Huntingtin-associated protein 1: A potential biomarker of breast cancer

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Abstract. It is reported that patients with Huntington's disease (HD) have a low incidence of cancer. In this study, we investigated the expression of huntingtin-associated protein 1 *(HAP1)*, the ligand of HD's production, in breast tumor and normal tissues. We found that *HAP1* expression was significantly lower in tumor compared to normal tissues. We then transfected the *HAP1* gene into the breast cancer lines MCF-7 and MDA-MB-231, and results showed that the overexpression of *HAP1* reduced the growth of the two cell lines. In addition, we observed that *HAP1* also reduced invasion and migration, and upregulated apoptosis in MCF-7 cells; however, these changes were not observed in MDA-MB-231 cells. We also demonstrated that the expression of EGFR and apoptosis-related genes might be involved in cell proliferation and apoptosis. In conclusion, overexpression of *HAP1* reduced *in vitro* cell growth in breast cancer cell lines, suppressed the migration and invasion, and promoted the apoptosis of certain cell lines. Therefore, *HAP1* is a potential molecular target for the diagnosis and treatment of breast cancer.

Introduction

Breast cancer is the most prevalent tumor in women, and its incidence accounts for 7-10% of all malignant tumors. Huntington's disease (HD) is an autosomal dominant hereditary neurodegenerative disorder characterized by late onset, progressive psychiatric disruption, cognitive deficits and loss of motor coordination (1). According to a previous study, the overall incidence of cancer is significantly lower among patients with HD, and this seems to be related to intrinsic biologic factors (2). HD is caused by a CAG triplet repeat expansion in exon 1 of the huntingtin (Htt) gene, encoding an abnormal expanded polyglutamine (polyQ) tract that confers toxicity to the mutant Htt protein. When the mutant Htt gradually accumulates in the cell, it can affect the function of several proteins and the conduction of some signaling pathways. Huntingtin-interacting protein 1 (HIP1) and huntingtin-associated protein 1 (HAP1) are two known ligands of Htt (3,4). Research on the relevance between HIP1 and cancer showed that HIP1 had a high expression level in breast, colon, prostate and other types of cancer (5,6). However, there are currently no studies regarding the association between HAP1 and cancer. In this study, we first examined the level of HAP1 in human breast tumor and normal breast tissues, and investigated the roles and possible mechanisms of HAP1 in human breast cancer cells. In addition, we selected ER positive MCF-7 and triple negative MDA-MB-231 cells, representing two main types of breast cancer, for research.

Materials and methods

Tumor samples. A total of 43 breast carcinoma specimens with non-neoplastic adjacent tissues from patients with primary breast tumor were collected during the surgical procedures at the Department of General Surgery, Jiangsu Cancer Hospital, Nanjing Medical University (Nanjing, China). The sample collection was carried out in accordance with the National Regulation of Clinical Sampling in China.

Cell culture. The human breast cancer cell lines MCF-7 and MDA-MB-231 were maintained in DMEM with high glucose (Gibco, Grand Island, NY, USA) and 10% FBS (Gibco).

Viral production and infection of target cells. Retrovirus was generated by cotransfection of pBabe-puro empty vector (as control) or pBabe-puro-*Hap1* plasmid along with pVSP-G (envelope) and pVSV-GP (packaging) plasmids in 293FT cells, growing at 85-90% confluency in 10-cm petri dishes. After 48 h, the culture medium containing the viral particles was harvested. Target cells were infected with virus containing medium in the presence of 10 μg/ml polybrene (Sigma, St. Louis, MO, USA). Then, changing back to fresh medium, cells were selected with 2 μg/ml puromycin (Fischer, USA) for 7-10 days, re-cultured in a larger dish containing puromycin

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Table I. Primers used for Q-PCR amplification.

