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ORIGINAL ARTICLE

Inhibition of CD44 expression in hepatocellular carcinoma cells enhances apoptosis, chemosensitivity, and reduces tumorigenesis and invasion

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Abstract
Purpose CD44 is overexpressed in various tumors including hepatocellular carcinoma (HCC). The purpose of this study was to examine the effects of CD44 antisense oligonucleotide (ASO) alone or combination with doxorubicin on HCC cells in vitro.
Methods Cytotoxicity was measured by use of a cell viability assay in HCC cell line SNU-449. Tumorigenesis and invasion were accessed by colony formation, growth in soft agar and ECMatrix invasion assay. Apoptosis and necrosis were evaluated by using double staining with Hoechst 33342 and propidium iodide. Protein expression and mRNA level were detected by Western blot and RT-PCR.
Results We have designed novel CD44 ASO, which can effectively down-regulate CD44 expression in SNU-449. Colony formation, growth in soft agar and invasion were significantly impaired after CD44 ASO treatment in SNU-499. In company with CD44 down-regulated by CD44 ASO, MDR-1 and Bcl-2 expression were also greatly reduced. CD44 ASO also increased chemosensitivity to doxorubicin significantly, lowered IC_{50} by one order of magnitude. Apoptosis and necrosis were also induced by CD44 ASO alone or in combination treatment with doxorubicin.
Conclusions Inhibition of CD44 expression by CD44 ASO significantly induced apoptosis, decreased tumorigenesis and invasion, and increased chemosensitivity. Thus, CD44 ASO is potentially a therapy that is worth investigating in the clinical setting.

Keywords CD44 antigen · Antisense oligonucleotides · Chemotherapy · Hepatocellular carcinoma

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Introduction

Hepatocellular carcinoma (HCC or hepatoma) is one of the most common tumors worldwide [1, 2]. HCC is often diagnosed at an advanced stage when most potentially curative therapies such as resection, transplantation or percutaneous and transarterial interventions are of limited efficacy [1, 3, 4]. The fact that HCC is resistant to systemic chemotherapy, and is rarely amenable to local regional radiotherapy, leaves this disease with no effective therapeutic options and a very poor prognosis [2, 5, 6]. Therefore, the development of more effective therapeutic tools and strategies is much needed. Recent insights into the biology of HCC suggest that certain signaling pathways (such as the Ras/Raf/Mek/Erk and PI3k/Akt/mTOR pathways) and molecular alterations are likely to play essential roles in HCC development by promoting cell growth and survival [4]. The identification of such mechanisms may open new avenues for the prevention and treatment of HCC through the development of targeted therapies. Recent data indicated that sorafenib,

a small molecule Raf kinase and VEGF receptor kinase inhibitor, is effective in prolonging survival of patients with advanced HCC [7].

CD44 is a highly glycosylated transmembrane protein, which composes of a distal extracellular domain, a membrane-proximal region, a transmembrane spanning domain, and a cytoplasmic tail [8–10]. CD44 contains 20 exons, many of which are subject to alternative mRNA splicing resulting in a large number of transcripts. The most common isoform of CD44, designated as CD44s (standard), is encoded by nine standard exons and has a molecular weight of approximate 90 kDa. A variant form of CD44 (CD44v) contains additional exons, referred to as variant exons (v2–v10 in humans) that result in additional protein sequences being inserted into the extracellular and membrane proximal region of the protein [9]. CD44 participates in a relatively diverse set of functions including lymphocyte homing, T-lymphocyte activation, signal transmission involved in cell proliferation, migration and apoptosis [9, 11, 12]. CD44 is essential to the physiological activities of normal cells, but it also associated with pathologic activities of cancer cells. Compared with control counterparts, enhanced expression of CD44 (CD44s and CD44v) in malignant tissues can be detected in breast cancer, colorectal cancer, thyroid carcinoma, lung cancer, hepatocellular carcinoma, renal cell carcinoma, gallbladder carcinoma, ovarian carcinoma, endometrial cancer and melanoma [12–15]. Endo et al. [16] compared CD44 (CD44s and CD44v) expressed in HCC tissue with those in the surrounding non-HCC hepatocytes from 107 patients. The result indicated overexpression of CD44 can be identified in 24–49% of HCC and is associated with poorly differentiated histology and a shortened survival. Overexpression of CD44 (often together with CD44v) is also correlated with hallmarks of cancer biology including tumorigenesis, tumor growth and metastasis. CD44 is emerging as an important metastatic tumor marker and is also associated with an unfavorable prognosis for a variety of cancers, including HCC [10–17]. Therefore, targeting agent specific for CD44 is potentially a promising method for tumor inhibition, especially in CD44 overexpressed tumors. It has been reported that targeting CD44 with specific anti-CD44 monoclonal antibodies (H90, A3D8, HI44a) can reverse the differentiation blockage of leukemic cell lines, and in some cases, inhibit proliferation and stimulate apoptosis [11, 17–20].

