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European Journal of Histochemistry 2021; volume 65:3207

Aberrant expression of *CCDC69* in breast cancer and its clinicopathologic significance

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ABSTRACT

Coiled-coil domain-containing protein 69 (*CCDC69*) is a novel gene and limited knowledge in known in breast cancer. In the present study, we aimed to explore the relationship between *CCDC69* and breast cancer, demonstrate the clinicopathological significance and prognostic role of *CCDC69* in breast cancer, and analyze the possible mechanism of *CCDC69* affecting the prognosis of breast cancer. First, from GEO database, TIMER, GEPIA, and OncoLnc, we selected *CCDC69* as the potential gene which closely involved in breast cancer progression. Next, by real-time PCR detection, the expression of *CCDC69* in breast cancer tissue was notably lower than that in normal breast tissues (p=0.0002). In addition, our immunohistochemistry indicated that the positive expression rate of *CCDC69* in the triple-negative breast cancer (TNBC) was lower than that in the non-TNBC (p=0.0362), and it was negatively correlated with the expression of Ki-67 (p=0.001). Further enrichment analysis of *CCDC69* and the similar genes performed on FunRich3.1.3 revealed that these genes were significantly associated with fat differentiation, and most of them were related to peroxisome proliferator-activated receptor (PPAR) signal pathway. Collectively, our findings suggest that *CCDC69* is down regulated in breast cancer tissue especially in TNBC which has higher malignant grade and poorer clinical prognosis.

Key words: Breast cancer; GEO database; *CCDC69*; SORBS1; PPAR.

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Contributions: JL and PZ contributed equally to this study. JL, YX, study concept and design, research work, research material, logistic support; PZ, data collection. All the authors have read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

Conflict of interest: No potential conflict of interest was reported by the authors.

Funding: This work was funded by the National Natural Science Fundation of China (No.81302309), and by the Natural Science Fundation of Hubei Province, China (No.2019CFB650)".

Availability of data and materials: The dataset is publicly available and can be used for data mining of various aspects of inorganic materials synthesis.

Ethical Approval: Ethical approval for the study was obtained from the Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

Patients consent and publication: Authors can use the BioMed Central consent form to obtain consent for publication, or a consent form from their own institution or region if appropriate.

Introduction

Breast cancer is the most common malignant tumor in women.[1] Detecting genes expression in breast cancer tissue, such as estrogen receptor (ER), human epidermal growth factor receptor 2 (HER2) and breast cancer susceptibility gene *BRCA1*/2, phosphatidylinositol-3 kinase catalytic subunit alpha (PIK3CA) and serine-threonine protein kinase 1 (*AKT1*) can effectively guide breast cancer treating.[2-6] However, tremendous functions of genes on the tumor progression remain unknown.[7] Thus, it is crucial to further explore genes that affect the prognosis of breast cancer to develop potent clinical targets.

With the development of gene sequencing technology, bioinformatics and big data, we are able to find essential genes related to breast tumor formation, invasion, and metastasis to provide a basis for accurate clinical treatment of breast cancer. This study chose coiled-coil domain-containing protein 69 (*CCDC69*) as the target gene through bioinformatics analysis. Researchers used *CCDC69* expression to predict tumor sample purity,[9] which is vital to immune infiltration.[10] Cui *et al.* found that *CCDC69* may reduce cisplatin resistance in ovarian cancer by activating P14ARF/MDM2/P53 signaling pathway.[11] Pal *et al.* proved that *CCDC69* engages in the assembly of control center spindles and the recruitment of central components. *CCDC69* can also reduce microtubule stability.[12] However, the connection between *CCDC69* and breast cancer is not clear. Survival analysis in OncoLnc (http://www.oncolnc.org/) database shows that breast cancer patients with high expression of *CCDC69* have a higher overall survival (OS) rate and better clinical prognosis. Therefore, we suppose that *CCDC69* may be a new biomarker and a potential therapeutic target for breast cancer.

In this study, we first analyzed the possible functions of *CCDC69* by bioinformatics analysis and found the *CCDC69* expression in breast cancer tissue was lower than that in normal breast tissue through GEO database and relative bioinformatics analysis. By real-time PCR and immunohistochemistry (IHC), we verified the difference and found that the expression of *CCDC69* in the triple-negative breast cancer (TNBC) tissue was lower than that in the non-TNBC tissue. We also showed a negative correlation between *CCDC69* and Ki-67. And the 4-year follow-up indicated a trend that TNBC patients with low *CCDC69* expression level had a lower disease-free survival (DFS). In addition, we analyzed the possible functions of *CCDC69* and its similar genes by bioinformatics analysis.

