

Activated interferon signaling by down-regulation of CENP-N contributing to inhibited tumor growth in breast cancer

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Research article

Keywords: CENP-N, Breast cancer, interferon signaling, Tumor growth

Posted Date: August 28th, 2019

DOI: <https://doi.org/10.21203/rs.2.12422/v1>

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Abstract

Background Centromere proteins (CENPs) are primary components for chromosomal segregation in the mitotic stage. CENP-N is a member of CENPs, and is a key factor for recruitment of other CENPs and formation of a link between the centromere and micro-tubules, which facilitate cell division. **Methods** In order to clarify the role of CENP-N in breast cancer, RNA sequences data were downloaded from TCGA online database and the CENP-N expression was knocked down in breast cancer cells. **Results** The results show that the expression of CENP-N was higher in breast cancer comparing with the paracancerous tissues. In breast cancer, patients with high expression of CENP-N have a short-term overall survival compared with low expression of CENP-N. Both in vitro and in vivo, the growth of breast cancer cells was inhibited by down-regulation of CENP-N. In the gene-chip analysis, it reveals that down-regulation of CENP-N is primarily associated with functions of immune response and anti-tumor effects. Of these changed canonical pathways, the activated interferon signaling was the most significant in CENP-N down-regulated breast cancer cells. In the western blot assay, up-regulated expressions of molecules involved in interferon signaling were also confirmed. **Conclusions** Our results suggest that CENP-N can be a potential therapeutic target in the treatment of breast cancer, and the involved interferon signaling needs to be mainly focused on. **Keywords:** CENP-N, Breast cancer, interferon signaling, Tumor growth

Background

During cancer development, successive generation and proliferation are dedicated to unfettered tumor growth. One of the distinguishing features of malignant with benign tumor cells is rapidly uncontrolled cell division and tumor growth. To date, high occurrence of aneuploidy that is result from failure of faithful chromosome segregation were found in tumor, and were considered to lead to carcinogenesis [1]. The phenomenon of chromosomal instability (CIN) refers to gains and loss of chromosomes which process is linked with aneuploidy in the context of cancer [2], and is believed to be associated with poor prognosis and resistance to therapy [3]. However, researchers recently found that different rates of chromosome missegregation have bidirectional role during tumor initiation and progression. Low rates of chromosome missegregation can contribute to tumorigenesis, however, missegregation of high numbers of chromosomes results in cell death and tumor suppression [4,5].

To ensure faithful chromosome segregation, precise assembly of kinetochore at the centromere of a chromosome during M (mitosis) - phase is required [6]. The 16-subunit constitutive centromere-associated network (CCAN) is a subcomplex in the kinetochore, and forms a base of the kinetochore to link between the centromere and microtubules [6]. The CCAN proteins can be grouped into five sub-complexes composed of centromere proteins (CENPs), such as CENP-C, CENP-L/N, CENP-H/I/K/M, CENP-T/W/S/X, and

CENP-O/P/Q/U/R [7]. Among these proteins, CENP-N has been confirmed to bind directly to CENP-A nucleosomes and participated in recruitment of other centromere proteins, formation of CCAN complex and assembly of kinetochore [8,9]. Additionally, CENP-A nucleosome retention at centromeres also requires being fastened by CENP-N [10]. Deficiency of CENP-N was demonstrated to lead to disrupted chromosome alignment and mitotic arrest [8,11].

As evidence accumulated, centromere proteins were linked with many cancer types, and consider as the potential targets for cancer treatment. High expressions of centromere proteins were found in many kinds of cancers, such as hepatocellular carcinoma [12,13], ovarian cancer [14], Lung adenocarcinoma [15], cervical cancer [16], and breast cancer [17-19]. CENP-A was indicated as a prognostic indicator for relapse in estrogen receptor (ER) positive breast cancer [18], and an independent prognostic factor in lung adenocarcinoma [15]. CENP-H was found to promote proliferation of gastric cancer cells, and highly expressed in hepatocellular carcinoma [12]. CENP-K was identified to be specifically up-regulated in ovarian cancer cells, and its overexpression was associated with poor patient survival [14]. These centromere proteins are all proved involving in assembly of kinetochore, in which process CENP-N is required [8].

However, the relationship between CENP-N and tumor is less understood. In a multivariable analysis, CENP-N, CETN1, CYP1A1, IRF2, LECT2, and NCOA1 were reported to be important predictors for both breast carcinoma recurrence and mortality among smokers [20]. For all the above supporting, we postulate CENP-N may associate with breast cancer malignant phenotype and progression, and can be a potential therapeutic target. In our study, we will focus on the expression of CENP-N in breast cancer tissues and the effect of reducing CENP-N expression on proliferation and -tumor formation of breast cancer cells in vitro and in vivo, and find the underling mechanisms by comparing the changed expression of genes after down-regulation of CENP-N using gene-chip analysis.

