E3 Ubiquitin Ligase NEDD4L Negatively Regulates Breast Cancer Metastasis By Downregulating SNAI2

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Abstract

Background

Accumulating evidence demonstrated that the abnormal expression of E3-ubiquitin ligase NEDD4L plays an important role in the biological process of carcinomas. However, its role in breast cancer (BRCA) remains elusive.

Methods

The expression of *NEDD4L* was analyzed using BRCA datasets from public database. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used for enrichment analysis. Both transwell assay and pulmonary metastasis model of BRCA were used to detect metastatic ability of cells. Western blotting, immunohistochemistry and immunofluorescence assay were used to detect the protein expression of target genes. The riskScore and nomogram were used to evaluate the prognosis of patients.

Results

NEDD4L was significantly downregulated in cancer tissues and positively correlated with the overall survival of patients. Knocking down NEDD4L could enhance the migration and metastasis ability of BRCA cells. SP1 promotes NEDD4L expression, resulting in SNAI2 downregulation in BRCA. A NEDD4L related to the prognostic model developed by LASSO Cox regression could be an independent predictive factor for BRCA. A nomogram combining riskScore and clinical indicators was established to evaluate the prognosis of BRCA quantitatively.

Conclusions

This study first reveals the role of the SP1/NEDD4L/SNAI2 axis in BRCA and establishes a reliable prognostic model, which provides a novel target and basis for clinical treatment and prognosis evaluation of breast cancer.

Introduction

The morbidity and mortality of breast cancer (BRCA) are first and second among US women [1, 2]. In 2021, 281,550 female BRCA will be newly diagnosed, while 43,600 women will die from BRCA in the United States. In 2021, about 281,550 females will be diagnosed with BRCA, and 43,600 women will die from BRCA in the US [2]. The 5-year survival rate of BRCA decreased significantly with the increase of pathological stage [2]. Metastasis is the leading cause of death from breast cancer [3]. Therefore, an in-depth study of the metastasis mechanism of BRCA is essential for the diagnosis and treatment of BRCA.

Tumor metastasis is a multi-step dynamic process, and TGF- β signaling plays a key role in the process. TGF- β can promote tumorigenesis by inducing epithelial to mesenchymal transition (EMT) via SMAD/non-SMAD pathways [4]. TGF- β /SMAD pathway induced EMT by upregulating the expression of E-cadherin transcriptional repressors, such as SNAIL1/2, ZEB1/2, and TWIST. TGF- β /non-SMAD pathway induced EMT by promoting cytoskeletal remodeling and resulting in activation of ERK[4]. Non-SMAD signaling can also be initiated directly downstream of the TGF- β receptor by activating the MAPK pathway, Rho-like GTPase pathway, and PI3K/AKT pathway [4]. Cumulative evidence showed that the activity of TGF- β signaling is inhibited by NEDD4L [6–8], which is a member of the NEDD4 E3 ubiquitin ligase subfamily [9].

Massive evidence shows that NEDD4L plays a vital role in regulating membrane proteins, crucial for maintaining cellular homeostasis. Previous studies have been confirmed that the epithelial sodium channel (ENaC) [10],renal Na⁺-Cl⁻ cotransporter (NCC) [11], voltage-gated sodium channels (Navs) [12], chloride channels [13], potassium channels [14], and surfactant protein C (SP-C) [15] were regulated by NEDD4L [9]. In addition, NEDD4L is considered to act as a tumor suppressor due to its potential functions in regulating EGFR [16], TGF- β [17], and Wnt [18] pathways. Previous studies have been showed that NEDD4L is down-regulated in colorectal cancer[19], non-small cell lung cancer [20], gastric cancer [21], ovarian cancer [22], glioma [23], prostate cancer [24], clear-cell renal cell cancer [25]. However, *Kito et al.* report that NEDD4L acted as an oncogene in melanoma [26]. These inconsistencies may be due to tumor specificity. However, the role of NEDD4L in BRCA remains unclear and needs to be further studied.

