Cancer Chemother Pharmacol (2012) 69:1079–1087
DOI 10.1007/s00280-011-1801-y

ORIGINAL ARTICLE

Gene therapy with RNAi targeting UHRF1 driven by tumor-specific promoter inhibits tumor growth and enhances the sensitivity of chemotherapeutic drug in breast cancer in vitro and in vivo

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Received: 14 September 2011 / Accepted: 8 December 2011 / Published online: 29 December 2011

Abstract UHRF1, also known as ICBP90 (inverted CCAAT box binding protein 90) in human, is a nuclear protein that acts as a fundamental regulator in cell proliferation and maintains DNA methylation. It is reported that UHRF1 is obviously upregulated in various human malignancies, but unchanged in differentiated tissues, suggesting that UHRF1 plays a crucial role in carcinogenesis and can be a useful anticancer drug target. In this study, we explored whether UHRF1 can be a therapeutic target for human breast carcinoma. We successfully constructed the tumor-specific shRNA expression vector driven by survivin promoter targeting UHRF1 gene. The tumor-specific RNA interference system efficiently and specifically knocked down UHRF1 expression, induced the apoptosis of tumor cells, and enhanced chemosensitivity of tumor cells to cisplatinum, but not in normal cells in vitro and in vivo. Therefore, the survivin promoter-driving shRNA expression system targeting UHRF1 may play a vital and potential role for the treatment of specificity and high efficacy in human breast carcinomas.

Keywords RNA interference · Survivin promoter · UHRF1 · Caner gene therapy · Chemosensitivity · Cisplatinum

Lin Fang and Li Shanqu should be regarded as joint first authors for equal contributions.

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Introduction

UHRF1 (ubiquitin-like containing PHD and RING finger domains 1) is a 90-kDa nuclear protein that plays an important role in the regulation of S phase, DNA damage control, and transformation to malignancy [1–5]. Recently, it is explored that UHRF1 is required for the maintenance of *CpG* methylation, directly interacts and colocalizes with DNMT1 [6]. UHRF1 levels fluctuate with cell cycle, which it peaks at late G1 phase and during G2/M phases in noncancerous human cells. However, in cancer cells, UHRF1 levels are obviously upregulated in various cancer cells, including breast, lung, prostate, and cervical cancer; meanwhile, UHRF1 expression remains at a high level in all phases of cell cycle of cancer cells. High levels of UHRF1 are identified as an important factor that can keep the cells in a proliferated state and prevent them from differentiation [7–12]. Moreover, it has been reported that UHRF1 localizes on the methylated CpG islands of some tumor suppressor genes' promoters including those of FHIT, RARb, p16INK4A, and APC, and proposed the possibility that UHRF1 may downregulate the expression of these genes [13]. Therefore, downregulation of UHRF1 levels can interfere with the proliferation and growth of tumor cells. Furthermore, UHRF1-depleted cells can suppress oncogenic properties and enhance sensitivity to anticancer drugs and gamma-irradiation in carcinoma cells [11, 14–16]. Specially, UHRF1 may be a new target in the chemotherapy and radiotherapy of breast cancer via affecting apoptosis and DNA damage repair [17, 18]. Therefore, in the treatment for human breast cancers, it is extremely urgent to find a new method of downregulating UHRF1 levels with high efficacy and tumor specificity, which can effectively inhibit tumor cell growth in cancer cells and maintain the activity of UHRF1 in normal cells.

At present, specific gene silencing techniques are used for the degradation of targeted gene in a variety of cell lines through RNA interference methods. These approaches mainly included a synthesized small interfering RNA (siRNA) in vitro or DNA vector–based shRNA [19]. In comparison with synthesized small interfering RNA, the silencing effect of DNA vector-based shRNA is more stable for gene therapy. But there are some limitations that many promoters to drive shRNA expression are short of specificity to deliver shRNA only into tumor cells not normal cells. The tumor-specific promoter has extensively used in the field of cancer gene therapy. Survivin is a novel member of the inhibitors of apoptosis (IAP) protein family, which is highly expressed in primary tumors and cancer cell lines but undetectable in most somatic tissues and normal differentiated cells [20, 21]. We identified a core promoter region of ~980 bp upstream of the transcription start site according to our previous studies, which has high transcriptional activity in tumor cells but not in normal differentiated cells [22, 23]. These results indicated that the survivin promoter may be useful for tumor-specific gene silencing.

In the present report, we constructed a novel tumor-specific RNAi vector targeting UHRF1 driven by survivin promoter, which were transfected into human breast carcinoma cell line (MCF-7 cells) and normal human mammary epithelial cell line (MCF210). UHRF1-shRNA obviously knocked down UHRF1 levels in MCF-7 cells, but not in MCF210 cells. Those stably transfected cells with minimal levels of UHRF1 expression showed a significant enhancement in chemosensitivity to cisplatinum besides the obvious growth inhibition and apoptosis induction in vitro and in vivo. Together, these results provided powerful evidences that RNAi targeting UHRF1 driven by survivin promoter can be used as a novel strategy of gene therapy for human breast carcinoma.

