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Ubiquitin ligase CHIP suppresses cancer stem cell properties in a population of breast cancer cells

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ABSTRACT

Cancer stem cells (CSCs) have several distinctive characteristics, including high metastatic potential, tumor-initiating potential, and properties that resemble normal stem cells such as self-renewal, differentiation, and drug efflux. Because of these characteristics, CSC is regarded to be responsible for cancer progression and patient prognosis. In our previous study, we showed that a ubiquitin E3 ligase carboxyl terminus of Hsc70-interacting protein (CHIP) suppressed breast cancer malignancy. Moreover, a recent clinical study reported that CHIP expression levels were associated with favorable prognostic parameters of patients with breast cancer. Here we show that CHIP suppresses CSC properties in a population of breast cancer cells. CHIP depletion resulted in an increased proportion of CSCs among breast cancers when using several assays to assess CSC properties. From our results, we propose that inhibition of CSC properties may be one of the functions of CHIP as a suppressor of cancer progression.

1. Introduction

Breast cancer is the most frequent cause of cancer death among women [1]. Although numerous therapeutic strategies have been developed for breast cancer, many problems with achieving complete remission remain to be resolved.

In a previous study we showed that a ubiquitin E3 ligase carboxyl terminus of Hsc70-interacting protein (CHIP) suppressed breast cancer malignancy [2]. CHIP is a tetratricopeptide repeat (TPR) containing protein that is expressed in a number of organs [3,4]. CHIP ubiquitinates misfolded proteins along with heat shock proteins and induces their degradation through the proteasome pathway, which is involved in protein quality control [5–7]. Moreover, CHIP participates in several biological processes through ubiquitination of specific target proteins [8–10]. In our previous study, we found that CHIP expression levels were negatively correlated with tumor malignancy in human breast tissues and suppressed tumor growth and metastasis of breast cancer cells [2]. In addition, a recent clinical study reported that CHIP expression levels were associated with favorable prognostic parameters of patients with breast cancer [11]. Thus, CHIP may be a novel target for breast cancer therapy. However, many issues remain to be addressed regarding the mechanism(s) of how CHIP is involved in breast cancer progression.

Recent studies suggested the importance of eliminating cancer stem cells (CSCs) to achieve complete remission [12,13]. CSCs have tumor-initiating potential [14] and high metastatic potential [15]. They also have properties that resemble those of normal stem cells, such as self-renewal, differentiation [16], and drug efflux [17]. Because of their self-renewal and differentiation properties, CSCs organize into a hierarchical structure and give rise to cancer heterogeneity [16], which hampers prediction of tumor behavior and clinical outcome [18]. Moreover, these cells' drug efflux capability results in resistance to conventional chemotherapies, which allows CSCs to survive even under anticancer drug treatment conditions [17]. Thus, understanding the biology of CSCs would contribute to the development of novel cancer therapies by overcoming problems encountered clinically, such as therapeutic resistance and relapse.

CSC properties can be assessed using different specific experimental systems; such as a sphere formation assay, a side

Abbreviations: CHIP, carboxyl terminus of Hsc70-interacting protein; CSC, cancer stem cell; SP, side population.

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population (SP) assay, and a limiting dilution xenograft assay. These assays are based on stem cell-like characteristics; including non-adherent growth capability [19] and drug efflux capacity [20], and on tumor-initiating potential [14]. To assess the non-adherent growth capability, the sphere formation assay is conducted [19]. In this assay, cancer cells are cultured under non-adherent conditions and form some spheres [21], after which the numbers of spheres are counted. To examine the drug efflux capacity mediated by transporters, the SP assay has been used [20]. In this assay, the proportions of SP cells are determined by evaluating the efflux capacity for the DNA-binding dye Hoechst 33342 using a fluorescence-activated cell sorter (FACS) [20]. To test the tumor-initiating potential, the limiting dilution xenograft assay is conducted [14,22]. While non-CSCs cannot form tumors by themselves, CSCs can initiate tumors *in vivo* [22]. This tumor-initiating potential is assessed by transplantation of serial diluted cancer cells into immunodeficient mice.

