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NDFIP1 as a Diagnostic Biomarker, Correlated with Immune Infiltrates in Breast Cancer

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Abstract

NDFIP1, an E3 ubiquitin ligase, plays a key role in multiple cancers. However, there is no detailed report on the role of NDFIP1 in Breast Cancer (BRCA). In this study, we used the Cancer Genome Atlas (TCGA) database to analyze the function of NDFIP1 in BRCA. NDFIP1 expression in clinical samples and cell lines was then validated using quantitative real-time PCR and immunohistochemistry. The ability of cells to proliferate, migrate and invade was determined with Cell Counting Kit-8 (CCK8), Transwell and wounding heal assays respectively. The results indicate that NDFIP1 expression was down-regulated and associated with the clinical characteristics of BRCA patients. In addition, the expression of NDFIP1 predicted poor immunotherapy response in specific patient groups; high NDFIP1 expression patients were with worse prognosis in four groups (CTLA4-PD1-, CTLA4-PD1+, CTLA4+PD1-, CTLA4+PD1+). Silencing NDFIP1, promoted the proliferation ability, migration ability and invasion ability of MCF-7 cells, MCF-7/ADM cells and MDA-MB-231 cells. In conclusion, NDFIP1 is a potential prognostic marker that can be used to determine immune infiltrates and BRCA's progress.

Keywords: Bioinformatics; Breast cancer; NDFIP1; Prognosis; Immune infiltrates

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Introduction

The annual incidence of Breast Cancer (BRCA) became the highest among cancers in 2021 [1]. About 10% of all BRCA cases are genetically-based and vary by region and ethnicity [2]. Depending on tumor characteristics, BRCA can be divided into three subtypes: Luminal, Human Epidermal Growth factor receptor (HER2+), and Triple-Negative Breast Cancer (TNBC) [3]. HER2+ BRCA and TNBC are more likely to express Tumor-Infiltrating Lymphocytes (TILs) compared to luminal BRCA. However, the classification of BRCA is complex and the incidence of postoperative recurrence is high. In recent years, antitumor immunotherapy using immune checkpoint inhibitors of Cytotoxic T-Lymphocyte-Associated protein 4 (CTLA-4) and Programmed Cell Death-1 (PD-1) has attracted much attention, although fewer immunotherapeutic agents have been proposed for breast cancer. Less than 20% of patients benefit from immunotherapy [4-6].

Therefore, new molecules are needed to improve the efficacy of BRCA treatment.

NDFIP1, a developmentally downregulated protein 4 (NEDD4) family interacting protein, can activate the E3 ubiquitin ligase of the NEDD4 family [7,8]. NDFIP1 protein is mostly expressed in various tissues, especially in brain tissues [9]. However, it has not been reported whether NDFIP1 is associated with immune infiltrates in BRCA. Thus, in this study we investigated the value of NDFIP1 in immune infiltrates in BRCA.

To determine whether NDFIP1 could act as a potential biomarker and is related to immune infiltrates, we obtained transcriptome data and clinical data from the Cancer Genome Atlas database (TCGA) and cBioPortal project. By using the CIBERSORT method and the TISIDB database, we explored the correlation NDFIP1 and immune infiltrates. *In vitro*, we detected NDFIP1 inhibited progress of BRCA or not *in vitro*. Our data demonstrated that NDFIP1 is related to immune infiltrates and progress of BRCA.

Methods and Materials

Data acquisition

We retrieved the transcriptome profiles of 1208 BRCA cases (only females; normal samples, 112 cases; tumor samples, 1096 cases) from TCGA database (https://portal.gdc.cancer.gov/) and the extracted the relevant clinical data based on the cBioPortal project (https://www.cbioportal.org/) (Table 1). All subsequent analyses were performed with R software (v 4.1.3).

The tissue samples used in this investigation was taken from the Affiliated Shandong Province Hospital of Shandong First Medical University. Tissue microarrays were constructed as a follow-up experiment, which included luminal A BRCA in 20 cases, luminal B BRCA in 26 cases, enriched HER2 in 30 cases, and TNBC in 34 cases (Table 2). The study was approved by the Ethics Committee of the Affiliated Shandong Province Hospital of Shandong First Medical University.

Table 1: Clinicopathological features of the BRCA patients.