In this study, bioinformatics was used to analyze the expression level of NEDD4L in multiple BRCA datasets. It was found that the expression level of NEDD4L in BRCA was significantly lower than normal breast tissues and was positively correlated with the prognosis of patients.

This study analyzed the expression of NEDD4L in multiple breast cancer datasets and found it was down-regulated and correlated to poor prognosis. Subsequently, we found that the expression of NEDD4L was regulated by transcription factor Sp1. Finally, we confirmed that NEDD4L inhibited TGF- β signaling by down-regulating SNAIL2 expression, thereby inhibiting EMT and metastasis of BRCA. This study is first to reveal the mechanism of the SP1/NEDD4L/SNAIL2 axis and provide a new potential target for the treatment of BRCA.

Materials And Methods

Data acquisition and processing: The BRCA project of the TCGA (TCGA_BRCA) dataset, METABRIC data, and GSE42568 dataset were acquired from the GDC hub of the UCSC Xena website (<http://xena.ucsc.edu/public>) and the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/gds/>), respectively. Data were processed as described in our previous study [25].

Online database analysis: A meta-analysis of NEDD4L transcriptional level in BRCA was performed by the Oncomine database (<https://www.oncomine.org/resource/login.html>). NEDD4L expression data were

retrieved from 31 BRCA datasets containing normal and cancer tissues. Data were processed as described in our previous study [25].

Functional analysis and enrichment analysis: Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed by the clusterProfiler package in R version 4.1.0. C2.cp.kegg.v7.1.symbols.gmt was chosen as the gene set database. Signaling pathways that meet the following criteria can be significantly enriched: nominal P -value < 0.05, P -value < 0.25, and normalized enrichment score > 1 [25].

Development of the prognostic model: The least absolute shrinkage and selection operator (LASSO) Cox regression analysis was performed using the glmnet package in R as described in our previous study [25]. A new score was calculated by the key genes and their corresponding coefficients generated by LASSO Cox regression analysis, as follows:

Score = $-0.3089 \times \text{CCDC24} + 0.2944 \times \text{CHORDC1} + 0.3609 \times \text{FAM114A1} + 0.2795 \times \text{EPB41L4B} - 0.4132 \times \text{GPR171}$. In order to make the results applicable to different datasets, the riskScore was calculated by the following formula:

riskScore = $(\text{Score} - \min(\text{Score})) / \text{abs}(\max(\text{Score}))$ [25].

Development and evaluation of the nomogram

The nomogram was developed by the independent risk factors determined by multivariate Cox regression analysis, used to predict the total overall survival (OS) probability described in the previous study [25].

Cell culture and treatment

Human BRCA cancer MD-MBA-231 and BT549 cells were obtained from Cell Bank of Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. Cells were cultured in L-15 (MD-MBA-231) and DMEM (BT549) medium, containing 10% FBS and maintained in an incubator with constant temperature and CO₂. Cells were treated with Mith A (HY-A0122, MCE).

Transfection and lentiviral transduction: The small interfering RNA of SP1 was synthesized by GenePharma Company (Shanghai, China), and the sequence was as follows: siSP1#1, 5'-UCCUUUUUCAAUUUUCACCAC-3'; siSP1#2, 5'-UUGAUACUGAAUAUUAGGCAU-3'; negative control, 5'-UUCUCCGAACGUGUCACGUTT-3'. Transfection was performed using Lipo8000™ (C0533, Beyotime). The lentivirus was acquired from Genechem company (Shanghai, China). The target sequence of shNEDD4L is as follow: 5'-GGAACUAAGCAGAAGGCUUTT-3'. Infection of the cells was performed according to the virus operation manual.

Western Blotting

Cells were washed with precooled PBS, then lysed with RAPA lysate on the vertical rotator at 4°C for 10 mins. Subsequently, the supernatant was obtained by centrifugation at 4°C for 10 mins (12000 rpm/min),

and the protein concentration was determined by the BCA protein concentration determination kit (Beyotime, P0010S). Finally, add loading buffer and cook in boiling water for 10 minutes, and the sample is stored at - 20°C. Western blotting was performed using antibodies against NEDD4L (ab46521, Abcam), SNAI2 (#9585S, CST), GAPDH (#5174S, CST), and SP1 (#9389S, CST). Anti-rabbit IgG, HRP-linked Antibody (#7074, CST) was used as secondary antibodies.