Materials and methods

Cell lines and culture conditions

MCF210 and MCF-7 were cultured in DMEM (Invitrogen, USA) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin in 37°C with 5% CO_2.

Plasmids construction

The survivin promoter gene (GenBank NM U75285) including 980 bp upstream from the transcription initiation site was amplified from our previously obtained plasmid. The oligonucleotides encoding UHRF1 shRNA were designed and synthesized as follows: UHRF1-shRNA1 (sense, 5′-GATCCGTCTTCAAGATTGAGCGGCTTCAAGAGA GCCGCTCAATCTTGAAGACTTA-3′), UHRF1-shRNA2 (sense, 5′-GATCCACTCACCAACACCAACAGGTT CAAGAGA CCTGTTGGTGTTGGTGAGTTTA-3′). The recombinant plasmids were named pSilencer4.1-SP-s1 and pSilencer4.1-SP-s2.

Transfection and selection of stable transfectants

The cells were seeded and transfected with different vectors, respectively, using Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer's instructions and were selected with 600 μg/ml of neomycin (Sigma, USA) after transfection. Stable transfectants were named MCF-7-s1 or MCF210-s1, MCF-7-s2 or MCF210-s2, MCF-7-NS or MCF210-NS, and MCF-7-parental or MCF210-parental, respectively.

Analysis of UHRF1 gene expression by semi-quantitative RT–PCR

Total RNA was extracted, and 5 μg of RNA was used to synthesize cDNA using Superscript First-Strand Synthesis Kit (Promega, USA), following the manufacturer's protocols. *One microliter of cDNA* was used to amplify specific genes. For normalization of cDNA loading, *β*-actin was also amplified from each sample. RT-PCR was performed by using primer set of UHRF1*: 5′-GTCCGCCAGAGCCT CGTGC-3′, 5′-TACCAGAAGCCCCGCTCCT-3′*; *β*-actin: *5′-CTACAATGAG-CTGCGTG-3′, 5′-GGTCTCAAACATG ATC-3′*; survivin: *5′-TTCTCAAGGACCACCGCATC-3′, 5′-AGAGGCCTCAATCCATGG-3′*. Signals were quantified by densitometric analysis using the Labworks Image Acquisition (UVP, Inc., Upland, CA). For the control, *β*-actin expression was used to normalize mRNA expression levels of other genes.

Western blot analysis

The cells were harvested and lysed on ice. Western blot assays were carried out using the following primary antibodies: anti-UHRF1, anti-cleaved caspase3, and anti-*β*-actin (Santa Cruz Biotechnology, USA) antibodies. The bands were detected by enhanced chemiluminescence (ECL, Amersham). The level of UHRF1 was determined and normalized to *β*-actin.

In vitro cell proliferation assay

The cells were seeded at 1.0×10^4 per well for MTT assay. Every day, one plate was used for MTT assay and 10 μL MTT (5 mg/mL) was added to each well. Finally, the absorbance was determined on a microreader (Bio-Rad Co.) at 490 nm.

In vitro colony formation assay

The cells were plated in 10-cm culture dishes, respectively. After 18 days of incubation, cells were fixed with methanol and stained with 0.1% crystal violet. Visible colonies were manually counted.

Analysis of cell cycle

Approximately 1.0×10^6 cells of each group were harvested, washed with ice-cold PBS twice, and fixed with 70% ethanol overnight at 4°C. Finally, the fixed cells stained with propidium iodide (PI) were analyzed by flow cytometry (BD, USA).

Flow cytometry for detection of apoptotic cells

The cells were harvested, washed with ice-cold PBS twice, resuspended, and stained with Annexin V-fluorescein isothiocyanate and propidium iodide following the manufacturer's protocols of apoptosis detection kit (Oncogene Research Products, Boston, MA). The stained cells were analyzed on flow cytometry (BD, USA).

Measurement of caspase3 activity

The activity of caspase3 was measured using caspase colorimetric assay kit (Sigma, USA). The activities were quantified spectrophotometrically at a wavelength of 405 nm.

Murine xenograft model for tumorigenicity assay and chemosensitivity to cisplatinum in vivo

The experiments on tumorigenicity were assessed by subcutaneous injection of stably transfected MCF-7 cells into 6–8-week-old male athymic nude mice. Approximately each aliquot of 1×10^7 cells was injected into the back of nude mice which were maintained under pathogen-free conditions, and tumor growth over a period of 4 weeks was monitored and measured with a digital caliper. Forty-two days after inoculation, all mice were killed, and s.c tumors were resected and fixed. In the assay of chemosensitivity to cisplatinum in nude mice, mice were treated with cisplatinum (5.0 mg/kg; thrice weekly) or with 0.1 mL N.S (PH 7.4; thrice weekly).

Immunostaining

Cells with apoptotic nuclei were visualized in cryosections by means of the TUNEL assay (In Situ Cell Death Detection kit; Keygene, Nanjing, China), following the manufacturer's instructions. Photomicrographs were taken on a Leica DMR microscope connected to a digital camera and the computer system.