Methods

Ethics approval and consent to participate

All animal procedures were supervised and approved by the Jinzhou Medical University Laboratory Animal Welfare and Animal Experimental Ethical Committee.

Study design

Downloaded clinical data of CENP-N mRNA expression on breast cancer and paracancerous tissues were analyzed to compare the differences. In order to confirm the potential role of CENP-N on breast cancer, we examined the malignant phenotype of breast cancer cells with or without deficient expression of CENP-N in vitro or in vivo. Finally, microarray analysis for genome-wide effects of silencing CENP-N in breast cancer MDA-MB-231 was performed to find the changed and associated signaling pathways, and diseases and functions.

Online data and analysis of breast invasive carcinoma (BRCA)

For the TCGA set, clinical data and CENP-N mRNA expression (RNA-seq and RNA-seq V2) were downloaded from the TCGA data portal (<http://tcga-data.nci.nih.gov/tcga/>). The data included 1094 samples with the CENP-N mRNA expression, of the 106 samples have their paired CENP-N mRNA data in the paracancerous tissues. The differential expression of CENPN between breast cancer and adjacent normal tissues were evaluated. Overall survival analyses between breast cancer patients separated by median of CENPN mRNA expression were performed using the downloaded data and online database (<http://kmplot.com>).

Cell culture

MDA-MB-231 and MCF7 human breast cancer cells were purchased from American Type Culture Collection (Manassas, VA, USA), and National Science & Technology Infrastructure (Beijing, China). Cells were maintained in DMEM (Coring, New Jersey, USA) medium containing 10% fetal bovine serum (Gibco, thermo Fisher Scientific, Inc., USA), and 1% penicillin and streptomycin in a 5% CO₂ humidified atmosphere. Trypsin-EDTA was used to detach cells.

Lentiviral transfection

10⁵ MDA-MB-231 or 25 × 10⁴ MCF7 cells was seeded in 6-well plates and incubated in 5% CO₂ at 37°C overnight. Afterwards, in order to construct experimental groups, cells was transfected with CENPN-siRNA GFP lentivirus (shCENPN) (Genechem, Shanghai, China) or a control GFP lentiviral vector (shCtrl) (Genechem, Shanghai, China), and the multiplicity of infection (MOI) was 20. 16h later, no obviously cytotoxic effects were

observed, and infection solution was replaced by standard cell culture medium. After another 72h, ~80% confluence was reached and cells were observed under fluorescence microscopy and harvested for further qRT-PCR and Western blot to evaluate the transfection efficiency.

Quantitative reverse transcription-PCR (qRT-PCR) analysis

Total RNA was extracted using a GeneJET RNA purification Kit (K0731, ThermoFisher Scientific, USA) according to manufacturer's instructions. For each reaction, a total of 2 µg purified RNA was reverse-transcribed into cDNA using the reverse transcription kit (A5001, Promega, Madison, WI, USA). Afterwards, using qRT-PCR Master Mix (A6001, Promega, Madison, WI, USA), mRNA expression of CENPN was evaluated. The primer sequence of Real-time PCR were: CENP-N upstream, 5'-ACAAACCTACCTACGTGGTGT-3'; downstream, 5'-CCAGAAGCGGTGTATTGCG, and GAPDH upstream, 5'-TGACTTCAACAGCGACACCCA-3'; downstream, 5'-CACCTGTTGCTGTAGCCAAA. Relative quantification was used to analyze mRNA expression of CENP-N.

Western blot assay

After lentiviral transfection, cells were harvested, and proteins were extracted using a radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). Lysates were centrifuged 12,000g for 30 min at 4°C, supernatants were collected. Using a BCA protein assay kit (PC0020, Solaria life sciences, China) the protein concentration was determined. A total of 80 µg of protein was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA). PVDF membrane were blocked in 5% non-fat milk for 1 hour, and incubated with primary antibodies against CENP-N (ab57660, abcam, China), STAT2 (#4594, cell signaling technology, China), IRF1 (ab55330, abcam), CCND1 (#2978, cell signaling technology), CASP1 (ab1872, abcam), ISG15 (#2758, cell signaling technology), IFIT1 (#12082, cell signaling technology) and GAPDH (sc-32233, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4°C. Subsequently, PVDF membranes were incubated with goat anti-mouse (sc-2005, Santa Cruz) or goat anti-rabbit (sc-2004, Santa Cruz) secondary antibody conjugated with horseradish peroxidase at room temperature for 1 to 2 hours. Protein levels were evaluated on a chemiluminescent imaging system (LAS4010, GE Healthcare, USA) after exposure to electrochemiluminescence reagent (TransGen Biotech, Inc., Beijing, China). Gray values of protein bands were analyzed by Image J software (National Institutes of Health, USA).