Transwell migration assay

Briefly, 2×10^4 MDA-MB-231 cells (starved for 12 h) suspended in medium without FBS were plated in the top chamber (24-well insert; pore size, 8 μ m; Corning Costar), and medium supplemented with 10% FBS was added in the lower chamber. Cell culture and staining refer to our previous studies[27].

Immunohistochemistry and immunofluorescence assay: Breast cancer and adjacent normal tissues were fabricated into a tissue chip. Immunohistochemical (IHC) staining of FFPE sections was performed as described [28]. The H-score system was used to assess the expression of target proteins, and the formula for the H-score is as follows: $\text{Histscore} = \sum (I \times P_i)$, where I = intensity of staining and P_i = percentage of stained tumor cells [28]. MDA-MB-231 cells were seed on the dish with a density of about 30% and treated with 400 nM Mith A for 24 h. Immunofluorescence (ICC) was performed as described in our previous study [29].

Luciferase reporter assays

For the promoter activity assay, a 2 kb fragment upstream of the transcription start site of NEDD4L (wild type and mutant) was cloned into the GP-Check2 vector (GenePharma). The resulting construct, together with a Renilla luciferase construct, was introduced into 293T cells. The preparation of cell extracts and the determination of luciferase activity were described in our previous study [28].

Patients and specimens

The 76 pairs of human BRCA and adjacent normal tissues were obtained from patients at Shanxi Provincial People's Hospital (Taiyuan, China). The use of clinical specimens was approved by the Ethics Committee of Shanxi Province Peoples Hospital. (2021196).

Pulmonary metastasis model of breast cancer

For the Pulmonary metastasis model of breast cancer, 6-week-old BALB/c nude mice were obtained from Weitong Lihua Experimental Animal Technology Co., Ltd (Beijing, China). MDA-MB-231 cells (2×10^6 per mouse) were injected into the peripheral blood of mice by tail vein injection. After three weeks, the mice were sacrificed to obtain lung tissues, and HE staining was performed to detect lung metastasis of breast cancer cells. All animal experiments were approved by the Ethics Committee of Shanxi Province Peoples Hospital. (2021191).

Statistical analysis

The difference analysis and correlation analysis was conducted by R (version 4.1.0) with pearson and wilcox test. Survival analysis of BRCA patients was also performed by R (version 4.1.0) using the Kaplan–Meier method and log-rank test. The "ggplot2", "ggpubr", "survival", "survminer", "clusterProfiler", "org.Hs.eg.db", "enrichplot", "DOSE", "GSVA" and "GSEABase" packages in R (version 4.1.0) were used for visualization. A p-value of less than 0.05 was considered to be statistically significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Results

NEDD4L was downregulated and correlated to poor prognosis in BRCA

To explore the expression of NEDD4L in tumors, we performed a pan-cancer analysis of NEDD4L using TCGA databases. We found that NEDD4L was significantly down-regulated in 9 cancers, significantly upregulated in 8 cancers, and no significant change in 7 cancers (Fig. 1A). The inconsistent expression of NEDD4L in different tumors is due to tumor specificity. We analyzed the expression of NEDD4L in TCGA_BRCA (113 normal tissues, 1104 cancer tissues) and GSE42568 (17 normal tissues, 104 cancer tissues) data sets, respectively. The results showed that NEDD4L is significantly down-regulated in tumor tissues compared to normal tissues (Fig. 1B-C). Subsequently, we detected the protein level of NEDD4L in 96 pairs of breast carcinoma and para-carcinoma tissues by IHC, and the result was consistent with the bioinformatics analysis results (Fig. 1D-E). Finally, we performed a meta-analysis of NEDD4L mRNA level in BRCA using 31 studies from the Oncomine database, and the result also showed that NEDD4L was significantly decreased in tumor tissues (Fig. S1). Survival analysis of patients from TCGA_BRCA, METABRIC, GSE24450, and GSE42568 datasets confirmed that patients with NEDD4L high expression had a longer overall survival (OS) time (Fig. 2). In addition, patients with high NEDD4L expression also had a longer progression-free interval (PFI) and disease-free survival (DSS) (Fig. S2). All these results suggested that NEDD4L might play as a tumor suppressor in BRCA.