Clinical characteristics		TCGA database (n=1084)	
		n	%
Age	<65	769	70.9
	≥ 65	315	29.1
Gender	Male	12	1.11
	Female	1072	98.9
Stage	I	181	16.7
	II	617	56.9
	III	249	23
	IV	19	1.75
	Unknow	18	1.66
T classification	T1	277	25.6
	T2	628	57.9
	T3	137	12.6
	T4	39	3.6
	Unknow	3	0.28
N classification	N0	512	47.2
	N1	357	32.9
	N2	119	11
	N3	76	7.01
	Unknow	20	1.85
M classification	M0	901	83.1
	M1	21	1.94
	Unknow	162	14.9
Race	Asian	60	5.54
	African American	183	16.9
	White	751	69.3
	Unknow	90	8.3
Molecular subtype	Normal	36	3.32
	Luminal	696	64.2
	Her2	78	7.2
	Basal	171	15.8
	Unknow	103	9.5

Immunohistochemistry staining

Immunohistochemistry (IHC) was performed as previously described [10]. All paraffin-embedded tumor tissues (FFEP) were transformed into microarray series and all slides were stained for NDFIP1 expression. We used the following antibodies to perform immunostaining: NDFIP1 antibody (1:100, ab236892, Abcam, USA) and goat anti-rabbit IgG H&L (HRP) (1:1000, ab6721, Abcam, USA). IHC scores were calculated from 0 to 12 by the Percentage of Positive (PP) cells (PP: 0, <5%; 1, 5-10%; 2, 11-50%; 3, 51-80%; 4, >80%) and intensity of staining (SI: 0, negative; 1, weak; 2, moderate; 3, strong) [11].

Kaplan Meier plotter database

The Kaplan-Meier plotter (http://kmplot.com/analysis/) was used to estimate the correlation between gene expression and survival in 21 types of tumors. Patient samples were divided into two groups (high and low expression) and selected with the best cutoff value.

Protein-Protein interaction network and functional annotations

We constructed the Protein-Protein Interaction (PPI) network using the STRING database (https://cn.string-db.org/). The minimum required interaction score between proteins was 0.4. R analyzed Gene Ontology Biological Process (GOBP).

The human protein atlas database

The Human Protein Atlas (HPA) database (https://www.proteinatlas.org/) is a Swedish-based program initiated in 2003 that

Table 2: Clinicopathological features of the BRCA patients.

Clinical characteristics		Sample (n=110)	
		n	%
Age	≤ 55	69	62.7
	>55	41	37.3
Gender	Male	0	0
	Female	110	100
T classification	T1a/b	8	6.7
	T1c	45	40.9
	T2	56	50.9
	T3	1	1.5
Nodal status	Positive	40	36.4
	Negative	59	53.6
	Unknow	11	10.0
ER status	Positive	65	59.1
	Negative	45	40.9
Her2 status	Positive	43	39.1
	Negative	67	60.9
Ki67 status	≤ 20%	41	37.3
	>20%	69	62.7
P53 status	Positive	64	58.2
	Negative	46	41.8
Molecular subtype	Luminal A	20	18.2
	Luminal B	26	23.6
	Her2	30	27.3
	TNBC	34	30.9