Antisense oligonucleotides (ASOs) have been considered potential strategies for down-regulating target proteins since the 1970s [21]. Several ASOs targeting genes involved in neoplastic progression have been evaluated as potential therapeutic agents [22–26]. In fact, the combined use of ASOs with chemotherapeutic agents has shown synergistic antineoplastic effects in several tumor models (non-small cell lung cancer, prostate carcinoma, non-Hodgkin lymphoma, gastric carcinoma, and others), and the efficacy is being evaluated in clinical trials (on malignant melanoma, chronic lymphocytic leukemia, non-small cell lung cancer, prostate carcinoma, and others) [27–29]. In this study, we have developed and tested the effect of novel CD44 ASO and found that it significantly suppresses CD44 expression, induces apoptosis, decreases colony formation and invasion, and increases chemosensitivity in SNU-449 cell, a CD44 over-expressing human HCC cell line. To our best knowledge, this is the first report investigating the therapeutic role of CD44 inhibition in HCC cells.

Materials and methods

Cell line and culture condition

Human HCC cell line SNU-449 was obtained from the American Type Culture Collection. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

Drug and oligonucleotides

Doxorubicin (D1515, Sigma, MO, USA) was dissolved in dimethylsulphoxide (DMSO) and stored at −20°C. Oligonucleotides were synthesized on an automated DNA synthesizer as phosphorothioate oligonucleotides in Sangon Biological Engineering Technology & Services Co. (Shanghai, China). Synthesized oligonucleotides were purified by polyacrylamide gel electrophoresis. ASO 4401 (5′ TGCCACCAA AACTTGTCCAT 3′), a CD44 ASO, targeted to 5′ region of human CD44 mRNA. RC was an 18-mer random oligonucleotide, which was used as a control ASO. Both of oligonucleotides were dissolved in saline and stored as frozen aliquots at −20°C.

Cytotoxicity assay

The cytotoxicity of ASO 4401 alone or combination with doxorubicin in SNU-449 cells was determined by measurement of cell viability by use of CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, WI, USA). SNU-449 cells were subcultured into 96-well plates at densities of 7×10^3 cells/well. After overnight growth, cells were transfected with ASO 4401 using siRNA Transfection Reagent (Santa Cruz, CA, USA) according to the manufacturer's instruction. For CD44 ASO alone treatment, cells were incubated at 37°C in a humidified, 5% CO_2 atmosphere for 48 h after transfection. For CD44 ASO combination with doxorubicin treatment, CD44 ASO transfected cells were incubated for 16 h first, and then treated with

doxorubicin for a further 48 h. At the end of incubation, 20 μL/well of One Solution reagent was added. After 1 h at 37°C in a humidified, 5% CO_2 atmosphere, the absorbance at 490 nm was recorded using an ELISA plate reader (Bio-Rad Laboratories, CA, USA).

Colony formation assay

SNU-449 cells were transfected as above, then cultured in normal growth medium for an additional 16 h. Cells were seeded in six-well plates at a density of 4,000 cells per well, and allowed to grow for 9 days. At the end of incubation, cell colony formation was assessed by a colorimetric assay using crystal violet (#C0775, Sigma, MO, USA).

Anchorage-independent growth assay

Anchorage-independent growth was assessed by comparing colony growth in soft agar as described by Bullard [30]. After the transfection, cells were cultured in normal growth medium for an additional 16 h. Cells were suspended in soft agar and growth medium in six-well plates at a density of 5×10^4 cells per well. After 12 days, colonies (>20 cells) were counted under the microscope.

Invasion assay

Invasion assays were performed using Cell Invasion Assay Kit (#ECM550, CHEMICON, CA, USA) according to the manufacturer's instruction. After the transfection, cells were cultured in normal growth medium for a further 16 h, then seeded in ECMatrix-coated inserts at a density of 1.5×10^5 cells per well, and allowed to grow for 48 h. At the end of incubation, gently removing non-invading cells as well as the ECMatrix gel from the interior of the inserts, invasion cells migrating through the ECMatirx were quantitated by dissolving stained cells in 10% acetic acid and reading OD at 550 nm.