NEDD4L was associated with the metastasis of BRCA

To explore the function of NEDD4L in BRCA, we performed GO and KEGG analysis using the TCGA_BRCA dataset, which was divided into two subgroups by the median value of NEDD4L. GO analysis showed that the differential genes ($FDR < 0.05$, $|\log FC| > 1$) between the two subgroups were mainly enriched in the endoplasmic reticulum and Golgi lumens and were involved in the formation of extracellular matrix and structural organizations and transmembrane receptor protein serine/threonine kinase signaling pathway (Fig. 3A). KEGG analysis showed that the differential genes between the two subgroups play a major role in TGF- β and PI3K-Akt signaling pathways (Fig. 3B). To confirm that NEDD4L can inhibit BRCA metastasis, we knocked down NEDD4L in MAD-MB-231 cells using lentivirus and performed a transwell assay. We found that the migration ability of MDA-MB-231 was significantly enhanced after the knockdown of NEDD4L (Fig. 3C-D). Subsequently, we constructed a pulmonary metastasis model of breast cancer as described in the method part. The results showed that after the knockdown of NEDD4L,

the number of metastases in the lung increased significantly (Fig. 3E). Altogether, these findings suggested that NEDD4L could inhibit BRCA metastasis.

NEDD4L could target SNAI2 in breast cancer

Bioinformatics analysis and interesting literature suggested that NEDD4L might regulate the TGF- β signaling pathway. SNAI2 is one of the key transcription factors of TGF- β signaling and plays a vital role in EMT and metastasis. Interestingly, when we knockdown or overexpress NEDD4L in MDA-MB-231 cells, SNAI2 has the opposite change, confirmed by Western blot and ICC assay (Fig. 4A-C). To further confirm that NEDD4L could downregulate SNAI2, we analyzed their mRNA levels using METABRIC and TCGA_BRCA datasets, respectively. The results showed that NEDD4L was significantly negatively correlated with SNAI2 (Fig. 4D-E). We also detected the protein level of SNAI2 by IHC and found that it was significantly upregulated in breast cancer tissues compared to normal tissues (Fig. 4F). In addition, IHC results also showed that NEDD4L was significantly negatively correlated with SNAI2 in breast cancer (Fig. 4G-H). All the above results suggested that NEDD4L might inhibit TGF- β signaling by decreasing SNAI2.

Sp1 regulated NEDD4L expression in BRCA

To explore the expression mechanism of NEDD4L, we performed bioinformatics analysis using METABRIC and TCGA_BRCA datasets. The results showed that compared to normal breast tissues, the expression of transcription factor SP1 was significantly reduced in breast cancer tissues and significantly positively correlated with NEDD4L (Fig. 5A-C). The IHC results showed that the protein level of SP1 was significantly decreased in BRCA tissues and was significantly positively correlated with NEDD4L (Fig. 5D-F). Based on the above results, we speculated that SP1 regulated the expression of NEDD4L. To confirm our hypothesis, we predicted the potential binding site of SP1 in the transcriptional regulatory region of NEDD4L using online tools (Fig. 6A). Therefore, we designed a double luciferase reporter gene experiment. The result suggested that SP1 could regulate NEDD4L expression (Fig. 6A-B). Subsequently, we treated the BRCA cells with SP1 inhibitor (Mith A) and performed WB and ICC assay. The results showed that the expression level of NEDD4L decreased significantly after Mith A treatment (Fig. 6C-E). Finally, we knocked down SP1 in MDA-MB-231 cells using lentivirus and then detected the expression level of target genes by WB and ICC. The results showed that the expression level of NEDD4L was significantly down-regulated after the knockdown of SP1 (Fig. 6F-H). Altogether, these findings suggested that SP1 positively regulates the expression of NEDD4L in breast cancer.

