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ORIGINAL ARTICLE

Knockdown of CETN1 inhibits breast cancer cells proliferation

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Summary

Purpose: *Breast cancer is a multifactorial disease and identification of the genes and proteins regulating breast carcinogenesis is critical in exploring novel targeted therapies. The aim of this study was to investigate the role CETN1 in breast cancer growth and metastasis.*

Methods: *CETN1 expression levels were measured in breast tissue samples by immunohistochemistry (IHC). We selected high CETN1-expressing cell lines from human breast carcinoma cells with metastatic potential. The proliferation of cells was evaluated by MTS assay. Changes in progression of cell division were assessed by cell cycle analysis. Changes in CETN1 levels in breast tumor cells were analysed by Western blot.*

Results: *Our results demonstrated that overexpression of CETN1 occurred mainly in breast cancer cells in which CETN1 expression was low. Knockdown of CETN1 by lentivirus-shRNA significantly inhibited breast cancer cells' proliferation, growth and metastasis.*

Conclusion: *CETN1 is overexpressed in breast cancer tissue and promotes cells'proliferation, tumor growth and metastasis.*

Key words: *breast cancer, CETN1, gene therapy, proliferation*

Introduction

Breast cancer, the most common malignancy in women, is a heterogeneous disease exhibiting substantial diversity of histological and molecular characteristics [1]. Most cases are classified as 'sporadic' breast carcinoma, caused by genetic changes that occur over time [2]. Despite continuing development of new therapies for patients with breast cancer, the prognosis remains rather poor. This prognosis may be related to molecular abnormalities of cancer cells. Moreover, higher percentage of metastasis and recurrence and poor prognosis have also been recorded in patients treated with local therapy. Identification of the genes and proteins regulating breast cancer evolution is critical in exploring novel targeted therapies [3,4].

Centrins are calcium-binding phosphoproteins with 4 Ca 2^{+}-binding EF-hand domains that are localized to the centrosome of all eukaryotes,including 3 separate Centrin genes; CETN1-3 have been identified in both mice and humans [5,6]. While CETN1 and CETN2 show substantial identity with each other (84%), they share only about 51% identity with CETN3. Inhibiting CETN2 in HeLa cells blocked centriole duplication, eventually leading to cell death [7]. In contrast to CETN2, not much is known about the human CETN1. CETN1 is an gene located on chromosome 18p11 that possesses all the sequence features of an expressed retroposon: no introns, coding region flanked by a pair of direct repeats due to the open reading frame without interruptions of stop codons [8]. CETN1 in humans is expressed specifically in some organs apart of breast tissue. However, whether it is expressed in breast cancer is unknown.

In this study, we performed a research to investigate the whether CETN1 can influence breast

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cancer cells' proliferation, growth and metastasis, in order to propose a new therapeutic strategy.

Methods

Cell lines

Human breast cancer cell lines MCF-7, Bcap-37, and mouse embryonic fibroblast 7 (MEF7) cell line were purchased from the ACTT (USA). The cells were cultured in Dulbecco's modified Eagle medium with 10% fetal bovine serum supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Eight-week-old female Lewis rats were purchased from HFK biology Co. Ltd, Beijing, China.

Immunohistochemistry (IHC) and immunocytochemistry (ICC)

The expression of CETN1 was measured by IHC in a retrospective cohort of breast cancer and matched adjacent non-tumor tissue samples from breast cancer patients after breast resection. The expression of CETN1 was also measured by ICC in MCF-7 cells. Paraffin-embedded tissue blocks were cut into 4 μm sections, and each one was incubated at 60 °C for 2 hrs before deparaffinization. Antigen retrieval was achieved by microwave exposure at 100 °C in citrate buffer for 15 min. After immersion in 3% hydrogen peroxide for 20 min, the sections and cells were incubated with goat serum for 1 h. Then, they were incubated with CETN1 (Santa, Los Angeles, USA) (1:80), and anti-phospho-CETN1 (Santa) (1:80) at 4 °C overnight. After 4 washings in phosphate twain buffer (PBST), sections were incubated with second antibody (K5007, Dako, Glostrup, Denmark) for 1 h (37 °C). Visualization was performed using DAB chromogen (K5007, Dako, Denmark). Sections were counterstained with hematoxylin, dehydrated, and mounted. Experiments were repeated twice, and the percentage of tumor cells or normal breast cells with obvious staining in the cytoplasm or nucleus was sought in all optical fields of the slices. A percentage exceeding 10% was considered as positive immunoreactivity. Two independent blinded investigators examined all tumor slides randomly.