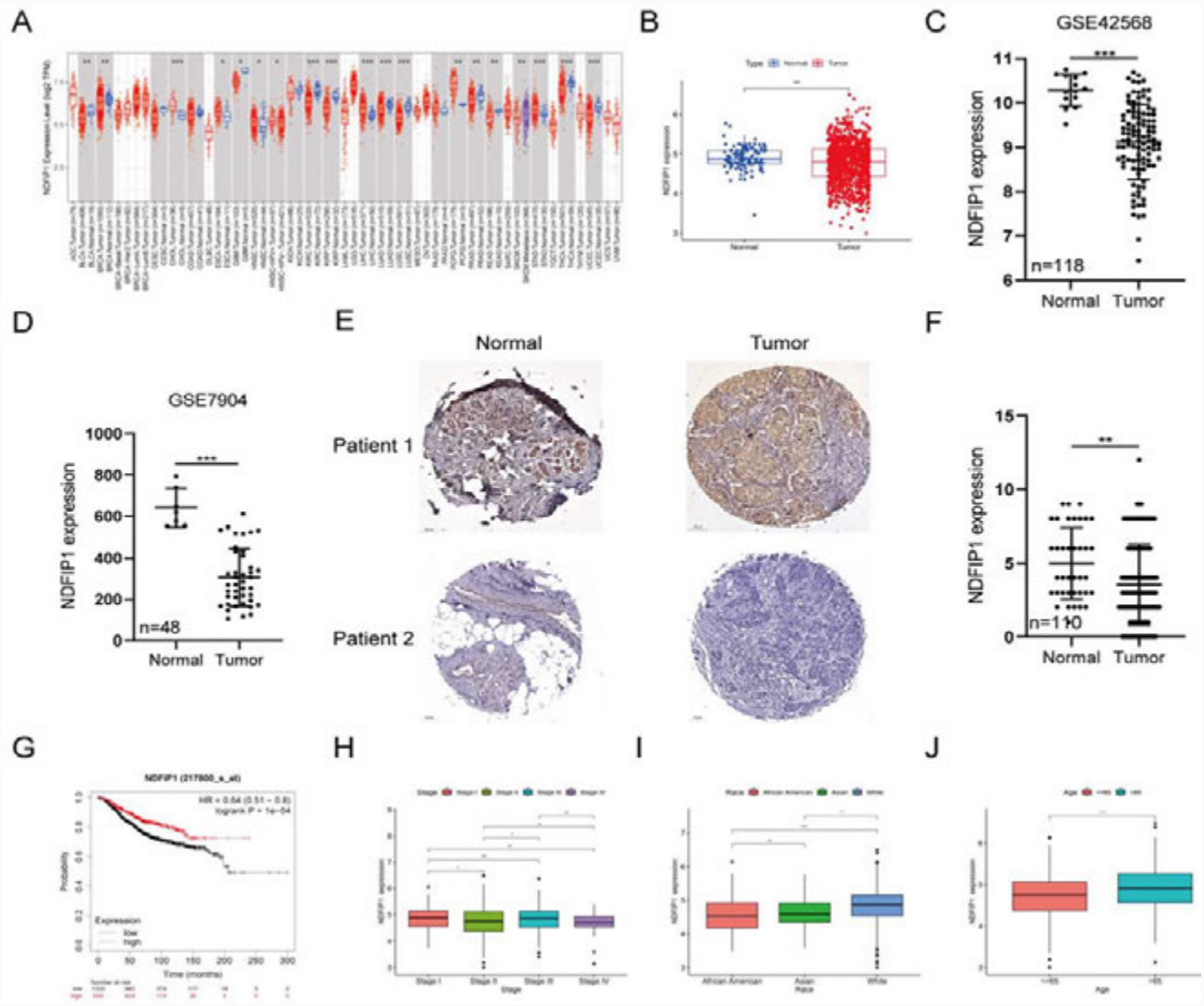

Figure 1: NDFIP1 expression is down-regulated in BRCA. NDFIP1 mRNA level in pan-cancer. (A) and BRCA (B-D) from TCGA and GEO database. (E, F) NDFIP1 protein levels in BRCA (Normal *vs.* Tumor), including luminal, her2 and TNBC. IHC score of NDFIP1 staining based on our own clinical samples. (G) OS of NDFIP1 in BRCA, as determined by the GEPIA database. (H-J) The correlation between NDFIP1 expression and clinical features.

focuses on mapping all human proteins [12]. Each slide containing IHC stained specimens was examined for intensity, quantity, location, and patient information.

TISIDB database

The TISIDB database was used to analyze the levels of NDFIP1 in multiple subtypes of BRCA and the correlations between NDFIP1 and immune-related molecules in this study (http://cis.hku.hk/TISIDB/index.php).

Cell culture and transfection

The MCF-7 cell line and MCF-7/ADM cell line obtained from Shanghai Biowing, Ltd., and the MDA-MB-231 cell line procured from Procell Life Science & Technology Co., Ltd. (China) were cultured at 37°C, and supplied with 5% CO_2 in a cell incubator. The MCF-7 cell line and MDA-MB-231 cell line were treated with DMEM (Gibco, USA), 12% fetal bovine serum (FBS, Cell-box, China), and 1% antibiotics (100 units/mL penicillin and 100 μg/mL; Solarbio, China). The MCF-7/ADM cell line were treated with RPMI 1640 (Gibco, USA), 12% FBS and 1% antibiotics. The expression of NDFIP1 was downregulated by transfection of small interfering RNA (siRNA) (RIBOBIO, China). The sequences were as follows: siRNA#1(Homo-NDFIP1-247), 5' - C A U U C C U C U U U A A C U G G A U U G G G U U - 3' and siRNA#2(Homo-NDFIP1-643), 5' - C A U U U C A G G A U U U G G U C U C U C U C U A - 3'.

Reverse transcription-PCR and quantitative real-time PCR

Total RNA was collected from cell lines using TRNzol (Tiangen, China), RNA used in the HisScript® III 1st Strand cDNA Synthesis Kit (Vazyme, China) was reversely transcribed into cDNA. The quantitative Real-Time PCR (qRT-PCR) was analyzed using ChamQ Universal SYBR qPCR Master Mix (Vazyme, China). The expression of NDFIP1 was computed using the $2^{-\Delta\Delta Ct}$ method. The primers were as follows: Homo-NDFIP1 forward primer, 5' - T A C T G G C T C T G G T G G G T G T T - 3'; Homo-NDFIP1 reverse primer, 5' - A C T C T G G T C C T G G G G A G A T T - 3'.