Development of a NEDD4L related prognostic model

We obtained 371 genes significantly related to NEDD4L from TCGA_BRCA, GSE24450, and GSE42568 datasets (Fig. 7A). Subsequently, we performed univariate Cox regression analysis on the above 371 genes and identified 11 genes with significant prognostic relevance ($P < 0.01$) to establish NEDD4L related prognosis model for BRCA. Patients from the TCGA_BRCA dataset ($n = 1082$) were used to establish a LASSO Cox regression model, and patients from the GSE24450 dataset were used to evaluate

the model's effectiveness. The above 11 genes were input into the lasso Cox regression model to obtain 8 key genes, namely *CCDC24*, *CHORDC1*, *FAM114A1*, *CD74*, *EPB41L4BLGALS2*, *LGALS2*, *GPR171*, and *GBP2* (Fig. 7B-D). The riskScore was calculated by the formula in the method part. The patients from both training and testing datasets were divided into low-risk and high-risk subgroups according to the median value of riskScore. Prognostic analysis results of both training and testing datasets showed that patients from low-risk subgroups had a longer OS (Fig. 7E-G). The analysis of the time-related receiver operating characteristic (ROC) in both pieces of training (AUC at 5 years = 0.636) and testing (AUC at 5 years = 0.644) datasets all showed that the riskScore had a favorable predictive value (Fig. 7F&7H). The above results suggested that the NEDD4L related prognostic model has a good predictive value.

Establishment of a nomogram based on NEDD4L

Univariate and multivariate Cox regression analyses were performed on the TCGA_BRCA data set to investigate whether the riskScore or the clinical characteristics were an independent predictor of prognosis in patients with BRCA. The adjustment of conventional clinical models, including age and TNM stage, indicated that riskScore, M stage, and N stage were independent prognostic factors, which confirmed that they had a strong ability to predict OS in patients with BRCA (Fig. 8A-B). A nomogram was developed using independent clinical risk factors, which could quantitatively evaluate the prognosis of BRCA (Fig. 8C). Based on the nomogram, the 1-, 3- and 5-year survival rates of BRCA patients could be estimated [30]. The analysis of the time-related ROC showed that riskScore + clinical had a better predictive value than all of the independent factors (Fig. 8D). In addition, we calibrated the nomogram and found that it was highly accurate in predicting 1, 3, and 5-year survival rates in patients with BRCA (Fig. 8E-G). Therefore, our results demonstrate that nomogram was a better model for predicting the prognosis of BRCA compared with a single risk factor.

Discussion

Although NEDD4L has been extensively studied in animal mammals, its function in the biological processes of BRCA is still unclear. Herein, we investigated the expression mechanism of NEDD4L and its function in the metastasis of BRCA and established a NEDD4L related prognostic model (Fig. 8C). We proposed that low expression of NEDD4L in BRCA could weaken its inhibitory effect on SNAI2, thereby promoting the metastasis of breast cancer. In this study, we found that patients with high NEDD4L expression had longer OS, and knocking down NEDD4L significantly enhanced the migration and metastasis ability of BRCA cells, implying that NEDD4L might play as a tumor suppressor in BRCA.

Originally, we analyzed different datasets by bioinformatics and found that NEDD4L was significantly downregulated in BRCA tissues (Fig. 1). To further enhance the reliability of the results, we used breast cancer samples to verify. In addition, Guarnieri's study in BRCA is consistent with our conclusion [31]. Subsequently, we performed survival analysis using four datasets with clinical information, and all the results showed that BRCA patients with high NEDD4L expression had a better prognosis (Fig. 2; Fig. S2). It suggested that NEDD4L acted as an anti-oncogene in BRCA, consistent with Lee's study [32]. Previous

studies have shown that NEDD4L plays an anti-cancer role in carcinomas, such as glioma, lung cancer, and colorectal cancer [23, 33, 34].