Materials and Methods

Tissue specimens

We collected fresh paired samples from resection specimens of patients who were admitted to the Department of Thyroid and Breast Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology in 2019 (n=24). All patients received surgical treatment with no chemotherapy, radiotherapy or any other adjuvant therapy. All patients were diagnosed as primary breast cancers (including a molecular classification) by the Department of Pathology, Tongji Hospital. All excised tissues were frozen immediately in liquid nitrogen. Breast cancer paraffin-embedded tissues were obtained from the Department of Pathology, Tongji Hospital in 2016. Follow-ups were terminated by September 2020.

Database and bioinformatics analysis

GEO Database Analysis and Venn Map: by searching in GEO (https://www.ncbi.nlm.nih.gov/gds), three datasets were obtained, including GSE22820, GSE29431 and GSE42568. More details of the series data are listed in Table 1. The threshold was determined as Log2-fold change (logFC) ≥ 2 or ≤ -2, p-value ≤ 0.05, and adjusted p-value ≤ 0.05. The selected different expressed genes were introduced into FunRich3.1.3 software to draw a Venn map, and the three groups of genes in the Venn map were intersected to get a new set of gene data.

OncoLnc Database Analysis: the Kaplan plot of each gene was drawn basing from the survival information of breast cancer patients in OncoLnc (http://www.oncolnc.org/). We identified the target gene that had not been studied in breast cancer.

Target gene expression analysis in TIMER and GEPIA: the different expression of the target gene in breast cancer tissue and normal breast tissue was analyzed by TIMER (https://cistrome.shinyapps.io/timer/) and GEPIA (http://gepia.cancer-pku.cn/detail.php?). We searched genes whose correlation coefficient with the target gene are higher than 0.8 in GEPIA. These genes were then inputted into the FunRich3.1.3 software as a data set for gene enrichment analysis (biological pathway, biological process) to explore the possible pathway of the target gene affecting the prognosis of breast cancer patients. The Pearson correlation between the target gene and similar genes was analyzed in GEPIA.

Quantitative real-time PCR

Total RNA extracted by TRIpureReagent (Adelai Biotechnology Co., Ltd., Beijing, China). Complementary DNA was prepared by using Random6 Primer. AceQ Universal SYBR qPCR Master Mix (Novozan Biotechnology Co., Ltd., Nanjing, China) was used to detect the expression of *CCDC69*. The PCR was performed as follows: 40 cycles at 95°C for 5 min, 95°C for 10 s, 60°C for 30 s. *GAPDH* was used as the internal reference gene. All amplifications were performed in triplicate. The sequence of primers used was as follows: CCDC69 (forward 5'-GTGGACAAACCCCGCAAATC-3', reverse 5'-CTGGCTACTGTCCCTTGGTG-3'); GAPDH (forward 5'-AATCCCATCACCATCTTCCAG-3'and reverse 5'-GAGCCCCAGCCTTCTCCAT-3'). The relative amount of target gene was calculated using the formula $2^{-\Delta\Delta Ct}$.

Immunohistochemistry

The dewaxed sections were repaired with EDTA (pH 9.0) antigen repair solution, sealed with BSA. The sections were incubated with anti-CCDC69 antibody (bs-6919R, Servicebio, Wuhan, China) overnight at 4°C, the slides were incubated with HRP goat anti-rabbit (GB23303, Servicebio, Wuhan, China) for 50 min. DAB staining, hematoxylin re-staining and dehydration sealing

Table 1. Information from the GEO datasets in the present study.

Series accession	Platform	Total genes	Sample (n)	Screening genes
GSE22820	GPL6480	41,000	Breast cancer (176) *vs* normal (10)	954
GSE29431	GPL570	54,675	Breast cancer (54) *vs* normal (12)	725
GSE42568	GPL570	54,675	Breast cancer (104) *vs* normal (17)	1195

tablets. Using HALO analysis software for automatic recognition, the cells stained blue are negative and the brown ones are positive (located in the nucleus). The positive rate (%) is equal to the number of positive cells / total cells x 100. To analyze SORBS1 expression by IHC in the same way, and the first antibody is anti-SORBS1 antibody (HPA027559, Promoter Biotechnology, Wuhan, China).

