Cancer Chemotherapy and Pharmacology (2019) 83:399–410
https://doi.org/10.1007/s00280-018-3737-y

ORIGINAL ARTICLE

Self-targeted knockdown of CD44 improves cisplatin sensitivity of chemoresistant non-small cell lung cancer cells

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Received: 12 August 2018 / Accepted: 25 November 2018 / Published online: 4 December 2018

Abstract

Background Chemoresistance remains a major challenge for effective chemotherapy of non-small-cell lung carcinoma (NSCLC). CD44 expression is related to the susceptibility of various cancer cell types to anticancer drugs. Here, we systematically investigated the CD44-dependent chemoresistance of NSCLC cells and developed a liposomal siRNA delivery system to overcome this chemoresistance by the self-targeted downregulation of CD44.

Methods We confirmed the relationship between the expression of CD44 and the chemosensitivity of NSCLC cells using flow cytometry and MTT assay. We then generated and characterized cisplatin-resistant cell lines and compared the expression of CD44 in resistant cells to that in parental cells using western blotting. To evaluate whether the chemosensitivity of resistant cells depends on CD44 expression, we performed CD44 knockdown using CD44 siRNA and detected the chemosensitivity of these cells. Additionally, we prepared hyaluronic acid (HA)-coated liposomes as a targeted delivery system to selectively deliver CD44-specific siRNA to chemoresistant NSCLC cells and observed whether the chemosensitivity of these cells was improved.

Results We found that CD44 expression is inversely proportional to the degree of cellular response to cisplatin chemotherapy and that CD44 is overexpressed in chemoresistant NSCLC cells. By performing CD44 knockdown using siRNA, we reconfirmed that the chemosensitivity of resistant cells depends on CD44 expression. We also observed that HA-liposome-mediated siRNA delivery prior to cisplatin chemotherapy significantly reduced CD44 expression and enhanced cisplatin sensitivity in chemoresistant NSCLC cells.

Conclusions These results suggest that self-targeted downregulation of chemoresistance-associated cell surface proteins during chemotherapy is an effective therapeutic strategy for overcoming the chemoresistance of NSCLC cells.

Keywords Chemoresistance · Non-small cell lung carcinoma · Hyaluronic acid-coated liposomes

Abbreviations

NSCLC	Non-small cell lung cancer
HA	Hyaluronic acid
CSC	Cancer stem cell
MTT	3-[4,5-Dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
SF	Surviving fraction
DiI	1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate
BAX	BCL2-associated X apoptosis regulator
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
DAPI	2-(4-Amidinophenyl)-1H-indole-6-carboxamidine
MDR1	Multidrug-resistant protein
MRP2	Multidrug resistance-associated protein 2

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00280-018-3737-y) contains supplementary material, which is available to authorized users.

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Background

Among the various forms of cancer treatment, chemotherapy is known to be the most effective [1, 2]. However, despite good initial response to chemotherapy, resistant cancer cells frequently develop during the administration of various chemotherapeutic agents. Additionally, the complete elimination of cancer remains difficult as 90% of drug failures in metastatic cancers are attributed to chemoresistance [3]. Therefore, tumor cell chemoresistance is recognized as a major limitation in the development of effective treatments for most cancers [4].

Chemoresistance in cancer is attributable either to the specific nature and genetic background of the cancer cell itself or to the genetic changes that follow toxic chemotherapy. Recently, a cancer stem cell (CSC) theory was proposed for chemoresistance development in cancer, which has received considerable attention in cancer treatments [5–10]. According to this theory, CSCs are naturally resistant to chemotherapy, can initiate tumor regrowth, and can differentiate via different multipotent pathways [11]. Since the first clear evidence of CSCs was provided in acute myeloid leukemia, similar tumor-initiating populations have been identified using various stem cell surface markers in multiple solid tumors. Among these markers, CD44 is closely related to the sensitivity of cancer cells to anticancer drugs in colon cancer, breast cancer, and multiple myelomas [12, 13]. Recent studies have shown that CD44 blockade with a monoclonal antibody against CD44 or CD44 knockdown with small interfering RNA (siRNA) increased sensitivity to gemcitabine in pancreatic cancer [14], trastuzumab in HER2-positive breast cancer cells [15], and lenalidomide in myeloma [16]. However, although chemoresistance is a major limiting factor in lung cancer treatment [17, 18], only a few studies have investigated whether CD44 is involved in chemoresistance in lung cancer. In patients with non-small-cell lung carcinoma (NSCLC), cisplatin resistance was detected in 63% of all samples, while carboplatin resistance was found in 68% of all samples [19]. Another chemotherapeutic approach for lung cancer patients with advanced adenocarcinoma is targeted therapy. However, studies have shown that nearly all patients succumb to drug-resistant recurrence within 2–3 years [19–21]. Therefore, it is important to elucidate the role of CD44 in the development of chemoresistant lung cancer and develop effective therapeutic strategies to overcome chemoresistance.

In this study, we systematically evaluated CD44-dependent chemoresistance in NSCLC cells and developed a self-targeted gene delivery system to effectively treat chemoresistant NSCLC. We took advantage of CD44 molecules overexpressed on the surface of chemoresistant NSCLC cells to achieve both targeting and therapeutic benefits. We prepared hyaluronic acid (HA)-coated liposomes to selectively deliver siRNA to CD44-overexpressing chemoresistant NSCLC cells as HA binds selectively to CD44 molecules on the cell surface to induce receptor-mediated endocytosis and has inhibitory effects on multidrug resistance [22–24]. We then evaluated the synergistic therapeutic effects of self-targeted CD44 knockdown in cisplatin-based therapy of chemoresistant NSCLC cells.

Methods

Cell lines and reagents

The human NSCLC cell lines H460, H522, H2087, H358, H1435, H1703, H1793, and H1299 were obtained from the Korean Cell Line Bank. Cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, ATCC® 30-2020™, ATCC, Manassas, VA, USA) and 1% antibiotics (Gibco) at 37 °C, and in an incubator containing 5% CO_2. Lipids were obtained from Avanti Polar Lipids (Alabaster, AL, USA), while 5-kDa and 700-kDa sodium hyaluronate were purchased from Lifecore Biomedical (Chaska, MN, USA).