Apoptosis and cell death assays

The occurrence of apoptosis and necrosis was visualized using double staining with Hoechst 33342-propidium iodide (PI) [31, 32]. After the transfection, cells were cultured in normal growth medium for 16 h, then cultured with 1,000 nmol/L doxorubicin for a further 24 h. The cells were removed from the culture dishes by trypsinization, centrifuged and suspended in PBS at the concentration 1×10^6 cells/mL. About 1×10^5 cells were stained for 10 min at 37°C with 2 μg/mL Hoechst 33342 (Hoechst, #14533, Sigma), followed by centrifugation and resuspension in 100 μL PBS containing 5 μg/mL PI (#81845, Sigma). After 5 min incubation at room temperature and centrifugation, the cells were resuspended in 10 μL PBS, dropped onto a slide, covered and examined by sequential use of filters appropriate for each dye on a Nikon fluorescence microscope at 400× magnification. In combination with a fluorescence microscopy technique, on the basis of the applied staining three types of cells were able to be identified: (1) normal cells (Hoechst positive/PI negative, Hoechst staining evenly); (2) early apoptotic cells (Hoechst positive/PI negative and the presence of apoptotic features: loss of the structural framework of the nuclei, condensation of chromatin, cell shrinkage, nuclear fragmentation and detachment of apoptotic bodies); (3) late apoptosis/necrosis cells (Hoechst positive/PI positive, with apoptotic morphology or swollen enlarged cells). Ten fields per sample areas were photographed and at least 270 cells were counted on each slide. All counted cells were taken as 100% and the cell number in each group was calculated as a percentage of the whole.

Western blot analysis

After the transfection, cells were cultured in normal growth medium for 16 h, then cultured with 1,000 nmol/L doxorubicin for a further 24 h. At the end, cells were harvested, washed two times with ice-cold PBS, and lysed with ice-cold lysis buffer (50 mmol/L Tric HCl pH 7.5, 150 mmol/L NaCl, 1% Triton-100) containing Halt Protease inhibitor cocktail (EDTA-free) (Pierce, IL, USA). Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto nitrocellulose membrane (Pall Corporation, NY, USA). The membrane was blocked in PBS containing 5% fat-free milk (Pierce). The proteins were detected by incubating with the appropriate monoclonal or polyclonal antibodies in TBS (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl), 0.05% Tween-20, and 1% BSA, followed by incubation with either anti-mouse or anti-rabbit immunoglobulin G (IgG) antibody coupled to horseradish peroxidase. Detection was achieved using SuperSignal® West Pico Chemiluminescent Substrate (Pierce). Antibodies used were as follows: CD44, MDR-1, Bcl-2, PARP, β-actin, horseradish peroxidase-conjugated secondary antibodies against mouse and rabbit IgG were from Santa Cruz Biotechnology (CA, USA); cleaved-PARP from Cell Signaling Technology (MA, USA).

RT-PCR

After the transfection, cells were cultured in normal growth medium for a further 16 h. Total RNA was extracted by using PURESCRIPT RNA Purification Kit (Gentra, MN, USA), and then 2.8 μg total RNA was reverse transcribed (in a volume of 20 μL) by using SuperScript III First-Strand

Synthesis SuperMix (Invitrogen, CA, USA). One microliter of the resulting cDNA was subjected to PCR by using primer pairs for CD44 (5′-ATGGACAAGTTTTGG TGGCACGC-3′ and 5′-AAGATGTAACCTCCTGAAGT GCTGC-3′), Bcl-2 (5′-ATGGCGCACGCTGGGAGAA CA-3′ and 5′-CCCAGCCTCCGTTATCCTGGA-3′) and β-actin (5′-GGCACCACACCTTCTACAATGAGCTG-3′ and 5′-CTCCTGCTTGCTGATCCACATCTGC-3′). Equal amounts of PCR products were electrophoresed on 1.5% agarose gels and visualized by GelRed (Biotium Corporation, CA, USA) staining. β-actin was used as an internal control.

Statistical analysis

Results were expressed as mean values ± standard deviation (SD), and one-way analysis of variance (ANOVA) was used for evaluating statistical significance. Results were considered to be significant when $P < 0.05$.

Results

Cytotoxicity assay

To determine whether the reduction of CD44 expression affects SNU-449 cell growth or chemosensitivity to doxorubicin, cells were treated with ASO 4401 alone or in combination with doxorubicin, then cell viability was measured. Figure 1a shows that treatment of SNU-449 cells with ASO 4401 results in a dose-dependent inhibition of cell growth which is significantly more effective when compared with the control oligonucleotide. Pre-treatment of SNU-449 cells with ASO 4401 also significantly chemosensitized SUN-449 cells to doxorubicin. Doxorubicin 500 nmol/L alone left 77% of cells surviving compared with 40% after ASO 4401 pre-treatment. The pre-treatment step lowered IC_{50} by one order of magnitude than doxorubicin alone in SNU-449 (Fig. 1b).