Gene	Forward	Reverse
HAP1	5'-ATGCGCCCGAAGAGGTTGG-3'	5'-CTGCAGATCGTCGTGCCGATGA-3'
Caspase-3	5'-GATGGAAGCGAATCAATGGACT-3'	5'-CTGTACCAGACCGAGATGTCA-3'
Caspase-8	5'-TCATGGACCACAGTAACATGGA-3'	5'-AGTGAACTGAGATGTCAGCTCAT-3'
Caspase-9	5'-CACTCCCCCTGAAGACGAGTC-3'	5'-GTGGGCAAACTAGATATGGCG-3'
Bcl-2	5'-GGTGGGGTCATGTGTGTGG-3'	5'-CGGTTCAGGTACTCAGTCATCC-3'
Bax	5'-CCCGAGAGGTCTTTTTCCGAG-3'	5'-CCAGCCCATGATGGTTCTGAT-3'
Survivin	5'-TGCGGGAATCCAAAGGATAATTCA-3'	5'-CTTCATCTTTGTCATACTTCATGGCT-3'
EGFR	5'-GAAGGAGCTGCCCATGAGAA-3'	5'-GACTATGTCCCGCCACTGGAT-3'
β-actin	5'-TTCTACAATGAGCTGCGTGTG-3'	5'-CAGCCTGGATAGCAACGTACA-3'

HAP1, huntingtin-associated protein 1.

and used for further assay. All plasmids and cells were kindly provided by Professor Jinrong Zhou, at Harvard University (Cambridge, MA, USA).

Cell viability assay. Cells (3,000/well) were plated in 96-well plates. Each group had five replicates. The growth was monitored 72 h later and cell viability was measured by CCK-8 (Dojindo, Kumamoto Prefecture, Kyushu, Japan).

Colony formation assay. Cells were seeded in six-well culture plates at 200 cells/well. After 10 days of incubation, the cells were stained with 0.1% crystal violet (Enox, Shanghai, China). The colonies with >50 cells were counted. The colony formation rate was acquired through colony numbers/total seeding cells.

Semiquantitative RT-PCR. Total RNA was extracted with the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and was then reverse transcribed using PrimeScript RT Master mix (Takara, Kumamoto Prefecture, Kyushu, Japan). Real-time PCR was performed using an ABI 7300 Real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) with the SYBR Premix Ex Taq (Takara). All samples were analyzed in triplicate and in optically clear 96-well plates (Corning Inc., Corning, NY, USA). The cycling parameters were: 95˚C for 30 sec followed by 40 cycles of 95˚C for 5 sec, 60˚C for 31 sec and 95˚C for 15 sec, 60˚C for 60 sec, 95˚C for 15 sec. The human β-actin transcript was used as an internal reference to control for variations in the total mRNA quantity of each sample. Each RNA sample was analyzed in triplicate. Primer sequences are listed in Table I.

Western blot analysis. Cells were collected and lysed with lysis buffer (Beyotime, Jiangsu, China), and concentrated to obtain the proteins. The amount of total proteins was estimated by Onedrop OD-1000+ Spectrophotometer (Onedrop, Shanghai, China), and then proteins were mixed with SDS-PAGE buffer (Beyotime) and boiled for 5 min. The proteins were loaded onto 10% SDS-PAGE gel and, following electrophoresis, the proteins were transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). Then, the membrane was blocked with nonfat-dried milk, incubated with primary and secondary antibody (Dako, Japan), and protein bands were visualized by ECL detection reagent (Millipore, Billerica, MA, USA).

In vitro migration and invasion assays. For the migration assays, the cells were detached and aliquots of $2x10^5$ cells/ml were plated onto the inserts of the 8-μm pore-sized Transwell chambers (Corning Inc.). For the Transwell invasion assays, $1x10^6$ cells were plated onto inserts containing a polycarbonate membrane with a thin layer of BD Matrigel Matrix (BD Biosciences, Franklin Lakes, NJ, USA). Both migration and invasion were assayed 24 h later by counting the cell numbers across the membrane.

Flow cytometric assay. For cell apoptosis, a total of $1x10^6$ cells were transferred to a tube in which 5 μl Annexin V and 1 μl propidium iodide (PI) were added after being resuspended with 100 μl binding buffer (Invitrogen). The cells were then allowed to incubate at room temperature for 15 min and were analyzed using flow cytometry. For cell cycle analysis, cells were collected, washed twice with PBS, supplemented with 1 ml 75% ethanol, and kept at -20˚C overnight. The cells were then resuspended with PBS, supplemented with PI and RNaseA, incubated for 30 min at 37˚C and analyzed using flow cytometry.

Statistical analyses. Data are expressed as the means ± SD, and statistical significance was assessed by Student's t-test and one-way ANOVA. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of HAP1 in breast tumor samples. *HAP1* expression was assessed by qRT-PCR in surgical specimens from patients with breast cancer. As shown in Fig. 1, *HAP1* expression was markedly reduced in most cancer tissues compared with normal breast tissues. Of 43 cases, ~70% showed *HAP1* expression levels in normal breast tissues >3-fold higher than those of matched tumors (Fig. 1).