Numerous studies analyzed the CSC properties using breast cancer cell lines [21,23]. Clinically, breast cancer cells with stem cell phenotypes were also detected in bone marrow of breast cancer patients [24]. Thus, the presence of CSCs among breast cancer cells is becoming clear, but the precise regulation and characteristics of CSCs remains to be determined.

Here we show that depleting CHIP increases the proportions of CSCs among breast cancer cells by examining CSC properties using these assays. Thus, CHIP may contribute to a favorable prognosis for patients with breast cancer through its inhibition of CSC properties.

2. Materials and methods

2.1. Cell culture and treatment

MCF-7, MDA-MB-231 and T47D cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and Penicillin-Streptomycin Mixed Solution (Nacalai Tesque).

2.2. RNA interference

RNA interference experiment was performed as described in Kajiro et al. [2], with minor modification. We used a retroviral expression system. The pVSV-G vector and pSINsi-hU6 (Takara) vector containing either the *CHIP* or *LacZ* (control) target sequence were co-transfected into GP2-293 cells (Clontech). MCF-7 and T47D cells were incubated with the retroviral supernatant in the presence of 8 μg/ml polybrene. Twenty-four hours after infection, the viral supernatant was replaced with fresh DMEM containing 10% FBS. The infected cells were selected with 1 mg/ml G418. The target sequences were 5′-gcacgacaagtacatggcgga-3′ for *CHIP*, and 5′-gctacacaaatcagcgatt-3′ for *LacZ*.

2.3. Immunoblotting

Immunoblotting experiment was performed as described in Kajiro et al. [2]. Cells were lysed in 0.5% Triton buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, and 0.5% TritonX-100]. Extracted proteins were separated by SDS–PAGE, transferred onto PVDF membranes (Millipore), immunoblotted with the indicated antibodies. The antibodies employed in this study included mouse monoclonal antibodies specific for β-Actin (1:1000; Santa Cruz, clone C4), and rat monoclonal antibodies against human CHIP (1:250; Green Space Biomed, Japan).

2.4. Sphere forming assay

MCF-7 cells were plated onto 6-well ultra-low-attachment plates (Corning Costar) at 1000 cells per well. MCF-7 cells were maintained in DMEM/Ham's F-12 (Nacalai Tesque) supplemented with 0.4% bovine serum albumin, 4 μg/ml bovine insulin (Sigma-Aldrich), 20 ng/ml EGF (Peprotech), and 1 × B27 supplement (Gibco). After 7 days, over 100 μm spheres were counted using BioZero BZ8000 microscope (Keyence). T47D cells were plated onto 6-well ultra-low-attachment plates at 5000 cells per well. T47D cells were maintained in CnT-27 medium (Cellntec). After 10 days culture, over 100 μm spheres were counted. MDA-MB-231 cells were plated onto 6-well ultra-low-attachment plates at 5000 cells per well. MDA-MB-231 cells were maintained in CnT-Prime medium (Cellntec). After 7 days culture, over 100 μm spheres were counted.

2.5. Side population (SP) analysis

Cells were removed from culture dish by Tripsin-EDTA solution. 1×10^6 cells were suspended in 970 μl Hanks' balanced salt solution (HBSS) with 2% fetal bovine serum (FBS) and 10 mM HEPES (pH 7.4). The cells were incubated at 37°C for 90 minutes with 17.5 μg/ml Hoechst 33342 (Sigma-Aldrich). Then, the cells were washed with 2% FBS in PBS, and resuspended in ice-cold HBSS with 2% FBS, 10 mM HEPES (pH 7.4), and 2 μg/ml propidium iodide (Sigma-Aldrich). Fumitremorgin C (FTC, 1 μM) was used to confirm the SP fraction. SP cells were analyzed with FACSAria (BD Biosciences). Collected events were analyzed using FlowJo (Tree Star).