Expression of CD44 in NSCLC cell lines

Flow cytometry

To evaluate the expression of CD44 in NSCLC cell lines, 1×10^6 cells were seeded into 100-mm cell culture dishes and cultured for 24 h at 37 °C until 80% confluence was reached. Cells were harvested using 0.05% trypsin–EDTA (Gibco), fixed with 70% ethanol for 15 min at 4 °C, and stained with antibodies against CD44 (Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4 °C. The cells were then incubated with a fluorescein isothiocyanate (FITC)-conjugated mouse IgG antibody (Santa Cruz Biotechnology) for 1 h at 4 °C. Fluorescent-labeled IgG isotype controls (BD Biosciences, Franklin Lakes, NJ, USA) were used as negative controls. CD44 expression in NSCLC cell lines was assayed by fluorescence-activated cell sorting (FACS, Beckman Coulter, Brea, CA, USA).

Confocal

To evaluate the expression of CD44 in NSCLC cell lines, 2×10^5 cells/well were seeded in 12-well plates that contained cover slides and then cultured for 24 h. The cover slides were fixed by 4% paraformaldehyde for 15 min in − 20 °C, and blocked with 3% bovine serum albumin in PBS for 2 h. The cells were incubated with an antibody against CD44 (Thermo, Rockford, USA) in blocking solution overnight. The slides were treated with the corresponding FITC-conjugated anti-mouse IgG antibody (Santa Cruz, CA. USA) for 1 h at 4 °C, and the expression of CD44 was investigated using the Zeiss observer z1 confocal laser scanning microscope (ZEISS, Oberkochen, Germany). The fluorescence intensity was measured on the Zen analysis program.

The results were obtained from at least three independent experiments.

MTT assay for cell proliferation

The proliferation rate and sensitivity to chemotherapy were assessed by the MTT assay (Calbiochem, San Diego, CA, USA). Cells were seeded into 96-well plates (5000 cells/well) for 24 h at 37 °C, and different concentrations of cisplatin (0, 5, 10, 20, 30, and 50 µg/mL) were then used to treat the cells for 48 h. Cells were incubated in 50 µL of MTT (0.5 mg/mL) for 4 h at 37 °C under light-blocking conditions. The culture medium was removed and 150 µL of dimethyl sulfoxide (Calbiochem) was added to each well. Absorbance was measured at 595 nm using the SPECTRAMAX Plus 384 (Molecular Devices, Sunnyvale, CA, USA) and cell viability was calculated as follows: cell viability (%) = $[OD_{595(\text{cisplatin treatment})} - OD_{595\ (\text{blank})}]/[OD_{595\ (\text{control})} - OD_{595\ (\text{blank})}] \times 100\%$. Results were obtained from at least three independent experiments [25].

Development of cisplatin-resistant NSCLC cells

The cisplatin resistance of each cell line (H522, H2087, H1299, and H1793) was determined after continuous exposure to *cis*-diammineplatinum(II) dichloride (cisplatin; Sigma, St. Louis, MO, USA) following an initial dose–response (0.05–10 µg/mL cisplatin) study over 72 h, from which IC_{50} values were determined. Resistant cells were treated with cisplatin (at IC_{50}) for 72 h, after which the culture medium was removed and cells were allowed to recover for 72 h. This cycle was performed for approximately 6 months, and IC_{50} concentrations were then reassessed for each resistant cell line. Cells were then maintained continuously in the presence of cisplatin at their new IC_{50} concentrations for an additional 6 months. Prior to each experiment, resistant cells were cultured in drug-free RPMI 1640 medium for 2 weeks.

Clonogenic survival assay

The survival rate of NSCLC cells treated with cisplatin was measured by a clonogenic assay. H522, H2087, H1299, and H1793 cells, as well as H522-resistant (H522-R), H2087-R, H1299-R, and H1793-R cells were seeded (1×10^3 cells/mL) into 6-well plates, incubated overnight at 37 °C, and treated with increasing concentrations of cisplatin for 9–14 days (0.5–10 µg/mL). Colonies were fixed and stained with crystal violet (0.05% w/v, Sigma) containing methanol (25% v/v) for 10 min. The staining solution was removed and the plates were washed with distilled water for 5 min. The surviving fraction (SF) was calculated using the formula: SF = number of colonies of cisplatin-treated sample/number of colonies of untreated sample. Survival curves were constructed to determine the survival ability of cisplatin-resistant cells compared to that of parental cells.

Expression of multidrug-resistant protein 1 (MDR1) and multidrug resistance-associated protein 2 (MRP2) in resistant cells

The expression of MDR1 in resistant NSCLC cells was measured by western blotting. The resistant NSCLC cells were seeded (5×10^5 cells/well) into cell culture dishes and cultured for 24 h. The cisplatin-treated cells were harvested, washed with phosphate buffered saline (PBS), and lysed using 20 µL of protein extraction reagent on ice for 30 min. Cell lysates were centrifuged at 13,000×*g* for 20 min at 4 °C to remove cell debris. Proteins in the lysate were quantified using the Bradford assay. From each lysate, 30 µg of protein was loaded for sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Amersham plc, Amersham, UK). Membranes were blocked by incubation with 1% bovine serum albumin (BSA) blocking buffer at 27 °C for 1 h with shaking. Primary antibodies against MDR1 (Santa Cruz) and MRP2 (Santa Cruz) were diluted at 1:1000 in tris buffered saline/Tween 20 containing 1% BSA. The secondary antibody was diluted in 1% BSA blocking buffer. Target proteins were visualized on X-ray films using ECL Plus Western Blotting Detection Reagents (Amersham plc). The results were obtained from at least three independent experiments.

Expression of apoptotic proteins in cisplatin-treated-resistant NSCLC cells

The resistant NSCLC cells and parental cells were seeded (5×10^5 cells/well) into cell culture dishes and cultured for 24 h. Following removal of the culture medium, the cells were treated with cisplatin (20 µg/mL) for 24 h and harvested. We detected the expressions of cleaved CASP3 (Cell Signaling Technology, Danvers, MA, USA), apoptosis regulators BCL2 and BCL2-associated X (BAX), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Cell Signaling Technology) in these cells by western blotting as described previously. The results were obtained from at least three independent experiments.

