number in each cell indicates how many datasets met the statistical criteria. P value is set to 0.05; fold change is set to 2; gene rank was set to top 10%. b The mRNA expression of CCNB1 in Curtis and TCGA Breast. c The mRNA expression of CCNB2 in Curtis and TCGA Breast. d The mRNA expression of CCNB3 in Curtis and TCGA Breast.

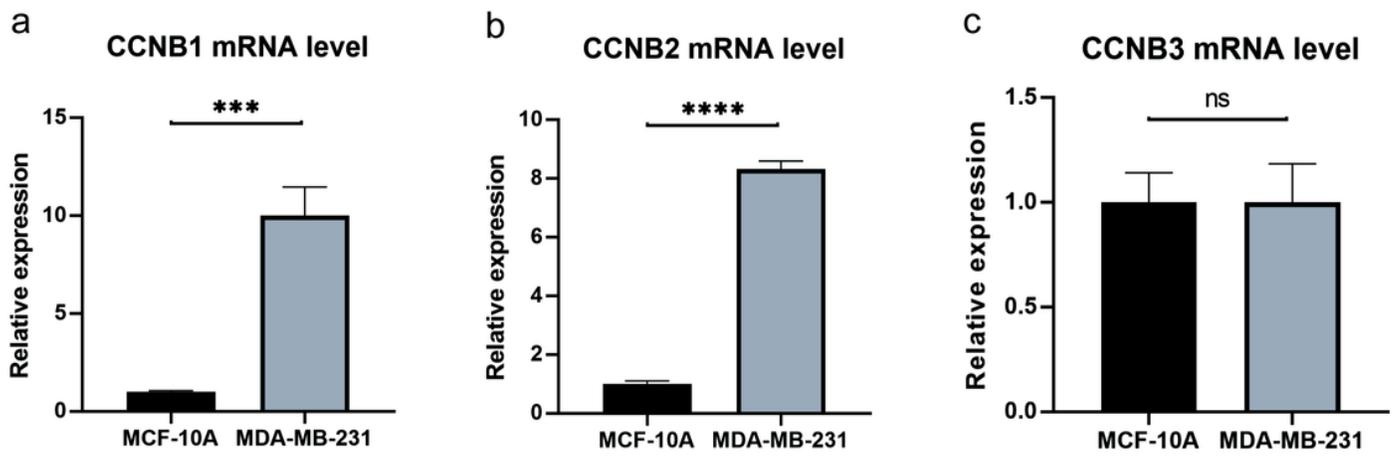


Figure 2

qPCR analysis of CCNBs mRNA expression levels.