2.6. Real-time RT-PCR

Real-time RT-PCR was performed as described in Kajiro et al. [2], with minor modification. Cells were homogenized in 1 ml of Sepasol-RNA I Super G (Nacalai Tesque) and total RNA was extracted according to the instruction manual and treated with DNase (Promega) for 30 min at 37°C. cDNA was synthesized from total RNA using RevatraAce reverse transcriptase (Toyobo) and random primers. The cDNA was amplified by rea-ltime PCR using Thermal Cycler Dice™ TP800 (Takara) and SYBR® Premix Ex Taq™ (Takara). Samples were normalized by *PPIA* mRNA levels. The primers for real-time PCR are as follows: 5′-acgtggtataaaaggggcgg-gag-3′ and 5′-tcaccaccctgacacataaaccctg-3′ for *PPIA*, 5′-atgcctgt-gatttgtgggcc-3′ and 5′-gccagttgtttttctgccac-3′ for *NANOG*, and 5′-ggacccattggcattctc-3′ and 5′-caggacacagcatagaataatc-3′ for *CD133*.

2.7. Limiting dilution transplantation assay

NOD.CB17-*Prkdc*^{scid}/J (NOD/SCID) female mice at 5 weeks of age were purchased from Charles River Laboratories US. MCF-7 cells were cultured as monolayers, trypsinized, and resuspended in Matrigel (BD Biosciences) at dose ranging from 1.0×10^6 to 5.0×10^2 cells/ml. Female NOD/SCID mice were given bilateral subcutaneous injections of 0.1 ml MCF-7 cells. The mice were kept in a pathogen-free environment. After 6 weeks all of the mice were sacrificed, and the tumor tissues were collected. All animal experiments were in accordance with institutional guidelines.

2.8. Statistical analysis

Student's *t*-test was used to compare number of sphere (Fig. 1C and E) and mRNA levels (Fig. 3). Standard deviation and sample number were abbreviated as "S.D." and "*n*", respectively.

