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Non-SMC Condensin I Complex Subunit D2 Is a Prognostic Factor in Triple-Negative Breast Cancer for the Ability to Promote Cell Cycle and Enhance Invasion

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Triple-negative breast cancer (TNBC) is a heterogeneous disease with an unfavorable prognosis and no specific targeted therapies. The role of non-SMC condensin I complex subunit D2 (NCAPD2), a regulatory subunit of the condensin I complex that mainly participates in chromosome condensation and segregation, has not been reported in cancer. We therefore evaluated the prognostic value and biological function of NCAPD2 in TNBC. The expression of NCAPD2 was studied in 179 TNBC tissues by immunohistochemistry, and associations among NCAPD2 expression, clinicopathologic features, and the prognosis information of patients with TNBC were analyzed. The mRNA expression profiles of 99 TNBC tissues were also studied, and cell biological behaviors were detected when *NCAPD2* was altered in three TNBC cell lines. NCAPD2 expression was positively associated with lymph node metastasis ($P = 3.84 \times 10^{-06}$), poor overall survival ($P = 0.0033$), and worse disease-free survival ($P = 0.0013$) of patients with TNBC. Moreover, knockdown of *NCAPD2* might cause G₂/M arrest through the p53 signaling pathway, which led to proliferation inhibition, polyploid cell production, and cell apoptosis and inhibited the invasiveness of TNBC cells. For the first time, we report the close association between NCAPD2 and cancer and demonstrate that NCAPD2 plays an important role in TNBC progression and acts as an independent poor prognostic factor and a potential therapeutic target for TNBC. (*Am J Pathol* 2020, 190: 37–47; <https://doi.org/10.1016/j.ajpath.2019.09.014>)

Triple-negative breast cancer (TNBC), which is the most aggressive subtype of breast cancer, accounts for 15% to 20% of all patients with breast cancer and is characterized by lack of expression of estrogen receptor (ER) and progesterone receptor (PR) and an absence of amplification of human epidermal growth factor receptor 2 (HER2).^{1–4} TNBCs are a heterogeneous group of tumors, and patients with this subtype are more likely to develop recurrence within the first 5 years; survival after metastatic relapse is shorter for patients with TNBC than patients with other breast cancer subtypes.^{5–7} Because no well-defined molecular targets have been established and no targeted therapies have been approved for TNBC, the only systemic treatment option is

cytotoxic chemotherapy, which has limited long-term success rates.^{8–10} Therefore, exploration of the molecular basis of TNBC and developing effective therapeutic strategies, which will eventually improve the survival rate of patients with TNBC, is urgently needed. The segregation of two copies of replicated genomic DNA into each daughter cell is the major

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feature of M phase during the cell cycle. During this period, the relaxed chromatin is converted into condensed chromosomes at the very beginning of mitosis, and the entangled sister chromatids resulting from DNA replication are decatenated.^{11,12} Failure to perform this process accurately can lead to detrimental chromosome segregation defects.¹³ One key factor involved in chromosome condensation and decatenation is a heteropentamer called condensin, which is evolutionarily conserved from bacteria to humans.

Non-SMC condensin I complex subunit D2 (NCAPD2) is a subunit of condensin I that is located on chromosome 12p13.3. Transfection and site-directed mutagenesis studies have confirmed that NCAPD2 is regulated by E2F.¹⁴ NCAPD2 contains a bipartite nuclear localization signal in its carboxyl terminus, which is necessary for the nuclear localization and chromosome targeting of condensin I.¹⁵ In addition, the HEAT repeat of NCAPD2, which is a bihelical structure arranged in tandem, may play a role in protein-protein interactions.^{16,17} However, the function of NCAPD2 is not well understood, and previous studies of NCAPD2 have primarily focused on its participation in chromosome condensation and segregation.^{18–20} These studies found that the recruitment of condensin I onto chromosomes depends on the interaction between the chromosome-targeting domain of NCAPD2 and the phosphorylated histone H3.²¹ Several observations have revealed the misalignment of chromosomes on the metaphase plate in the absence of NCAPD2. Moreover, depletion of NCAPD2 affects both the individualization of mitotic chromosomes and the resolution of sister chromatids.^{13,22} Recent research has found that NCAPD2 also interacts with rootletin, which functions in centrosome cohesion and restrains DNA damage–induced centriole splitting.^{23,24} However, as a pivotal participant in the cell cycle, the role of NCAPD2 in cancer has not yet been reported.