Cell counting assay

Cells transfected with lentivirus carried with green fluorescence protein (GFP) can be captured and counted by Celigo Image Cytometer (Nexcelom, USA). After lentiviral transfection, MDA-MB-231 (1500 cells in 100 μ l per well) and MCF7 (2000 cells in 100 μ l per well) cells were seeded into 96 well plates. In the next five days, the number of cells was counted using Celigo Image Cytometer each time a day.

MTT assay

Lentivirus transfected MDA-MB-231 (2×10^4 cells/ml) and MCF7 (3×10^4 cells/ml) cells suspension were prepared and seeded into 96 well plates (100 μ l per well). After attachment, cells were incubated in 5% CO₂ at 37°C for 1 to 5 days. Next, cells were added with 10 μ l MTT reagent (5mg/ml in PBS) and incubated with cells for 4h. In order to dissolve the formazan product, 100 μ l of dimethyl sulfoxide (DMSO) was added. At 490 nm, the absorbance was measured using a microplate reader, and continuous 5-day changes of the optical density (OD) value were analyzed.

Apoptosis assay

Cells of MDA-MB-231 and MCF7 were trypsinized, counted and planted in 6-well plates. In the second day, cells were transfected with shCENPN and shCtrl lentivirus. 72 h later, according instruction of Apoptosis detection kit (88-8807, eBioscience, USA), transfected cells reach ~80% confluence, were trypsinized, washed, and 10^6 cells were resuspended in 200 μ l $1 \times$ binding buffer. Afterward, 10 μ l Annexin V-APC staining solution was added and incubated with cell suspension at room temperature avoiding from light for 10-15 min. Finally, 800 μ l $1 \times$ binding buffer was added and cell apoptotic percentage was examined within 1 h.

Caspase3/7 assay

Cells were transfected with lentivirus as described in apoptosis assay. Afterward, cells were trypsinized, counted and seeded into each 96 well plate at a density of 10000 cells, and were cultured at 37°C in a humidified incubator containing 5% CO₂ overnight. Caspase3/7 activity was examined by Caspase-Glo[®] 3/7 assay kit (G8901, Promega, USA). According to

manufacturer's instruction, Caspase-Glo solution was prepared. After, each well was added 100 μ l Caspase-Glo solution and shaking at a speed of 300-500 rpm using a 96-well plate shaker for 30 min. For another 2 h incubation at room temperature, micoplate reader was used to determine intensity of fluorescence signals. Caspase3/7 activity was evaluated by analyzing the examined data.

Nude mice and in vivo Tumor growth assay

4-week old female BALB/c nude mice averagely weighted 21 gram were obtained from Shanghai Lingchang Biotechnology Co., Ltd. The mice were bred in specific pathogen free housing, and 3-5 female mice were kept in a cage, which was away form light. The bedding material and food were all sterilized and provided by BEIJING HFK BIOSCIENCE CO., LTD. All experiments were conducted under the approval of Jinzhou Medical University Laboratory Animal Welfare and Animal Experimental Ethical Committee.

A total of 20 mice were randomized and allocated to two groups using statistical software (JMP software). MDA-MB-231 cells were transfected with shCENPN and shCtrl lentivirus. Afterwards, 10^7 transfected MDA-MB-231 cells were inoculated subcutaneously into right axillae of nude mice (10 miles for each group). When tumor could be observed, tumor length and width were measured and tumor volume was calculated as volume =length \times width² /2. The measurement was conducted each three days until tumor volume up to 1cm³. At this time, nude mice were killed by cervical dislocation, and tumor were removed and weighted up. Photos of nude mice and tumor were taken and preserved.

Microarray analysis

Using a GeneChip® PrimeView™ Human Gene Expression array (901838, Affymetrix, USA), which can measure gene expression of more than 36,000 transcripts and variants per sample, the genome-wide effects of CENP-N knockdown were evaluated. Total RNA of transfected MDA-MB-231 cells were extracted, and examined for quality control using Thremo Nanodrop 2000 spectrophotometer and Alient Bioanalyzed 2100. Amplified RNA (aRNA) was then prepared using GeneChip® 3' IVT labeling kit (Affymetrix) according to manufactuters' instructions. Next, GeneChip Hybridization Wash and Stain Kit (Affymetrix) were used for target hybridization and fluidics setup, according to manufacturers' instructions. Finally, array scanning was performed using CeneChip Scanner 3000

(Affymetrix). Data were collected and normalized to find the differentially expressed gene that the absolute of gene expression fold change is more than 1.5.