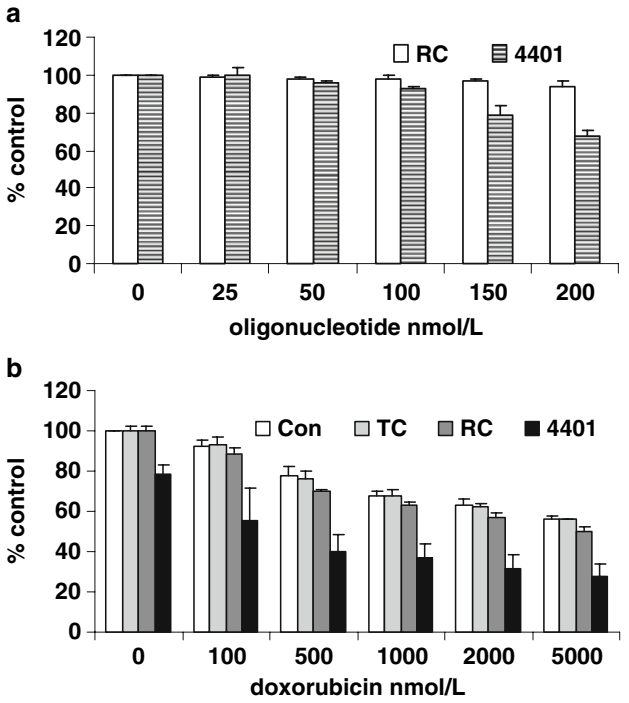

Fig. 1 Cytotoxic effect of ASO 4401 alone or combination with doxorubicin on SNU-449 cells. Cytotoxicity was determined by measurement of cell viability by use of CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, WI, USA). **a** Cells were treated with various concentration of ASO 4401 and incubated 48 h, then 20 μL per well of One Solution reagent was added. After 1 h at 37°C in a humidified, 5% CO_2 atmosphere, the absorbance at 490 nm was recorded using an ELISA plate reader. *RC* control oligonucleotide, *4401* CD44 ASO. The ODN 4401 inhibited the growth of HCC cells significantly ($P < 0.05$ at concentration 150 nmol/L, $P < 0.01$ at concentration 200 nmol/L). **b** Cells were transfected with 150 nmol/L ASO 4401 and cultured for 16 h, then treated with various concentration of doxorubicin for 48 h. *Con* untransfected control, *TC* transfection reagent control, *RC* control oligonucleotide, *4401* CD44 ASO. Pre-treatment of SNU-449 cells with ASO 4401 significantly chemosensitizes SUN-449 cells to doxorubicin ($P < 0.05$ at concentration 100 nmol/L doxorubicin, $P < 0.01$ at other concentrations). The pre-treatment lowered IC_{50} by one order of magnitude than doxorubicin alone. Data represent the mean ± SD derived from three separate experiments with triplicate wells per condition

CD44 ASO treatment decreased tumorigenesis and inhibited invasion

To determine whether CD44 ASO can inhibit tumorigenesis of HCC cells, we did colony formation and anchorage-independent growth assay. Compared to the control oligonucleotide, ASO 4401 significantly inhibited the capacity of producing colonies in SNU-449 cells ($P < 0.01$, Fig. 2a, b). In addition, ASO 4401 treatment led to smaller and fewer numbers of tumor colonies in soft agar than control oligonucleotide treatment ($P < 0.01$, Fig. 2c). Invasion through the extracellular matrix (ECM) is an important step in tumor metastasis [33]. Reconstituted basement membrane (ECMatrix) has been used to model the naturally occurring basement membrane to assess cellular invasion [34, 35]. In this assay, ASO 4401 treatment significantly decreased ECMatrix invasion when compared with control oligonucleotide treatment in SNU-449 cells ($P < 0.01$, Fig. 3).