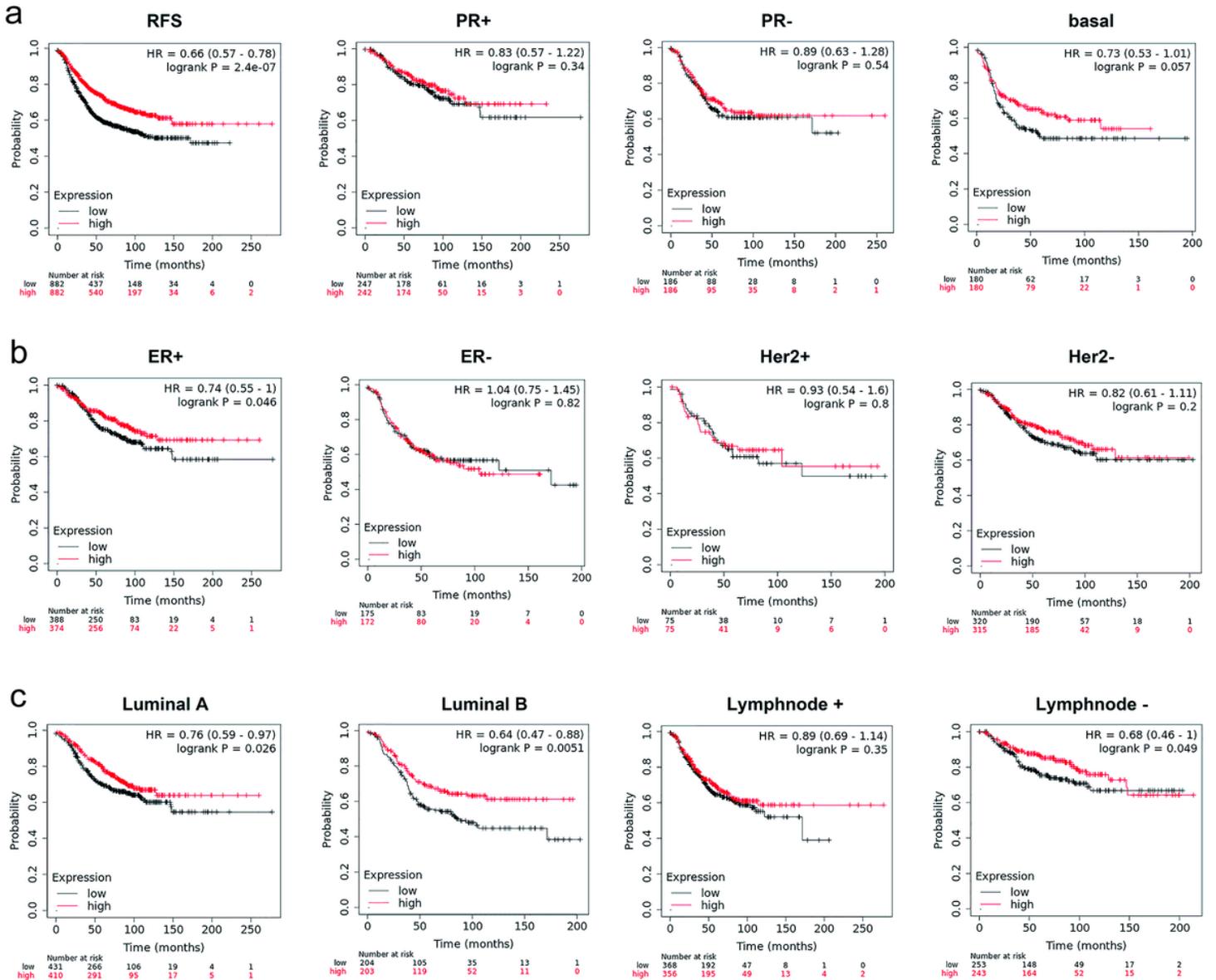


Figure 3

Analysis of the prognostic value of CCNBs in breast cancer. The correlation of CCNBs expression with relapse free survival was analyzed by the Kaplan-Meier plotter in a all patients with breast cancer, and various subtypes of BC, including progesterone receptor-positive (PR-positive), progesterone receptor-negative (PR-negative), basal-like b estrogen receptor-positive (ER-positive), estrogen receptor-negative (ER-negative), human epidermal growth factor receptor 2-positive (Her2-positive), human epidermal growth factor receptor 2-negative (Her2-negative), c Luminal A, Luminal B, lymph node-positive and lymph node-negative.

