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HAP1 gene expression is associated with radiosensitivity in breast cancer cells

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ABSTRACT

Objectives: The purpose of this study was to investigate the relationship between *huntingtin-associated protein1* (*HAP1*) gene and radiation therapy of breast cancer cells.
Methods: *HAP1* gene was transfected into breast cancer MCF-7 cells, which was confirmed by quantitative reverse transcription-polymerase chain reaction analysis (qRT-PCR) and Western blot *in vitro*. The changes of cell radiosensitivity were assessed by colony formation assay. Apoptosis were examined by flow cytometry. The expressions of two radiation-induced genes were evaluated by Western blot. Tumor growth was investigated in nude mice xenograft models *in vivo*.
Results: Our data showed that *HAP1* gene expression was significantly increased in *HAP1*-transfected MCF-7 cells in comparison with the parental cells or negative control cells. The survival rate in MCF-7/HAP1 cells was significantly decreased after irradiation (0, 2, 4, 6, 8 Gy), compared to cells in MCF-7 and MCF-7/Pb groups *in vitro*. *HAP1* gene increased apoptosis in MCF-7 cells after irradiation. Additionally, the tumor volume and weight in MCF-7/HAP1 + RT group were observably lower than in MCF-7/HAP1 group and MCF-7/Pb + RT group.
Conclusion: The present study indicated that *HAP1* gene expression was related to the radiosensitivity of breast cancer cells and may play an important role in the regulation of cellular radiosensitivity.

1. Introduction

Breast cancer (BC) is one of the most prevalent malignancies that affects women's health [1]. Radiotherapy (RT) is the most important and effective treatment for BC [2,3]. However, radioresistance is the main reason that lead to treatment failure. Therefore, how to increase the radiosensitivity in breast cancer is very important. Although many studies have been conducted to further explore the mechanisms of radiosensitivity, the molecular mechanism underline BC radiosensitivity is still poorly understood. Therefore, it is worthwhile to further understand the molecular mechanisms of BC radiosensitivity and to explore valuable predictive markers as a new treatment.

Huntingtin-associated protein1 (*HAP1*) gene is a protein-coding gene which was initially found in nervous system in patients with Huntington's disease (HD) [4]. With the increasing understanding of this gene, it is not only found in nervous system but also in other systems [5]. Some reports showed that the incidence of tumor among patients with Huntington's disease was much lower than the healthy ones, and apoptosis was the main reason [6]. Others showed that the relationship between cancer and Huntington's disease may share a common mechanism of disease spread via extracellular vesicles [7]. Moreover, previous studies showed that *HAP1* overexpression resulted in suppression of tumor growth [8]. However, the role of *HAP1* in radiosensitivity of BC has not been studied.

In the current study, we investigated the relationship between the *HAP1* gene expression and the radiosensitivity of breast cancer

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cells. In addition, expressions of Bax and Bcl-2 were also studied and correlated with *HAP1* gene expression.

2. Materials and methods

2.1. Ethics statement

Animal protocols were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (Nanjing, China). All animal experiments were performed under the guidelines of humane use and care of laboratory animals for biomedical research published by National Institutes of Health. All efforts were made to reduce animals' suffering.

2.2. Cell culture

The human BC cell lines MCF-7 and transfected cell lines MCF-7/Pb, MCF-7/HAP1 were originated from Research Center of Clinical Oncology of the Affiliated Jiangsu Cancer Hospital, Nanjing Medical University, Nanjing, China. The MCF-7/Pb and MCF-7/HAP1 cells were established with transfected Pb-puro empty vector and Pb-puro-Hap1 plasmid, respectively. All cells were cultured in DMEM (Corning, Manassas, VA, USA) supplemented with 10% FBS (Gibco, USA) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Real-time PCR assays