Furthermore, we performed GO and KEGG analysis and found that the differential genes between the two subgroups enriched in the TGF- β pathway, which plays a vital role in the EMT and metastasis of BRCA [4]. SNAI2 is a key transcription factor of the classical TGF- β /Smad pathway, which plays a vital role in EMT and metastasis [35]. Rajesh *et al.* reported that E3 ubiquitin ligase FBXO31 could mediate the ubiquitination pathway of SNAI2, thereby inhibiting the EMT and invasion of BRCA [36]. Accordingly, we speculated that NEDD4L might also inhibit the progression of BRCA by targeting SNAI2. To confirm our conjecture, we performed correlation analysis between NEDD4L and SNAI2 using TCGA_BRCA and METABRIC datasets, and we found a significant positive correlation between them (Fig. 4D-4E).

Moreover, the IHC result was consistent with the bioinformatics result. Furthermore, Western blot and ICC assays confirmed that the expression level of SNAI2 increased after NEDD4L knockdown and vice versa. Finally, the pulmonary metastasis model of breast cancer and transwell assay confirmed that the migration and metastasis ability of MDA-MB-231 cells was significantly enhanced after knocking down NEDD4L. Zhang's study in lung adenocarcinoma [37] and Cui's work in melanoma confirmed that NEDD4L could inhibit tumor metastasis, consistent with our conclusion [38].

In addition, we confirmed that NEDD4L expression was regulated by transcription factor Sp1. Firstly, bioinformatics analysis showed that NEDD4L was significantly positively correlated with SP1. Secondly, the luciferase reporter assays confirmed that SP1 regulated the expression of NEDD4L. Finally, the expression level of NEDD4L was significantly down-regulated after inhibiting SP1 activity or knocking down SP1. SP1 plays an important role in various tumors and can activate or suppress the expression of many oncogenes and tumor suppressors [39]. However, its expression pattern and function in breast cancer remain divergent. Li *et al.* reported that the expression of SP1 was downregulated in advanced BRCA and highly aggressive BRCA cell lines, which is consistent with our findings [40]. Additionally, Cui *et al.* reported that in melanoma, NEDD4L could mediate ubiquitination degradation of SP1 [38]. The specific relationship between SP1 and NEDD4L still needs to be further studied.

Finally, we established a NEDD4L related prognostic model by LASSO Cox regression, which could act as an independent prognostic factor for breast cancer. To quantitatively evaluate the prognosis of BRCA, we constructed a nomogram combining riskScore and clinical indicators (Fig. 8C). Compared with a single risk factor, the nomogram is a better prognostic model. Although this study revealed the new mechanism SP1/NEDD4L/SNAI2 axis in breast cancer progression and established a reliable prognostic model, it still has breast limitations. First, we need to investigate the driving signal on the SP1/NEDD4L/SNAI2 axis in cancer. Secondly, we need to study further the underlying mechanism of SNAI2 degradation mediated by NEDD4L.

Conclusions

This study first reports the mechanism of SP1/NEDD4L/SNAI2 axis in the process of BRCA metastasis. This study shows that NEDD4L is positively correlated with the OS of BRCA and constructs a NEDD4L related prognostic model. The nomogram constructed with riskScore, M stage, and N stage can effectively quantitatively evaluate the prognosis of BRCA. This study provides a new target for clinical treatment of BRCA, and provides a reliable model for prognosis evaluation of BRCA.

Declarations

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Availability of data and materials

The datasets generated during and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

GYL and HHZ were responsible for the conceptual design of the study. GYL, BLZ, YL, XYL, LPZ, KXY, BHT, ZNG, MQS, ZZ and HNZ were responsible for data processing and statistical analysis. BLZ, HHZ and GYL wrote and reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Ethics Committee of Shanxi Province Peoples Hospital. (2021191). The use of clinical specimens was approved by the Ethics Committee of Shanxi Province Peoples Hospital. (2021196).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Figures

Figure 1

The expression level of NEDD4L in BRCA (A) Expression level of NEDD4L in pan-cancer. (B-C) Relative mRNA levels of NEDD4L in samples from TCGA_BRCA dataset (B) and GSE42568 dataset (C). (D-E) Expression level and representative pictures of NEDD4L in normal and BRCA tissues. *P < 0.05; **P < 0.01; ***P < 0.001

Figure 2

Prognostic analysis of NEDD4L in BRCA (A-D) Kaplan-Meier curves of the OS of the patients from TCGA_BRCA, METABRIC, GSE24450, and GSE42568 databases.