CD44 siRNA transfection

H522-R, H2087-R, H1299-R, and H1793-R cells were seeded and maintained in 6-well plates at a density of 2×10^5 cells/well for 24 h using 2 mL of growth medium. Further, 5 µM siRNA solution was prepared in 1 × siRNA buffer (5 × siRNA Buffer, Thermo Scientific, Waltham, MA, USA). In separate tubes, 200 pmol of siRNA and 3 μL of Lipofectamine™ 2000 were added to 25 μL of Opti-MEM medium (Thermo Scientific) and incubated for 5 min at 25 °C. Pre-incubated siRNA and Lipofectamine™ 2000 were mixed gently and incubated for 25 min at 25 °C to allow complex formation to occur. After washing with PBS, cells were treated with ON-TARGETplus SMARTpool siRNA (non-targeting control) or ON-TARGETplus SMARTpool CD44 siRNA (Thermo Fisher) prepared in 1 mL of Opti-MEM medium. Transfected cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 48 h, after which the treatment medium was replaced with RPMI 1640 medium containing 10% FBS. Cells were incubated for an additional 24 h and the apoptotic response to cisplatin was determined by western blotting and MTT assay.

Cellular uptake of HA-liposomes

H522, H522-R, H2087, H2087-R, H1299, H1299-R, H1793, and H1793-R cells were seeded (1×10^5 cells/well) on round cover slides in 12-well plates and cultured for 24 h. After washing with PBS, the cells were incubated with 50 μg/mL liposomes or HA-liposomes labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Thermo Fisher Scientific) for 30 min. Following incubation, the cells were washed three times with cold PBS and fixed with 4% formaldehyde. The nuclei were stained using mounting medium with 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI, VECTASHIELD, Vector Labs, Burlingame, CA, USA). The intracellular distribution of DiI-labeled liposomes was examined using a confocal laser scanning microscope (Zeiss, Wetzlar, Germany).

Cellular toxicity of HA-liposomes

H522, H522-R, H2087, H2087-R, H1299, H1299-R, H1793, and H1793-R cells were seeded (5×10^3 cells/well) into 96-well plates and cultured for 24 h. These cells were treated with liposomes and HA-liposomes at concentrations of 50 μg/mL (1×) or 100 μg/mL (2×) for 48 h, and the proliferation of these cells was measured by the MTT assay, as described previously. Results were obtained from at least three independent experiments.

Treatment with siRNA-loaded HA-liposomes and cisplatin

NSCLC cells were seeded into 6- and 96-well plates at a density of 1×10^5 cells/cm^2 in growth medium and incubated for 24 h. Cells were treated with siRNA(CD44)-loaded liposomes or HA-liposomes, as described previously, and incubated at 37 °C for 48 h in a humidified atmosphere containing 5% CO_2. The cells were then treated with cisplatin (20 μg/mL) for 48 h. The apoptotic response to cisplatin was determined by western blotting and MTT assay. Results were obtained from at least three independent experiments.

Expression of pro-apoptotic proteins

NSCLC cells were seeded (5×10^5 cells/well) into cell culture dishes and cultured for 24 h. Following removal of the culture medium, the cells were treated with cisplatin (10 μg/mL) for 24 h and harvested. The expressions of cleaved-CASP3, BAX, BCL2, and GAPDH were investigated by western blotting as described previously.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad, Inc., La Jolla, CA, USA). The overall significance for parent and resistant groups was calculated by two-tailed Student's *t*-test. The correlation between the level of CD44 and IC_{50} was tested by calculating the Spearman's rank correlation coefficient, which is a nonparametric test correlation method. *P* values of less than 0.05 were considered statistically significant. For all experiments with error bars, the standard deviation was calculated to indicate the variation within each experiment and dataset, and values are represented as the mean ± standard deviation. This statistic was tested by rho test of spearman, which is a nonparametric test correlation method.

Results

Cisplatin sensitivity and resistance of CD44-expressing NSCLC cells

We first examined the relationship between CD44 expression and chemosensitivity in NSCLC using a panel of eight NSCLC cell lines. Chemosensitivity to the anticancer drug cisplatin was determined by the MTT assay, and CD44 expression was measured by FACS analysis. Cell lines expressing relatively high levels of CD44 (H1435, H1703, H1793, and H1299) showed significantly lower chemosensitivity to cisplatin compared to those of cell lines expressing low levels of CD44 (H460, H522, H2087, and H358) (Table 1). Therefore, the proliferation rate of NSCLC cells in response to cisplatin may depend on the CD44 expression level.

To better understand the molecular mechanism underlying CD44-dependent chemoresistance of NSCLC cells, we developed cisplatin-resistant cell lines by repeatedly exposing the cells to low doses of cisplatin. H1299 and H1793 were chosen from the CD44-overexpressing cell lines, while H522 and H2087 were chosen from the cell lines with low CD44 expression. Over a period of 6 months, cells were

Table 1 Inverse relationship between CD44 expression and chemosensitivity in parental non-small cell lung cancer (NSCLC) cells

Number	Cell line	CD44 Level (%)	Fluorescent intensity	IC_{50}	Inner cell line		Inter cell line	
					r (Spearman)	*P* value	*r* (Spearman)	*P* value
1	H1703	96.37	59.2 ± 1.57	9.89	−0.800	0.200	0.786 R^2 = 0.813	< 0.001
2	H1793	94.05	49.7 ± 1.14	16.58	0.600	0.400		
3	H1435	93.4	69.8 ± 4.99	9.89	0.000	> 0.999		
4	H1299	92.83	56.9 ± 4.36	13.56	0.200	0.800		
5	H460	17.18	6.3 ± 0.58	6.89	−0.800	0.200		
6	H358	5.55	4.0 ± 1.16	5.69	−0.400	0.600		
7	H2087	2.92	3.1 ± 0.72	6.88	0.999	–		
8	H522	0.61	0.6 ± 0.23	3.21	−0.400	0.600		

treated with increasing doses of cisplatin for 72 h and incubated in fresh medium for an additional 72 h to recover and re-populate (Fig. 1a). These chemoresistant cells showed significantly higher cell survival upon cisplatin treatment than their respective parental cells (Fig. 1b, c). We also examined the expression levels of MRP2, a major ATPase responsible for increased cisplatin efflux, and MDR1 in cisplatin-resistant NSCLC cells. Western blot analysis revealed that the expression levels of MDR1 and MRP2 increased significantly in cisplatin-resistant NSCLC cells compared to those of their parental cells (Fig. 1d). As shown in Fig. 1e, the cisplatin-resistant NSCLC cells expressed lower levels of the pro-apoptotic proteins cleaved-CASP3 and BAX, and higher levels of the anti-apoptotic protein BCL2 than did the parental cells.