In this study, the protein expression level of NCAPD2 was evaluated in 179 primary TNBC samples by immunohistochemistry, and the association among the protein expression, the clinicopathologic features, and the prognosis of these patients was determined. Moreover, mRNA expression profiles of TNBC tissues and *NCAPD2* low-expression TNBC cell models were used to investigate the role of NCAPD2 in TNBC.

Materials and Methods

Patients and Specimens

All tissue samples were obtained from 179 female patients who were diagnosed with primary breast cancer and underwent surgery at the Department of Pathology, Tianjin Medical University Cancer Hospital (Tianjin, China) between 2003 and 2006. No patients received chemotherapy or radiotherapy before surgery. Surgically resected tissue samples were fixed in 10% formaldehyde and then embedded in paraffin for 4- μ m histologic sectioning. The histopathologic examination was performed by two independent and

experienced pathologists (L.Fu and F.L., who work in the Department of Breast Cancer Pathology and Research Laboratory, Tianjin Medical University Cancer Institute and Hospital, Tianjin, China, and have been engaged in pathologic diagnosis for the last 5 years) who were blinded to the groups in this study. All samples were identified as TNBC by ER, PR, and HER2 staining. The study was reviewed and approved by the Institutional Ethics Committee of Tianjin Medical University Cancer Institute and Hospital, and all patients provided written informed consent. All methods were performed in accordance with the approved guidelines.

Immunohistochemistry and Scoring

After routine deparaffinization in xylene and rehydration in a graded ethanol of decreasing concentrations, the tissue sections were incubated in 3% H₂O₂ for 10 minutes to block endogenous peroxidase activity. Then the sections were subjected to antigen retrieval by heating in citrate buffer pH 6.0 at 121°C for 150 seconds. After cooling to room temperature, the sections were treated with 5% normal goat serum to block nonspecific binding and incubated with a mouse monoclonal antibody against NCAPD2 at a 1:100 dilution (catalog number ab56885; Abcam, Cambridge, UK) overnight at 4°C. Then a biotin-conjugated secondary antibody was applied, and NCAPD2 expression was detected using a streptavidin-peroxidase complex (catalog number SP-9000; Zhongshan Golden Bridge Biotechnology Company, Zhongshan, China). The chromogenic reaction was performed using a commercial diaminobenzidine kit (catalog number ZLI-9550; Zhongshan Golden Bridge Biotechnology Company). Finally, the sections were counterstained in hematoxylin.

The immunohistochemistry-stained sections were analyzed and scored independently by two experienced pathologists (L.Fu and F.L.) who were blinded to the patients' clinicopathologic data. The staining intensity was scored as 0, 1, 2, or 3 based on color (no color, ecru, brown, and puce, respectively), and the percentage of positively stained tumor cells was determined, with a staining percentage $\geq 1\%$ considered positive. The staining intensity scores and the percentage of the positive tumor cells were used to calculate H-scores with the following formula:

$$\text{H-score} = \sum (\text{percentage of positively stained tumor cells}) \times (\text{staining intensity})$$

H-scores were then used to categorize the samples into four groups as follows: negative (–), score 0; mild (+), score of >0 to 1; moderate (++), score of >1 to 2; and marked (+++), score of >2 to 3.

Cell Culture and siRNA Knockdown

The human TNBC cell lines MDA-MB-231, MDA-MB-453, and MDA-MB-468 were obtained from the National Infrastructure of Cell Line Resource (Beijing, China). MDA-MB-231 and MDA-MB-453 cells were maintained in

RPMI 1640 medium (catalog number 21875109; Gibco, Waltham, MA) supplemented with 10% fetal bovine serum (DMEM; catalog number 12484-028; Gibco). MDA-MB-468 cells were maintained in Dulbecco's modified Eagle's medium (catalog number 11966025; Gibco) supplemented with 10% fetal bovine serum and 2 mmol/mL of L-glutamine. All the cell lines were cultured in a humidified incubator at 37°C with 5% CO₂.