Figure 3

The migration and metastasis ability of BRCA enhanced after knocking down NEDD4L (A-B) GO and KEGG enrichment analysis of NEDD4L. (C-D) The migration ability of MDA-MB-231 cells was enhanced after knocking down NEDD4L. (E) MDA-MB-231 cells formed more metastases in the lung after knocking down NEDD4L.

Figure 4

NEDD4L can downregulate SNAI2 in BRCA (A&B) Western blot analysis of MDA-MB-231 cells stably knocking down or overexpressed NEDD4L. (C) ICC analysis of MDA-MB-231 cells stably knocking down or overexpressed NEDD4L. (D-E) There was a significant negative correlation between NEDD4L and SNAI2 in patients from the TCGA_BRCA dataset (D) and METABRIC dataset (E). (F) The IHC assay result of SNAI2 in normal and BRCA tissues. (G) There was a significant negative correlation between NEDD4L and SNAI2. (H) Representative pictures of NEDD4L and SNAI2 in BRCA tissues.

Figure 5

NEDD4L was positively correlated with SP1 in BRCA (A-B). There was a significant positive correlation between NEDD4L and SNAI2 in patients from TCGA_BRCA (A) and the METABRIC dataset (B). (C) The expression of SP1 was downregulated in patients from the TCGA_BRCA dataset. (D) The IHC assay result of SNAI2 in normal and BRCA tissues. (E) There was a significant positive correlation between NEDD4L and SP1. (F) Representative pictures of NEDD4L and SP1 in BRCA tissues.

Figure 6

SP1 regulated the expression of NEDD4L in BRCA (A-B) Luciferase reporter assays with the vector containing wildtype or mutant sequence of NEDD4L transcriptional regulatory region. (C-D) MDA-MB-231 and BT549 cells were treated with Mith A for 48 h and subject to Western blot analysis. (E) MDA-MB-231 cells were treated with Mith A for 48 h and subjected to ICC analysis. (F-G) MDA-MB-231 and BT549 cells were transfected with siSP1 for 48 h and subjected to Western blot analysis. (H) MDA-MB-231 cells were transfected with siSP1 for 48 h and subject to ICC analysis.

Figure 7

Construction of LASSO Cox regression model and verification (A) Venn of NEDD4L related genes in three datasets. (B-D) A LASSO Cox regression model was developed from NEDD4L and 11 related genes, calculating the tuning parameter (λ) based on the partial likelihood deviance with tenfold cross-validation. The vertical black line indicates the optimal $\log \lambda$ value in the plot. (E) Kaplan-Meier plot of the OS in the high-risk and low-risk groups of the TCGA_BRCA dataset. (F) Time-dependent ROC analysis of the riskScore for the OS and survival status in the TCGA_BRCA dataset. (G) Kaplan-Meier plot of the OS in the high-risk and low-risk groups of the GSE24450 dataset. (H) Time-dependent ROC analysis of the riskScore for the OS and survival status in the GSE24450 dataset.

Figure 8

Integration of risk score and clinical characteristics (A-B) Univariate and multivariate regression analysis of the relation between the riskScore and clinical features regarding OS in the TCGA_BRCA dataset. (C) Nomogram developed to predict the 1-, 3-, and 5-year OS in the TCGA_BRC data set. (D) Time-dependent ROC analysis of the riskScore and clinical characteristics for the OS and survival status in the TCGA_BRCA dataset. (E-G) The calibrated plots of a nomogram predict 1, 3, 5-year survival rates in patients with BRCA.

Supplementary Files

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