CD44-dependent chemosensitivity of NSCLC cells

We assessed CD44 expression in cisplatin-resistant NSCLC cells and found that it was significantly enhanced compared to that in parental cells, regardless of the original expression levels of CD44 (Fig. 2a). These results demonstrated that the cisplatin resistance of NSCLC cells is induced by blocking the apoptotic cell death pathway and increasing cisplatin efflux, which may be related to CD44 expression. After observing the upregulation of CD44 expression in cisplatin-resistant NSCLC cells, we examined whether the disruption of CD44 expression affects the sensitivity of chemoresistant cells to cisplatin treatment. The cisplatin-resistant NSCLC cells were treated with CD44-specific siRNA, and the reduction of CD44 expression in these cells was confirmed by western blot analysis (Fig. 2b). In the cell survival study, the chemosensitivity of cisplatin-resistant NSCLC cells was improved effectively after knocking down CD44 expression with siRNA (Fig. 2c). We also found that siRNA treatment significantly increased the expression of cisplatin-related apoptotic proteins (BAX and cleaved-CASP3) in cisplatin-resistant NSCLC cells, and decreased BCL2 protein expression (Fig. 2d). In addition, CD44-specific siRNA treatment alone exerted slight cytotoxicity to cisplatin-resistant NSCLC cells, likely reflecting the biological roles of CD44 in cell survival and chemosensitivity. Collectively, these results suggest that the level of CD44 expression in NSCLC cells played an important role in regulating the cisplatin resistance of NSCLC cells.

CD44-targeted siRNA delivery using HA-liposomes

Previous experiments showed that the chemosensitivity of cisplatin-resistant NSCLC cells was significantly increased by downregulating CD44 expression. To achieve more selective and efficient knockdown of CD44 in cisplatin-resistant NSCLC cells, we took advantage of CD44 overexpression on the surface of chemoresistant cells for the targeted delivery of siRNA. We prepared liposomes coated with 5- or 700-kDa HA because HA binding to CD44 on the cell surface induces receptor-mediated endocytosis and binding may depend on the molecular weight of HA. The hydrodynamic sizes of 5- and 700-kDa HA-liposomes were approximately 144.2 and 156.4 nm, respectively (Supplementary Fig. S1A). Both displayed negatively charged surfaces, but the surface charge of the 700-kDa HA-coated liposome was greater than that of the 5-kDa liposomes. We then examined the binding affinity of HA-liposomes to CD44-expressing NSCLC cells using fluorescently labeled HA-liposomes. The 700-kDa HA-liposomes showed notably higher targeting towards CD44-overexpressing H1299 and H1793 cells than did the 5-kDa HA-liposomes (Supplementary Fig. S1B). HA-liposome binding to H522 and H2087 cells, which expressed low levels of CD44, was negligible. We also found that cellular binding of HA-liposomes was higher in cisplatin-resistant NSCLC cells compared to that in parental cells, regardless of the cell type (Fig. 3a). This enhanced targeting efficiency in cisplatin-resistant NSCLC cells was well correlated with the increased CD44 expression (Figs. 2a, 3a), confirming the CD44-dependent targeting of HA-liposomes. In addition, control liposomes did not bind to any cells regardless of the CD44 expression level.