Expression of HAP1 in transfected and control cells. The MCF-7 and MDA-MB-231 cells transfected with pBabe-puro-HAP1

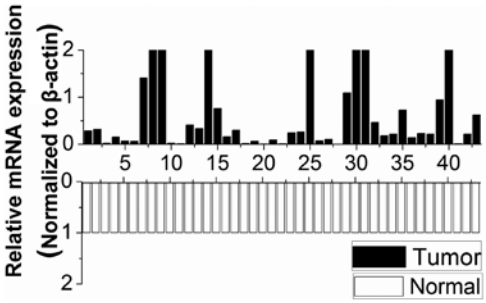

Figure 1. Huntingtin-associated protein 1 *(HAP1)* expression in breast tumor and matched normal breast tissues. *HAP1* expression in 43 cases of breast tumor and matched normal breast tissues was evaluated. The *HAP1* mRNA expression of the majority of the normal breast tissue (70%) was >3-fold higher compared with matched tumors.

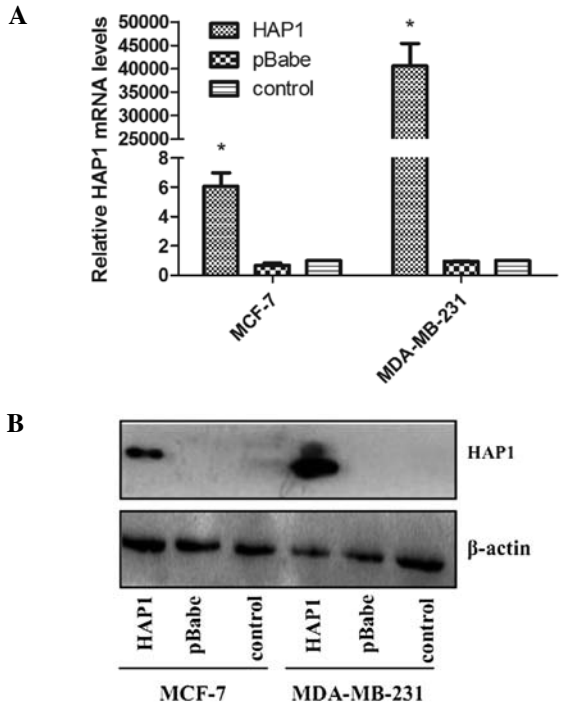

Figure 2. Huntingtin-associated protein 1 *(HAP1)* expression in transfected and control cells lines. (A) Relative mRNA levels of *HAP1* in all cell lines. (B) Western blot analysis of *HAP1* levels in all cell lines. *P<0.05.