For the knockdown experiments, cells were transfected with siRNA targeting *NCAPD2* (siNCAPD2; catalog number HSS115010; Invitrogen, Carlsbad, CA) or nontargeting control (NC; catalog number 12935100; Invitrogen) using the Lipofectamine RNAiMAX transfection reagent (catalog number 13778150; Invitrogen) according to the manufacturer's instructions.

RNA Isolation and Real-Time RT-PCR

Total RNA was extracted using TRIzol reagent (catalog number 15596018; Thermo Fisher Scientific, Waltham, MA) and reverse transcribed using a SuperScript II reverse transcription kit according to the manufacturer's protocol (catalog number 18064014; Thermo Fisher Scientific). Then real-time RT-PCR was conducted as described in our previous report.²⁵ Melting curves were generated, and the relative mRNA expression level of the target gene was normalized to that of 18S ribosomal RNA. All data are presented as the means of three replicates. The primers used for the amplifications are as follows: *NCAPD2* forward, 5'-TGGAGGGGTGAATCAGTATGT-3'; *NCAPD2* reverse, 5'-GCGGGATACCACTTTTATCAGG-3'; 18S forward, 5'-CTGAGAAACGGCTACCACATCC-3'; 18S reverse, 5'-GCACCAGACTTGCCCTCCA-3'.

CCK-8 Assay

The proliferation abilities of three TNBC cell lines were measured using the Cell Counting Kit 8 (CCK-8) assay (catalog number CK04; Dojindo, Kumamoto, Japan). Briefly, cells were seeded in 96-well plates at a density of 2000 (MDA-MB-231 and MDA-MB-453) or 4000 (MDA-MB-468) cells per well. After adhesion for 24 hours, the cells were transfected with siNCAPD2 or NC. After incubation for 0 to 120 hours at an interval of 24 hours, the cells were stained with 10 µL of CCK-8 solution in 90 µL of culture medium for 2.5 hours at 37°C at each time point. Cell proliferation was measured as the absorbance at 450 nm using a microplate reader (BioTek, Winooski, VT).

In Vitro Invasion Assay

Corning BioCoat Matrigel Invasion Chambers (24 wells, 8.0-µm pore size, Corning, Corning, NY) were used for Transwell invasion assays according to the manufacturer's instructions. Briefly, after 48 hours of transfection, 1×10^5

cells suspended in 500 µL of serum-free medium were seeded into the upper chambers, whereas 750 µL of complete medium with 10% fetal bovine serum was injected into the lower chambers as a chemoattractant. After incubation for 24 hours, the cells that had invaded through the membrane were fixed in cold methanol followed by staining with 0.1% crystal violet, and then cells were counted in at least five fields under a light microscope.

Western Blotting

Cells were harvested and total protein was extracted using radioimmunoprecipitation assay lysis buffer (catalog number C1053; Applygen, Beijing, China) supplemented with protease inhibitor cocktail (catalog number 78410; Thermo Fisher Scientific). The Pierce BCA Protein Assay Kit was used to quantify the protein concentration (catalog number 23225; Thermo Fisher Scientific). Subsequently, equal amounts of the proteins were separated by SDS-PAGE and transferred onto a 0.25-µm polyvinylidene difluoride membrane (catalog number IPFL00010; Millipore, Burlington, MA) via a wet trans-blot system (BioRad, Hercules, CA). The membranes were then blocked and incubated with an antibody against *NCAPD2* (catalog number ab56885; Abcam, Cambridge, UK) overnight at 4°C, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (catalog number ZDR-5307; Zhongshan Golden Bridge Biotechnology Company) and analyzed using an enhanced chemiluminescence detection kit (catalog number P1050; Applygen). Finally, images were obtained with an ImageQuant LAS 4000 mini system (GE Healthcare, Chicago, IL). β-actin (catalog number sc-58673; Santa Cruz Biotechnology, Santa Cruz, CA) was used as an internal control.

Cell Cycle Assay

The effect of *NCAPD2* on cell cycle distribution was determined by flow cytometry. Briefly, after transfection with siNCAPD2 or NC for 96 hours, cells were harvested, fixed in 70% ice-cold ethanol, and incubated overnight at -20°C. Cells were washed with phosphate-buffered saline and resuspended in 500 µL of propidium iodide (PI)/RNase Staining Buffer (catalog number 550825; BD, Franklin Lakes, NJ). After incubation for 15 minutes in the dark at room temperature, cells were analyzed by flow cytometry using a LSRII flow cytometer (BD). Each experiment was performed at least three times. The fractions of cells in G₀/G₁, S, and G₂/M phases and the ratio of polyploid cells were analyzed using ModFit LT software version 3.2 (BD).