Statistical analyses

SPSS26 was used to analyze the data, and GraphpadPrism was used to draw statistical charts. The differential expression level of *CCDC69* in breast cancer and its adjacent normal tissues was analyzed using Wilcoxon matched pairs signed rank test. The correlation between CCDC69 and SH3 Domain-containing Protein 1 (SORBS1) and clinicopathological parameters was analyzed using Spearman correlation analysis. Differences of *CCDC69* expression among the five breast cancer types were analyzed using Univariate ANOVA analysis, and multiple LSD comparisons were made to compare the intra-group differences. The histogram of *CCDC69* of five breast cancer types and the bar chart of *CCDC69* of TNBC and non-TNBC groups was made in GraphpadPrism, and statistical methods were respectively univariate ANOVA analysis and unpaired t-test. The difference was statistically significant when $p<0.05$. Besides, this study counted the number of DFS events in the 4-year disease-free survival of the 101 breast cancer patients.

Results

Bioinformatics analysis and *CCDC69* expression in breast cancer

We searched certain genes which were significantly differently expressed between breast cancer and normal breast tissues from three breast cancer datasets in the GEO database, as shown in Table 1.

We inputted the differential genes into FunRich3.1.3 software to draw a Venn map to get a new data set containing 170 genes (Figure 1A). Through OncoLnc survival analysis and literature reviews of these genes, we found that the OS rate of breast cancer patients those with lower expression of *CCDC69* was lower, and there was no report about *CCDC69* in breast cancer research. Thus, we selected *CCDC69* as the target gene of this study. *CCDC69* expression was analyzed on TIMER and GEPIA. *CCDC69* expression in breast cancer tissue was significantly lower than that in normal breast tissue (Figure 1B, $p<0.001$, $p<0.05$). The Kaplanplot map of the effect of *CCDC69* on the survival rate of breast cancer patients in OncoLnc showed that breast cancer patients with low expression of *CCDC69* had a lower OS rate (Figure 1C, Logrank p-value=0.000698). Differential expression of *CCDC69* in breast cancer and its adjacent tissues was further verified by RT-PCR test. $2^{-\Delta\Delta Ct}$ values of 24 pairs of mRNA values obtained by RT-PCR test