In vitro chemosensitivity assay

The chemosensitivity of MCF-7 cell to cisplatinum (Sigma, USA) was evaluated by MTT analysis and apoptosis detection assay. Firstly, the IC_{50} of cisplatinum to MCF-7 cells was examined by MTT assay according to the previously described methods. Then, MCF-7 cells in the 96-wells were treated with various concentrations of cisplatinum at 0, 2.0, 4.0, 8.0, 16.0, and 32.0 μg/ml. After 48 h of incubation, the cell viability and cell apoptosis was detected.

Statistical analysis

All experiments were performed in triplicate, and the results are expressed as Means ± standard deviation (SD). Statistical analyses were performed using SPSS statistical software. Statistical significance was accepted at the level of $P < 0.05$ and indicated by asterisks in the figures.

Results

Survivin promoter-driven shRNA efficiently knocked down UHRF1 gene expression

We first analyzed the endogenous expression level of UHRF1 and survivin gene in both MCF210 and MCF-7 cells, as shown in Fig. 1a, and MCF-7 cells show higher levels of UHRF1 than MCF210 cells. Furthermore, the survivin gene was only detectable in MCF-7 cells. The silencing effects of two different specific shRNAs targeting UHRF1 were firstly determined respectively. In comparison with those in the control cells, the levels of UHRF1 mRNA and protein expression in the MCF-7-s1 and the MCF-7-s2 cells were all decreased, and the levels of UHRF1 were noticeably lower in the MCF-7-s1 cells than in the MCF-7-s2 cells (Fig. 1b, c). In addition, we observed that the levels of UHRF1 showed no significant difference in MCF210-s1, MCF210-s2, MCF210-NS, MCF210-parental, and untransfected MCF210 cells (Fig. 1d, e). The results indicated that UHRF1-shRNA system driven by survivin promoter could efficiently and specifically downregulate UHRF1 expression in breast carcinoma cells but not in normal mammary epithelial cells. So we chose pSilencer4.1-SP-s1 plasmid (UHRF1-shRNA1) for further assays.

Downregulation of UHRF1 gene expression significantly inhibited the growth of cancer cells in vitro

To investigate whether the potential of UHRF1-shRNA might inhibit the growth of cancer cells, we evaluated the proliferation of cancer cells and normal cells by MTT assay and colony formation assay. According to the cell growth

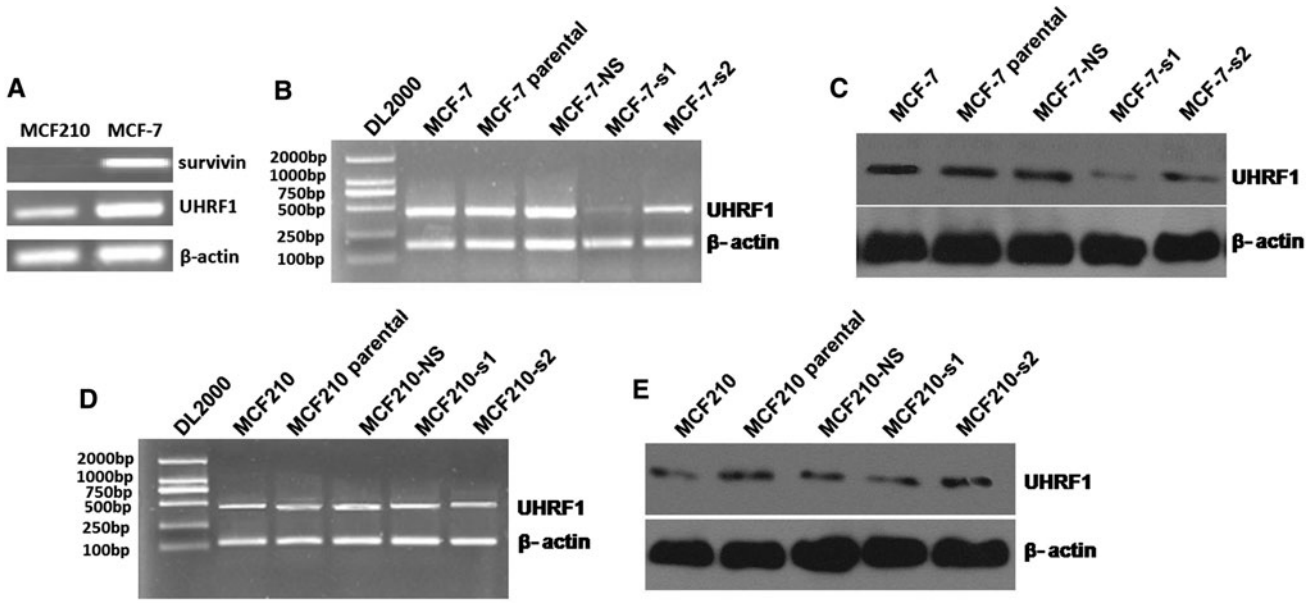

Fig. 1 Survivin promoter-driven shRNA efficiently knocked down UHRF1 gene expression. **a** The expression levels of UHRF1 and survivin in MCF-7 and MCF210 cells were detected by RT-PCR method. **b–e** UHRF1 in MCF-7 cells (**b** and **c**) and MCF210 cells (**d** and **e**) was detected by RT–PCR and Western blot analysis. Compared with the control cells, UHRF1 of MCF-7-s1 was obviously decreased ($P < 0.05$)