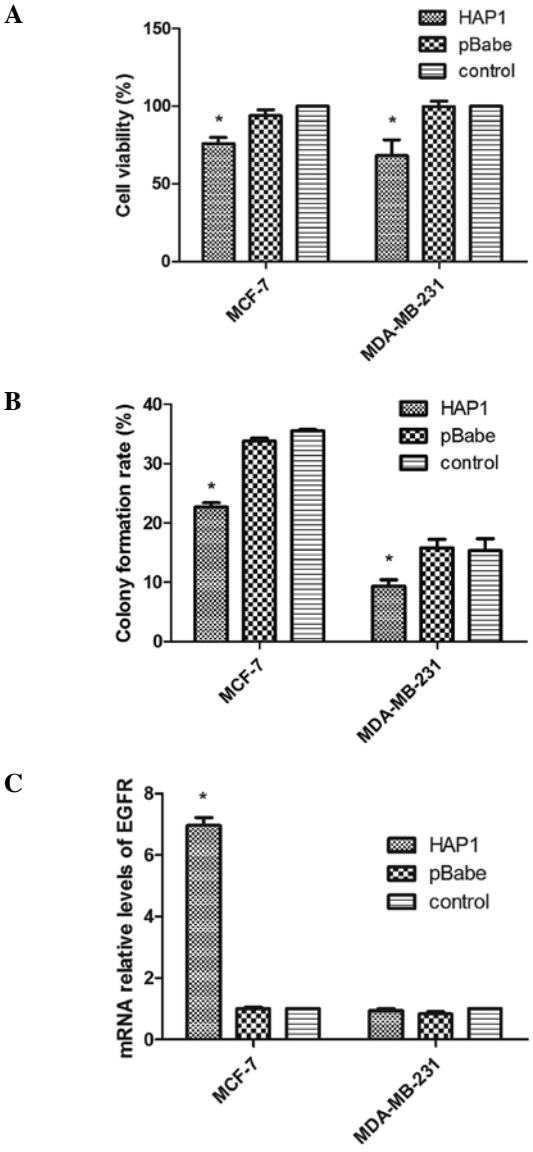

Figure 3. Growth and proliferation ability of cells and EGFR expression levels in cells. (A) Cell viability test of the two cell line groups. (B) Quantitative evaluation of colony formation in the two cell line groups, indicated as the ratio to control cells. (C) Significantly higher expression of EGFR in MCF-7/HAP1, but not in MDA-MB-231/HAP1. *P<0.05. HAP1, huntingtin-associated protein 1.

and pBabe-puro were labeled MCF-7/HAP1, MCF-7/pBabe and MDA-MB-231/HAP1, MDA-MB-231/pBabe, respectively. Western blotting and qRT-PCR results of different cells showed that MCF-7/HAP1 (P<0.006) and MDA-MB-231/HAP1 (P=0.001) had a significantly higher expression of HAP1 than their control groups. Moreover, there was no obvious difference between pBabe and negative control groups (P>0.05) (Fig. 2A and B).

Effects of HAP1 on breast cancer cell growth and expression of EGFR in cells. At 72 h after cells were plated in 96-well plates, upregulation of *HAP1* significantly increased the cell viability of both *HAP1* overexpression cells in MCF-7 [HAP1 mean, 76.0±6.8 vs. pBabe mean, 94.0±6.6 (P=0.03) vs. control mean, 100±0.0 (P=0.004)] and MDA-MB-231 [HAP1 mean, 68.2±17.4 vs. pBabe mean, 99.8±6.0 (P=0.041) vs. control mean, 100±0.0 (P=0.034)] compared with empty vector plasmid transfected cells and negative control cells (Fig. 3A). In addition, colony formation rates were significantly decreased in MCF-7 cells with HAP1 overexpression [HAP1 mean, 22.7±1.3% vs. pBabe mean, 33.8±0.8% (P=0.000) vs. control mean, 35±0.5% (P=0.000)] and MDA-MB-231 [HAP1 mean, 9.3±1.9% vs. pBabe mean, 15.8±2.5% (P=0.022) vs. control mean, 16±2.6% (P=0.024)] when compared with those of pBabe and control cells (Fig. 3B). We also compared the expression of EGFR in three groups of the cell lines. We observed that in the MCF-7 cell lines, EGFR expression of MCF-7/HAP1 [HAP1 mean, 7.0±0.4 vs. pBabe mean, 1.0±0.1 (P=0.000) vs. control mean, 1.0±0.0 (P=0.000)] was ~7-fold higher than that of the other two groups. However,there was no difference in EGFR expression between the three groups in the MDA-MB-231 cell lines (F=3.2; P=0.1) (Fig. 3C).