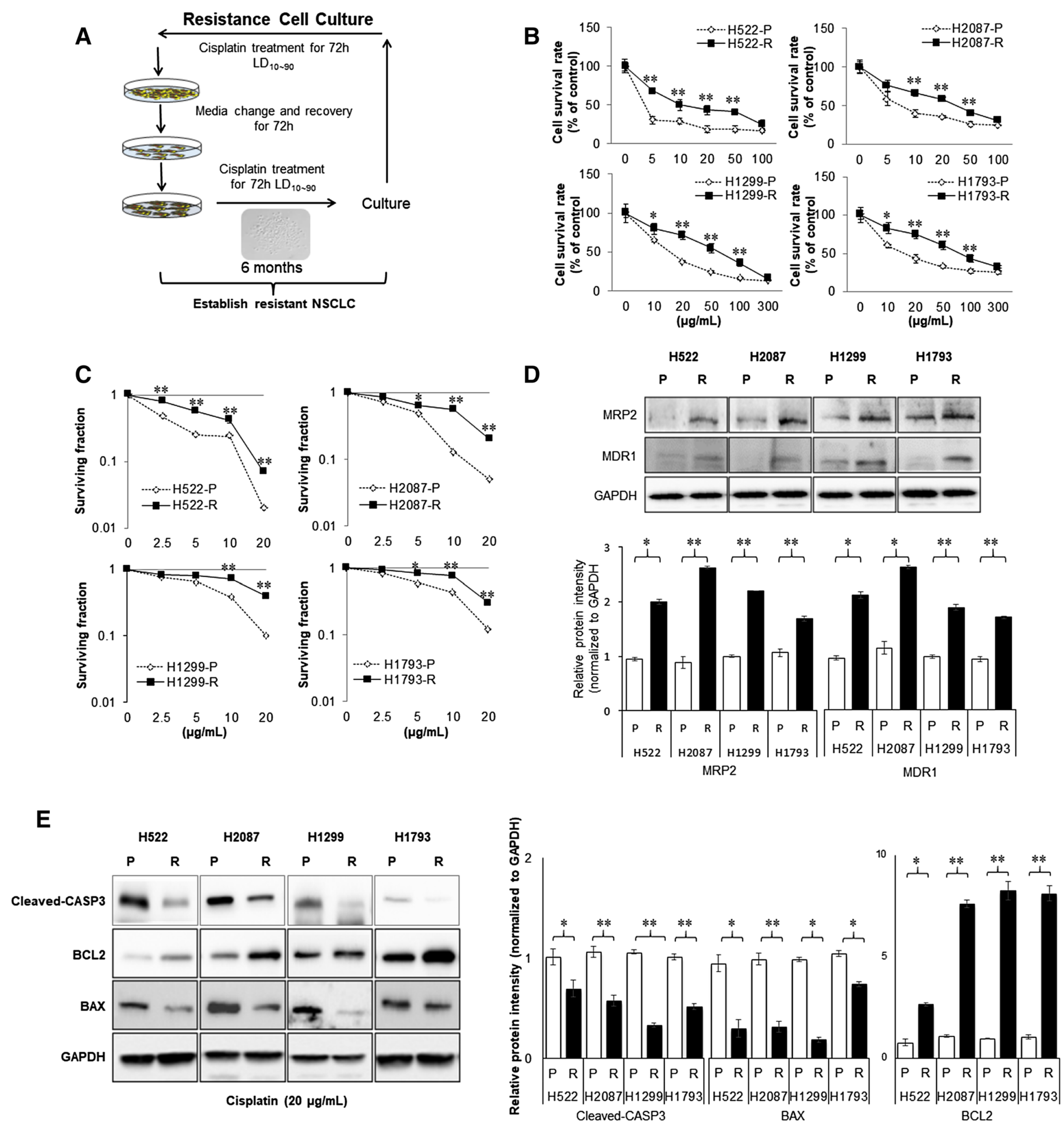

Fig. 1 Establishment and characterization of cisplatin-resistant NSCLC cells. **a** Procedure for establishment of cisplatin-resistant NSCLC cells. Cell lines with high (H1299 and H1793) and low (H522 and H2087) CD44 expression were treated with increasing doses of cisplatin. The cisplatin-containing medium was removed after 72 h, and cells were allowed to recover and re-populate. This cycle was repeated for at least 6 months. **b** The chemosensitivity of cisplatin-resistant cells significantly decreased compared to that of parental NSCLC cells. **c** The clonogenic survival ability of cisplatin-resistant cells increased in response to increasing doses of cisplatin compared to that in parental cells. **d** Significant upregulation of MRP2 and MDR1 proteins was observed in cisplatin-resistant NSCLC cells. The GAPDH was used as a loading control. **e** Significant downregulation of the pro-apoptotic proteins (cleaved-CASP3 and BAX) and upregulation of BCL2 were observed in cisplatin-resistant NSCLC cells. GAPDH was used as a loading control. The density of the specific bands was quantified using the ImageJ software. *t* tests showed statistically significant differences. $*P<0.05$ and $**P<0.01$