curve (Fig. 2a, b), we concluded that the proliferation of MCF-7-s1 cell was obviously inhibited in a time-dependent manner ($P < 0.01$) in comparison with untransfected MCF-7 cells, MCF-7-NS and MCF-7-parental cells, while UHRF1-shRNA1 could not induce the inhibition of cell growth in MCF210-s1 cells. In the colony formation assay (Fig. 2c, d), the numbers of colonies were significantly decreased in MCF-7-s1 cells compared with untransfected MCF-7 cells, MCF-7-NS, and MCF-7-parental ($P < 0.01$). However, there were no obvious differences in the numbers of colonies among MCF210, MCF210-parental, MCF210-NS, and MCF210-s1 cells, respectively. The above results indicated that tumor-specific RNA interference system driven by survivin promoter could noticeably inhibit tumor cell proliferation in vitro through UHRF1 downregulation.

UHRF1-shRNA obviously induced the arrest of MCF-7 cells at G_2/M phase

In order to determine the effects of UHRF1-shRNA on the cell cycle of MCF-7 cells, we performed cell cycle analysis by flow cytometry assay. In comparison with untransfected MCF-7, MCF-7-parental and MCF-7-NS cells, the population of G_2/M phase cells in MCF-7-s1 cells was significantly increased ($P < 0.01$), while the percentage of MCF-7-s1 cells in S phase was obviously reduced (Fig. 3a). According to the results of cell cycle analysis, we found a typical G_2/M arrest in MCF-7-s1 cells, which suggested that the growth inhibition induced by UHRF1-shRNA might be as a result of perturbed cell cycle.

UHRF1-shRNA-induced apoptosis of MCF-7-s1 cells

In order to confirm whether the proliferation inhibition of UHRF1-shRNA on MCF-7 cells was induced by cell apoptosis, we tested the changes of apoptosis in stably transfected MCF-7-s1 cells and control cells using flow cytometry assay. The result of apoptosis analysis indicated that the apoptotic rate of MCF-7-s1 cells was markedly increased ($P < 0.05$), while there were no differences in cell apoptosis among MCF-7, MCF-7-parental and MCF-7-NS cells (Fig. 3b). The results revealed that UHRF1-shRNA treatments could accelerate the apoptosis of human breast cancer cells.

UHRF1-shRNA-induced apoptosis through caspase3 pathway

To further confirm whether caspase3 pathway was involved in the process of cell apoptosis induced by UHRF1-shRNA, we used anti-cleaved caspase 3 antibodies to perform Western blot analysis and detected the activity of caspase3 on MCF-7-s1 cells and control cells (MCF-7, MCF-7-parental, and MCF-7-NS). The results showed that the presence of cleaved caspase3 was markedly detected in MCF-7-s1 cells compared with control groups (Fig. 4a, b), which suggested that caspase3 was involved in the process of this apoptosis.

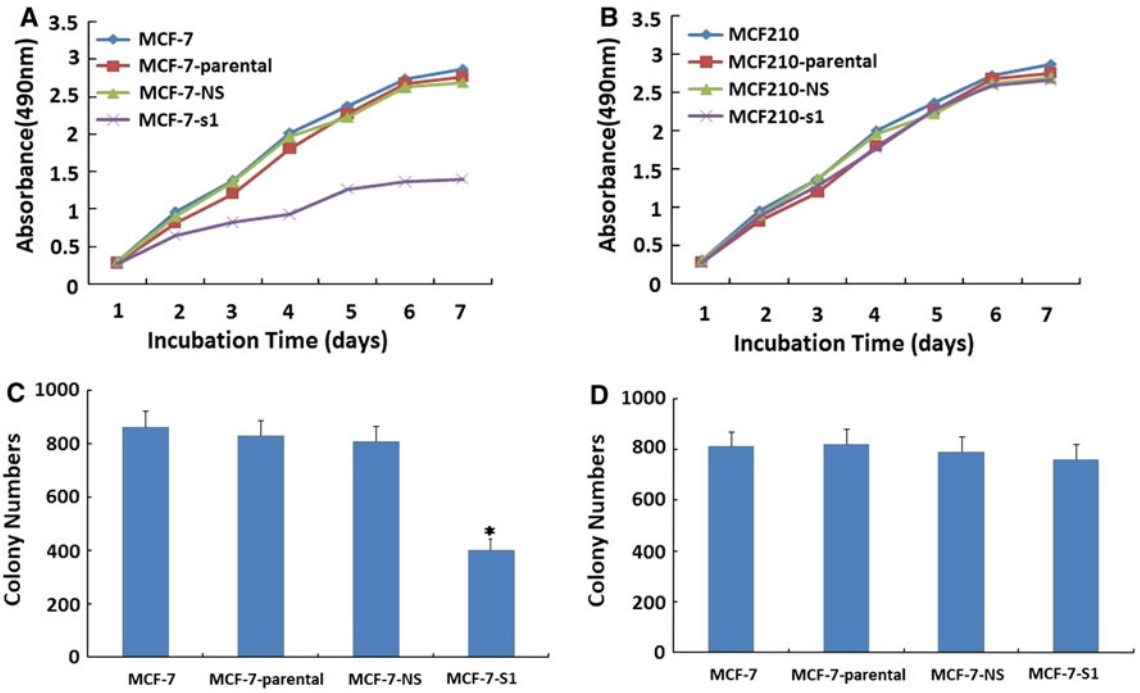

Fig. 2 Downregulation of UHRF1 expression obviously suppressed the proliferation of cancer cells. **a** and **b** UHRF1-shRNA inhibited the proliferation of MCF-7 cells not MCF210 cells proliferation. **c** and **d** the colony numbers of MCF-7-s1 were obviously lower than those of the control groups ($P < 0.05$)