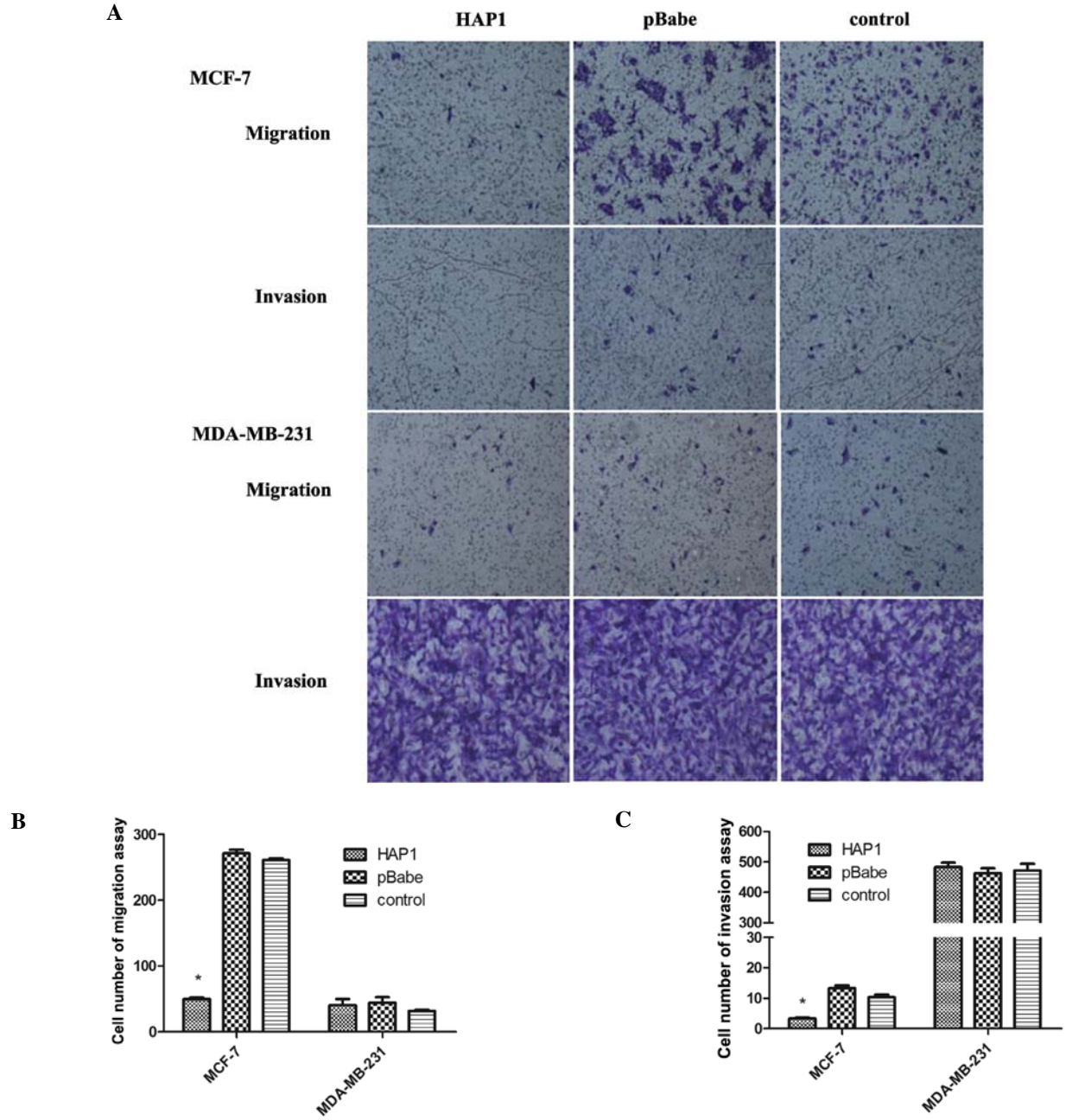

Figure 4. Effect of huntingtin-associated protein 1 *(HAP1)* overexpression on the migration and invasion ability of MCF-7 and MDA-MB-231 cells. (A) Images of representative crystal violet-stained membrane of migration and invasion, x200. In MCF-7 cells, migration and invasion in the HAP1 group were reduced compared with the other two groups. No changes were observed in MDA-MB-231 cells. (B) Quantitative evaluation of cell migration ability (chambers without Matrigel); the migrating cells were counted in six predetermined fields. (C) Quantitative evaluation of cell invasion ability (chambers with Matrigel); the invading cells were counted in six predetermined fields. *P<0.05.

Overexpression of HAP1 impairs migration and invasion. We observed that MCF-7/HAP1 had fewer cells across the membrane than MCF-7/pBabe and MCF-7 in migration assays [HAP1 mean, 50.0±3.6 vs. pBabe mean, 271±9.5 (P=0.000) vs. control mean, 261±4.6 (P=0.000)] (Fig. 4A and B). In addition, compared to the number of the other two cell groups, the number of MCF-7/HAP1 cells that invaded the Matrigel-coated filter was significantly lower [HAP1 mean, 3.3±0.6 vs. pBabe mean, 13.3±1.5 (P=0.000) vs. control mean, 10.3±1.5 (P=0.000)] (Fig. 4A and C). Thus, the overexpression of *HAP1* in MCF-7 can curb tumor cell migration and invasion. However, in MDA-MB-231 cells, there was no significant difference in the number of cells across the membrane in both cell migration (F=0.8; P=0.5) and invasion (F=0.3; P=0.7) (Fig. 4A-C).