Lentivirus production and transduction

Firstly, we selected the sequence for targeting the CETN1 gene in the short hairpin RNA (shRNA) Clone Library [http://cgap.nci.nih.gov/RNAi/RNAi2]. The effective target point sequence of CETN1 gene was: 5'-CATTGCTGCTGTTAATGTA-3'. Human GAPDH:-Forward primer: 5'-AGAAGGCTGGGGCTCATTTG-3', Reverse primer:5'-AGGGGCCATCCACAGTCTTC-3'. The lentivirus (pMagic 4.1) construct was provided by Shanghai Sunbio Medical Biotechnology Co. Ltd, China (SB1262-C). MCF-7 cells were transfected with lentiviral vectors and control lentivirus in complete medium containing polybrene (8 mg/ml). At 72 hrs after the first transfection, total RNA and protein were extracted from cells and real time PCR and Western blot analysis were employed to evaluate the inhibitory effect.

Flow cytometry analysis for cell cycle

Cells were harvested at the exponential growth phase, and single-cell suspension solutions containing 1×10^6 cells were fixed with 75% alcohol for 48 hrs in 4 °C. Cell cycle was monitored using propidium iodide (PI) 50 μg/ml (Sigma, San Diego, USA) staining. The fluorescence of DNA-bound PI in cells was measured with a flow cytometer (BD Biosciences, New Jersey, USA).

In vitro cell growth assay

A novel reagent (Cell Titer 96 AQueous One SolutionCell Proliferation Assay, Promega, Cat # 3582), which contains a tetrazolium compound (MTS) was used in this part of the study [9]. The cells were prepared at a concentration of 1×10^4 cells/ml. Aliquots (100 μl) were dispensed into 96-well microtiter plates. The cells were incubated for 1, 2, 3, 4 and 5 days, respectively. MTS assay was performed by adding 20 μl of MTS reagent into each well and incubating the plate at 37 °C for 3 hrs in a humidified 5% CO_2 atmosphere. The absorbance value (optical density/OD) of each well was measured with a microplate reader set using ELISA (Model 550, Bio-Rad, USA) at 490 nm. The absorbance value on day 1 was defined as 1. The data of the other days were reported relative to that on day 1.

Real-time quantitative PCR

Total RNA was extracted with TRIzol (No: 1382739; Invitrogen, San Diego, USA). The total RNA concentration was measured by spectrophotometry, then 300 ng of total RNA from each sample was reverse-transcribed to cDNA using the PrimeScript RT reagent Kit (San Diego, USA) (Code: DRR037A, TaKaRa). The cDNAs were amplified by RT quantitative (q) PCR (SYBR, Code: DRR041A, TaKaRa). The amplification process was applied in a LightCycle system (Roche 2.0, software: version 3.5). Amplified conditions were: 95 °C for 30 sec, 40 cycles at 95 °C for 5 sec, and 64 °C for 30 sec. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. Primers (Forward Primer: 5'-GCAGAAGATGTCCGAGAAGG-3'; Reverse Primer: 5'-CACACGCTTCAGGTTTTTGA-3') were designed by the Sangon Co. (Shanghai, China). Primer specificity was checked using Primer BLAST (NCBI). Relative quantification was accomplished by double standard curve method or the $2^{-\Delta\Delta Ct}$ method [10]. The Ct values of the samples were normalized to an appropriate endogenous housekeeping gene GAPDH. Each measurement was repeated in triplicate.