Proliferation assay, Transwell assay and wounding heal assay

CCK8 Kit was purchased from NCM Biotech (China). Cell

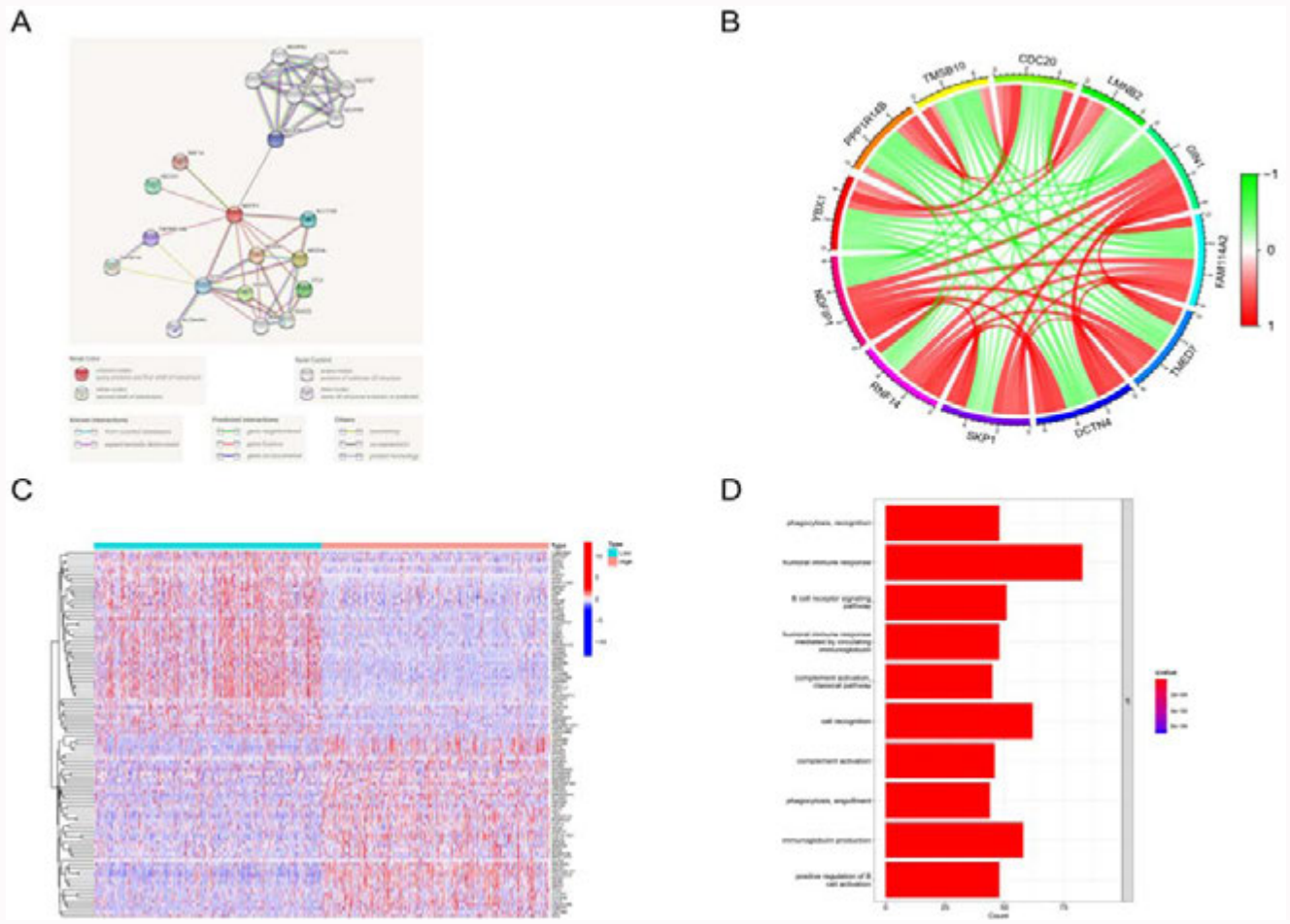

Figure 2: Co-expression and enrichment analysis of NDFIP1. (A) NDFIP1 PPI network produced by the STRING online server. (B) Co-expression loop map of NDFIP1. Red means positive correlation and green means negative correlation. (C) The heatmap of the top 100 genes with positive and negative correlation in the high and low expression groups of NDFIP1. (D) The bar graph of the GOBP enrichment analysis.

proliferation, migration and invasion were carried out as previously studied [13].

Statistical analysis

Statistical analysis was implemented using GraphPad Prism 8.0 (USA). Data are presented as mean ± Standard Deviation (SD). Comparison of the IHC score between two groups was performed using the Mann-Whitney U test. The downregulation of NDFIP1 in cells was verified using a student's t-test. The correlation analysis was estimated using the Spearman correlation test. Two-tailed p-value <0.05 was considered statistically significant (*p<0.05, **p<0.01, ***p<0.001).