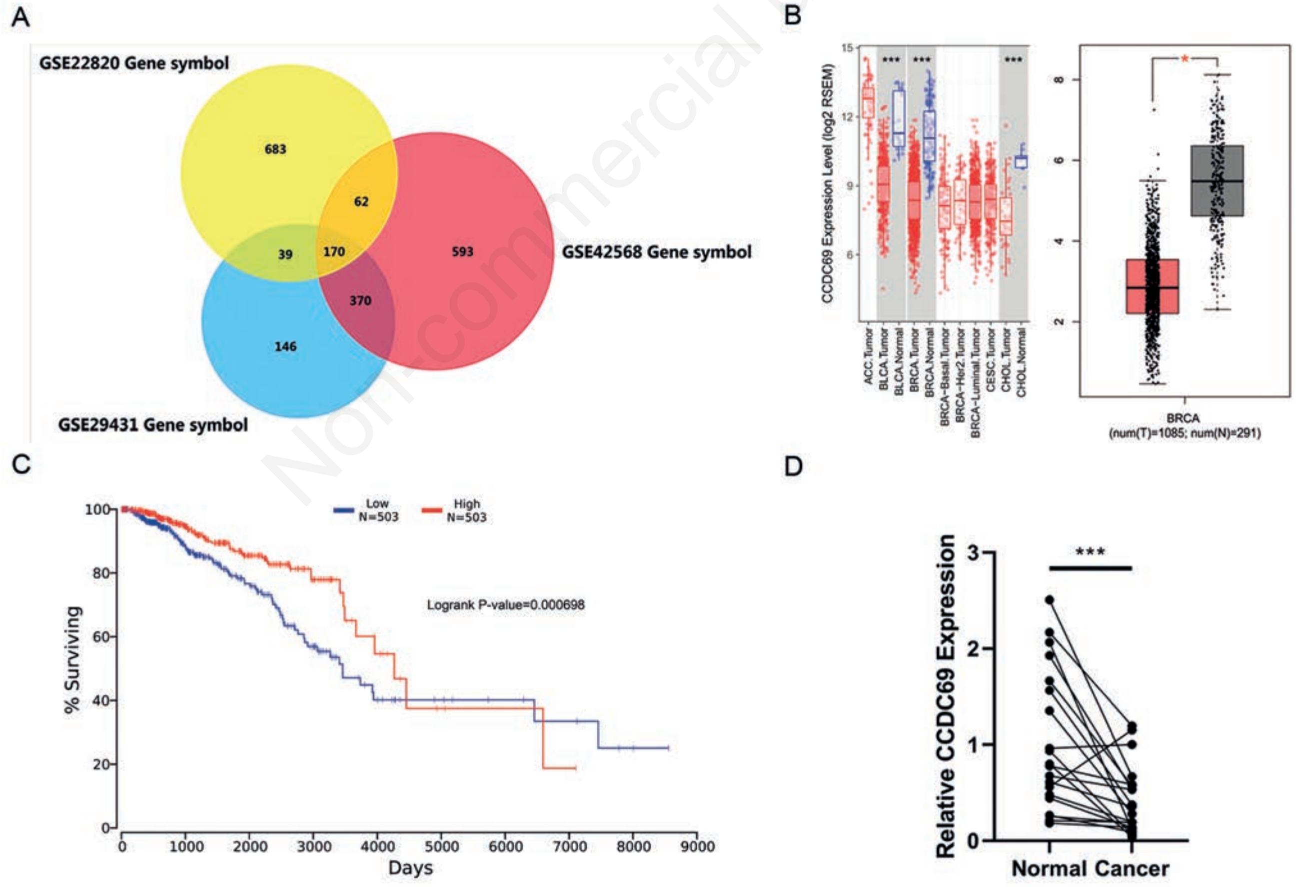

Figure 1. **Bioinformatics analysis of *CCDC69* expression in breast cancer. A) Wayne diagram of three groups of screened genes in FunRich3.1.3. B) Differential expression of *CCDC69* in TIMER (left, $p<0.001$) and GEPIA (right, $p<0.05$). C) *CCDC69* survival analysis diagram in OncoLnc (Logrank p-value=0.000698). D) The relative mRNA levels of *CCDC69* in breast cancer tissues and adjacent normal tissues (Wiring diagram, $p=0.0002$).**

indicated that *CCDC69* expression in breast cancer tissues was significantly lower than that in normal breast tissues (Figure 1D).

In this study, we found 117 similar genes with a correlation coefficient of more than 0.8 with *CCDC69* in GEPIA. Then 98 genes, including *CCDC69*, were identified by inputting them into FunRich3.1.3 software. These 98 genes were then analyzed by gene enrichment analysis (biological pathway, biological process), and we found that they were significantly associated with transcriptional regulation of white fat differentiation, lipid digestion, mobilization and transport, lipid metabolism, energy pathway (Figure 2 A,B). One of the similar genes is SORBS1, also known as c-Cbl-associated protein (CAP), an adaptor protein of the SOHO family. It is mainly expressed in adipose tissue, heart, skeletal muscle, and macrophages,[13,14] and is functioned through the PPAR signal pathway.[15] The correlation between *SORBS1* and *CCDC69* was analyzed on GEPIA, and the Pearson correlation coefficient was 0.89 (Figure 2C). Lipoprotein lipase (LPL) is another similar gene of *CCDC69*, and its Pearson correlation coefficient with CCDC69 is 0.82 (Figure 2D).