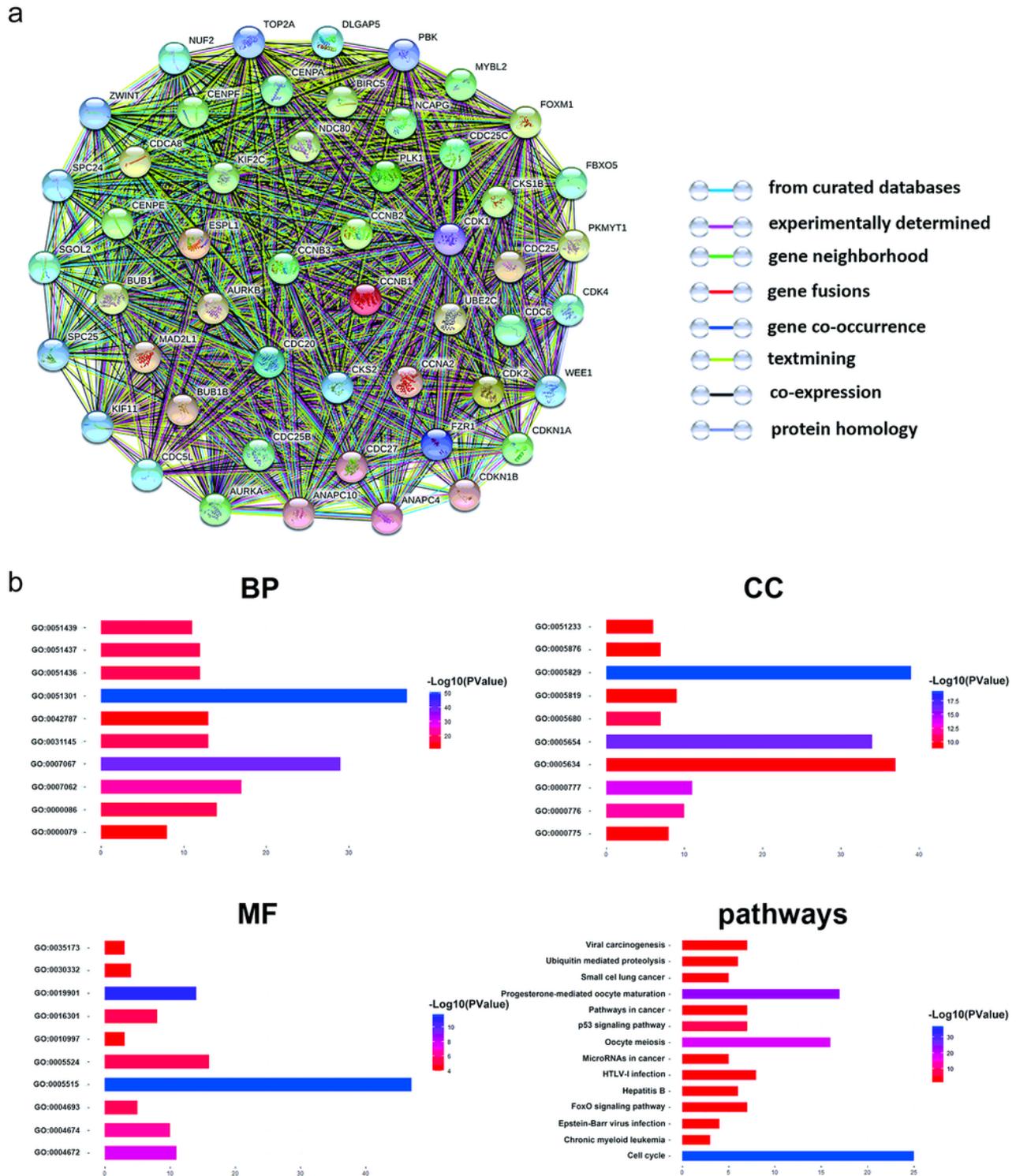


Figure 4

Protein-protein interaction network and gene oncology enrichment analysis of CCNBs in breast cancer. a The protein-protein interaction network of CCNBs and frequently altered interaction genes was established based on STRING database. b The potential functions of CCNBs and interaction genes significantly related to CCNBs were predicted through the DAVID database, including biological process (BP), cellular component (CC), molecular function (MF), and KEGG pathways.