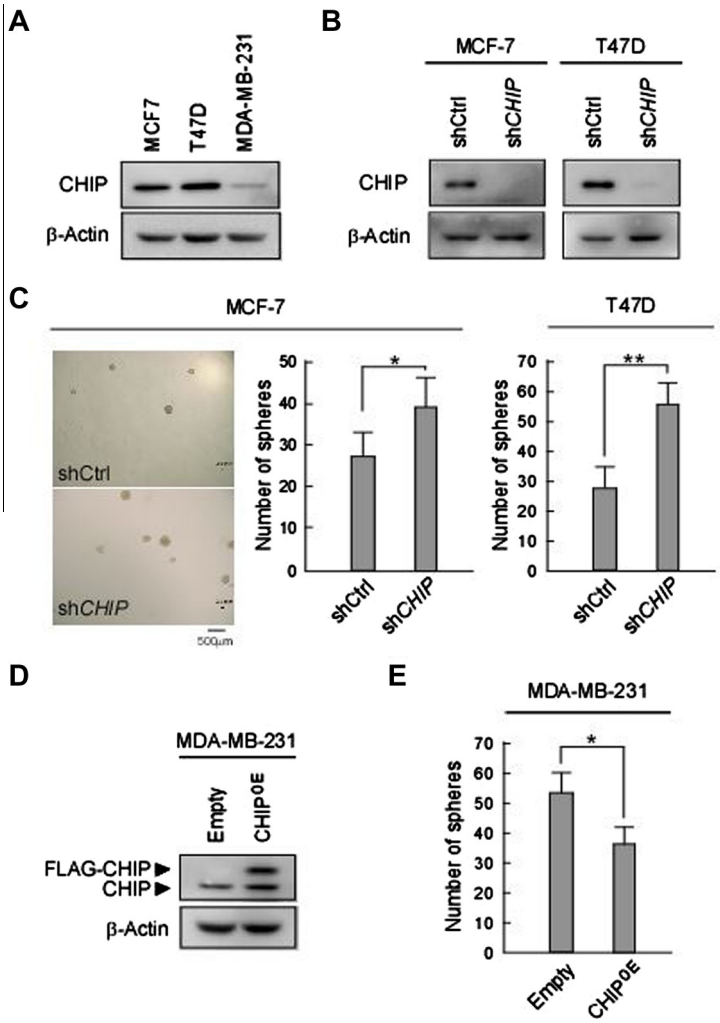

Fig. 1. CHIP inhibits sphere-forming capability. (A) Endogenous CHIP expression levels in MCF-7, T47D, and MDA-MB-231 cells. Total cell lysates of these cells were analyzed using the indicated antibodies. (B) CHIP depletion efficiency by shRNA. CHIP protein levels in MCF-7 and T47D cells infected with a retro virus containing shRNA against *LacZ* (shCtrl) and *CHIP* (sh*CHIP*) were analyzed by immunoblotting using the indicated antibodies. (C) Sphere-forming capabilities of MCF-7 and T47D shCtrl and sh*CHIP* cells. Representative photomicrographs (left panels) and numbers of spheres (right graphs) are shown. (D) CHIP protein levels in MDA-MB-231 cells introduced FLAG-CHIP ($CHIP^{OE}$) or empty vector (empty). Cells were generated as described by Kajiro et al [2]. CHIP expression levels were assessed using the indicated antibodies. (E) Sphere-forming capabilities of MDA-MB-231 empty and $CHIP^{OE}$ cells. Numbers of spheres (right graphs) are shown. Error bars are + S.D.; $n = 3$ for C and E. $^{*}p < 0.05$; $^{**}p < 0.01$.

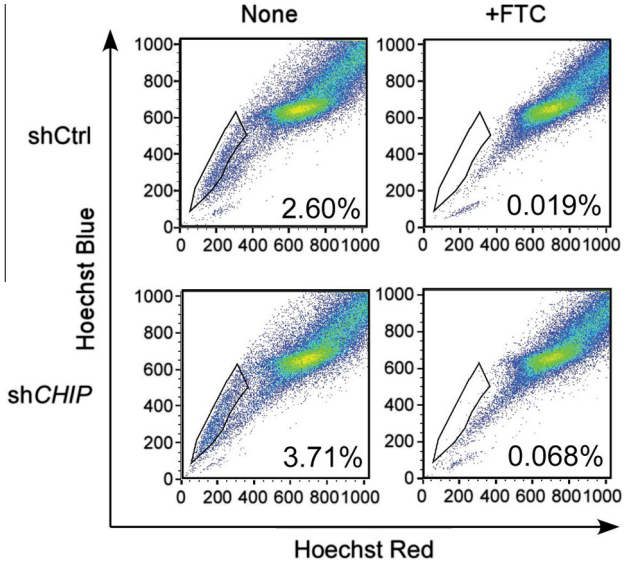

Fig. 2. CHIP depletion increases the proportion of SP cells. Fumitremorgin C (FTC) was used to confirm the SP fraction. SP cells were detected using FACS analysis.

3. Results

CHIP expression levels were previously found to be significantly associated with the prognosis of patients with breast cancer [11]. Consistent with this clinical observation, CHIP expression levels in MCF-7 and T47D cell lines, both non-aggressive human breast cancer cell lines, were much higher than in the MDA-MB-231 cell line, an aggressive triple-negative human breast cancer cell line (Fig. 1A). However, little is known regarding the mechanism(s) of how CHIP is involved in breast cancer progression. Therefore, we investigated the relationship between CHIP expression levels and CSC properties, as recent evidence suggests that CSCs are responsible for cancer progression and recurrence [12,13].