Ingenuity Pathway Analysis

Datasets of differentially expressed genes from microarray analysis were imported into the Ingenuity Pathway Analysis (IPA) tools (Ingenuity® Systems, USA). According to manufactures' instructions, IPA is an integrative online analysis software containing more than 5 millions of biological information from a number of literatures and more than 30 public databases. Different functional modules were set to help understand interaction network among different molecules and diseases and functions. Classical pathway analysis was performed to find the possible activated or inhibited signaling pathways involved in CENP-N knockdown. In the present study, CENP-N associated diseases and functions were also analyzed. During the analytic process, two statistical indicators were used, Z-score was to evaluate which process is activated ($Z\text{-score} > 2$) or suppressed ($Z\text{-score} < -2$), as for P value was to assess whether the difference is significant.

Statistical analyses

JMP software (version 11, SAS Institute Co. Ltd, China) was used for statistical analysis. Student's t test was used to analyze difference between two groups. One-way ANOVA followed by Turkey-Kramer HSD method was used to determine differences among multiple groups. Analysis of variance of repeated measures was conducted to determine differences of tumor growth in continuous 5 days. Kaplan-Meier method was performed to examine differences of overall survival between patients in low-expression CENPN group and high-expression CENPN group. $P < 0.05$ was identified as a statistical significance.

Results

Up-regulation of CENP-N in breast cancer tissues and correlated with decreased overall survival

To determine the role of CENP-N on progression of breast cancer, we downloaded RNAseq and clinical characteristic data of 106 paired breast cancer and adjacent noncancerous breast tissues (Table1), as well as of 1094 breast cancer tissues from TCGA database (Table 2). Differential expression of CENP-N between breast cancer and normal tissues was significant ($P < 0.0001$) (Fig.1a), and fold change (FC) calculated as Log_2 (cancer/Normal) was plotting in Fig.1b, with an average value of 2.177. Kaplan-Metier analysis showed higher expression of CENP-N was associated with a short-term overall

survival for patients with breast cancer ($P=0.042$) (Fig.1c). Consistently, the same result was also found in the online database (<http://kmplot.com>) (Fig.1d-g). Moreover, CENP-N expression on tumor with negative expression of estrogen receptor (ER) ($Z=-16.58$, $P<0.0001$) or progesterone receptor (PR) ($Z=-15.35$, $P<0.0001$) or positive expression of human epidermal growth factor receptor-2 (HER2) ($Z=3.71$, $P =0.0006$) was higher (Table 2). Altogether, these results identified a significantly positive association between progression of breast cancer and CENP-N expression.

Table 1 Clinical information of 106 paired samples

Characteristics	No. (percentage)
Age	
<35	4 (3.77)
≥35	102 (96.23)
TNM Stages	
I	18 (16.98)
II	60 (56.60)
III	25 (23.58)
IV	2 (1.89)
Not sure	1 (0.94)

Table 2. mRNA level of CENPN stratified by clinical characteristics in breast cancer

Characteristics	No. (percentage)	CENPN expression (Median (IQR))	P value
Age			
<35	28 (2.65)	324.46 (232.37)	0.08
≥35	1065 (97.35)	280.04 (289.30)	
T stage			
T1	279 (21.56)	248.08 (242.22)	<0.0001*
T2	633 (62.41)	301.88 (321.35)	
T3	138 (11.91)	260.41 (293.58)	
T4	40 (3.84)	343.18 (349.72)	
Not sure	3 (0.28)	417.37 (414.00)	
N stage			
N0	909 (84.25)	289.69 (297.25)	0.27
N1	22 (2.33)	298.98 (476.70)	
N2	162 (13.41)	226.66 (243.41)	
N3	77 (6.04)	261.27 (238.77)	
Not sure	20 (1.69)	295.72 (425.79)	
M stage			
M0	909 (84.25)	289.69 (297.25)	-
M1	22 (2.33)	298.98 (476.70)	
Not sure	162 (13.41)	226.66 (243.41)	
TNM Stage			
I	181 (14.34)	248.08 (236.74)	0.01*
II	620 (60.1)	290.62 (321.64)	
III	250 (21.98)	290.14 (276.66)	
IV	20 (2.04)	296.23 (441.48)	
Not sure	22 (1.53)	208.45 (254.97)	
ER status			
Positive	806 (58.03)	228.83 (195.06)	<0.0001*
Negative	237 (36.98)	552.54 (390.00)	
Not sure	50 (4.99)	315.97 (355.25)	
PR status			
Positive	698 (47.75)	218.26 (185.17)	<0.0001*
Negative	342 (47.24)	491.46 (414.64)	
Not sure	53 (5.01)	313.22 (258.64)	
HER2 status			
Positive	197 (19.04)	332.13 (246.71)	0.0005*
Negative	122 (10.45)	242.67 (320.24)	