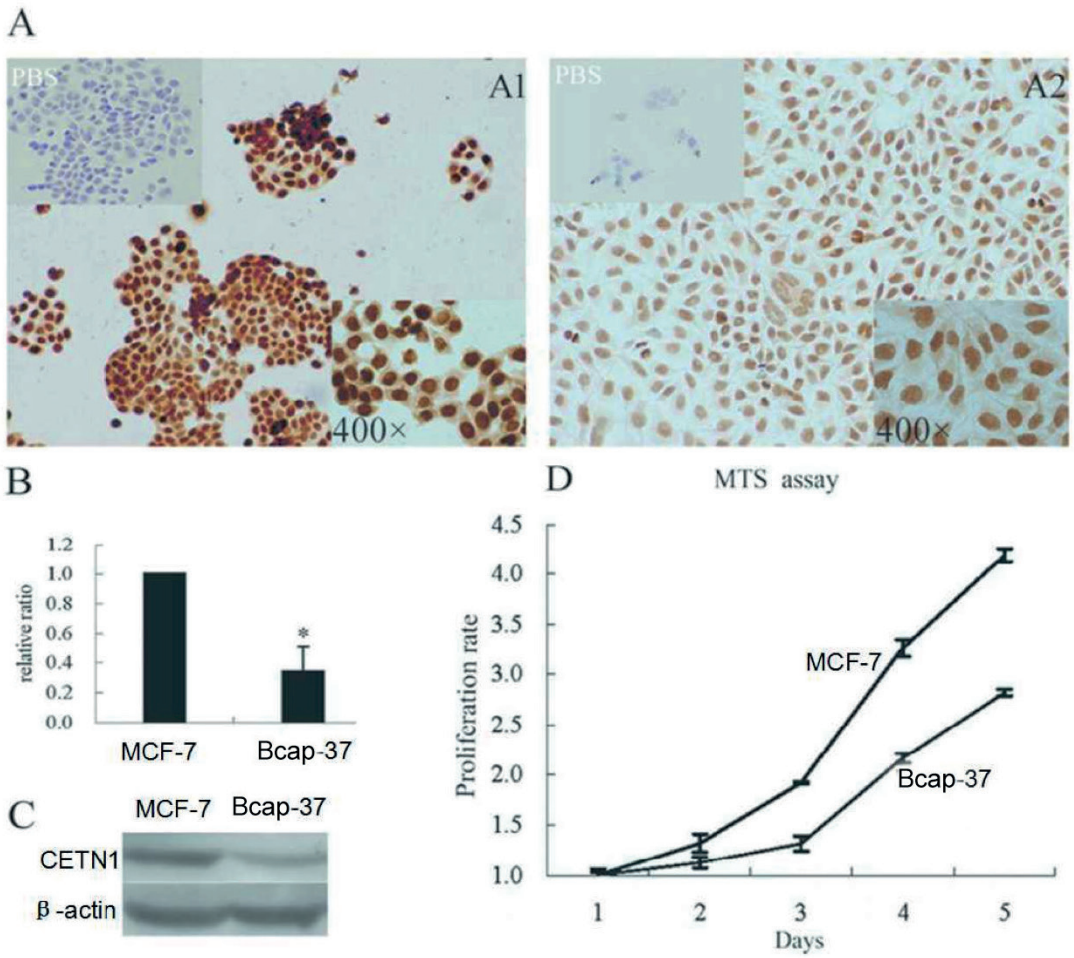

Figure 1. Expression of CETN1 in MCF-7 and Bcap-37 cell lines. **A:** immunohistostaining of CETN1 in MCF-7 and Bcap-37 cells (x100). Negative controls: PBS instead of first antibody. A1 shows MCF-7 cells, A2 Bcap-37 cells. Upper left of A1 and A2: shown is the expression of CETN1 in MCF-7 and Bcap-37 cells after treatment, respectively. Down right of A1 and A2: shown is the expression of CETN1 in MCF-7 and Bcap-37 cells after CETN1, respectively. **B:** CETN1 mRNA levels in MCF-7 and Bcap-37 cell lines as determined by real-time PCR. *Paired samples Student's t-test, p=0.021. Relative quantification was accomplished by double standard curve method, and normalized by GAPDH mRNA. Experiments were performed in triplicate. **C:** CETN1 protein levels in MCF-7 and Bcap-37 cells as determined by Western blot analysis ($p<0.05$ vs MCF-7). β-actin was used to normalize the data. **D:** Cell proliferation rates were measured using the MTS assay. Results are presented as means±SD. The proliferation rates in 2-5 days were relative to the value of the 1st day ($p<0.05$).