We first analyzed sphere-forming capability, which is one of the major indices used to define CSCs [19], to test the effects of CHIP depletion on CSC properties. When CHIP expression levels were reduced using shRNA for *CHIP* (sh*CHIP*) in MCF-7 and T47D cells (Fig. 1B), sphere formation assay results showed that sh*CHIP* cells more frequently formed spheres than did shCtrl cells both for MCF-7 and T47D cells (Fig. 1C). Alternatively, we used FLAG-tagged CHIP over-expressed MDA-MB-231 cells ($CHIP^{OE}$; Fig. 1D), as

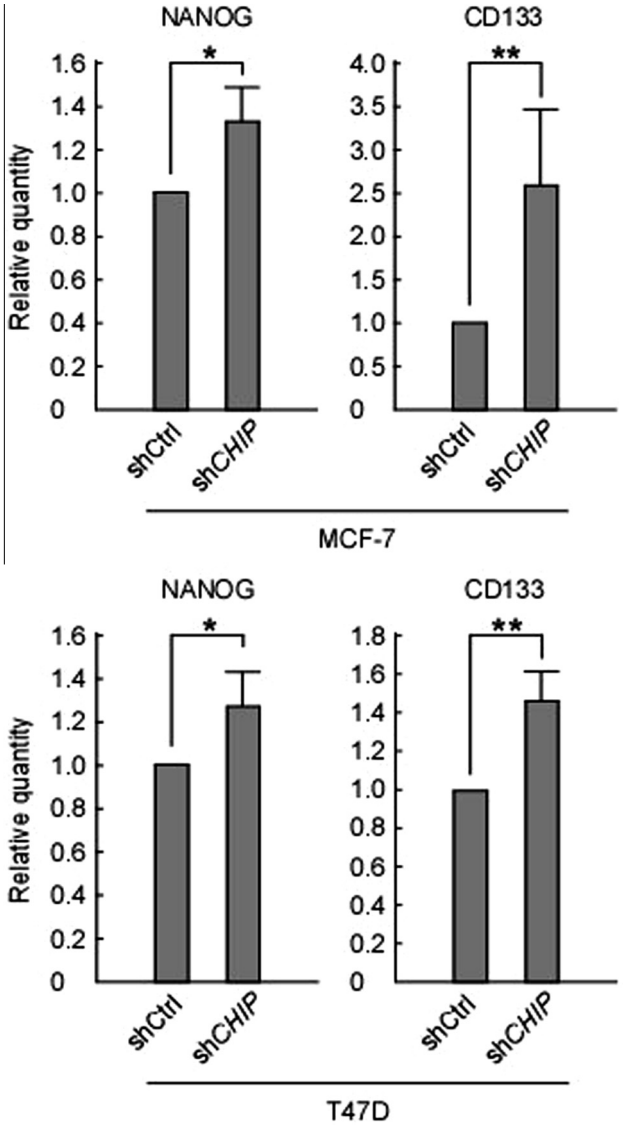

Fig. 3. mRNA levels of CSC related genes are enhanced in spheres after CHIP depletion. Error bars are +S.D.; $n \geqslant 3$. $^{*}p < 0.05$; $^{**}p < 0.01$.

described by Kajiro et al. [2], to further test the effect of CHIP expression on sphere-forming capability. Consistent with the CHIP depletion results, sphere-forming capability was reduced for CHIP^{OE} MDA-MB-231 cells (Fig. 1E). Taken together, these results (Fig. 1) indicated that CHIP expression levels were negatively correlated with sphere-forming capability, which suggested that CHIP suppressed CSC phenotypes.

We next analyzed the proportion of the SP fraction in sh*CHIP* cells to further assess whether CHIP suppressed CSC phenotypes. SP assay is one method used to assess CSC enrichment in several cancers, including breast cancer [25], hepatocellular carcinoma [26], ovarian cancer [27], and lung cancer [28]. SP cells, which have the ability to efflux the DNA-binding dye Hoechst 33342 out of the cell membranes, were detected using FACS analysis [20]. This showed that the proportion of the SP fraction in sh*CHIP* cells was markedly higher than that of shCtrl cells (Fig. 2); thus, CHIP depletion had increased the SP fraction in breast cancer cells.