Apoptosis and necrosis

To assess the effect of CD44 ASO alone or combination with doxorubicin at the single cell level, we applied simultaneous staining of cells with Hoechst and PI. Cells were classified as live, apoptotic or necrotic on the basis of their morphological and staining characteristics and the percentages

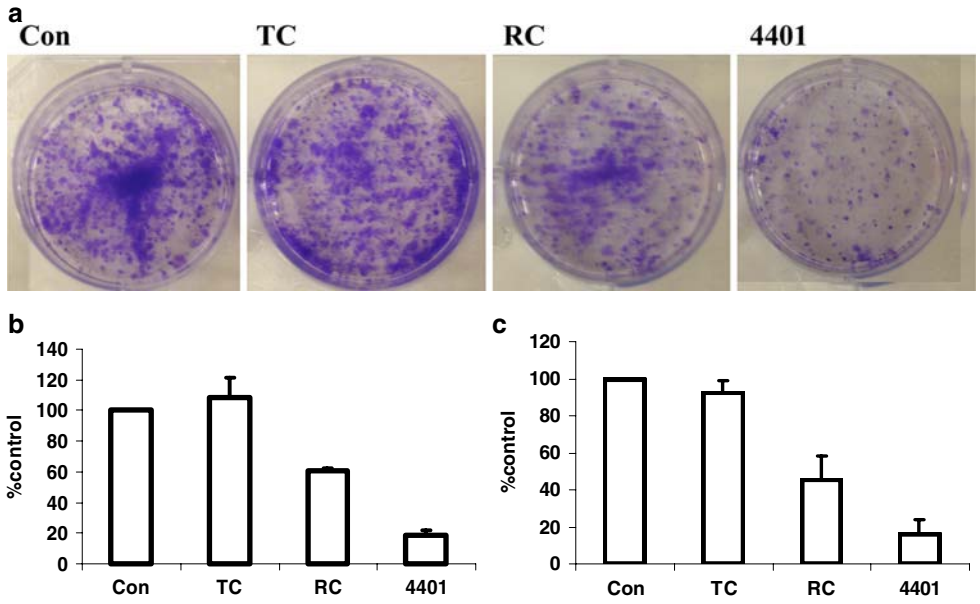

Fig. 2 Tumorigenesis assay in SNU-449 cells treated with CD44 ASO. **a**, **b** Colony formation assay. SNU-449 cells were transfected with ASO 4401, then seeded in six-well plates at a density of 4,000 cells per well, and cultured for 9 days. At the end, cells were stained with crystal violet, photographed. Colony formation was quantitated by dissolving stained cells in Sorenson's buffer (0.1 mol/L sodium citrate, 50% ethanol, pH 4.2) for colorimetric reading of OD at 550 nm. **c** Anchorage-independent growth assay. Cells transfected were suspended in growth medium with 0.3% agar, then plated onto a 0.6% agar foundation in six-well plates at a density of 5×10^4 cells per well. After 12 days, colonies (>20 cells) were counted under the microscope. *Con* untransfected control, *TC* transfection reagent control, *RC* control oligonucleotide, *4401* CD44 ASO. Compared with control oligonucleotide, CD44 ASO treatment decreased colony formation and anchorage-independent growth ($P < 0.01$). Data represent the mean ± SD derived from three separate experiments with triplicate wells per condition

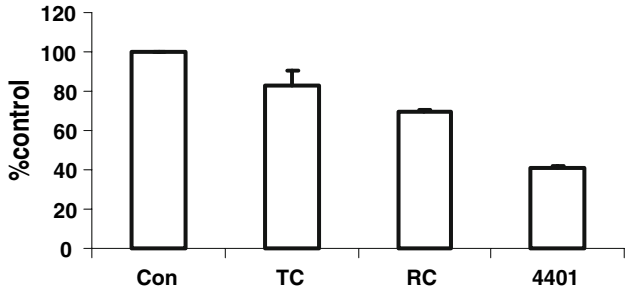

Fig. 3 Invasion assay in SNU-449 cells treated with CD44 ASO. Invasion was assessed in ECMatrix-coated invasion chambers. After the transfection, cells suspending in serum-free medium were seeded onto the ECMatrix-coated inserts (1.5×10^5 cells per insert), and the lower champers were filled with normal growth medium. The chambers were incubated for 48 h at 37°C. The non-invading cells as well as the ECMatrix gel were then removed and stained. Cells that migrated to the lower surface of the inserts were considered to have invaded through the overlying ECMatrix. Invasion was quantitated by dissolving stained cells in 10% acetic acid and reading OD at 550 nm. *Con* untransfected control, *TC* transfection reagent control, *RC* control oligonucleotide, *4401* CD44 ASO. Compared with control oligonucleotide, CD44 ASO treatment decreased ECMatrix gel invasion ($P < 0.01$). Data represent the mean ± SD derived from two separate experiments with triplicate wells per condition

of particular cell types were determined from the total number of cells. Figure 4 indicated that treatment with ASO 4401 alone induced apoptosis and necrosis significantly. Furthermore, comparing with the control oligonucleotide, ASO 4401 alone or combination with doxorubicin treatment induced a higher level of apoptosis/necrosis ($P < 0.05$, and $P < 0.01$, respectively).