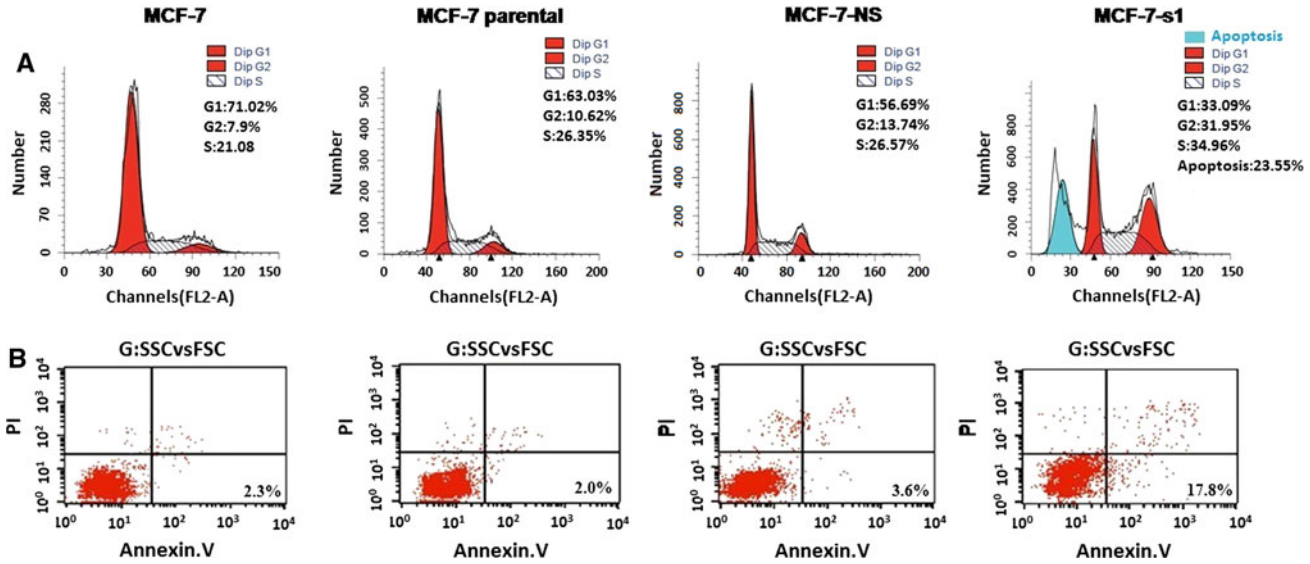

Fig. 3 UHRF1-shRNA obviously induced the arrest of MCF-7 cells at G2/M phase and apoptosis of MCF-7-s1 cells. **a** Compared with control cell, MCF-7-s1 cells were obviously arrested at G_2/M phase. **b** The apoptotic rate of MCF-s1 cells obviously increased compared with that of the control cells

In order to further define the above results, we performed the MTT assay to compare the cell viability of MCF-7-s1 cells with the cell viability of MCF-7-s1 treated with a pancaspase inhibitor. These data of MTT analysis suggested that the cell viability of MCF-7-s1 cells could be partially restored through a pancaspase inhibitor, which demonstrated that UHRF1-shRNA caused the apoptosis of MCF-7 cells in a caspase-dependent manner (Fig. 4c).