We then tested whether CHIP depletion affected the expression levels of CSC-related genes. We isolated total RNAs from spheres derived from MCF-7 or T47D shCtrl and sh*CHIP* cells and analyzed the expression levels of some CSC-related genes. As a result, CHIP depletion increased the expression levels of self-renewal gene NANOG [29] and cell surface marker gene CD133 [23,29,30] in spheres (Fig. 3), suggesting that CSC-related genes such as NANOG and CD133 were involved in enhanced CSC properties by CHIP depletion.

Table 1
Tumor initiation capability of MCF-7 cells is enhanced by CHIP depletion.

Number of cell line	Number of cells injected				
	10^5	10^4	10^3	10^2	50
shCtrl	4/4	4/4	3/4	1/4	0/4
sh*CHIP*	4/4	4/4	4/4	4/4	1/4

Finally, we examined whether tumor-initiating potential was affected by CHIP expression levels, as numerous studies have indicated that CSCs have tumor-initiating potential [14]. To examine this, we conducted a limiting dilution xenograft assay using NOD/SCID mice. This showed that tumors were more effectively initiated in MCF-7 sh*CHIP* cells than in shCtrl cells (Table 1), which demonstrated that the tumor-initiating capability was increased in CHIP-depleted cells.

All of these results showed that CHIP depletion increased the CSC population among breast cancer cells (Figs. 1–3 and Table 1). Thus, CHIP may reduce breast cancer malignancy by suppressing CSC properties.

4. Discussion

In our previous study, we found that CHIP suppressed breast cancer metastasis and tumor growth. Moreover, a clinical study reported that CHIP expression levels were associated with a favorable prognosis for patients with breast cancer [11]. All of these findings suggest that CHIP suppresses breast cancer malignancy.

In this study, using several different assays, we found that CHIP depletion increased the CSC population among non-aggressive breast cancer cell lines that had higher CHIP protein levels, whereas CHIP over-expression reduced the CSC population in an aggressive breast cancer cell line that had lower CHIP protein expression. Thus, inhibition of CSC properties by CHIP may account for one aspect of CHIP as a suppressor of cancer progression, because CSCs have high metastatic [15] and tumor-initiating potentials [14]. Thus, it is possible that inhibition of CSC properties by CHIP could result in a favorable prognosis for patients with breast cancer with higher CHIP expression. However, we have not yet determined the molecular mechanism(s) of how CHIP suppresses CSC properties.

We consider that there are two possibilities for inhibition of CSC properties by CHIP, one is by quantity control and the other is quality control of proteins due to its ubiquitinating potential. Numerous studies have identified CSC-related genes, including transcription factors [31,32], receptors [33,34], and signaling factors [35,36]. Thus, the abundance of one or some of these CSC-related factors may be regulated by CHIP (quantity control), as CHIP regulates the quantity of its specific substrates by inducing protein degradation [2,9,10]. Alternatively, the accumulation of misfolded proteins due to CHIP depletion may cause for the changes in the expression of genes that function to maintain CSC properties, as CHIP regulates protein quality by inducing the degradation of misfolded proteins [5–7]. Our next goals will be to identify the target proteins of CHIP and elucidate the molecular mechanisms of how CHIP suppresses CSC properties.

In this study, we showed that CHIP suppressed not only breast cancer metastasis and tumor growth [2], but also CSC properties. Thus, developing methods to enhance CHIP expression levels may provide an efficient therapy for patients with breast cancer.

Therefore, understanding the relationship between CHIP expression and CSC properties and regulating its expression may provide important clues for developing a novel breast cancer therapy.

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