In vivo assay

After approximately one week of acclimatization after female Lewis rats arrival, 5×10^6 MCF-7 cells in 100mL PBS were injected s.c. into the right and left flanks of the rats.

Western blot

Cells were lysed in RIPA (P0013B, Beyotime, Suzhou, Jiangsu, China) with a protease inhibitor (No.11873580001, Roche, Penzberg, Germany). The bicinchonic acid disodium (BCA) method (P0010, Beyotime, Suzhou, Jiangsu, China) was used to determine the protein concentration [11]. A total of 40 μg of protein was boiled for 10 min before loading, was then separated using 10% SDS-PAGE. Then the proteins were transferred onto nitrocellulose membranes (0.45 μm, Whatman, Clifton, NJ, USA) by the semi-dry transfer system (Bio-Rad, USA). After blocking with 5% bovine serum albumin (BSA), the membrane was probed with CETN1 (1:300), and β-actin antibody (1:500) (Immunocreate,USA). Secondary antibodies against rabbit (Thermo Fisher, USA) (1:40,000) and mouse (Jackson, USA) (1:40,000) IgG were used. Protein band signals were amplified by chemoluminescence detection reagents (Thermo Fisher, USA). Protein levels were determined semi-quantitatively using the image software (Quantity one, version 4.4.0).

Statistics

Pearson's Chi-Square test, and the Independent-Sample t-test were used. All statistical analyses were performed using the SPSS 13.0 program. A p value <0.05 was considered to be statistically significant.