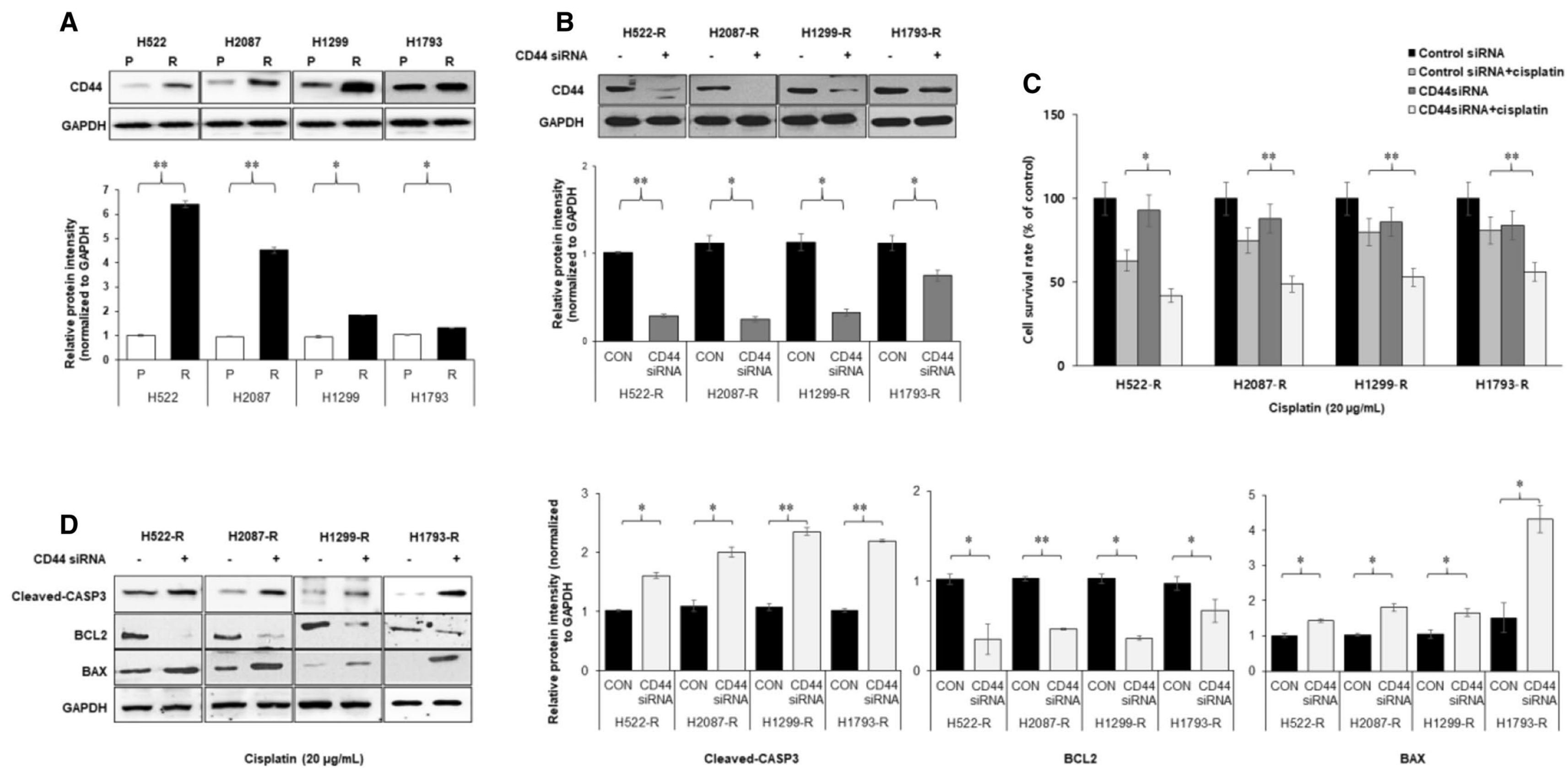

Fig. 2 CD44-dependent chemoresistance of NSCLC cells. **a** Comparative western blot analysis of CD44 between cisplatin-resistant NSCLC cells and these parental cells. The CD44 expression was significantly increased in cisplatin-resistant NSCLC cells. **b** The CD44 expression in cisplatin-resistant NSCLC cells was reduced after CD44-specific siRNA treatment. **c** The chemosensitivity of cisplatin-resistant cells significantly improved after siRNA treatment compared to that before siRNA treatment. **d** The CD44 siRNA treatment significantly increased the expression of cisplatin-related apoptotic proteins (BAX and cleaved-CASP3) in cisplatin-resistant NSCLC cells, and decreased BCL2 protein expression. GAPDH was used as a loading control. The density of the specific bands was quantified using the ImageJ software. *t* tests showed statistically significant differences. *$P<0.05$ and **$P<0.01$

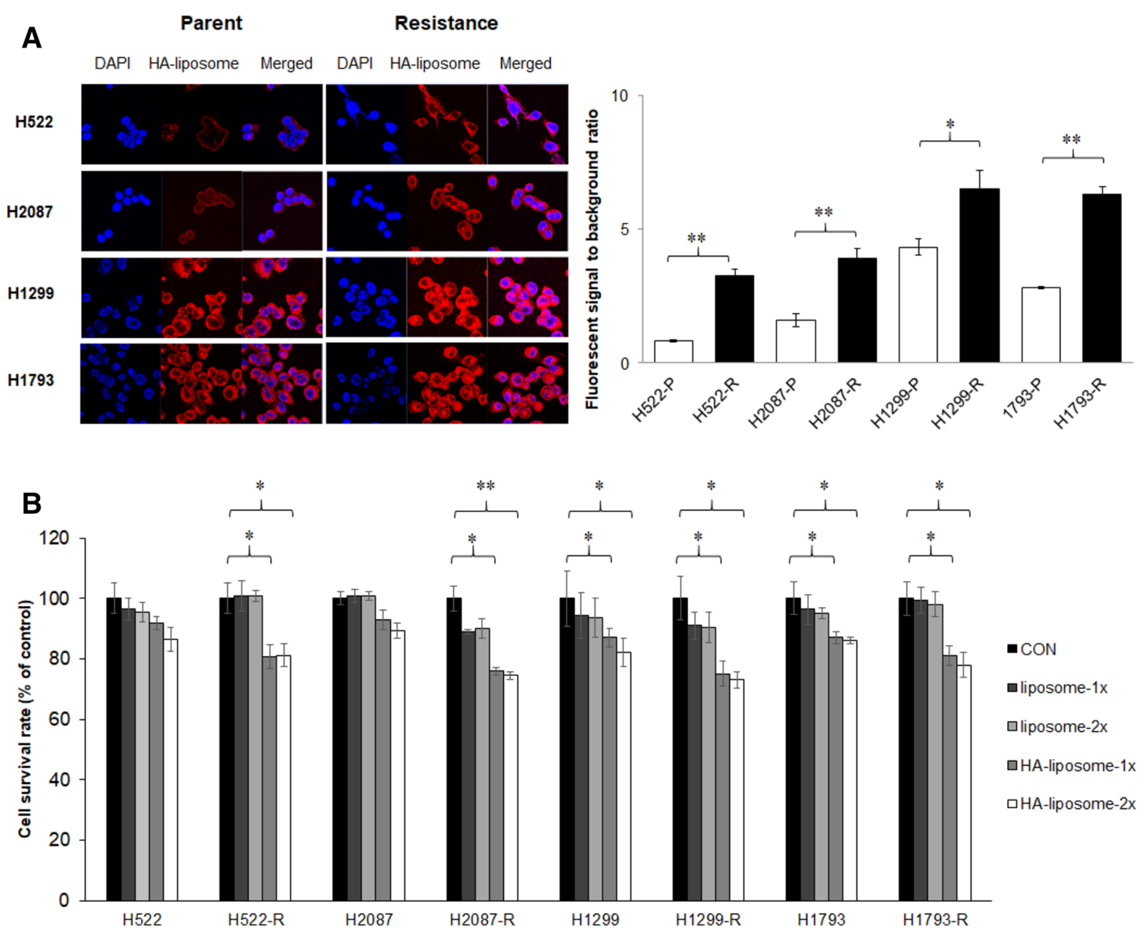

Fig. 3 CD44-dependent targeting and therapeutic effects of siRNA-loaded HA-liposomes to chemoresistant NSCLC cells. **a** Confocal microscopic images and fluorescence quantification of NSCLC cells treated with HA-liposomes. Cells were treated with fluorescently labeled HA-liposomes (red) and imaged by confocal microscopy. The nuclei were stained with DAPI (blue). **b** The MTT assay for cisplatin-resistant NSCLC cells treated with liposomes and HA-liposomes at concentrations of 50 µg/mL (1×) or 100 µg/mL (2×) for 48 h. The HA-liposome itself has cytotoxic effects towards cisplatin-resistant NSCLC cells which overexpress CD44. *t* tests showed statistically significant differences. *$P < 0.05$ and **$P < 0.01$

Before conducting the siRNA encapsulation, we evaluated whether the HA-liposome itself is cytotoxic towards cisplatin-resistant NSCLC cells. We analyzed the anti-proliferation effects of liposomes or HA-liposomes at different concentrations and showed that the cytotoxicity in resistant NSCLC cells treated with HA-liposomes increased significantly compared to that in cells treated with the same concentration of liposomes (Fig. 3b). This therapeutic benefit of CD44-HA binding can be combined with chemotherapy to further improve overall cancer treatment.

Additionally, for the loading of siRNA into HA-liposomes, we prepared a nanocomplex of siRNA and protamine with an average size of 100 nm by mixing them at a 1:1 molecular ratio. This size was appropriate for subsequent liposomal encapsulation (Supplementary Fig. S1C). The lyophilized HA-liposomes were rehydrated with an aqueous solution containing the siRNA–protamine complex and sucrose. The hydrodynamic size of HA-liposomes encapsulated with siRNA was measured to be approximately 164 nm. Collectively, these results suggest that the cell surface protein CD44 can be used to improve the targeted liposomal delivery of siRNA to cisplatin-resistant NSCLC cells (Supplementary Fig. S1D).