Inhibited proliferation and expansion of breast cancer cells by silencing of CENP-N in vitro and in vivo

To investigate effect of CENP-N on tumorigenesis of breast cancer, CENP-N expression was assayed in human breast cancer cell lines. It was found that CENP-N expression was great, and then lentivirus packaged plasmid incorporated with EGFP element for knocking down of CENP-N (LV-CENP-N-RNAi) was constructed. MDA-MB-231 and MCF7 cells were infected with LV-CENP-N-RNAi (shCENP-N) and vector virus (shCtrl), then CENP-N mRNA and protein expression were examined to confirm efficiency of CENP-N silencing (Fig. 2a-f). Findings of MTT, colony formation and proliferation assays were extraordinarily similar, these results all indicated that interference of CENPN-N expression lead to inhibited cell proliferation, viability and colony formation (Fig. 2g-l). Moreover, apoptosis assay revealed that apoptotic percent of MDA-MB-231 and MCF7 cells were increased by down-regulation of CENP-N, and caspase3/7 assay conferred to the same result (Fig.2m-p). Consistently, in vivo experiment, the result showed inoculated tumor growth was dramatically slowed in CENP-N knocking-down group (shCENP-N) compared with control group (shCtrl) in nude mice (Fig.3). Taken together, we could conclude that CENP-N may be a key regulator during breast cancer proliferation and expansion.

Microarray analysis for genome-wide effects of silencing CENP-N in MDA-MB-231 cells

To disclose mechanisms underlying influences causing by CENP-N knockdown, differentially expressed gene profiles were examined by GeneChip® PrimeView™ Human Gene Expression assay. Compared with control group, 199 up-regulated and 278 down-regulated genes were found in MDA-MB-231 cells with CENP-N knockdown. To further illustrate the potential biological interactions among these differently regulated genes, the Ingenuity Pathway Analysis (IPA) were conducted. The results showed these differentially expressed genes were largely enriched in cancer, cell death and survival, and infectious diseases with the absolute value of Z-score more than 2 which indicated theses functions and diseases were significantly activated ($Z\text{-score} > 2$) or suppressed ($Z\text{-score} < -2$) (Fig. 4a). In the canonical pathway analysis, activated Interferon signaling and inhibited PI3K/AKT signaling were highlighted (Fig. 4b). Moreover, up-regulated interferon signaling was more significant than depressed PI3K/AKT signaling in CENP-N silenced MDA-MB-231 cells (Fig.

4b). These results suggested that activated Interferon signaling causing by down-regulated CENP-N might be the main factor contributing to inhibited cell growth of breast cancer cells.

Activation of interferon signaling after down-regulation of CENP-N

In the canonical signaling pathway analysis, we found that genes involved in interferon signaling, such as STAT2, IRF1, IFIT1, CCND1, CASP1, ISG15 were all increased in the microarray analysis (Fig. 4c). Western blot assay was performed to determine the protein expressions of these factors. In accordance with canonical pathway analysis, compared with shCtrl group, molecules of STAT2, CASP1, and ISG15 were all upregulated in shCENP-N group, which is accompanied by decreased expression of CCND1 (Fig. 4d). These results indicate that inhibited breast cancer cell growth by knocking down CENP-N might be partially attributed to activated interferon associated signaling.

Discussion

Tumor development is partially dictated by rapid proliferation that associated with uncontrolled cell cycle process [21]. Several centromere proteins (CENPs), which are necessary for cell division, are highly expressed in malignant tumors, and targeting CENPs have been demonstrated to inhibit tumor growth or enhance effect of chemotherapy in cancer cells [22,23]. As the role of CENP-N that is required for recruitment of many other CENPs and formation of the platform for chromosomal separation [8], we investigate the effect of deficiency of CENP-N in breast cancer cells. Our findings provided evidence supporting the hypothesis that targeting CENP-N led to inhibited cell proliferation and viability coupled with increased apoptosis by activating interferon associated signaling in breast cancer cells.

We showed that the RNA expression of CENP-N was overexpressed in breast cancer tissues and associated with unfavorable overall survival. Grouped by status of ER, PR, or HER-2, higher expression of CENP-N was found in the groups of negative ER, negative PR or positive HER-2. For patients with ER and PR negative or HER-2 positive breast cancer usually predict poor prognosis [24,25], our results suggest that CENP-N may be an indicator of aggressive phenotype of breast cancer cells. As expected, reducing the expression of CENP-N in breast cancer cells lead to inhibited cell proliferation and increased apoptosis, which was in accordance with the finding that tumor growth was slowed in vivo.