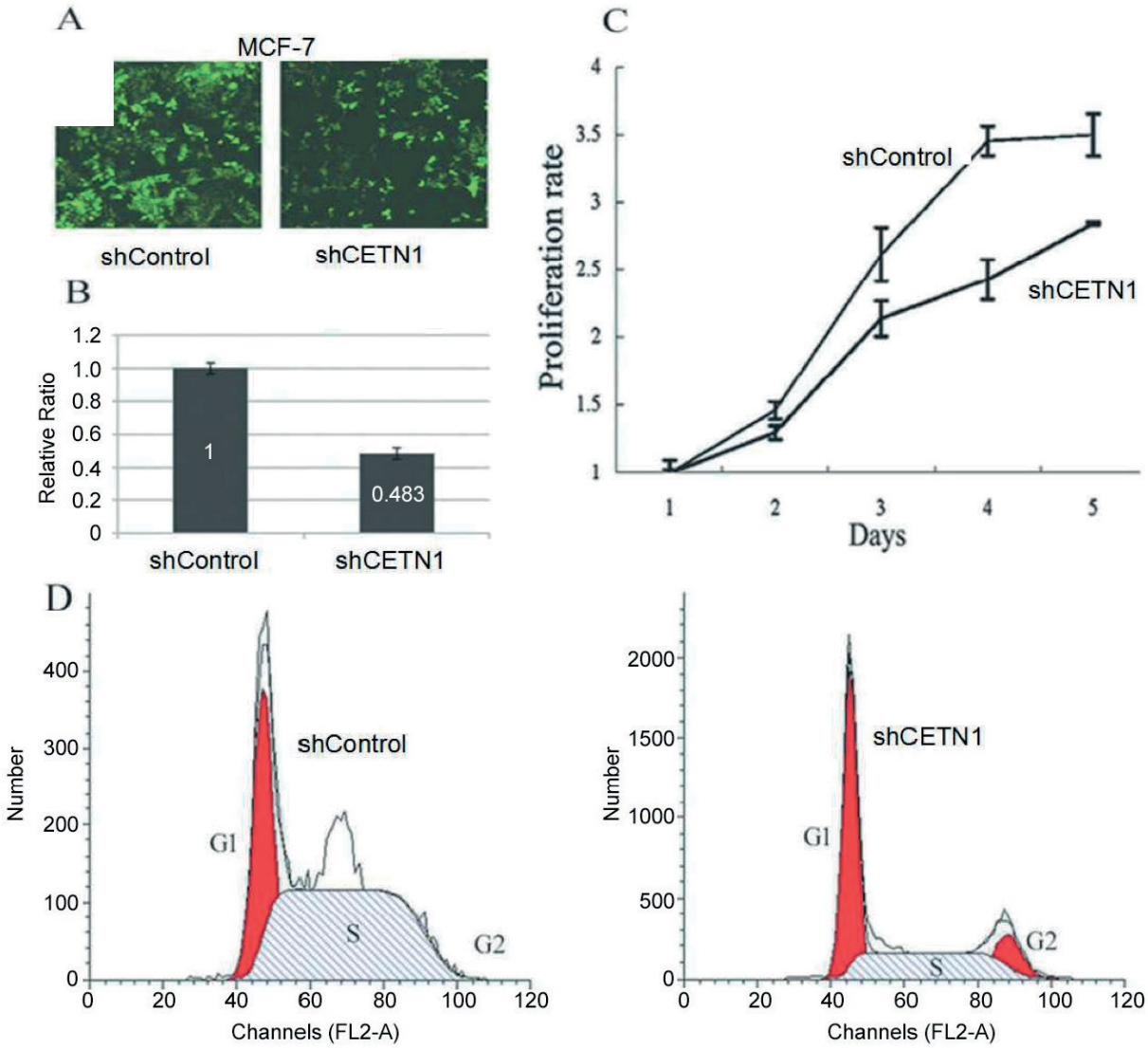

Figure 2. Effects of lentivirus-mediated RNAi on CETN1 expression and cell proliferation. **A:** Fluorescence photomicrographs of MCF-7 cells show transduction efficiency of lenti-shRNA and control lentivirus. Images were taken at 72 hrs after transfection with same cell density in 6-well plates. Cell counts in shCETN1 wells were lower than shControl wells. **B:** Effects of knockdown of CETN1 mRNA and CETN1 gene as determined by real-time PCR in MCF-7 cells. Relative quantification of mRNA was accomplished by the $2^{-\Delta\Delta Ct}$method ($p<0.05$). **C:** Cell proliferation rate was measured using the MTS assay. Results are presented as means±SD ($p<0.05$). **D:** Effects of CETN1 knockdown on the cell cycle progression as detected by flow cytometry analysis.

Results

Breast cancer tissues expressed higher levels of CETN1 compared with the adjacent tissues

We investigated CETN1 protein levels in 40 cases of breast cancer tissue and 15 cases of para-cancerous breast tissues (PCT) by IHC. The positivity rate of CETN1 protein in breast cancer tissue (29/40, 72.5%) was higher than in PCT (3/15, 20.0%; $p<0.01$). Compared with adjacent tissues, breast cancer tissues had higher levels of CETN1 protein (32/40, 80%; $p<0.05$). Furthermore, CETN1 was found to be concentrated in the nuclei of breast cancer cells.

The MCF-7 cell line with higher level CETN1 had higher proliferating capacity

The proliferation activity of MCF-7 cell line with various CETN1 levels differed. CETN1 expression in the two breast cancer cell lines with different metastatic properties was examined using immunohistochemistry and Western blot analysis (Figure 1A,C). The MCF-7 cells aggregated during growth, while the Bcap-37 cells spread out during growth. MCF-7 cells, which have a high metastatic potential, expressed higher levels of CETN1 protein than Bcap-37 cells. As shown in Figure 1B, MCF-7 cells had higher levels of CETN1 mRNA than Bcap-37 cells ($p=0.021$). More

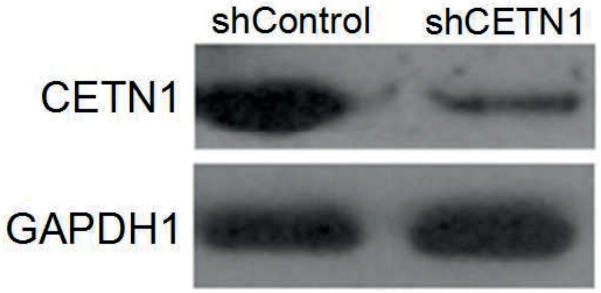

Figure 3. Effects of lentivirus-mediated RNAi on CETN1 expression in Mouse Embryonic Fibroblast-7 (MEF-7) cells. Effects of knockdown of CETN1 mRNA, and CETN1 protein as determined by Western blot, respectively, in shControl MEF-7 cells and shCETN1 MCF-7 cells.