Effects of HAP1 on cell cycle, apoptosis and expression of relative genes. According to cell cycle distribution analysis by flow cytometry, HAP1 increased the percentage of cells in the G_2M phase. In MCF-7 cells, the percentage in the G_2M phase of the HAP1 group was 30.84%, higher than the 13.17% and the 10.61% in the pBabe and control groups. In MDA-MB-231 cells, the percentage in the G_2M phase of the HAP1 group was 14.84%, also higher than the other two groups (Table II). The results indicated that HAP1 induced G_2M arrest in both

Table II. Cell cycle distribution in MCF-7 and MDA-MB-231 cells.

	MCF-7			MDA-MB-231		
	HAP1	pBabe	Control	HAP1	pBabe	Control
G_0G_1 (%)	36.53±0.96	46.04±2.22	58.11±2.18	56.69±0.83	56.79±1.08	55.63±1.02
S (%)	32.63±0.34	40.78±0.41	31.27±0.66	28.46±0.22	31.45±0.22	33.17±0.17
G_2M (%)	30.84±1.09[a]	13.17±1.83	10.61±1.53	14.84±0.90[a]	11.76±0.93	11.19±0.94

[a]P<0.05.

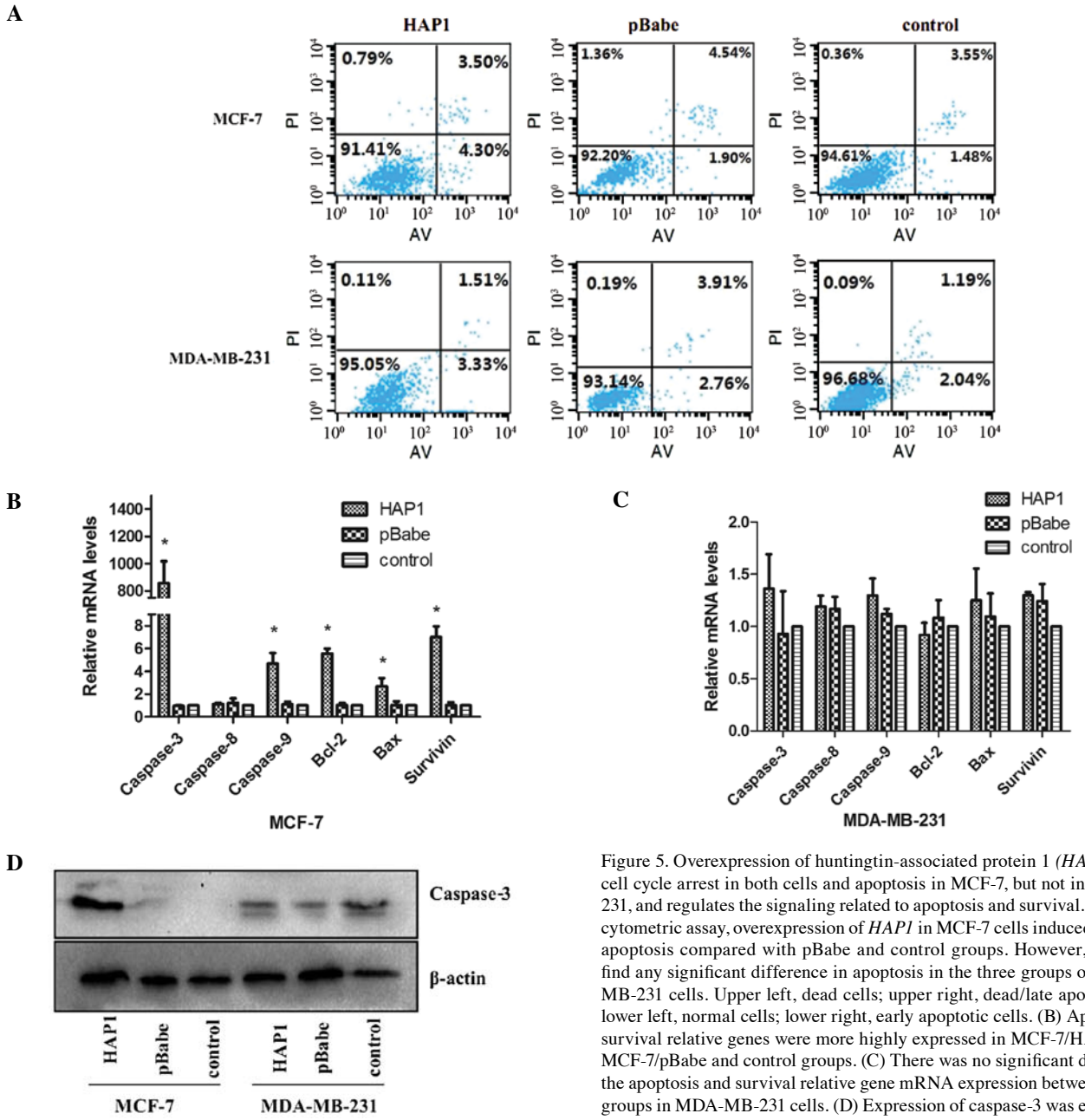

Figure 5. Overexpression of huntingtin-associated protein 1 *(HAP1)* induces cell cycle arrest in both cells and apoptosis in MCF-7, but not in MDA-MB-231, and regulates the signaling related to apoptosis and survival. (A) By flow cytometric assay, overexpression of *HAP1* in MCF-7 cells induced significant apoptosis compared with pBabe and control groups. However, we did not find any significant difference in apoptosis in the three groups of the MDA-MB-231 cells. Upper left, dead cells; upper right, dead/late apoptotic cells; lower left, normal cells; lower right, early apoptotic cells. (B) Apoptosis and survival relative genes were more highly expressed in MCF-7/HAP1 than in MCF-7/pBabe and control groups. (C) There was no significant difference in the apoptosis and survival relative gene mRNA expression between the three groups in MDA-MB-231 cells. (D) Expression of caspase-3 was examined by immunoblotting, which was in accordance with the RT-PCR results. *P<0.05.

cell lines. Furthermore, *HAP1* overexpression significantly induced apoptosis of MCF-7 cells compared with pBabe and control groups, and there was also no significant difference in MDA-MB-231 cells (Fig. 5A). Therefore, we detected the expression of genes, including caspase-3, -8, -9, Bcl-2, Bax and survivin, involved in cell apoptosis and survival, by RT-PCR (Fig. 5B and C). The results showed that the expression of caspase-3, -9, Bcl-2, Bax and survivin in MCF-7/HAP1 was