As considerable role of CENPs has been reported, CENPs were identified as therapeutic targets in cancer treatment. High expression of CENP-A was reported in ER negative breast cancer compared with ER positive subjects, and positively correlated with Ki67 expression [18]. By targeting CENP-A, the aggressive phenotype of lung adenocarcinoma cells could be attenuated [23]. Using a lung cancer mouse model with down-regulation of CENP-E, researchers found that low rates of chromosome missegregation can promote tumorigenesis, however, missegregation of high numbers of chromosomes results in tumor inhibition [26]. Knockdown the expression of CENP-K decreased cell viability in triple negative breast cancer cells [27]. On account of the core status of CENP-N during CCAN assembly, kinetochore formation and chromosome segregation [8,9,11], CENP-N may be an effective therapeutic target in the treatment of breast cancer, especially in the aggressive subjects.

Gene profiling was evaluated to clarify the changed molecules involving in down-regulating expression of CENP-N in breast cancer cells. Analyses of diseases or functions annotation using differential expressions of molecules after CENP-N knocking-down showed that many tumor development associated diseases or functions were significantly suppressed, which were accompanied by activated functions of immune response, cell apoptosis and cell death. Within these diseases and functions, the mostly affected is the immune response. Recently, enhanced tumor immunity has been considered to be associated with the anti-cancer effect [28]. Patients with more tumor-infiltrated lymphocytes (TILs) usually have a long-term overall survival, especially in estrogen and progesterone receptor negative breast cancer [29,30]. Moreover, more infiltration of immunologic effector cells after chemotherapy indicates a good response to the treatment [31]. Thus, the enhanced immune response is closely associated with inhibited tumor progression and increased therapeutic effect. Therefore, significantly activated immune response after down-regulation of CENP-N may be potential stimuli to enhance anti-tumor effect. Moreover, increased apoptosis and cell death effect by down-regulation of CENP-N may coordinately inhibit breast tumor growth. These findings support the notion that targeting CENP-N can block breast cancer development by promoting apoptosis and enhancing immune response for killing cancer cells.

In our study, further analysis reveal that significantly activated Interferon signaling by reducing the expression of CENP-N may contribute to blocked tumor growth. Interferon was found to be against tumor primarily owing to their anti-proliferation and immune-stimulating activities, which can function through activation of specific signal transducers

and activators of transcription (STATs) and interferon stimulated genes (ISGs) [32-34]. In our study, by knocking down of CENP-N, STAT2 and a number of ISGs were identified, such as interferon-induced transmembrane protein 1 (IFITM1), interferon induced protein with tetratricopeptide repeats 3 (IFIT3), interferon induced protein with tetratricopeptide repeats 1 (IFIT1), Interferon alpha-inducible protein 6 (IFI6) and interferon regulatory factor 1 (IRF1), which indicated the activation of interferon signaling. Consistent the anti-tumor effect by knocking down of CENP-N, IRF1 was reported to be associated with the growth inhibitory activity of IFN- γ in human breast carcinoma cells [35-37]. Additionally, IRF1 is widely considered as a tumor suppressor to promote apoptosis, inhibit tumor growth and progression in breast cancer. Furthermore, high IRF1 expression is associated with prolonged survival in patients with breast cancer. In addition, down-regulated cyclin D1 (CCND1), up-regulated caspase 1 (CASP1) and interferon-stimulated gene 15 ubiquitin-like modifier (ISG15) were also found, which all could be regulated by IRF1. Taken together, activated interferon signaling through down-regulation of CENP-N, which is characterized by up-regulated IRF1, may attribute to inhibited tumor growth in breast cancer.

In addition to anti-tumor effect on tumor growth, the interferon signaling also participates in immune responses. Free ISG15 is found to show anti-tumor activity by increasing NK cell infiltration, and therefore suppress tumor growth. Thus, our study provide the concept that targeting CENP-N may be used in the treatment of breast cancer, which is dependent on its role in both directly inhibiting tumor growth and indirectly promoting immune responses. However, overexpression of ISG15 is also linked to tumorigenesis and unfavorable prognosis in breast cancer. Though the targeting CENP-N therapy could be a promising therapeutic regimen, the mechanisms still need to be further examined and elucidated.

Conclusion

Centromere proteins are essential for mitosis and cell proliferation, and associated with tumor initiation and progression. In the present study, we found that targeting CENP-N could promote apoptosis and inhibit tumor growth, which could be partially attributed to the activated interferon signaling that is characterized by increased expression of IRF1. Our study provides a new strategy for the treatment of breast cancer, and targeting CENP-N therapy may not only have a function on breast cancer, but also on immune responses.