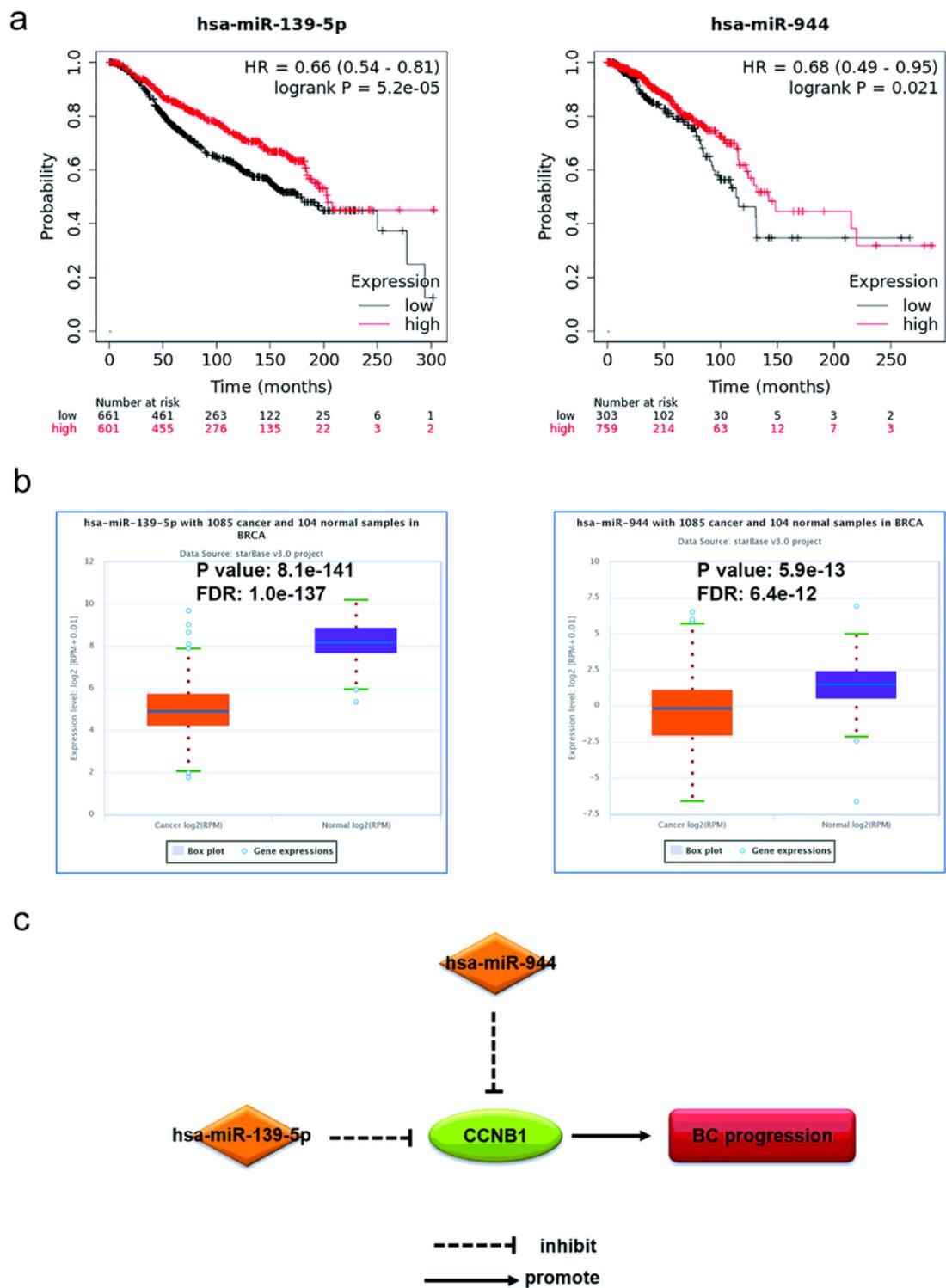


Figure 5

hsa-miR-139-5p, hsa-miR-944 and their relationships with BC. a, b high level of hsa-miR-139-5p and hsa-miR-944 were related to longer overall survival in BC patients. c hsa-miR-139-5p and hsa-miR-944 play an antitumor role in BC.

Supplementary Files

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