importantly, the MTS assay showed that the proliferative activity of MCF-7 cells was higher than that of Bcap-37 cells (Figure 1D), although Bcap-37 cells grew rapidly. The proliferation of MCF-7 and Bcap-37 on the 5th day were 4.12 ± 0.06 and 2.82 ± 0.03, respectively ($p<0.05$). So, the proliferation activity of these breast cancer cells paralleled the levels of CETN1.

RNAi mediated CETN1 gene silencing inhibits MCF-7 cells proliferation and cell cycle

The effects of knockdown of CETN1 expression in MCF-7 cells on cell proliferation and cell cycle were obvious. Lentivirus-mediated RNAi efficiently downregulated CETN1 expression as reflected by mRNA (Figure 2A and B) and protein levels (Figure 3). Cell proliferation was suppressed and cell cycle progression was altered in MCF-7 cells (Figure 2C and D). The proliferation of shCETN1 cells and shControl cells on the 4th day were 2.42 ± 0.15 and 3.45 ± 0.11 ($p<0.05$), respectively. Cell cycle analysis of MCF-7 cells by flow cytometry showed that the proportion of shCETN1 cells in the G1, G2 and S phases were 50.33, 13.94 and 35.73%, respectively, and the proportion of shControl cells were 33.67, 0.94 and 65.39%, with significant differences between the two groups ($p<0.001$). The proportion of cells in the G1 and G2 phase was higher in the shCETN1 cells, whereas the proportion cells in S phase was higher in the shControl cells. CETN1 silencing cells showed G1, G2 phase arrest and a decrease of cells in S phase.

Discussion

In this study, we found that CETN1 is overexpressed in breast cancer tissue compared to non-breast cancer tissue. Moreover, silence of CETN1 can inhibit the cancer cells' proliferation and tumor growth and metastasis.

Cancer cell lines from different origins showed very little expression of CETN1 [12]. However, our research has shown that CETN1 is overexpressed in breast cancer tissue. Overexpression of CETN1 promotes the MCF-7 breast cancer cells proliferation, and tumor growth and metastasis and has been identified as an oncoprotein in breast cancer. As critical constituents of the centriole, centrins play an important role in cytokinesis and cell cycle progression [13]. For example, gene disruption experiments in yeast demonstrate that centrin (CDC31) is essential for cell viability [14]. In addition, in yeast and other eukaryotes, deletions and mutations in the calcium binding domains can cause a no-intact basal body, cell separation, and polarization, important in ciliary function [15]. Finally, siRNA-mediated silencing of CETN1 in HeLa cells blocked centriole duplication, eventually leading to cell death [16]. CETN1 mRNA and protein levels were both higher in breast cancer than in para-cancerous tissue. Surprisingly, a previous study has shown that CETN1 is also overexpressed in prostate and pancreatic cancer [12]. Taken together, CETN1 may be an important tumor antigen.

Emerging evidence also suggests that human CETN1 plays a regulatory role in the recognition of DNA damage [17]. CETN1 promotes DNA binding by xeroderma pigmentosum cells (XPC) and increases the specificity of the heterotrimer for damaged DNA [18,19]. Finally, data from yeast and Xenopus indicate additional functions of the centrins that include mRNA transport from the nucleus [20,21]. Considered together, centrins are multifunctional proteins that are critical to normal cellular function. Therefore, CETN1 promotes tumor progress by modulating the gene expression, including genetic damage.

In conclusion, our results show that CETN1 is overexpressed in breast cancer tissue, and promotes cancer cells' proliferation, tumor growth and metastasis. These findings may form the basis for inhibition of CETN1 as targeted therapy for breast carcinoma in the future.

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