Results

Comparison of NDFIP1 expression

Compared to breast normal tissues, we found that the level of NDFIP1 protein was down-regulated in tumor tissues (Figure 1A, 1B). Simultaneously, the results of two GEO datasets, namely GSE42568 and GSE7904, showed the same trend (Figure 1C, 1D). We could see that NDFIP1 protein level in breast normal tissues was higher than in tumor tissues by using IHC staining (Figure 1E, 1F). We also investigated OS of NDFIP1 in BRCA and found that there was a poor prognosis with high-expression NDFIP1 from Kaplan-Meier plotter online tool (Figure 1G). To explore the association between NDFIP1 and clinical characteristics of patients with BRCA, we downloaded clinical data from cBioPortal database. The results indicated that NDFIP1 expression was related to age (p<0.001), tumor stage (stage II *vs.* stage I, p=0.016; stage III *vs.* stage II, p=0.041), race (African American *vs.* White, p<0.001; Asian *vs.* White, p=0.0024) (Figures 1H-1J).

Functional annotations and co-expression analysis

The PPI network of NDFIP1 was built using the STRING online server (PPI enrichment p-value <0.001) was shown in Figure 2A. We drew an NDFIP1 co-expression loop map using TCGA dataset (Figure 2B), including the top six strongest positively expressed genes (DCTN4, FAM114A2, GIN1, RNF14, SKP1, TMED7) and the top five strongest negatively expressed genes (CDC20, LMNB2, PPP1R14B, TMSB10, YBX1). Moreover, median NDFIP1 expression was used as the cut-off value and was divided into high- and low-expression groups. A total of 1982 Differential Expression Genes (DEGs) were obtained from TCGA dataset, including 1525 downregulated DEGs and 457 upregulated DEGs (|Log2 FC|>1, p<0.05). According to |Log2 FC| values, a heatmap of the top 50 downregulated DEGs and upregulated DEGs was constructed (p<0.05) (Figure 2C). The Biological Process analysis of Gene Ontology (GOBP) is shown in Figure 2D. We found that GOBP enrichment results focused mainly on immune-related function.