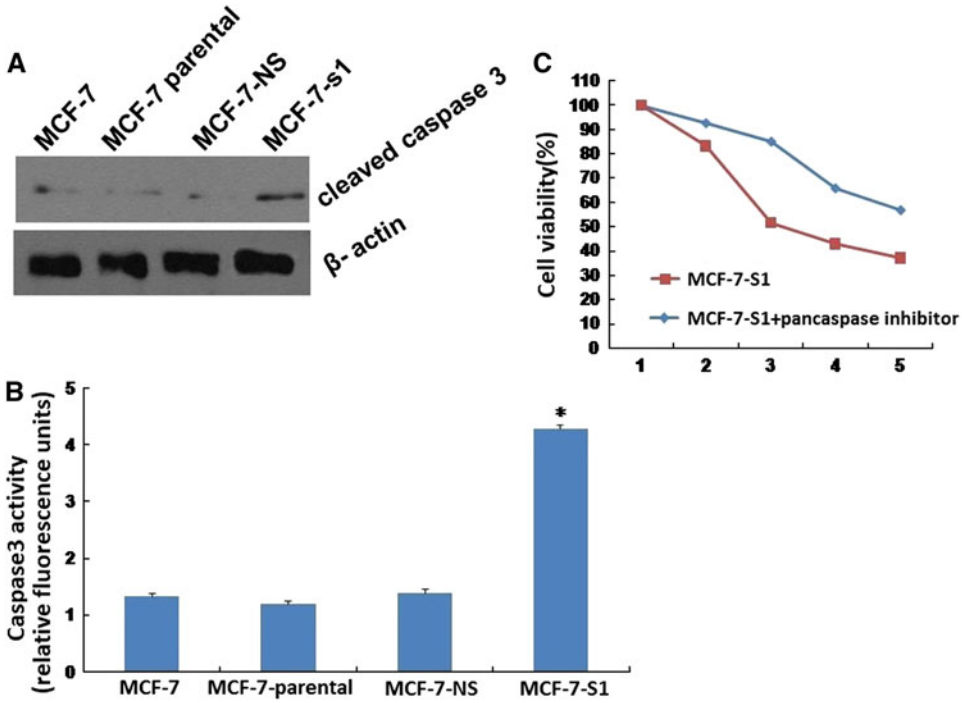

Fig. 4 UHRF1-shRNA-induced apoptosis through caspase3 pathway. **a** Compared with the control cells, the levels of caspase3 in MCF-7-s1 cells was obviously increased ($P < 0.05$). **b** The activity of caspase3 in MCF-7-s1 cells is increased by UHRF1-shRNA. **c** The MCF-7-s1 cells were treated with and without caspase inhibitor. Cell viability of MCF-7-s1 cells was partially restored. (*$P < 0.05$)

UHRF1-shRNA inhibited tumorigenicity in vivo

In order to determine whether the treatment of UHRF1-shRNA had an effect on tumor growth in nude mice, we performed Western blot analysis and tumor formation assay. We found that the levels of UHRF1 protein in tumors from MCF-7-s1 cells was significantly lower than those in tumors from other control cells by Western blot analysis (Fig. 5a). Furthermore, we performed tumor formation assay in nude mice to further explore the possible effect of UHRF1-shRNA on tumor growth in vivo. Forty-two days after inoculation, we measured tumor size and found that the average size of the xenografts formed from MCF-7-s1 cells was obviously smaller than that of the xenografts formed from control cells (Fig. 5b).Taken together, all these results demonstrated that the treatment of UHRF1-shRNA exerted a strong effect on the inhibition of tumor growth in vivo.

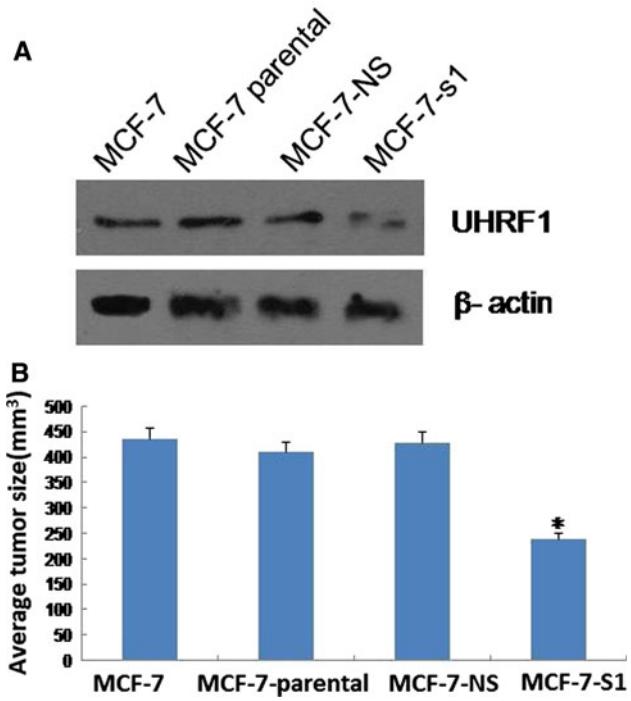

Fig. 5 Downregulation of UHRF1 expression inhibited tumorigenicity in vivo. **a** The levels of UHRF1 protein in tumors from MCF-7-s1 cells were significantly lower than those in tumors from the control cells. **b** The average size of the xenografts formed from MCF-7-s1 cells was obviously smaller than that of the xenografts formed from control cells. (*$P < 0.05$)

UHRF1-shRNA driven by survivin promoter enhanced the sensitivity to cisplatinum in vitro and in vivo

The previous studies have shown that there was an increased sensitivity to chemotherapeutics in the absence of mUHRF1 and that the treatment with cisplatinum could result in the dramatic decrease in UHRF1 level in human tumor cells [11, 24]. In this experiment, we explored whether the downregulation of UHRF1 expression by survivin promoter-driven shRNA could affect the sensitivity to cisplatinum in vitro and in vivo. The cisplatinum IC_{50} and the survival index of MCF-7-s1 cells markedly reduced in comparison with control cells ($P < 0.05$) (Fig. 6a, b). Furthermore, the apoptotic fraction of MCF-7-s1 cells significantly increased compared with that of control cells (Fig. 6c). These results suggested that UHRF1-shRNA could enhance the sensitivity to cisplatinum