significantly higher than in the other two groups. Moreover, the expression of caspase-3 was >800 times higher in MCF-7/HAP1 compared with the other two groups [HAP1 mean, 857.7±278.9 vs. pBabe mean, 1.0±0.1 (P=0.000) vs. control mean, 1.0±0.0 (P=0.000)], and it was confirmed by western blotting (Fig. 5D).

Discussion

HAP1 is a type of cytoplasmic protein distributed mainly in the microtubules and membranous organelles, including the mitochondria, endoplasmic reticulum, tubulovesicles, endosomal and lysosomal organelles, and synaptic vesicles (7,8). HAP1 affects the synthesis of certain proteins and the conduction of signals (9-11). As a ligand of the production of HD, it was initially studied in the nervous system, whereas later research showed that it was involved in the other systems.

Herein, we detected the expression of *HAP1* in breast tumor and normal breast tissues. In contrast to normal breast tissues from breast cancer patients, we observed that *HAP1* expression was reduced in the majority of breast tumor samples. To the best of our knowledge, this is the first published study to show that *HAP1* is downregulated in breast tumor tissues and, based on these findings, it is suggested that *HAP1* plays a role in the pathogenesis of breast cancer.

HAP1 is involved in numerous cellular functions, including cell proliferation and apoptosis (12,13). In this study, upregulation of *HAP1* resulted in a significant decrease in the cell growth and colony formation rate of both breast cancer cells. Furthermore, cell cycle results showed that the percentage of G_2M phase increased in both MCF-7 and MDA-MB-231 cells, indicating that cell growth could be arrested at the G_2M phase by HAP1. Moreover, apoptosis may be involved in the inhibition of cell growth. It has been reported that apoptosis plays an important role during the malignant transformation of normal cells. The above studies, along with ours, indicate that loss or downregulation of *HAP1* may disrupt the balance between proliferation and apoptosis and may represent a key pathogenic step in the development of breast cancer. The cell apoptosis assay confirmed that HAP1 induced cell apoptosis and increased expression of relative genes in MCF-7 cells, particularly caspase-3. Caspase-3 is located at the downstream of the apoptosis signaling pathway, and plays a key role in cell apoptosis (14,15). Collectively, HAP1-induced apoptosis in MCF-7 cell lines may be mainly mediated by the caspase-dependent pathway; in the MDA-MB-231 cells, however, the three groups showed no difference in the expression of those genes.