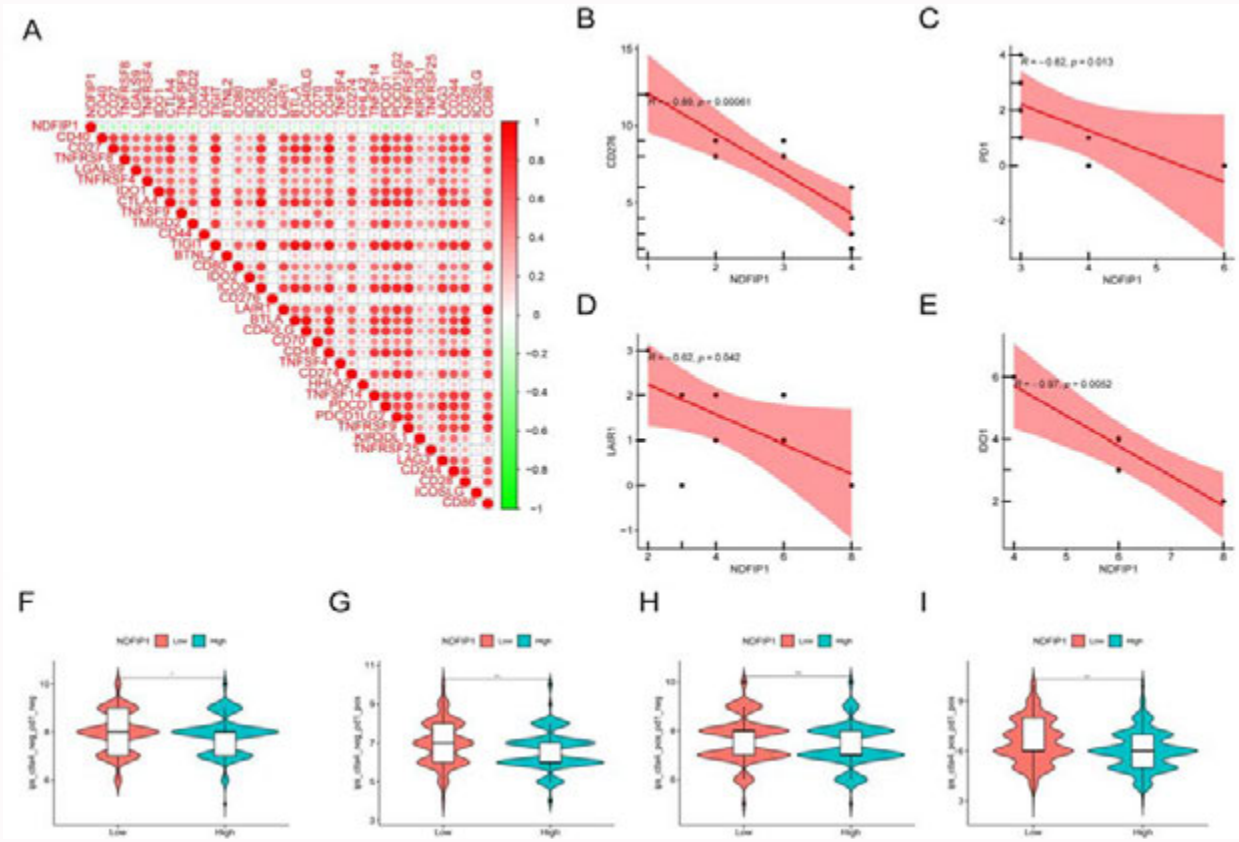

Figure 3: Immune checkpoints and immunotherapy analysis for NDFIP1. The relationship between NDFIP1 expression and immune checkpoint from TCGA database (A) and HPA database (B-E) and immunotherapy (F-I) in BRCA.

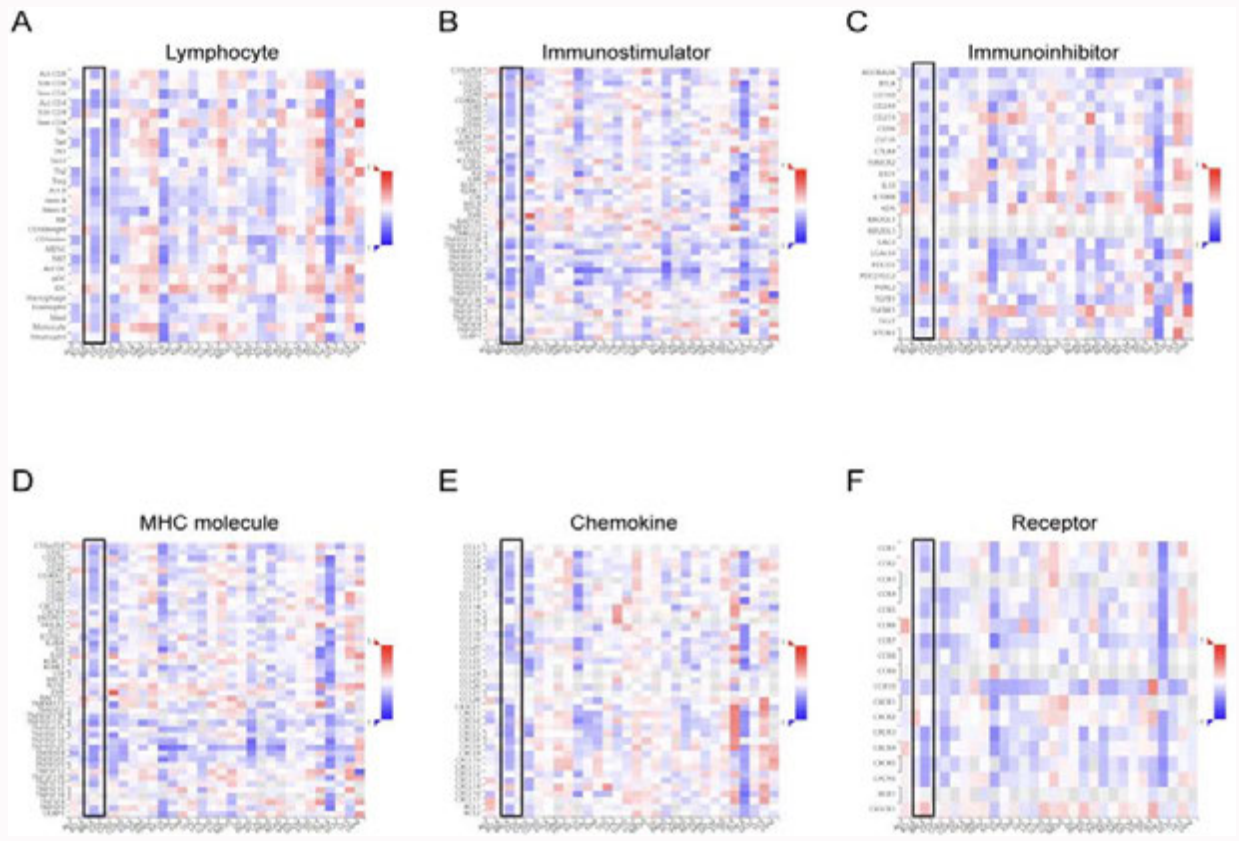

Figure 4: The correlation of NDFIP1 expression with immune-related molecules from the TISIDB database in cancer. (A) Lymphocyte. (B) Immunostimulator. (C) Immunoinhibitor. (D) MHC molecule. (E) Chemokine. (F) Receptor.