Total RNA was extracted from MCF-7, MCF-7/Pb, and MCF-7/HAP1 cells using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed using PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Dalian, China), according to the manufacturer's instructions. Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Bio-systems, Carlsbad, CA, USA) on the ABI7300 (Applied Bio-systems). *HAP1* mRNA expression was examined using the following specific primers: forward, 5′-ATGCGCCCGAAGAGGTTGG-3′ and reverse, 5′-CTGCAGATCGTCGTGCCGATGA-3′; primers for *β-actin* (used as an internal control): forward, 5′-TTCTACAATGAGCTGCGTCTG-3′ and reverse, 5′-CAGCC TGGATAGCAACGTATC-3′. Cycling parameters were followed as protocol described. All reactions were repeated 3 times for each sample. Primer quality was analyzed by dissociation curves. Data were analyzed by comparing Ct values.

2.4. Colony-forming assay

Clonogenic survival assays were actualized according to the literature [9]. Briefly, cells were plated in 60 mm^2 culture dishes at various cell densities (3×10^2–1×10^3 cells/well) for 12 h and were exposed to IR in doses of 0, 2, 4, 6 and 8 Gy. Then the cells were cultured for an additional 10 days, and colonies were stained with Giemsa. The surviving colonies (a colony was defined as >50 cells) were counted. Lastly, plating efficiencies (PE) were calculated as the number of colonies divided by the number of cells seeded. Survival fraction (SF) was calculated as the PE of the group with radiation divided by the non-irradiated one. The experiments were performed for three times.

2.5. Flow cytometric analysis

Flow cytometric analysis was performed as characterized formerly [10]. MCF-7, MCF-7/Pb, and MCF-7/HAP1 cells were seeded in 6-well plates (3.5×10^5 cells/well and each cell line has a parallel hole) for 12 h and three parallel holes were exposed to 4 Gy IR, then cells were harvested after culture for 7 h and washed twice with ice-cold PBS. AnnexinV-FITC/PI staining was used to detect apoptotic cells. Three individual experiments were repeated.

2.6. Western blotting analysis

Total cell proteins were extracted, equivalent amounts of protein were applied for 10% SDS–PAGE gels. And then proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated subsequently with the relevant primary antibodies and secondary antibodies. The primary antibodies used in this study included polyclonal anti-HAP-1 (1:1000; Abcam, HK), anti-Bcl-2 (1:500; Santa Cruz, CA), anti-Bax (1:1000; Cell Signaling Technology, USA). β-Actin was used as a loading control and blotted protein bands were visualized by ECL detection reagent (Millipore, Billerica, MA, USA).

2.7. Animal experiment

Six week old female nude mice (BALB/c; Medical Center of Yangzhou University, Yangzhou, China) were subcutaneously injected into the left inguinal with MCF-7/Pb and MCF-7/HAP1 cells (5×10^6), respectively. At the 21th days after injection, all mice were divided into 4 groups, including Pb group (MCF-7/Pb group), HAP1 group (MCF-7/HAP1 group), combination group 1 (MCF-7/Pb + RT group), combination group 2 (MCF-7/HAP1 + RT group). Meanwhile, the combination group 1 and combination group 2 received xenograft irradiation (5 Gy, once), and all groups followed for about 3 weeks. Tumor growth was examined every 4 days; tumor volume (mm^3) was monitored by gauging the length (mm) and width (mm) of the tumors and calculated as length (mm) × width (mm^2) × 0.5. Mice were sacrificed by decapitation and xenograft tumors were immediately removed after death.

2.8. Statistical analysis

The data were expressed as means ± SD. Statistical analysis was conducted using Student's *t*-test and one-way ANOVA with Graphpad 5.0 and SPSS 13.0. $P < 0.05$ was set as significant level of difference.

3. Results

3.1. Overexpression of HAP1 gene was confirmed in vitro

HAP1 gene was significantly upregulated in MCF-7/HAP1 cells compared with MCF-7 and MCF-7/Pb cells ($P < 0.01$). There was no difference between MCF-7/Pb and MCF-7 cells (Fig. 1A and B).