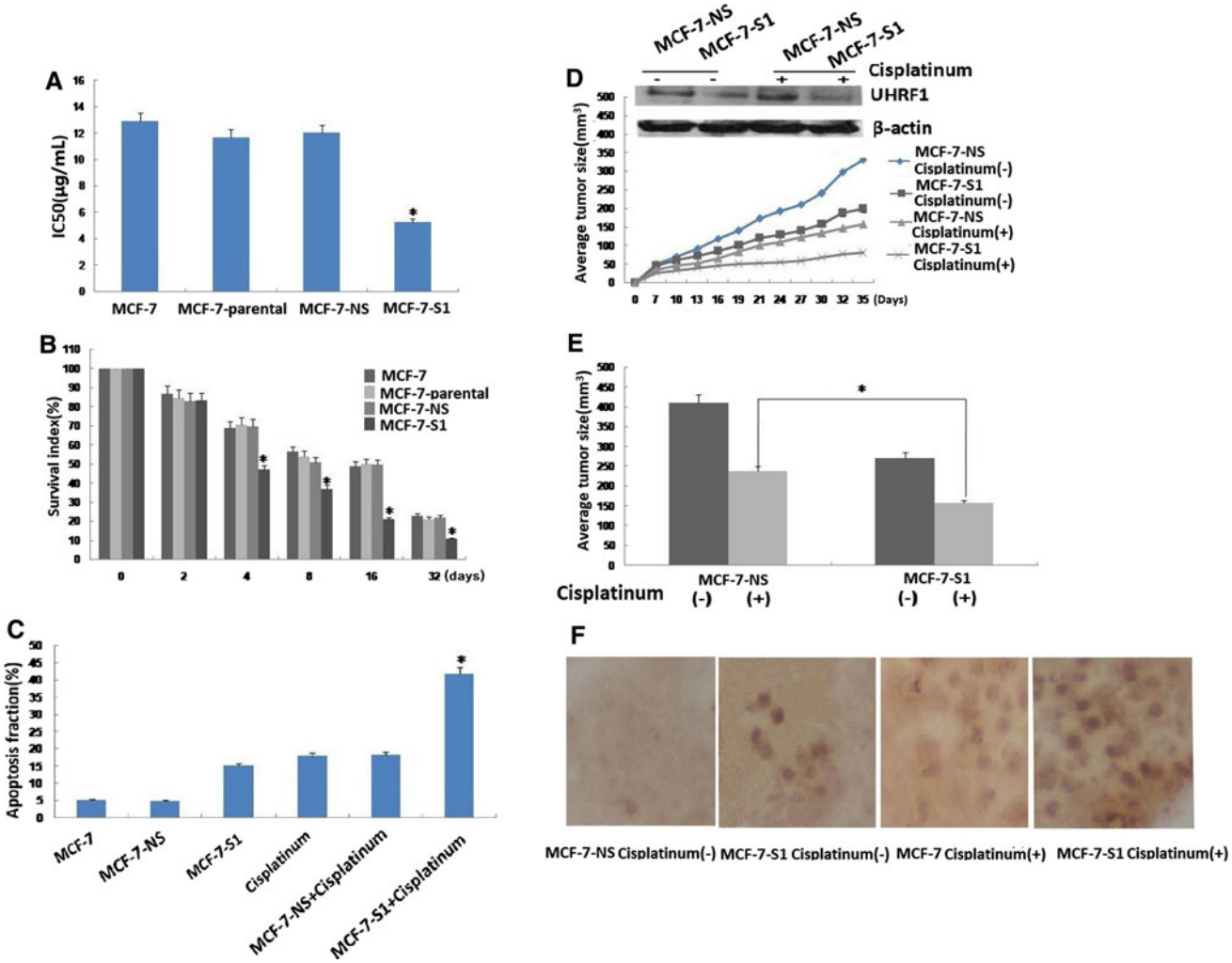

Fig. 6 Downregulation of UHRF1 expression enhanced the sensitivity to cisplatinum in vitro and in vivo. **a** The cisplatinum IC_{50} of MCF-7-s1 cells markedly reduced in comparison with control cells. **b** The survival index of MCF-7-s1 cells obviously decreased compared with that of the control cells. **c** The apoptotic fraction of MCF-7-s1 cells was significantly higher than that of control cells. **d** After treatment, the growth of tumors developed from MCF-7-s1 cells was markedly inhibited compared to those developed from control cells, and the expression level of UHRF1 protein was significantly lower in tumors developed from MCF-7-s1 cells with cisplatinum treatment than other groups. **e** Tumor size after 5 weeks of treatment with cisplatinum was detected. (*$P < 0.05$) (**f**) The number of apoptotic cells was much more higher in tumors developed from MCF-7-s1 cells with cisplatinum treatment than any other groups

in vitro. After treatment with cisplatinum, we found that the growth speed of tumors formed from MCF-7-s1 cells was markedly inhibited compared to control cells (Fig. 6d, e). We also detected the expression levels of UHRF1 and the apoptosis fraction in the xenografts 35 days after inoculation, as shown in Fig. 6d; the level of UHRF1 protein in tumors from MCF-7-s1 cells with cisplatinum treatment was significantly lower than those in tumors from other groups by Western blot analysis. Fig. 6f also showed that the number of apoptotic cells in tumors from MCF-7-s1 cells with cisplatinum treatment was much more higher than that of any other groups.