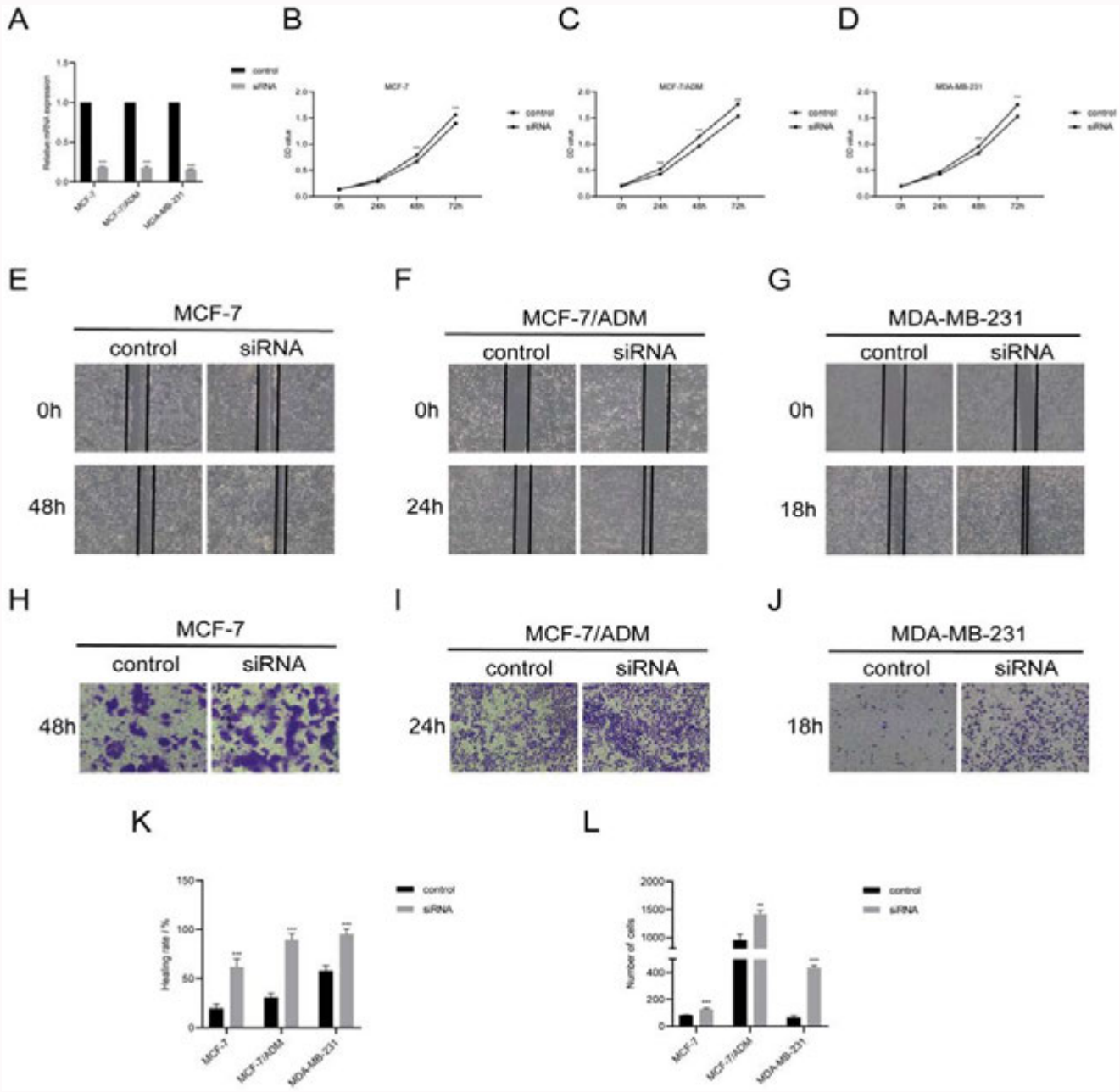

Figure 5: NDFIP1 inhibited BRCA development *in vitro*. (A) Ectopic NDFIP1 mRNA expression of three cell lines. (B-D) CCK8 assay, (E-F, K) wounding heal assay and (H-J, L) Transwell assay transfected with siRNA in three cell lines.