3.2. HAP1 gene overexpression influenced the sensitivity of MCF-7 cells to irradiation

The survival rate of cells in MCF-7/HAP1 group was significantly decreased after irradiation (0, 2, 4, 6, 8 Gy), compared with MCF-7 and MCF-7/Pb cells (Fig. 2A–C). The survival fraction of 2 Gy (SF2) and the values of D_0 and D_q were 0.39, 1.15 and 1.8 in MCF-7/HAP1 cells, 0.61, 1.64 and 3.64 in MCF-7/Pb, and 0.69, 1.68, 3.63 in MCF-7, respectively (Table 1) [11,12]. These results indicated that overexpression of *HAP1* gene could increase cell sensitivity to irradiation.

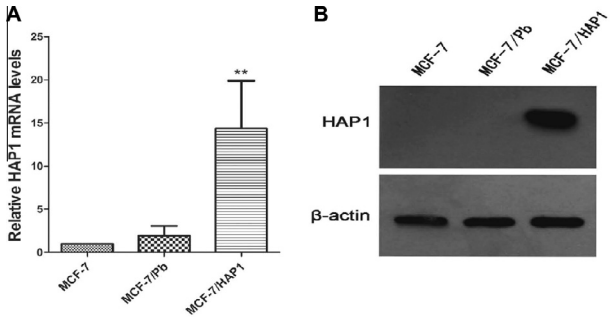

Fig. 1. *HAP1* gene overexpression was verified *in vitro*. (A) Relative mRNA levels of *HAP1* gene by quantitative RT-PCR analysis. $n = 3$ for each groups. (B) Western blot analysis of HAP1 expression changes in all cell lines. $^{**}P < 0.01$ vs MCF-7/Pb and MCF-7.

3.3. Influence of HAP1 gene on cell apoptosis and protein expression

After radiation of 4 Gy, the apoptosis rate was significantly higher in MCF-7/HAP1 group than in MCF-7/Pb and MCF-7 groups ($P < 0.001$) (Fig. 3A and B).

To investigate the potential mechanism by which *HAP1* gene regulates radiosensitivity, we detected the expression of Bax and Bcl-2 proteins which were involved in cell apoptosis by Western blotting. Our results showed that Bax expression was much higher while Bcl-2 expression was much lower in MCF-7/HAP1 cells than in MCF-7/Pb and MCF-7 cells (Fig. 3C). These results indicated that overexpression of *HAP1 gene* could increase cell apoptosis after radiotherapy.

3.4. Overexpression of HAP1 gene influenced radiosensitivity in vivo

The *in vitro* results suggested that overexpression of *HAP1* gene can influence radiosensitivity in BC MCF-7 cells, we investigated whether *HAP1* gene has a similar effect *in vivo*. Nude mice were injected with MCF-7/Pb and MCF-7/HAP1 cells, respectively. After irradiation, the tumor volume in MCF-7/HAP1 + RT group was smaller than in MCF-7/HAP1 group ($P < 0.001$) and MCF-7/Pb + RT group ($P < 0.01$). The tumor weight in MCF-7/HAP1 + RT group was lower than in MCF-7/HAP1 group ($P < 0.01$) and MCF-7/Pb + RT group ($P < 0.05$) (Fig. 4A–C). Synergistic Indices of combination treatment with *HAP1* gene and RT *in vivo* xenograft models

Table 1
Comparison of radiosensitivity in different groups.

	D_0	D_q	SF2	N
MCF-7	1.68	3.63	0.69	3.16
MCF-7/Pb	1.64	3.64	0.61	3.22
MCF-7/HAP1	1.15	1.8	0.39	2.57

SF, survival fraction = number of colonies formed/(number of cells seeded plating efficiency of the control group) [12].
D_0, lethal radiation dose.
D_q, quasi-threshold dose.
SF2, fraction of cells surviving after 2 Gy irradiation.
N, cell repair capacity.
High D_0, D_q and *N* values indicate increased radioresistance of cells.