All these results demonstrated that the UHRF1-shRNA driven by survivin promoter resulted in a suppression of tumor growth in vivo.

Discussion

The uncontrolled proliferation that is a characteristic of tumorigenesis is usually caused by lots of changes in cell growth signaling pathways or cell cycle checkpoints. UHRF1, a nuclear ring finger protein, involves in cellular proliferation. Some groups also confirmed that UHRF1 is overexpressed in various cancers, and overexpression of UHRF1 in breast tumor is associated with malignant potential of the tumors as defined by the grade and stage [6, 13, 25]. Moreover, the downregulation of UHRF1 in breast cancer cells can suppress cell proliferation and induce cell apoptosis. Therefore, UHRF1 may be an ideal anticancer target for the treatment of human breast cancer. In the field of cancer gene therapy, it is vital to efficiently decrease the

expression of target genes in tumor tissue while reduce adverse effects in normal tissues. Tissue- or cell-specific promoters that can specifically drive the expression of target genes in tumor tissue have been extensively used for gene therapy. However, at present, the promoters employed in DNA vector-based shRNA system can silence a target gene in all cell types and produce adverse effect in non-target cells. According to our previous studies, survivin promoter belongs to tumor-specific promoter and displays high activity in human cancer cells but not in differentiated cells [22, 26, 27]. Therefore, we adopted a novel strategy to knock down UHRF1 expression in a tumor-specific manner by constructing survivin promoter-driven shRNA eukaryotic expression vector. To acquire better effect of cancer therapy, we combined this system with the chemotherapeutic agents. Cisplatinum as a chemotherapeutic drug that has been used as a first-line therapy to metastatic breast carcinoma, but its severe toxicity limits its use in routine clinical practice. Therefore, it is necessary to explore a new way of enhancement to conventional chemotherapy of breast carcinoma [28, 29]. Other reports have shown that there was an increased sensitivity to the effects of chemotherapeutics in the absence of mUHRF1, and the treatment with the antitumor agent cisplatinum could result in the dramatic decrease in UHRF1 level in human tumor cells [11, 24]. Therefore, we attempt to confirm the synergistic effect of cisplatinum and DNA vector-based UHRF1-shRNA system driven by survivin promoter in breast cancer treatment in this study.

To achieve a stable and specific supply of silencing effect in tumor cells for cancer gene therapy, we successfully constructed the plasmid vectors with small hairpin RNA expression cassettes targeting UHRF1 under the direction of survivin promoter. We designed sequences of UHRF1 shRNAs and transfected them into breast carcinoma cells (MCF-7 cells) and normal mammary epithelial cells (MCF210 cells). Two shRNAs could specifically knock down the levels of UHRF1 in MCF-7 cells, but not in MCF210 cells. Therefore, UHRF1-shRNA system driven by survivin promoter could only have a silencing effect in tumor cells not in normal cells, which suggested that the design of this system was effective and specific. We selected one shRNA with better silencing effect to perform further experiments. It has been reported that UHRF1 downregulation can lead to a G_2/M arrest and cell apoptosis [30]. Our results also demonstrated that the downregulation of UHRF1 expression induced the arrest of cell cycle in G_2/M phase and obvious cell apoptosis in MCF-7 cells. The arrest of cell cycle can suppress the proliferation and growth of stably transfected breast cancer cell, which is in accordance with the results we acquired in this study. We also found that the apoptosis in MCF-7 cells knocking down UHRF1 was concerned with the activation of caspase3, but the mechanism of this regulatory effect needs to be further examined. Besides, animal experiments confirmed that the downregulation of UHRF1 expression could inhibit the tumorigenicity of the mouse xenograft model in vivo. All the above results demonstrated that UHRF1-shRNA system driven by survivin promoter could specifically and efficiently suppress the proliferation of breast cancer in vitro and in vivo. Therefore, this system might play an important role for gene therapy of breast cancer.

In order to enhance the therapeutic potential of cisplatinum and reduce the adverse effects of cisplatinum, we explored whether cisplatinum could be combined with UHRF1-shRNA system driven by survivin promoter in the therapy of breast cancer. The results indicated that this synergism could enhance the cytotoxicity of cisplatinum in vitro and in vivo.

In summary, we provide potent evidence with a new strategy in breast cancer therapy. The results suggest that UHRF1-shRNA system driven by survivin promoter can specifically and efficiently suppress the proliferation of breast cancer and enhance the chemosensitivity of cisplatinum in breast cancer in vitro and in vivo. The further study should be performed whether this new strategy is applied for a wide range of cancers.

Acknowledgments Thanks to every one of the Department of Clinical Laboratory for their sincere help and technical support. This study was supported by grants from National Natural Science Foundation of China (30901579).

Conflict of interest The authors declare that no conflict of interest exits in the submission of this manuscript.

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