Cell Apoptosis Assay

Cell apoptosis was detected using the fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit I

(catalog number 556547; BD) according to the supplier's protocols. Ninety-six hours after transfection, cells were collected, centrifuged, and resuspended in 500 μ L of $1\times$ binding buffer. Then 5 μ L of Annexin V–FITC and 10 μ L of PI were added to each tube. The tubes were incubated in the dark at room temperature for 15 minutes. Cell apoptosis assays were performed immediately on an LSRII flow cytometer (BD).

Analysis of the Public mRNA Expression Data Set

Normalized mRNA expression data of The Cancer Genome Atlas (TCGA), including 99 TNBC samples and six normal breast tissues from patients with TNBC, were obtained from the cBioPortal for Cancer Genomics database (<http://www.cbioportal.org>, last accessed April 28, 2019). Pearson's product-moment correlation test was performed to identify genes that were significantly correlated with *NCAPD2* ($P < 0.001$ and $|\text{cor}| > 0.3$), and DAVID version 6.8 and R package clusterProfiler was applied to analyze the Gene Ontology (GO) enrichment of gene functions and KEGG pathways (<https://david.ncifcrf.gov>, last accessed April 28, 2019).

Statistical Analysis

All results are the means of at least three independent experiments with separately treated and transfected cells. Data are expressed as the means $\pm$ SEM. Statistical analyses were performed using SPSS software version 17.0 (SPSS, Chicago, IL). The correlations between *NCAPD2* expression and the clinicopathologic parameters were evaluated using the χ^2 test. Survival curves were obtained by the Kaplan-Meier method, and the differences in survival were analyzed by the log-rank test. Multivariate analyses were conducted using a Cox proportional hazards model. Differences were considered statistically significant at $P < 0.05$.

Results

NCAPD2 Is an Independent Prognostic Predictor for TNBC Patients

In TNBC tissues, the staining pattern of *NCAPD2* was intense and clear in the cytoplasm. In general, the rates of negative, mild, moderate, and marked expression were 25.69% ($n = 46/179$), 60.33% ($n = 108/179$), 8.93% ($n = 16/179$), and 5.11% ($n = 9/179$), respectively (Figure 1,

Figure 1 Non-SMC condensin I complex subunit D2 (*NCAPD2*) expression in triple-negative breast cancer (TNBC) tissues and the association between *NCAPD2* expression and prognosis of patients with TNBC. **A–D:** Representative immunohistochemical staining results for *NCAPD2* protein in TNBC tissues with negative (**A**), mild positive (**B**), moderate positive (**C**), and marked positive (**D**) staining. **E and F:** A total of 179 patients with TNBC were divided into two groups based on *NCAPD2* expression: negative staining represents the *NCAPD2*-negative group (black line), and mild, moderate, and marked positive staining represents the *NCAPD2*-positive group (red line). Positive *NCAPD2* protein expression is a poor prognostic factor for overall (**E**) and disease-free (**F**) survival of patients with TNBC as indicated by Kaplan-Meier analysis. $n = 46$ (**E** and **F**, *NCAPD2*-negative group); $n = 133$ (**E** and **F**, *NCAPD2*-positive group). Original magnification, $\times 200$ (**A–D**). –, negative staining; +, mild positive staining; ++, moderate positive staining; +++, marked positive staining.

A–D). According to the staining intensity, the samples were divided into two groups, with negative staining defined as negative NCAPD2 expression group (NCAPD2 negative) and mild, moderate, and marked staining defined as positive NCAPD2 expression group (NCAPD2 positive). The correlations between NCAPD2 expression and a variety of clinical features were further analyzed. NCAPD2 expression was frequently associated with lymph node involvement ($P = 3.84 \times 10^{-06}$) (Table 1). However, the expression of NCAPD2 was not associated with patient age ($P = 0.3042$), tumor size ($P = 0.2358$), or pathologic grade ($P = 0.3018$).