Western blot and RT-PCR

As shown in Fig. 5, CD44 ASO significantly down-regulated CD44 level, which was reduced by >90% after ASO 4401 treatment. MDR1 protein, a transmembrane ATPase that serves as an efflux pump for a wide range of drug molecules, is often over-expressed in tumor cells thus contributing to the multi-drug resistant phenotype commonly seen in cancers [36]. MDR1 is regulated by hyaluranan through its receptor CD44 [37], so we accessed the effect of ASO 4401 on MDR1. The level of MDR1 was significantly reduced to <10% of controls after treatment. Down-regulation of Bcl-2 by drugs has strong potential to induce apoptosis and enhance chemosensitivity [23–26]. ASO 4401 also down-regulated Bcl-2 level by 90% when compared with controls. In SNU-449 cells, doxorubicin treatment increased the level of poly ADP-ribose polymerase (PARP), which is involved in DNA repair predominantly in response to environmental stress. However, combination of doxorubicin and ASO 4401 induced cleaved-PARP significantly, which serves as a marker of cells undergoing

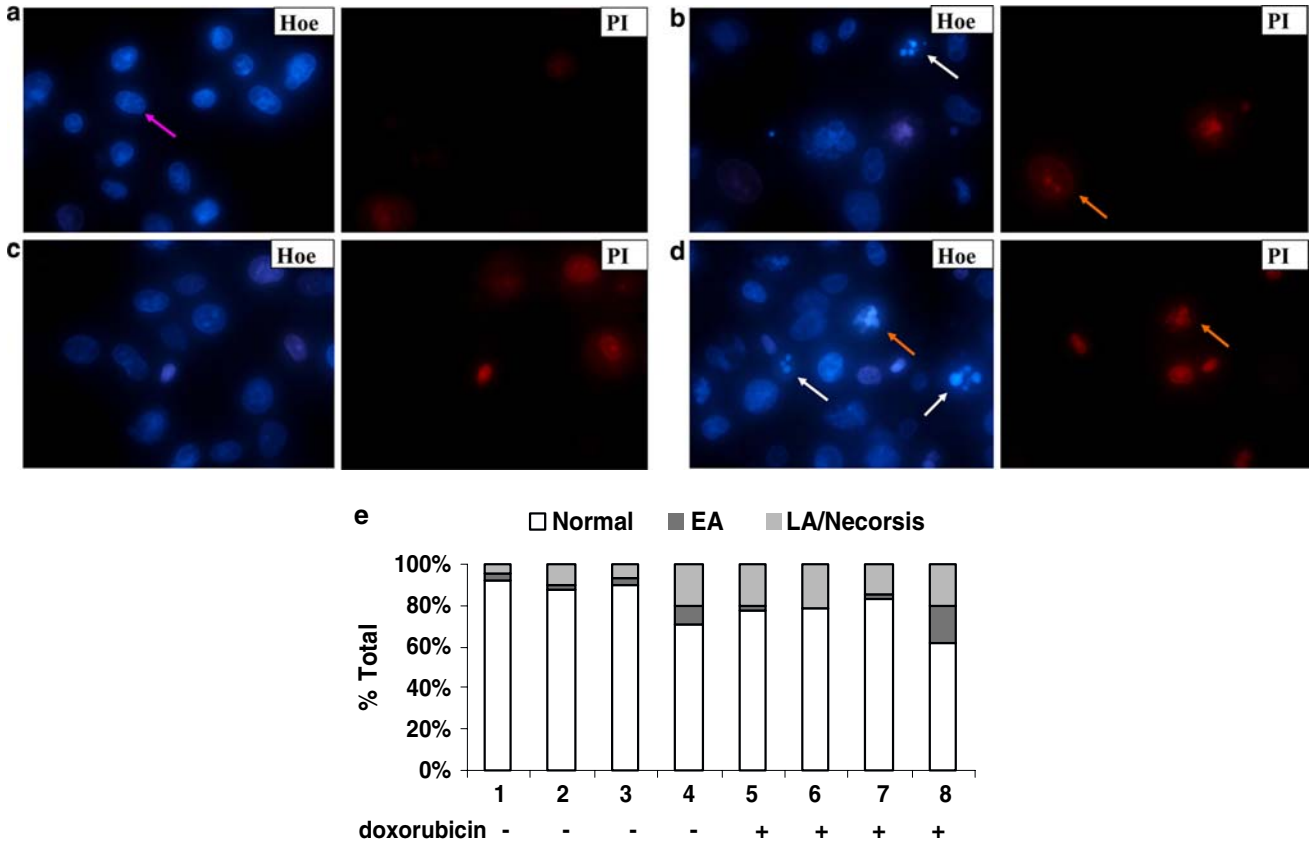

Fig. 4 Apoptosis and necrosis were induced by the CD44 ASO alone or combination with doxorubicin in HCC cells. Cells were transfected with the CD44 ASO and incubated at 37°C in a humidified, 5% CO_2 atmosphere for 16 h, and then doxorubicin was added. After a further 24 h incubation, cells were harvested by trypsinization, stained with PI and Hoechst 33342 (*Hoe*). After centrifugation, the cells were resuspended in PBS, dropped onto a slide, covered and examined on a Nikon fluorescence microscope at 400× magnification. Ten fields per sample areas were photographed sequentially with the filter appropriate for each dye. On the basis of the applied staining procedure three types of cells were able to be identified: (1) normal cells (Hoe positive/PI negative, Hoe staining evenly, *pink arrows*); (2) early apoptotic cells (*EA*) (Hoe positive/PI negative and the presence of apoptotic features, *white arrows*); (3) late apoptosis (*LA*)/necrosis cells (Hoe positive/PI positive, *orange arrows*). Representative areas were showed in **a**–**d**. **a** Untreated control. **b** CD44 ASO alone. **c** Doxorubicin alone. **d** CD44 ASO combination with doxorubicin. **e** At least 270 cells were counted in each slide and the percentages of particular cell types were determined from the total number of cells. *1*, *5* Untransfected control; *2*, *6* transfectin