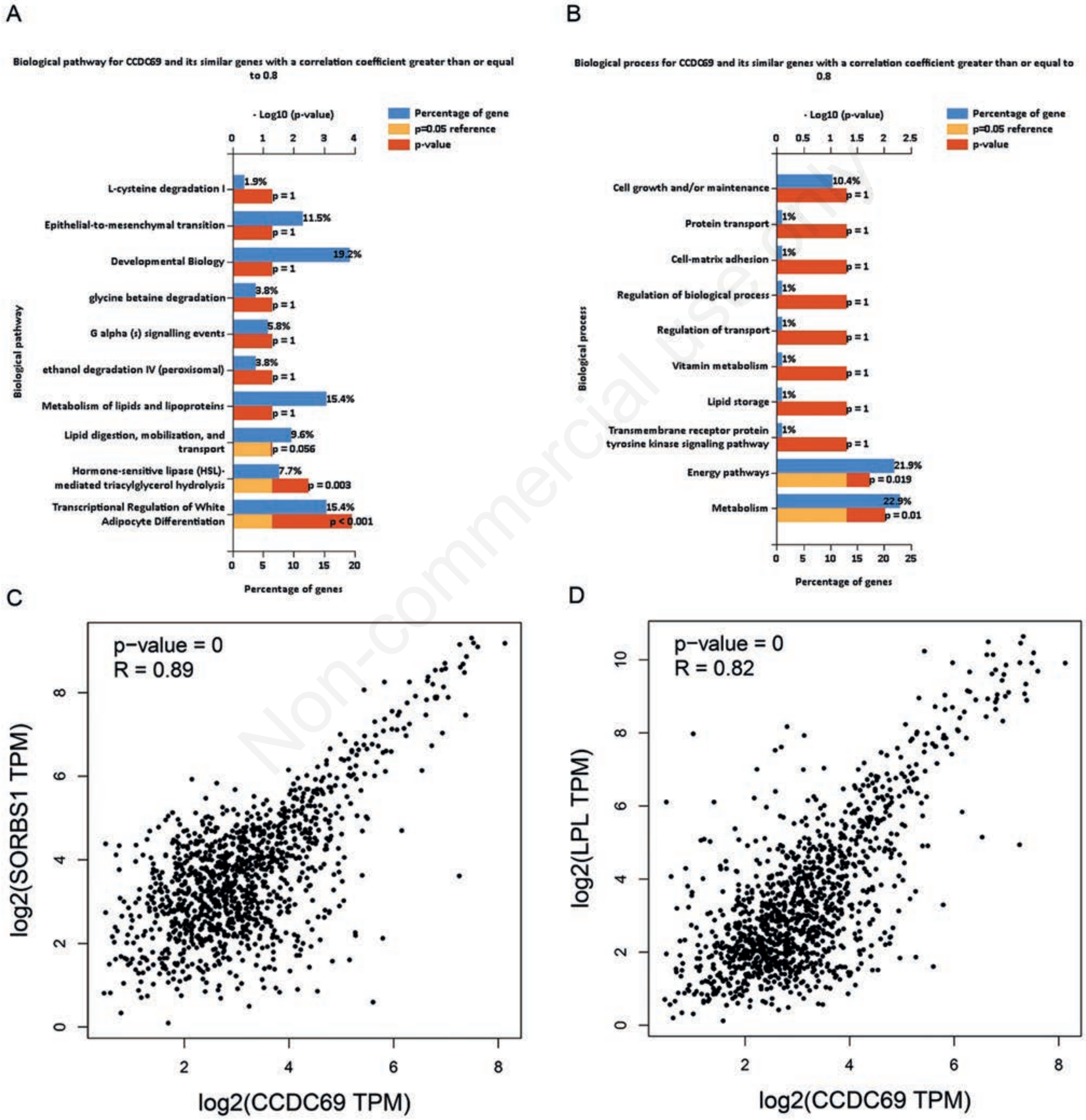

Figure 2. **Bioinformatics analysis of related pathways of *CCDC69*. A) Biological pathway of 98 genes. B) The biological process of 98 genes. C) Correlation between *CCDC69* and *SORBS1* in GEPIA database (R=0.89). D) Correlation between *CCDC69* and LPL in GEPIA database (R=0.82).**

Relationship between *CCDC69* expression and breast cancer

Correlations between *CCDC69* and *SORBS1* and clinicopathological parameters were analyzed in SPSS26 (Spearman correlation test). The results showed a positive correlation between *CCDC69* and *SORBS1* (p=0.001, Figure 3 A,B). The correlation analysis of clinicopathological parameters with CCDC69 in the SPSS26 (Spearman correlation test) showed a negative correlation between *CCDC69* and Ki67 (p=0.001, Table 2). Next, we used the One-sample Kolmogorov-Smirnov test to find whether *CCDC69* conformed to the normal distribution. The results showed that *CCDC69* was under normal distribution since p-value was 0.2. Then univariate ANOVA analysis was used to analyze the effect of five breast cancer types in the positive rate of *CCDC69*. The results showed no significant difference among the five types (p>0.05; Table 3, Figure 3C). However, when making multiple LSD comparisons, we found that the expression of CCDC69 in

Table 2. **Immunohistochemical staining of CCDC69 and its correlation with clinicopathological parameters of the BRCA cases.**

Characteristics	R	P (Spearman)
Weight	0.147	0.144
Age	0.130	0.194
WHO rating I, II / III	-0.066	0.523
Size (≤2 cm and >2 cm)	0.049	0.629
ER	0.060	0.550
PR	0.108	0.282
HER2	0.147	0.144
Ki67	-0.317	0.001
P53	0.160	0.116
N stage N0 / N1 / N2, N3	0.112	0.267
Clinicopathological staging I / II, III	0.043	0.666