Statistical analysis revealed that positive NCAPD2 expression was significantly correlated with poor overall survival (Figure 1E). A Cox proportional hazards model was applied to estimate the effect of NCAPD2 expression on overall survival. Both univariate and multivariate Cox regression analyses found that positive NCAPD2 expression increased the risk of TNBC-related death compared with negative NCAPD2 expression (Supplemental Table S1). Moreover, patients with positive NCAPD2 expression had significantly worse disease-free survival than those with negative expression (Figure 1F). Both univariate and multivariate Cox regression analyses found that positive NCAPD2 expression could increase the risk of TNBC progression (Supplemental Table S2). Taken together, these results indicate that NCAPD2 is an independent predictor of poor survival for patients with TNBC.

NCAPD2 Participates in Multiple Aspects of Tumorigenesis in TNBC

To determine the associations between NCAPD2 expression patterns and the biological features of cancer, the mRNA expression profiles of 99 TNBC tissue samples from TCGA program were analyzed. Pearson's product-moment correlation test was performed to identify genes that were significantly correlated with NCAPD2. Overall, 1728 genes were recognized to be closely correlated with NCAPD2 ($P < 0.001$ and $|\text{cor}| > 0.3$), including 956 positively correlated genes and 772 negatively correlated genes. Through GO enrichment analysis, it was found that these positively correlated genes were associated with cell proliferation (cell proliferation, cell division, cell cycle), RNA processing (mRNA processing, mRNA splicing, rRNA processing, mRNA and tRNA export from the nucleus), and DNA replication and repair (ontology: biological process) (Figure 2A). These positively correlated genes were mainly located in the nucleoplasm and cytoplasm (ontology: cellular component) (Figure 2C). On the contrary, the negatively correlated genes were associated with angiogenesis, cell migration, cell proliferation, and signal transduction (ontology: biological process) (Figure 2B) and mainly located in extracellular exosomes and extracellular space (ontology: cellular component) (Figure 2D). KEGG pathway analysis revealed that multiple cellular pathways,

including DNA replication, cell cycle, spliceosome, RNA transport, complement and coagulation cascades, and lysosome, were significantly enriched (Figure 2, E and F). These data indicated that NCAPD2 participates in multiple aspects of TNBC tumorigenesis. Therefore, the differential expression of NCAPD2 may correspond to the different prognostic states of patients.

NCAPD2 Knockdown Inhibits the Proliferation and Invasion of TNBC Cells

The NCAPD2 mRNA expression of six normal and 99 tumor breast tissues from TCGA patients with TNBC was analyzed. The mRNA expression of NCAPD2 in TNBC tissues was significantly higher than in the normal breast tissues that from patients with TNBC (Supplemental Figure S1). To further investigate the biological roles of NCAPD2 in TNBC tumorigenesis and to uncover the veil of the reason why NCAPD2 differential expression caused different prognostic states of patients with TNBC, TNBC cell models were constructed in which NCAPD2 was knocked down. NCAPD2 expression levels were first examined in the three human TNBC cell lines MDA-MB-231, MDA-MB-468, and MDA-MB-453. The results indicate that the expression levels of NCAPD2 were varied in

Table 1 Association between Non-SMC Condensin I Complex Subunit D2 (NCAPD2) Expression and the Clinicopathologic Characteristics of 179 Patients with TNBC

Characteristic	NCAPD2 negative, <i>n</i> (total = 46)	NCAPD2 positive, <i>n</i> (total = 133)	<i>P</i>
Age, years (median = 52 years)			0.3042
≤52	23	80	
>52	23	53	
Tumor size			0.2358
T1	6	17	
T2	34	82	
T3/T4	4	25	
Pathologic grade			0.3018
1	0	5	
2	21	70	
3	18	44	
Lymph node involvement			3.84×10^{-06}
N0	36	43	
N1	6	47	
N2	4	28	
N3	0	10	

Two cases in the NCAPD2-negative group and nine cases in NCAPD2-positive group have missing tumor size characteristics. Seven cases in the NCAPD2-negative group and 14 cases in NCAPD2-positive group have missing pathologic grade characteristics. Five cases in the NCAPD2-positive group have missing lymph node involvement characteristics.

these three TNBC cell lines and that the expression levels were consistent at the RNA and protein levels (Supplemental Figure S2, A and C).