reagent control; *3*, *7* control oligonucleotide; *4*, *8* CD44 ASO and *5*–*8* were treated with 1,000 nmol/L doxorubicin. Compared with the control oligonucleotide, CD44 ASO alone or combination with Doxo treatment induced a higher level of apoptosis/necrosis ($P < 0.05$, $P < 0.01$, respectively). Data represent the mean ± SD derived from two separate experiments

apoptosis. We examined whether the observed CD44 ASO-mediated reduction in CD44 expression were accompanied by similar changes at the mRNA level. As seen in Fig. 6, treatment of SNU-449 cells with ASO 4401 resulted in sharp reduction of CD44 mRNA, suggesting that the one of actions of the CD44 ASO is affecting at the level of mRNA degradation. Bcl-2 mRNA was also investigated after the CD44 ASO treatment, which did not show any modulation.

Discussion

In this study, we identify, for the first time, the profound biological effects of CD44 down-regulation by CD44 ASO in HCC cells. CD44 knockdown by CD44 ASO decreased growth, induced apoptosis, and impaired colony formation, growth in soft agar and invasion in SNU-449 cells. The combination treatment of CD44 ASO and doxorubicin significantly inhibited growth and enhanced apoptosis compared with doxorubicin treatment alone in SNU-449 cells. MDR1 protein, a transmembrane ATPase that serves as an efflux pump for a wide range of drug molecules, is often over-expressed in tumor cells thus contributing to the multi-drug resistant phenotype commonly seen in cancers [36]. Companied with CD44 down-regulation by CD44 ASO, MDR-1 level was also significantly reduced. This could partially explain the enhanced chemosensitivity to doxorubicin provided by CD44 targeting. Overexpression of antiapoptotic genes in cancer cells mediates drug resistance through the inhibition of apoptosis induced by

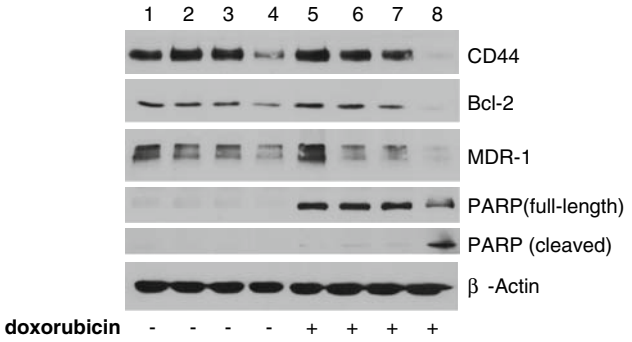

Fig. 5 Western blot analysis of protein expression in SNU-449 cells following treatment with CD44 ASO alone or combination with doxorubicin. Cells were transfected with CD44 ASO and incubated at 37°C in a humidified, 5% CO_2 atmosphere for 16 h, and then treated with doxorubicin. After a further 24 h incubation, total protein was extracted and Western blot was performed. *1*, *5* Untransfected control; *2*, *6* transfectin reagent control; *3*, *7* control oligonucleotide; *4*, *8* ASO 4401 and *5–8* were treated with 1,000 nmol/L doxorubicin. *β*-actin was as an internal control