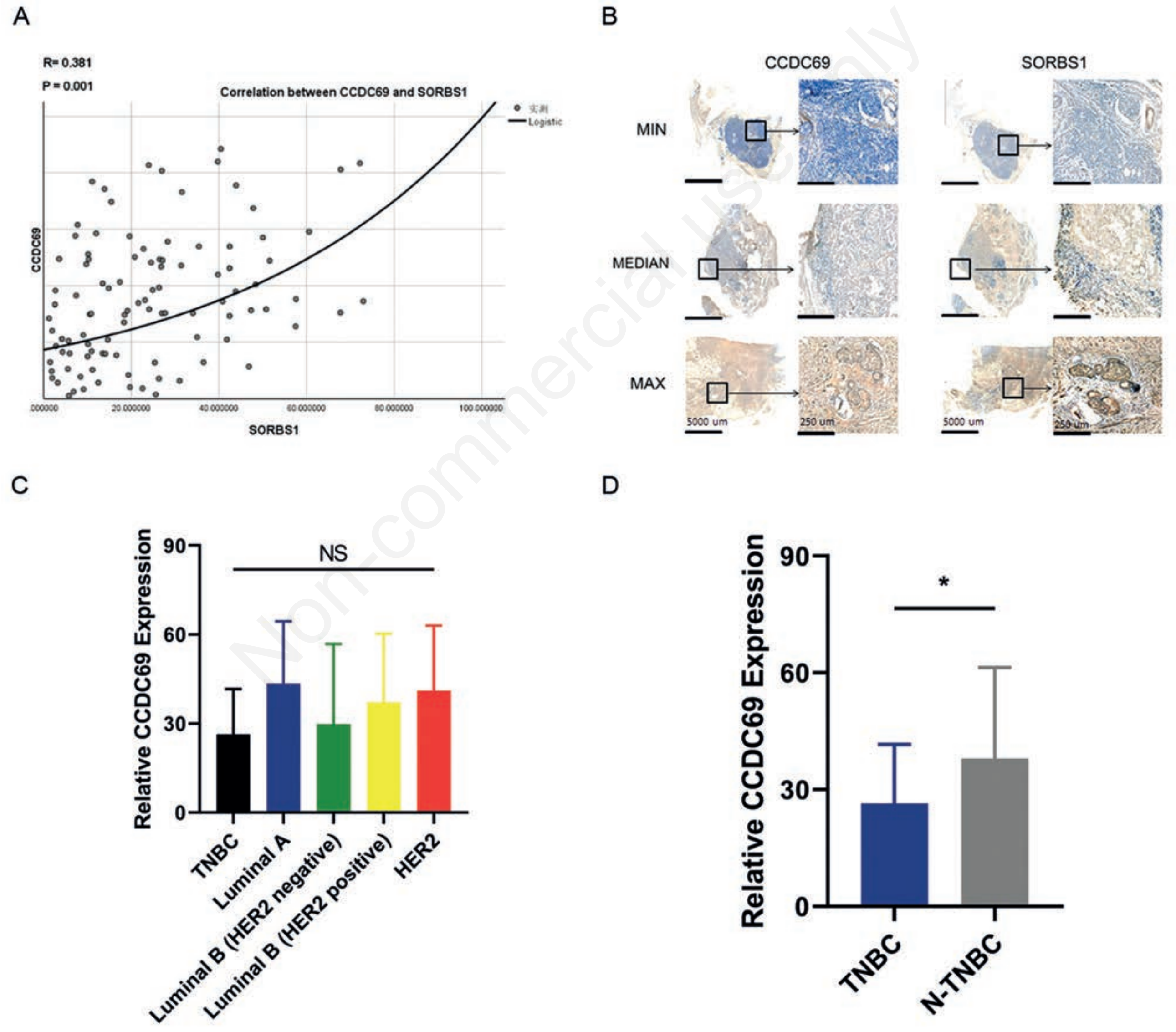

Figure 3. **The relationship between *CCDC69* expression and breast cancer. A) Correlation between *CCDC69* and *SORBS1* (r=0.381, p=0.001). B) The corresponding immunohistochemical images of *CCDC69* and SORBS1 when *CCDC69* were the MIN, the median, and the MAX (corresponding to the same breast cancer tissue section, diaminobenzidine staining, and hematoxylin re-staining) showed consistent expression. C) Histogram of *CCDC69* in five types of breast cancer, the overall difference was not statistically significant. D) *CCDC69* positive rate in triple-negative and non-triple negative breast cancer (unpaired t-test, p=0.0362).**

TNBC was significantly lower than that in Luminal A and HER2 breast cancer (p=0.014, p=0.034; Table 4). We made the histogram of *CCDC69* positive rate in five types of breast cancer and the bar chart of *CCDC69* positive rate in TNBC group and non-TNBC group in GraphPad Prism. The results showed that the expression of *CCDC69* in the TNBC group was lower than that in the non-TNBC group (unpaired *t*-test, p=0.0362, Figure 3D).

Furthermore, basing on the preliminary analysis of the 4-year DFS of these 101 breast cancer patients, we found that there were only 16 DFS events during the 4-year follow-up. A longer follow-up time was required to meet the statistical requirement. However, we found a trend that among the 16 DFS events, 5 DFS events occurred in TNBC. The *CCDC69* in these 5 cases of TNBC was low expression level (Table 5), and the positive rate of *CCDC69* was divided into high expression level and low expression level according to the median.