TNBC cells were transfected with either siNCAPD2 or NC to deplete *NCAPD2*. The efficiency of siRNA-mediated knockdown of *NCAPD2* was validated by real-time PCR and Western blotting. TNBC cells transfected with siNCAPD2 showed a noteworthy decrease in the *NCAPD2* mRNA and protein expression levels compared with the NC group (Supplemental Figure S2, B and D). The effect of siRNAs on cell proliferation was evaluated by CCK-8 assays. The results indicate that the proliferation of TNBC cells was significantly inhibited at 96 and 120 hours after *NCAPD2* knockdown compared with that of the NC group (Figure 3A). Moreover, Transwell assays revealed that down-regulation of *NCAPD2* resulted in a significant decrease in the invasive capabilities of TNBC cells (Figure 3B), which fits better with the finding that low expression of *NCAPD2* is associated with less lymph node metastasis and better overall survival.

NCAPD2 Knockdown Induces G₂/M Arrest in TNBC Cells, which Generates Polyploid Cells and Enhances Apoptosis

To address how *NCAPD2* contributes to cell proliferation, the cell cycle of TNBC cells was analyzed by flow cytometry after siNCAPD2 or NC transfection. Down-regulation of *NCAPD2* caused an accumulation of cells in G₂/M phase compared with the NC group of TNBC cells (Figure 4A). In addition, a significant increase of polyploid cells was observed after *NCAPD2* depletion (Figure 4B).

Furthermore, the rate of cell apoptosis was studied after *NCAPD2* knockdown. TNBC cells were stained with Annexin V–FITC/PI after transfection with siNCAPD2 or NC and then analyzed by flow cytometry. An increased fraction of apoptotic cells after *NCAPD2* knockdown was found compared with the NC group (Figure 5). These results indicate that the growth-inhibitory effect of *NCAPD2* depletion on TNBC cells was attributed to G₂/M arrest during the cell cycle, polyploid cell formation, and cell apoptosis.

Figure 2 Non-SMC condensin I complex subunit D2 (*NCAPD2*) participates in multiple aspects of triple-negative breast cancer tumorigenesis. Representative Gene Ontology (GO) biological process (A), GO cellular component (C), and KEGG pathway terms (E) for genes positively correlated with *NCAPD2* expression. Representative GO biological process (B), GO cellular component (D), and KEGG pathway terms (F) for genes negatively correlated with *NCAPD2* expression.

NCAPD2 Participates in p53 Signaling Pathway

To further explore mechanisms by which NCAPD2 applied to execute biological functions, a protein-protein interaction network was constructed using *NCAPD2* and the 1728 aforementioned correlated genes. The positively correlated genes constituted a network with 909 nodes and 23,436 links (positive network), and negatively correlated genes constituted a network with 652 nodes and 2282 links (negative network) (Supplemental Figure S3). Intriguingly, the negative network did not contain *NCAPD2*, suggesting

that these negatively correlated genes might have a low influence on the biological function implementing of NCAPD2. Afterward, MCODE was applied to identify highly interconnected clusters in the positive network and in the network constructed by all 1729 genes, and the cluster with the highest score was chosen from each network. Surprisingly, the two selected clusters were totally identical and included *NCAPD2* (Figure 6A), which further verified the negatively correlated genes might not interact with *NCAPD2*. Cluster 1 consisted of 134 nodes that all were positively correlated genes and 7536 links. GO analysis

Figure 3 Down-regulation of non-SMC condensin I complex subunit D2 (NCAPD2) inhibits the proliferation and invasion of triple-negative breast cancer (TNBC) cells. **A:** Cell Counting Kit 8 assays were used to evaluate the proliferation of TNBC cells after transfection with siRNA targeting *NCAPD2* (siNCAPD2) or nontargeting control (NC). **B:** Transwell invasion assays of TNBC cells 48 hours after transfection with siNCAPD2 or NC. Data are expressed as means $\pm$ SEM. $n = 3$ independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus siNCAPD2. Original magnification, $\times 200$.

found that the 134 nodes were enriched in many aspects of cell cycle, and KEGG enrichment found that the 134 nodes were significantly enriched in p53 signaling pathway and other pathways (Figure 6, B and C).