Abbreviations

List of Abbreviations

Abbreviations

CENPs	Centromere proteins
CIN	Chromosomal instability
M phase	Mitosis phase
CCAN	Centromere associated network
STATs	Specific signal transducers and activators of transcription
ISGs	Interferon stimulated genes
IFTM1	Interferon-induced transmembrane protein 1
IFIT3	Interferon induced protein with tetratricopeptide repeats 3
IFIT1	Interferon induced protein with tetratricopeptide repeats 1
IFI6	Interferon alpha-inducible protein 6
IRF1	interferon regulatory factor 1
CCND1	cyclin D1
CASP1	Caspase 1
ISG15	interferon-stimulated gene 15 ubiquitin-like modifier

Declarations

Acknowledgements

The authors thank Dr. Bin Yang for statistical analysis of data in this study.

Funding

This study was sponsored by the National Natural Science Foundation of China (grant no. 81472460).

Authors' contributions

All authors have read and approved the manuscript. ZZ, and HS designed this study. All authors participated in doing experiments, and data collection and analysis, also contributed to write the manuscript. All authors revised and approved the final manuscript.

Consent for publication

Not applicable.

Competing interests

There are no competing interests to declare.

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Figures

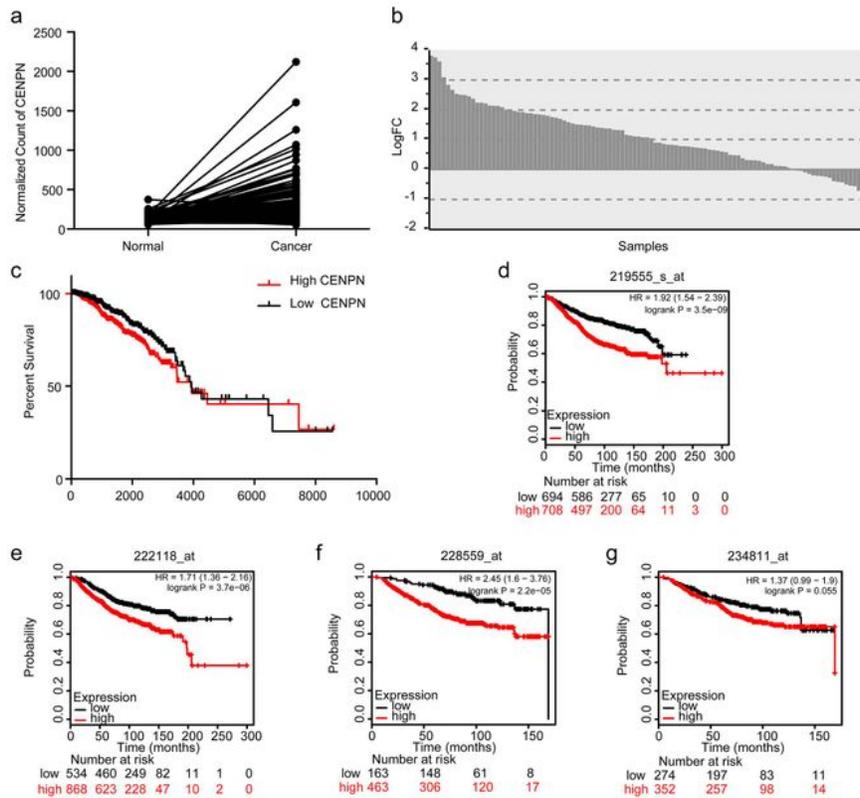


Figure 1

Higher CENPN in breast cancer compared with normal and associated with short-term overall survival. Higher normalized RNA count in breast cancer compared with paired adjacent noncancerous breast tissues from TCGA database (a). Fold change of cancer to normal in each paired group were plotted (b). Survival curve showed patients with high CENPN associated with a decreased overall survival compared with low CENPN ($P=0.042$) (Data were from TCGA database) (c). Survival curves downloaded from

website (<http://kmplot.com>) (d-g). Group of High or low CENPN was split by median value of CENPN on mRNA level.