Discussion

More effective targets are in urgent need to improve TNBC treatment. In this study, *CCDC69* was our target gene. *CCDC69* expression in breast cancer tissue was significantly lower in the GEO database. Our RT-PCR results further confirmed that the expression of *CCDC69* in clinical breast cancer tissue was significantly lower than that in its adjacent normal tissue (p=0.0002). Simultaneously, the Kaplanplot map indicated that breast cancer patients with lower *CCDC69* expression showed a lower OS rate in OncoLnc. One study demonstrated that *CCDC69* is a potential downstream target of paired-liked homeodomain transcription factor 2 (Pitx2a). Up-regulation of Pitx2a increases the mRNA level of *CCDC69*,[12] while the high expression of Pitx2a is negatively correlated with breast cancer progression.[16] So, the high expression of *CCDC69* is also negatively correlated with breast cancer progression. Recently, some researchers have built a prognostic risk score system for Her2-positive breast cancer patients using the TCGA database's information. *CCDC69* expression is related to the OS of Her2-positive breast cancer patients. The univariate analysis shows that *CCDC69* is a low-risk factor for breast cancer.[17] It can be seen that the high expression of *CCDC69* is a protective factor for the prognosis of breast cancer patients.

Ki-67 is a protein encoded by the *MKi67* gene, which is closely related to cell proliferation and a predictive valuable parameter for breast cancer prognosis and treatment.[18,19] The TEXT and SOFT have shown that higher Ki-67 is a high-risk factor for breast cancer patients.[20] Patients with no decrease in Ki-67 after neoadjuvant chemotherapy have poor DFS and OS.[21] High expression of Ki-67 is an independent risk factor for poor prognosis of breast cancer.[22] The results of our study showed that there was a negative correlation between *CCDC69* and Ki-67 (p=0.001). Patients with lower expression of *CCDC69* had a higher Ki-67 index, stronger proliferation ability of breast cancer cells, and worse prognosis. The TNBC treating is limited, with high the risk of recurrence and metastasis.[23-26] The OS of patients with TNBC is worse than that of non-TNBC in all stages.[27] There is an urgent need for new molecular targeted drugs to treat TNBC. In this study, the positive rate of *CCDC69* in TNBC was lower. *CCDC69* may be one of the potential therapeutic targets for TNBC. Of the 16 DFS events we followed up, 5 DFS events occurred in TNBC The *CCDC69* expression in these 5 cases of TNBC showed a low level, indicating that the clinical prognosis of TNBC with low expression of *CCDC69* was worse. And we further confirmed that breast cancer patients with lower expression of *CCDC69* have lower survival rate on OncoLnc.

In order to further explore the possible mechanism of *CCDC69* in breast cancer, similar genes were detected by GEPIA. Through gene enrichment analysis, we found that these genes were significantly related to transcriptional regulation of white fat differentiation, lipid digestion, mobilization, transport, lipid metabolism, and energy pathway. We also found that most of these genes are related to the PPAR pathway. For example, retinal dehydrogenase 5 (RDH5) is associated with PPAR signal transduction.[28] *SORBS1*, perilipin 1 (*PLIN1*), and fatty acid binding protein 4 (*FABP4*) are biomarkers

Table 3. Univariate ANOVA analysis of *CCDC69* positive rate in five types of breast cancer.

Typing	Cases	*CCDC69* expression (%)	p
TNBC	21	26.41±15.29	0.068
Luminal A	20	43.51±20.96	0.068
Luminal B1	20	29.80±26.97	0.068
Luminal B2	20	37.22±23.07	0.068
HER2	20	41.11±21.96	0.068

Luminal B1, HER2 negative; Luminal B2, HER2 positive.

Table 4. The positive rate of *CCDC69* in five types of breast cancer: a post-mortem LSD multiple comparison.

Type one	Type two	95% CI	p
TNBC	Luminal A	-30.696～-3.505	0.014
	Luminal B1	-16.983～10.209	0.622
	Luminal B2	-24.405～2.787	0.118
	HER2	-28.293～-1.101	0.034
Luminal A	Luminal B1	-0.047～27.474	0.051
	Luminal B2	-7.469～20.052	0.366
	HER2	-11.357～16.164	0.730
Luminal B1	Luminal B2	-21.183～6.339	0.287
	HER2	-25.071～2.451	0.106
HER2	Luminal B2	-9.873～17.649	0.576

Luminal B1, HER2 negative; Luminal B2, HER2 positive.

Table 5. The occurrence of DFS events in five types of breast cancer.