Eight of the 134 genes of cluster 1 were enriched in p53 signaling pathway, including *CCNB1*, *CCNB2*, *CCNE2*, *CDK1*, *CDK2*, *CHEK1*, *GTSE1*, and *RRM2*. Thereinto, *CCNB1*, *CCNB2*, and *CDK1* play crucial roles in G₂/M transition phase of the cell cycle, which was consistent with our knockdown result that down-regulation of *NCAPD2* caused an accumulation of TNBC cells in G₂/M phase. *CCNE2*, *CDK1*, and *CDK2* play roles in cell cycle G₁/S transition. *CHEK1* is required for checkpoint-mediated cell cycle arrest, and *GTSE1*, expressed only in the S and G₂ phases of the cell cycle, participates in the DNA

damage—induced apoptosis, which was consistent with the result that knockdown of *NCAPD2* enhanced apoptosis. All the above results indicate that *NCAPD2* might participate in p53 signaling pathway to execute cell cycle—related biological functions.

Discussion

TNBC accounts for 15% to 20% of all breast cancers; however, it causes a disproportionate percentage of breast cancer—related deaths. The aggressive phenotype, the high molecular heterogeneity, and the absence of targeted therapies of TNBC make it intractable. Patients with TNBC have a high risk of both local and distant recurrence and metastases,

Figure 4 Down-regulation of non-SMC condensin I complex subunit D2 (NCAPD2) induces G₂/M phase blockage and polyloid cell formation in triple-negative breast cancer (TNBC) cells. TNBC cells were transfected with siRNA targeting *NCAPD2* (siNCAPD2) or nontargeting control (NC). Then 96 hours after transfection, the fixed and permeabilized cells were stained for DNA content with propidium iodide (PI) and analyzed by flow cytometry to detect the cell cycle (A) and the polyloid cells (B). The dot within the black frame in B represents polyloid cells that have the same PI width value as normal cells but a higher PI area value. Data are expressed as means $\pm$ SEM. $n = 3$ independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

which lead to a poor prognosis.^{26,27} Therefore, it is necessary to elucidate the molecular mechanisms and to identify potential therapeutic targets for TNBC. The expression level of NCAPD2 is constant during the cell cycle, but its localization changes with different cell cycle phases. From mitotic interphase to early prophase, most NCAPD2 is soluble in the cytoplasm, whereas small amounts are associated with insoluble nuclear components. After breakdown of the nuclear envelope, NCAPD2 climbs up on the chromosome arms in late prophase and is not separated from the chromosome until the end of M phase. NCAPD2 is important for the assembly and segregation of mitotic chromosomes, which is consistent with its localization during the cell cycle.^{13,15,21} Recently, a study found that polymorphisms of *NCAPD2* are associated with Parkinson disease.²⁸ Nonetheless, no study has linked this gene to cancer. In our study, we found a

close association between NCAPD2 and cancer for the first time. All four subtypes of breast cancer, including the luminal subtype (ER and/or PR positive, HER2 negative), HER2 subtype, luminal (HER2 positive and ER and/or PR positive), HER2 subtype, nonluminal (HER2 positive and ER and PR negative), and basal-like subtype (HER2 negative and ER and PR negative; TNBC), contain NCAPD2-positive and NCAPD2-negative protein expression cases. The rates of NCAPD2-positive expression cases in luminal, HER2 subtype luminal, HER2 subtype nonluminal, and basal-like (TNBC, reported in our article) subtypes were 32.99% ($n = 64/194$), 39.39% ($n = 13/33$), 44.12% ($n = 15/34$), and 74.30% ($n = 133/179$), respectively. In patients with TNBC, NCAPD2 was an independent predictor of prognosis. Positive NCAPD2 protein expression was significantly correlated with poor overall survival and

Figure 5 Down-regulation of non-SMC condensin I complex subunit D2 (NCAPD2) induces cell apoptosis in triple-negative breast cancer (TNBC) cells. TNBC cells were transfected with siRNA targeting *NCAPD2* (siNCAPD2) or nontargeting control (NC). Then 96 hours after transfection, cells were stained with Annexin V–fluorescein isothiocyanate (FITC) and propidium iodide (PI) and then analyzed by flow cytometry. Box Q3 points represent living cells; box Q1 points, dead cells caused by mechanical damage; and box Q2 and Q4 points, apoptotic cells. Data are expressed as means $\pm$ SEM. $n = 3$ independent experiments. * $P < 0.05$, ** $P < 0.01$.

worse disease-free survival of patients with TNMB. In addition, the risk of TNBC-related death in the NCAPD2-positive group was nearly fivefold greater than that in the NCAPD2-negative group.