Association of NDFIP1 expression with immune-related function

Based on TCGA database, we analyzed the relationship between NDFIP1 and immune checkpoints in BRCA. There was a negative correlation between the two, including PDCD1/PD1 and CTLA4 (Figure 3A). We then obtained IHC scores to verify the correlation between NDFIP1 and four immune checkpoints (CD276, IDO1, PD1 and LAIR1) using the HPA database (Figures 3B-3E). We observed the same trend as in Figure 3A. The immunotherapy effect of the NDFIP1 low expression group was superior in the four groups (CTLA4-PD1-, CTLA4 PD1+, CTLA4+PD1-, CTLA4+PD1+) (Figures 3F-3I). To reveal whether NDFIP1 expression was related to immune-related molecules in BRCA, we also correlated the expression of NDFIP1 with six factors from the TISIDB database (Figure 4). There was a strongly negative correlation between NDFIP1 expression and immune-related molecules, including lymphocyte (Figure 4A), immunostimulatory (Figure 4B), immunoinhibitory (Figure 4C), MHC molecule (Figure 4D), chemokine (Figure 4E) and receptor (Figure 4F). The above results showed that NDFIP1 expression was correlated with immune-related function in BRCA.

Function of NDFIP1 in BRCA cells

MRNA level of NDFIP1 were down-regulated in three cancer cells transfected with siRNA (Figure 5A). Silencing NDFIP1, promoted proliferation ability of three cells using CCK8 assay (Figures 5B-5D). Wounding heal assay demonstrated that migration ability of three cell lines was higher in transfected groups than in control group (Figures 5E-5G, 5K). Transwell assay proved that invasion ability of

three cell lines was increased after transfected siRNA (Figures 5H-5J, 5L). Overall, these findings suggested that NDFIP1 inhibited process of BRCA *in vitro*.

Discussion

In recent years, emerging therapies, such as immunotherapy, have been used in clinical practice, but drug resistance often occurs during treatment due to tumor heterogeneity [14-16]. This has posed a significant challenge on the treatment of BRCA. Therefore, additional biomarkers need to be introduced into clinical practice. In this study, we provide experimental data supporting NDFIP1 as a prognostic biomarker and its potential association with immune infiltrates and progress of BRCA. We used bioinformatics analysis from TCGA database and experimental *in vitro* validation.

Herein, we showed that NDFIP1 expression was related to the prognosis of patients with BRCA. High expression of NDFIP1 mRNA was associated with shorter OS. As shown in Figures 1A-1G, compared with regular group, the expression of NDFIP1 protein measured by IHC staining was higher in tumor group. NDFIP1 expression was strongly correlated with race, age and stage. Collectively, our experimental data implied that NDFIP1 could be a potential prognostic biomarker in BRCA.

Our results also demonstrated that NDFIP1 was correlated with immune infiltrates. The results of GOBP were mainly enriched in immune-related functions, comprising the humoral immune response, B-cell receptor signaling pathway, complement activation, immunoglobulin production, and positive regulation of B-cell activation. Then we observed a negative correlation between NDFIP1 and the expression of immune checkpoints, indicating that the immunotherapy response of patients with lower expression of NDFIP1 may be superior to than that of patients with high expression. Lu et al. [17] found that an innovative nanotherapy approach can improve the immune response by combination therapy with IDO1 inhibitors and immune checkpoint blocking antibodies. In recent years, phase I trials have shown that immune checkpoint inhibitor therapy combined with conventional chemotherapy is more effective in the treatment of BRCA [18]. Furthermore, our study revealed that NDFIP1 expression was negatively correlated with immune-related molecules using the TISIDB database. Comparably, a negative relationship has been reported between SYT16 expression and the degree of immune cell infiltration in lower-grade glioma [19]. A similar observation was documented by Dong et al., whereby IL27 was positively correlated to the levels of antitumor immune cells [20]. Therefore, we can conclude that there was a strong relationship between NDFIP1 and immune infiltrates in BRCA.

NDFIP1 is a transmembrane binding protein and an important member of the NEDD4 family. Although the mechanism of NDFIP1 activity in BRCA is unclear, it has been shown that NEDD4 is closely related to the development of BRCA. NEDD4 overexpression also promotes the malignant phenotype of MDA-MB-231 cells [21]. However, there have been no detailed reports on NDFIP1 activity in BRCA. Transwell assay and wounding heal assay proved that NDFIP1 can promote progress of BRCA. Based on our results and those of previous studies of NEDD4, we believe that NDFIP1 can influence the occurrence and development of BRCA.

Our study had several limitations. First, all bioinformatics data were derived from public databases, which may have additional results if others used different dataset. Second, a tumorigenesis was not evaluated in terms of dox sensitivity in nude mice, and there was a lack of *in vivo* experimental evidence.

In summary, our study demonstrated that NDFIP1 expression was closely related to immune infiltration and progress of BRCA. However, considering that NDFIP1 has not been described in BRCA and our data are limited, we cautiously draw the conclusion that NDFIP1 may serve as a prognostic biomarker in BRCA, and may provide a new target for the clinical treatment of BRCA.

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