Previous studies showed that inhibition of *HAP1* expression decreased EGFR signaling and cell viability, whereas overexpression of *HAP1* enhanced this signaling activity (12). EGFR is expressed in various types of tissue, including epithelial, mesenchymal and of neuronal origin, and plays a major role in normal cellular processes such as proliferation, differentiation and development. EGFR is also highly expressed in a number of solid tumors and its expression correlates with tumor progression, resistance to chemotherapy and a poor prognosis (16,17). Our detection of EGFR showed that EGFR expression was higher in MCF-7/HAP1, which negatively correlated with the results of cell proliferation. Therefore, we considered that EGFR might play a role in MCF/HAP1, although not a major one; on the contrary, the effect of EGFR was counteracted by others.

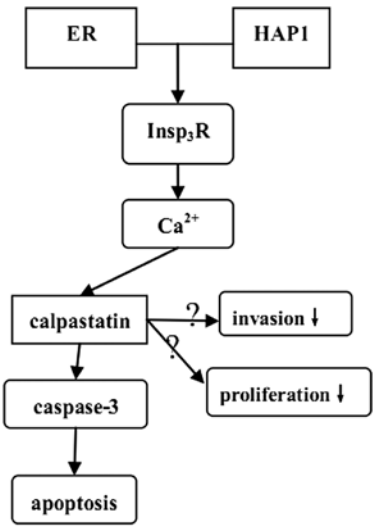

Figure 6. Model of our hypothesis showing how huntingtin-associated protein 1 (HAP1) affects MCF-7 but not MDA-MB-231 cells.

Although it is unclear why the effect of HAP1 is significant in MCF-7 cells and not in MDA-MB-231 cells, we propose a hypothesis (Fig. 6). It was reported that more than 90% sporadically lurking HAP1-immunoreactive (HAP1-ir) cells expressed nuclear estrogen receptor (ER) (18). Therefore, we hypothesized that ER-positive MCF-7 cells gathered more HAP1 than triple negative MDA-MB-231 cells and interacted with them.

In various cells, the Ca^{2+} signaling process is mediated by the endoplasmic-reticulum-membrane-associated 1,4,5-trisphosphate ($Insp_3$) receptor ($Insp_3R$), which has a critical role in the control of cellular and physiological processes as diverse as cell division, cell proliferation and apoptosis (19,21). Tang *et al* found that $Insp_3R$ could bind with HAP1A *in vitro* and *in vivo*, suggesting that HAP1 may impact the function of $Insp_3R$ (20). When $Insp_3R$ is activated by HAP1, the Ca^{2+} release is increased, subsequently inducing the activation of calpains.

The calpains are a family of neutral cysteine proteases that require calcium for their catalytic activity. The cellular activity of calpain is, in part, regulated by their endogenous inhibitor calpastatin (22,23). The calpains function in the controlled proteolysis of a large number of specific substrates involved in various cellular processes such as migration, cell signalling and apoptosis. In addition, previous studies showed that calpain cleaved caspase-9 and therefore blocked the caspase-3 activation (24). Furthermore, Storr *et al* suggested that ER-positive breast cancer had a significantly high calpastatin expression compared with ER-negative breast cancer, and the expression of calpastatin in non-triple negative breast cancer was also significantly higher than in triple-negative breast cancer (22). The expression of calpain 1, however, was the opposite of calpastatin. Based on these data, we deduced that in ER-positive or (and) non-triple negative breast cancers, more apoptosis was induced than in ER-negative or (and) triple-negative breast cancer.

Collectively, ER, expressed in MCF-7, induced HAP1 assembly, then sequentially activated $Insp_3R$, calpastatin and caspase-3, finally increasing apoptosis. Moreover, the procedure may also impact invasion. This pathway does not exist in MDA-MB-231. However, further research is required to verify the hypothesis and to clarify the mechanism.

In summary, our findings demonstrated for the first time that *HAP1* downregulation occurs in breast tumor, and that HAP1 suppresses breast cancer cell growth. However, there were also some different effects of *HAP1* between ER-positive MCF-7 and triple negative MDA-MB-231 cells, which we hypothesize may reflect the different types of breast cancer in the clinic. The findings of this study may provide a new therapeutic target for the treatment of breast cancer patients.

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