(endpoint tumor volumes) was 1.42 (Table 2) [13]. Taken together, there is synergistic effect between *HAP1* gene and radiotherapy.

4. Discussion

Radiosensitive and radioresistant of tumor cells play important roles in the clinical treatment [14,15]. However, the accurate molecular mechanisms underline radiosensitivity and radioresistant remains unclear. Some studies reported that many oncogenes and tumor suppressor genes are related to the radiosensitivity in cancer [16–20]. *HAP1* gene localize on human chromosome 17q21.2–21.3 [21]. HAP1 is a cytoplasmic protein associated with mitochondria, endoplasmic reticulum, tubulovesicles, endosomal and lysosomal organelles, and synaptic vesicles [22]. Some reports showed that HAP1 reactivity was associated with angiogenesis and metastasis [23–25]. Furthermore, previous studies revealed that the expression of *HAP1* gene was reduced in BC tissues, and *HAP1* gene played an important role in pathogenesis of BC [8].

In the present study, we showed that in a given dose of irradiation, the survival fraction significantly reduced in MCF-7/HAP1 cells in comparison with MCF-7/Pb group and MCF-7 group. Therefore, *HAP1* gene has the effect of radiosensitization when concurrent with radiotherapy. Colony formation assay is one of the most important methods to assay cell survival and is the golden standard for testing the radiosensitivity of cells [26]. In this study, all radiosensitization parameters were calculated by single-hit multi-target model. The survival fraction of 2 Gy (SF2) and the values of D_0 and D_q were 0.39, 1.15 and 1.8 in MCF-7/HAP1 cells after irradiation. So *HAP1* gene can modulate the radiosensitivity of BC MCF-7 cell lines.

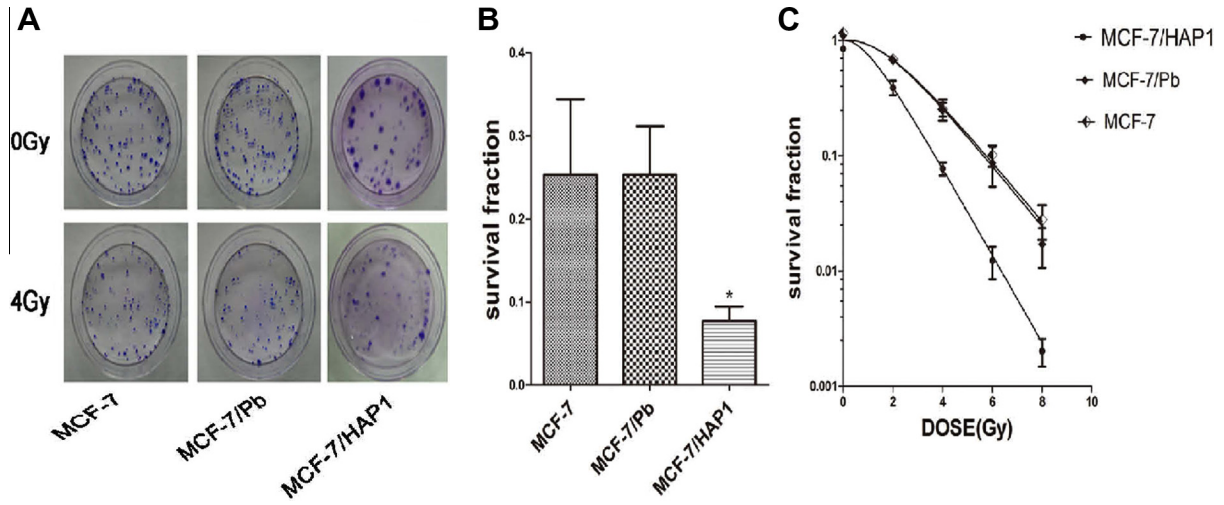

Fig. 2. Overexpression of *HAP1* gene influenced the radiosensitivity of MCF-7 cells *in vitro*. (A and B) The colony formation assay in MCF-7 cells, MCF-7/Pb and MCF-7/HAP1 cells with or without irradiation and survival fractions with irradiation (4 Gy). (C) Survival fractions at different irradiation doses. $n = 3$ for each groups. $^{*}P < 0.05$ vs MCF-7 and MCF-7/Pb groups.