Last, we tested whether the self-targeted downregulation of CD44 by HA-liposomes enhances cisplatin sensitivity in cisplatin-resistant NSCLC cells. We confirmed that the CD44-targeted delivery of siRNA using HA-liposomes significantly knocked down CD44 expression in cisplatin-resistant NSCLC cells compared to the case of non-specific siRNA delivery using untargeted liposomes (Fig. 4a). Additionally, in the cisplatin cytotoxicity experiment, the chemosensitivity of cisplatin-resistant NSCLC cells was dramatically enhanced following the CD44-targeted delivery of siRNA using HA-liposomes (Fig. 4b). As expected, the expression of cleaved-CASP3 and BAX significantly increased, while that of BCL2 decreased in

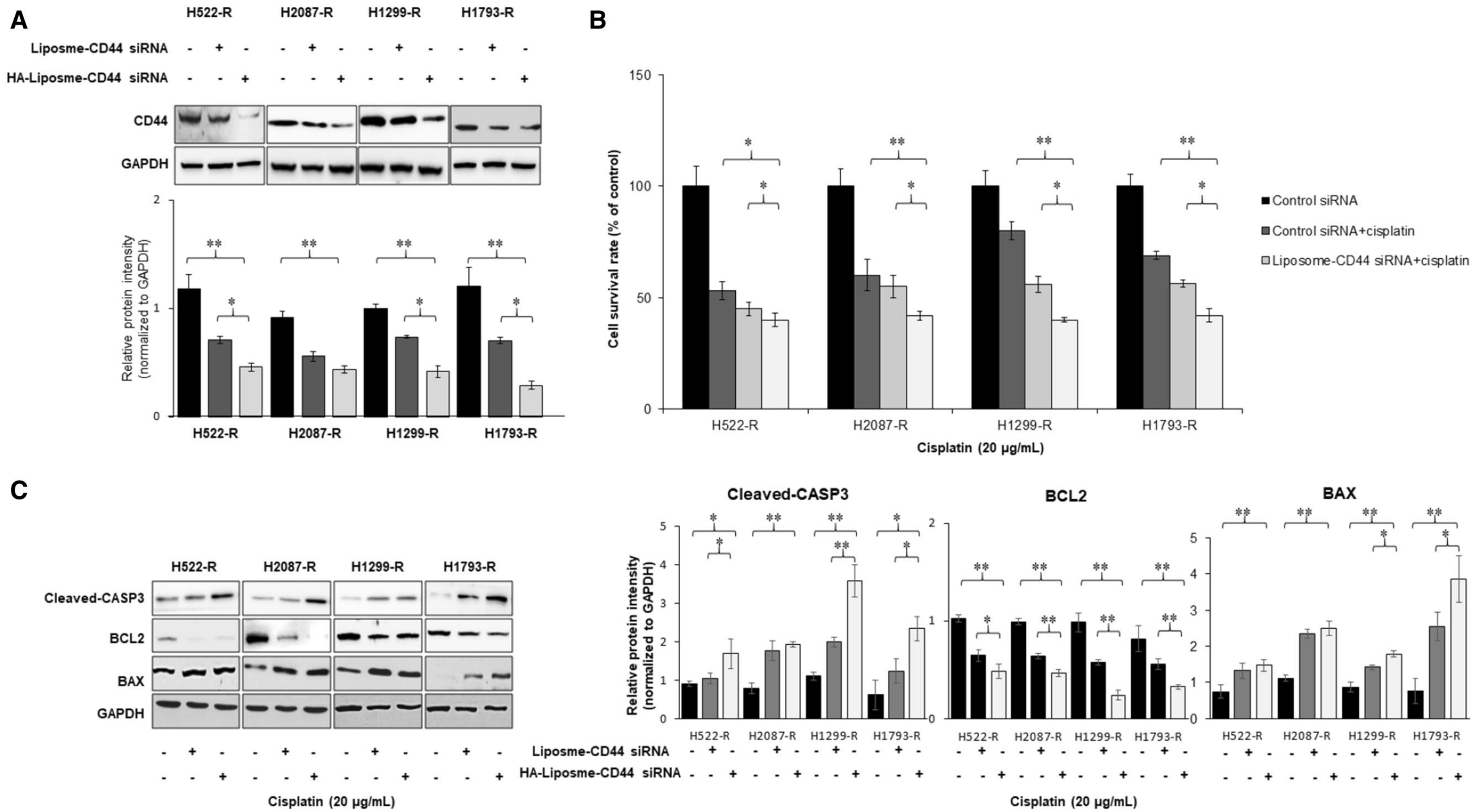

Fig. 4 Self-targeted CD44 knockdown for the effective treatment of chemoresistant NSCLC. **a** The CD44-targeted delivery of siRNA using HA-liposomes significantly knocked down CD44 expression in cisplatin-resistant NSCLC cells compared to untargeted liposomes. **b** The chemosensitivity of cisplatin-resistant cells significantly decreased after HA-mediated liposomal siRNA delivery. **c** Significant downregulation of BCL2 and significant upregulation of cleaved-CASP3 and BAX were observed in cisplatin-resistant NSCLC cells after HA-mediated liposomal siRNA delivery. GAPDH was used as a loading control. The density of the specific bands was quantified using the ImageJ software. *t* tests showed statistically significant differences. *$P<0.05$ and **$P<0.01$

HA-liposome-treated-resistant NSCLC cells (Fig. 4c). Collectively, these results suggest that the chemoresistance of NSCLC cells can be overcome by the self-targeted downregulation of chemoresistance-associated cell surface proteins prior to chemotherapy (Fig. 5).

Discussion

During the clinical treatment of NSCLC, patients typically show good initial response to cisplatin-based chemotherapy, but cancer recurrence may occur because of the development of chemoresistance. Chemoresistance frequently develops during NSCLC treatment [8, 20, 26], markedly reducing the drug's clinical effectiveness. Therefore, new strategies are needed to address this significant problem associated with cancer treatment and to improve the survival rate of patients with NSCLC [17, 20, 21].