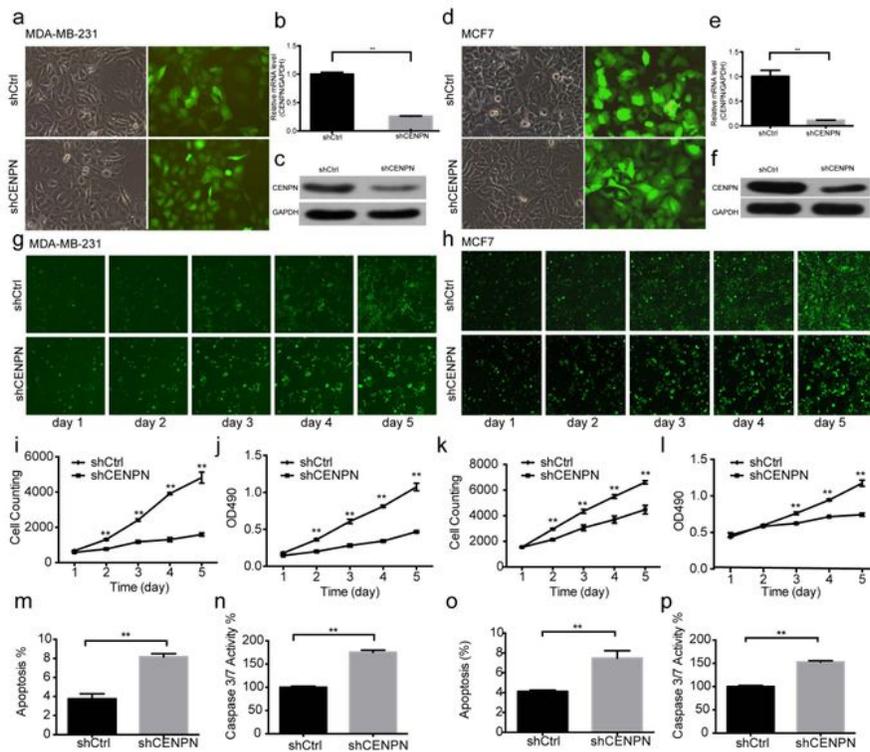


Figure 2

CENPN down-regulation inhibited proliferation and promote apoptosis of breast cancer cells. Immunofluorescent pictures of MDA-MB-231 and MCF7 breast cells indicated efficiency of transfection of lentivirus packaged plasmid in both shCtrl and shCENPN groups (a, d). Down-regulated relative mRNA

level and protein expression of CENPN were confirmed in shCENPN group compared with shCtrl (b-c, e-f). Down-regulation of CENPN decreased cell proliferation, cell viability in MDA-MB-231 (g, i-j) and MCF7 cells (h, k-l). Down-regulation of CENPN increased apoptosis and caspase3/7 activity percentage in MDA-MB-231 (m, n) and MCF7 cells (o, p).

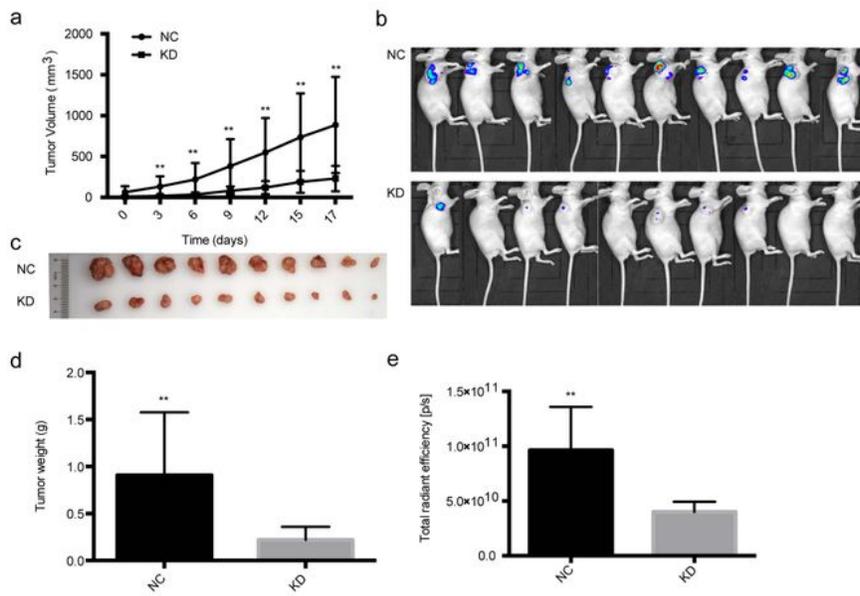


Figure 3

score, and effect direction was dependent on positive or negative values of Z-score (a). Significant activated or inhibited ingenuity canonical pathway after downregulation of CENP-N (b). Heatmap of molecules involved in significantly activated or inhibited signaling in shCtrl (NC) and shCENPN (KD) group (c). Protein expressions involved in interferon signaling in shCtrl (NC) and shCENPN (KD) group (d). Red color show activated diseases or functions, canonical pathways, or up-regulated molecules. Blue color show inhibited diseases or functions, canonical pathways, or downregulated molecules.

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