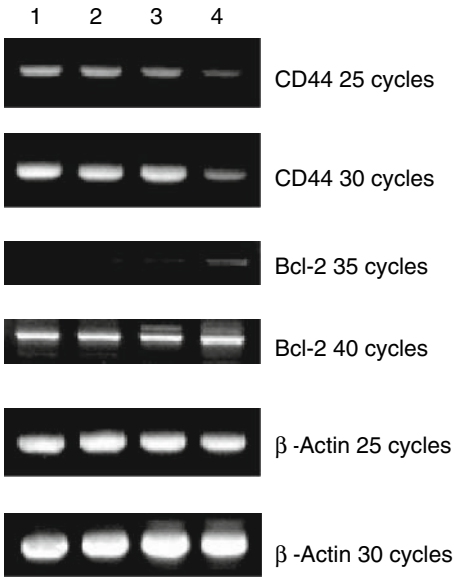

Fig. 6 Analysis of CD44 ASO effects by RT-PCR. SNU-449 cells were transfected with ASO 4401 and cultured for a further 16 h. RT-PCR was performer to determine levels of CD44 and Bcl-2 mRNA, *β*-actin as an internal control. *1* Untransfected control; *2* transfection reagent control; *3* control oligonucleotide; *4* ASO 4401

chemotherapeutic agents. Therefore, reducing the expression of antiapoptotic genes could be a rational strategy for enhancing chemosensitivity [28, 38, 39]. The Bcl-2 ASO (Genasense, Genta Inc., NJ, USA) has been shown to suppress Bcl-2 protein in association with proapoptotic effects in vitro. Preclinical and clinical studies using Genasense in combination with cytotoxic agents have revealed potential therapeutic benefit in some hematologic and solid neoplasms [24–26, 28, 29, 40]. In this study, associated with CD44 down-regulation, Bcl-2 was also inhibited significantly by CD44 ASO that could explain enhanced apoptosis and chemotherapy sensitivity when combined with doxorubicin. Our data on RT-PCR indicated that Bcl-2 mRNA was not affected by the CD44 ASO. The down-regulation of Bcl-2 protein by CD44 ASO may very well be a distinctive mechanism from Genasense and deserved further investigation. Although the ASO 4401 alone may seems to be modest in cell viability assay in vitro, but our studies on colony formation, growth in soft agar and invasion assays suggested that targeting CD44 by ASO 4401 impacted multiple critical biology features in cancers, which may not be revealed in cell viability assay alone.

It is now well established that the extracellular matrix component, hyaluronan (HA), is one of the primary ligands recognized by surface CD44 molecules [37]. The external portion of CD44 binds to HA, whereas its intracellular domain interacts with specific signaling molecules such as RhoA-activated Rho kinase, c-Src kinase, HSP90/CDC37, IQGAP1, $p185^{HER2}$, and transforming growth factor-*β* receptors. CD44 also binds directly to cytoskeletal proteins such as ankyrin and ezrin, radixin, and moesin [37, 41, 42]. The interaction of CD44 with the cytoskeleton and various signaling molecules plays a pivotal role in promoting metastatic-specific tumor phenotypes such as matrix metalloproteinase (MMP)-mediated matrix degradation, tumor cell growth, migration, and invasion. Furthermore, recent studies also indicate transfection of CD44s into CD44s (−) cell lines induced the expression of MDR1 [43]. Together with published data, our results show that the expression of CD44 and MDR1 are co-regulated and it may explain the association of HCC with drug resistance. Thus, CD44 is potentially an attractive therapeutic target because its inhibition would impact multiple different pathways implicated in cancer cell survival and resistance as opposed to targeting on single pathway.

In summary, our present study suggests that CD44 ASO down-regulation of CD44 in HCC cells induces apoptosis, decreases tumorigenesis and invasion, and more importantly, enhances chemosensitivity of HCC to doxorubicin. Our data provided valuable information to the development of potential effective therapy for HCC using CD44 ASO in combination with doxorubicin. Questions remain as to the signaling pathways linking CD44 to Bcl-2 and the in vivo efficacy of CD44 ASO. These will be further explored in future work.

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