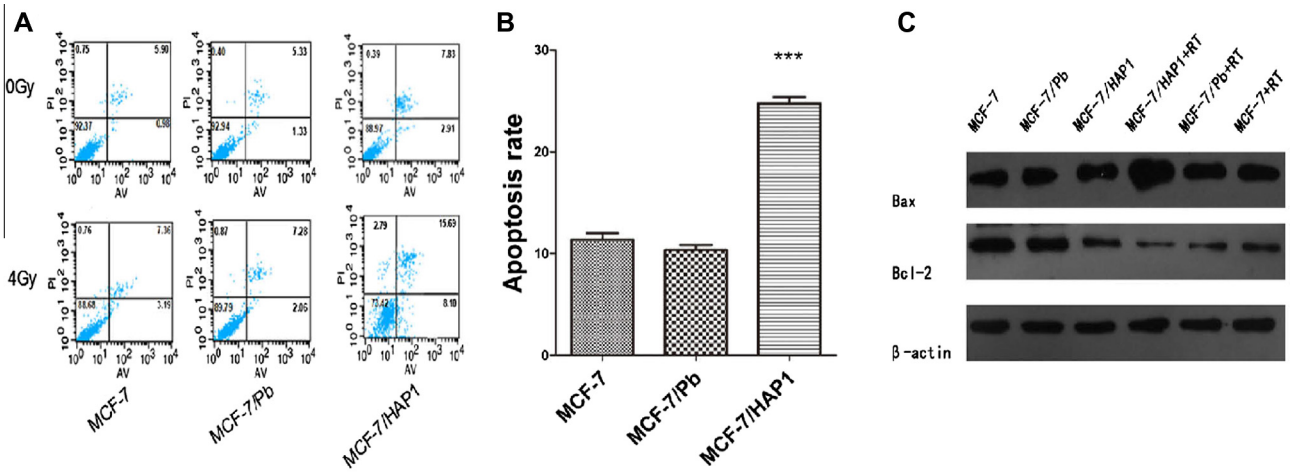

Fig. 3. Overexpression of *HAP1* gene influenced radiation-induced apoptosis *in vitro*. (A and B) Apoptotic changes in MCF-7 cells, MCF-7/Pb and MCF-7/HAP1 cells with irradiation (4 Gy) for 7 h. $n = 3$ for each groups. (C) Equivalent amounts of protein were applied for 10% SDS–PAGE gels, Changes of Bax and Bcl-2 in different groups with RT (4 Gy). $^{***}P < 0.001$ vs MCF-7 and MCF7/Pb groups.