Typing	Cases	DFS events (%)	*CCDC69* (%)	
			Low expression	High expression
TNBC	21	5 (31.25)	5 (31.25)	0 (0.00)
Luminal A	20	2 (12.50)	1 (6.25)	1 (6.25)
Luminal B1	20	2 (12.50)	2 (12.50)	0 (0.00)
Luminal B2	20	2 (12.50)	0 (0.00)	2 (12.50)
HER2	20	5 (31.25)	2 (12.50)	3 (18.75)
Total	101	16 (100.00)	10 (62.50)	6 (37.50)

Luminal B1, HER2 negative; Luminal B2, HER2 positive.

closely related to PPAR γ signaling pathway in breast cancer.[29] Besides, *SORBS1* and *LPL* are PPAR pathway genes.[15]

Breast cancer is a heterogeneous malignant tumor caused by various pathogenic reasons.[30] The plasticity of cancer cells plays a crucial role in the heterogeneity of tumors.[31] Epithelial-mesenchymal transition (EMT) refers to the biological process of transforming epithelial cells into phenotypic stromal cells. EMT can increase the ability of invasion and metastasis of breast cancer.[32] However, it is worth noting that EMT can also increase the plasticity of cancer cells, gain stem cell-like characteristics, and have the potential to transform into various cells.[33] PPAR is a group of nuclear protein receptors, involving in regulating stem cell EMT.[34-36] Recently, Ishay-Ronen *et al.* combined rosiglitazone (an agonist of PPARγ), and trametinib (an inhibitor of mitogen activation) to transform epithelial-mesenchymal transformed breast cancer cells into post-mitotic adipocytes to inhibit the invasion and metastasis of breast cancer.[37] Another similar method was reported to make breast cancer cells receiving EMT differentiate into adipocytes.[38] Besides, the ligand activation of PPAR γ receptor can induce the terminal differentiation of malignant breast epithelial cells and reduce the growth rate of cancer cells and the ability of Ketron formation.[39] Mycophenolic acid (MPA) can induce adipose terminal differentiation of breast cancer cell lines MDA-MB-231 and mcf-7 by activating PPAR γ and permanently withdrawing from the cell cycle G_0/G_1 phase.[40] Many studies have confirmed that the PPAR signal pathway is related to adipocyte differentiation and lipid metabolism.[41-44] It can be seen that the plasticity of breast cancer cells in the process of EMT make them have the potential of transdifferentiation therapy and is expected to become a new treatment for breast cancer, and PPAR γ is one principal target.

It has been reported that *CCDC69* localizes in the antiparallel overlapping microtubules of the central spindle in the later stage of the cell cycle, which may act as a microtubule destruction factor and a scaffold to control the assembly of the central spindle and recruit microtubules to the central spindle, which is related to the cytokinesis of animal cells.[12] Previously, we described the transdifferentiation effect of PPARγ agonists on breast cancer cells. We concluded that *CCDC69* might be involved in transforming cancer cells to adipocytes after the forced arrest of mitosis by the PPAR pathway. Upregulation of *CCDC69* can weaken the invasion and metastasis of breast cancer by activating PPARγ-induced lipid differentiation. Here, this study proposed a possible pathway of *CCDC69* on breast cancer worthy of further study.

SORBS1 is a gene in the PPAR signal pathway.[15] Studies have found that *SORBS1* is under expressed in breast cancer.[29] Yu *et al.* demonstrated that miR-142-5p promotes proliferation, invasion, and migration of breast cancer by targeting *SORBS1*.[45] Song *et al*. revealed that *SORBS1* silencing increases the migration and invasion of breast cancer cells by activating JNK/cJun and promotes EMT. The chemosensitivity was reduced by inhibiting p53.[46] In this study, the GEPIA database analysis showed a significant correlation between *CCDC69* and *SORBS1*, and immunohistochemical results confirmed a positive correlation between the expression of *CCDC69* and *SORBS1*. It is further suggested that the upregulation of *CCDC69* may improve breast cancer patients' prognosis through the PPAR signal pathway.

To sum up, breast cancer cells with lower expressions of *CCDC69* have stronger proliferation ability, worse pathological classification, and poorer clinical prognosis. *CCDC69* may affect the prognosis of breast cancer patients by inducing lipid differentiation through the PPAR signal pathway. This study provides a starting point for the study of *CCDC69* in breast cancer. We can further carry out cell experiments *in vitro* and animal experiments *in vivo* to study the biological cytological functions and related mechanisms such as the effect of *CCDC69* on the proliferation of TNBC cells, which can help to develop new target therapies for this cell population in the future.

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Received for publication: 10 December 2020. Accepted for publication: 27 January 2021.

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European Journal of Histochemistry 2021; 65:3207
doi:10.4081/ejh.2021.3207