In this study, the correlation analysis of the mRNA expression data of 99 TNBC samples from TCGA found that *NCAPD2* was associated with the cell cycle, cell proliferation, cell division, and cell migration, which was further validated by *NCAPD2* low-expression cell models. In MDA-MB-231, MDA-MB-468, and MDA-MB-453 TNBC cells, the proportion of cells in G₂/M phase changed significantly after *NCAPD2* depletion. Compared with the NC group, the cell proportion of G₂/M phase was increased twofold to threefold in the siNCAPD2 group,

which was consistent with the previous report that *NCAPD2* depletion caused an abnormal accumulation of cells in prometaphase.¹³ Moreover, the G₂/M block caused by *NCAPD2* down-regulation could significantly suppress the proliferation of TNBC cells and lead to the failure of complete mitosis in some cells. For cells that were unable to successfully complete mitosis, they either quit the process of mitosis and became polyploid cells or underwent programmed cell death, which might be realized through p53 signaling pathway. The down-regulation of *NCAPD2* suppressed invasiveness of TNBC cells, which may be attributable to the ability of *NCAPD2* to stabilize the centrosome and promote microtubule formation.^{23,24} In addition, low expression of *NCAPD2* was associated with less lymph node metastasis. Hence, the expression of *NCAPD2* might be related with epithelial to mesenchymal transition (EMT). Twenty-three genes that significantly co-expressed with *NCAPD2* (*FAM83D*, *HDAC2*, *TRIM28*, *EZH2*, *NOLC1*, *STRAP*, *BMP5*, *LRP6*, *TGFB111*, *MSX1*, *DACT3*, *PDCD4*, *HGF*, *MSX2*, *ACVRL1*, *TGFB2*, *ENG*, *DAB2*, *BMP4*, *EPHA3*, *SPRY1*, *TGFB3*, *SNAI2*) in the 136-gene set related to EMT (downloaded from GO: 0001837, EMT) were found by analyzing 99 TNBC mRNA expression data from TCGA. *SNAI2* (alias *SNAIL2* or *SLUG*) is an important transcription factor that can induce EMT. The *SNAI2*-induced EMT is partially attributable to the direct repression of E-cadherin expression.²⁹ Many signaling molecules can induce *SNAI2* gene expression, including *BMP5*, *HGF*, *BMP4*, and *TGFB3*, which appear in our data.³⁰ *FAM83D* was reported as an oncogene, and overexpression of *FAM83D* was reported to induce EMT in breast cancer cells through the mTOR signaling pathway.³¹ Although the pivotal role of *NCAPD2* in TNBC progression was found in this study, the underlying specific molecular mechanisms and the potential use of *NCAPD2* as a prognostic marker in TNBC require further investigation.

In conclusion, for the first time, we report the close association between *NCAPD2* and TNBC. We found that *NCAPD2* played an important role in the cell cycle and migration of TNBC cell lines and potentially participated in p53 signaling pathway. Positive *NCAPD2* expression was frequently associated with lymph node involvement. Importantly, our results indicate that *NCAPD2* expression was significantly correlated with the overall survival and disease-free survival of patients with TNBC and acted as an independent poor prognostic factor.

Author Contributions

S.C. and L.F. conceived and designed the project; F.L., Z.Y., C.Z., and M.K. performed the experiments and analyzed the data; Y.Z., C.Z., and M.K. wrote the manuscript; F.L., M.R., and X.D. collected the clinical samples and conducted the follow-up; T.X. and L.F. revised the manuscript.

Figure 6 MCODE cluster from non-SMC condensin I complex subunit D2 (*NCAPD2*) protein-protein interaction (PPI) network and functional enrichment of cluster genes. **A:** MCODE cluster 1 with an MCODE score of 113.323 from *NCAPD2* PPI network includes 134 nodes and 7536 links. *NCAPD2* is involved in the cluster 1. **B** and **C:** Representative Gene Ontology (GO) biological process (**B**) and KEGG pathway terms (**C**) of the 134 nodes in cluster 1.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.09.014>.

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