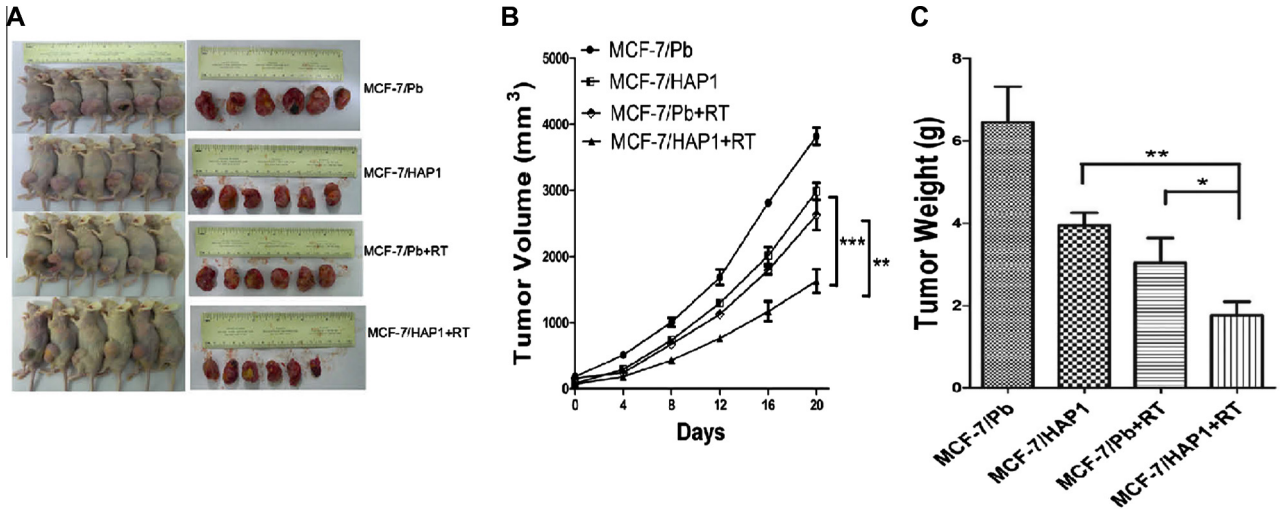

Fig. 4. Overexpression of *HAP1* gene influenced the radiosensitivity of BC MCF-7 cells *in vivo*. (A) Representative images of xenograft tumors in different groups of nude mice with or without irradiation, before (left) or after (right) excision. (B) Tumor growth after transplantation in different groups. The day before the mice were irradiated was specified as 0 day. (C) Quantitative determination of tumor weight of the excised tumors in different groups at the end of the research. $n = 3$ for each groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 2
Synergistic Indices of combination treatment with *HAP1* gene and RT *in vivo* xenograft models (endpoint tumor volumes).

MCF-7/HAP1	MCF-7/Pb + RT	MCF-7/HAP1 + RT			SI
MGI	MGI	Expected‡	Observed§	*P*†	
0.78	0.69	0.54	0.38	<0.001	1.42

MGI, mean growth inhibition rate = growth rate of treated group/growth rate of untreated group.
SI, synergistic index = expected growth inhibition rate/observed growth inhibition rate. An index more than 1 indicates synergistic effect and <1 indicates less than additive effect.
† *P* value (two-sided) was calculated by one-way ANOVA compared with no treatment.
‡ Expected growth inhibition rate = MGI MCF-7/HAP1 × MGI MCF-7/Pb + RT.
§ Observed growth inhibition rate = growth inhibition rate of combined treatment/growth rate of untreated group.

As reported by previous studies, increasing cell apoptosis was one of the most important mechanisms leading to radiotherapy caused tumor regression [27,28]. For example, the silence of anti-apoptotic protein *survivin* expression enhanced the radiosensitivity of gastric cancer SGC7901 cells through inducing apoptosis [29]. Liu et al. demonstrated that *p73* played an important role in regulating the cellular response in cervical cancer to radiotherapy through apoptosis signaling pathway [30]. Moreover, several studies also reported that Bcl-2 and Bax played an important role in cell apoptosis [31,32]. We found an enhancement of apoptosis rate in MCF-7/HAP1 group after irradiation, with increased Bax and decrease Bcl-2 protein expression in MCF-7/HAP1 cells. So we considered this to be one mechanism of radiosensitization.

Furthermore, we confirmed that the growth of tumor was significantly inhibited in MCF-7/HAP1 group after irradiation in comparison with other groups *in vivo*. The results revealed that *HAP1* gene may play an important role in regulating the cellular response of MCF-7 cells to radiotherapy. However, the exact mechanism of *HAP1* gene in mediating tumor cells response to radiosensitivity is still unknown, which need to be further studied.

In summary, our study proved a significant association between the overexpression of *HAP1* gene and the cellular radiosensitivity

in BC MCF-7 cells. However, the detailed mechanism on how *HAP1* gene affect the apoptotic pathway in the consequence of irradiation requires further investigation.

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