CD44 is a putative CSC marker and may play an important role in the development of chemoresistance [27, 28]. Although the role of CD44 in promoting tumor metastasis and invasion aligns with its natural role in motility, activation, adhesion, and localization, the mechanism underlying the involvement of CD44 in drug resistance is not yet completely understood. In the present study, we examined the chemosensitivity and chemoresistance of NSCLC and their relationship with CD44 expression. Based on the FACS and MTT analysis (Table 1), CD44 expression was inversely related to chemosensitivity in NSCLC cells. Cells expressing low levels of CD44 were more sensitive to cisplatin treatment than were cells that overexpressed CD44. Our findings are consistent with those of a previous study, which reported that CD44-negative-sorted cells were more sensitive to cisplatin chemotherapy than were CD44-positive cells [26]. Next, to determine whether CD44 expression contributes to chemoresistance, we induced cisplatin resistance in NSCLC cells that originally expressed low or high levels of CD44 (Fig. 1). The cisplatin-resistant NSCLC cells developed in our study showed an increased expression of MRP2 compared to their parental cells, which is consistent with the results of previous studies [29]. Our results also showed increased CD44 expression in cisplatin-resistant NSCLC cells compared to that in parental cells (Fig. 2a, b), which is consistent with the results of a study by Bjorklund et al. [30]. Furthermore, the western blot analysis of intracellular proteins showed that CD44 expression is closely linked with apoptosis signaling (Figs. 2e, 4c). Finally, the specific knockdown of CD44 significantly enhanced the sensitivity of cisplatin-resistant NSCLC cells to cisplatin treatment (Fig. 2). Collectively, our observations suggest that chemoresistance in NSCLC cells

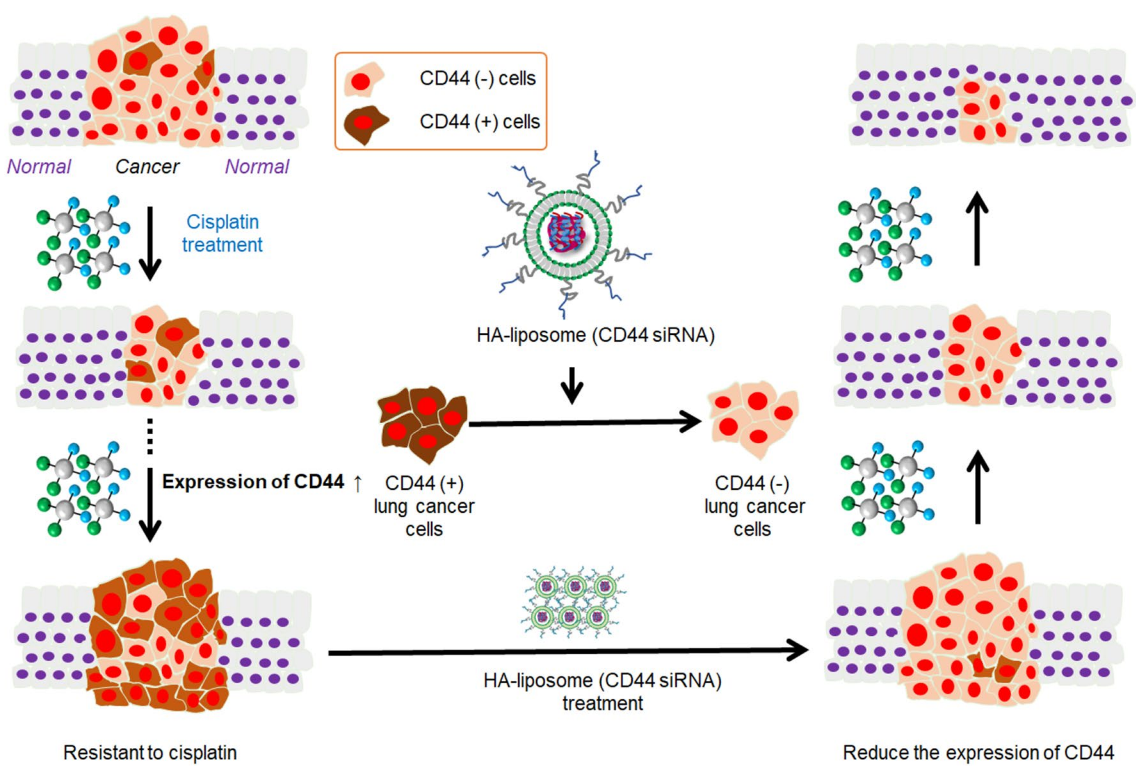

Fig. 5 Proposed mechanism of synergistic therapeutic effects of self-targeted CD44 downregulation for treatment of chemoresistant NSCLC. The self-targeted downregulation of chemoresistance-associated cell surface proteins CD44 during chemotherapy is an effective therapeutic strategy for overcoming the chemoresistance of NSCLC cells

is associated with increased expression of the cell surface receptor CD44.

To further enhance the chemosensitivity of cisplatin-resistant NSCLC cells, we used CD44 molecules overexpressed on the cell surface as molecular receptors for targeted siRNA delivery. To achieve self-targeted CD44 knockdown, we employed HA-liposomes to deliver CD44-specific siRNA as HA interacts selectively with the cell surface receptor CD44 and liposomal formulations secure siRNA in their cores during intracellular delivery. These HA-liposomes bound selectively to cisplatin-resistant NSCLC cells expressing high levels of CD44 (Fig. 3). Additionally, HA-liposomes binding to CD44 on the surface of cisplatin-resistant NSCLC cells alone induced cytotoxicity, which was not observed with untargeted liposomes. Furthermore, we found that CD44-targeted liposomal delivery of siRNA resulted in markedly improved CD44 knockdown and chemosensitivity in cisplatin-resistant NSCLC cells compared to that observed following untargeted liposomal delivery of siRNA (Fig. 4). Thus, these results suggest that chemoresistance-targeted siRNA delivery prior to chemotherapy can significantly improve chemotherapeutic outcomes in the treatment of chemoresistant NSCLC.

Conclusions

We demonstrated that the self-targeted downregulation of CD44 can significantly enhance the cisplatin sensitivity of chemoresistant NSCLC cells. We successfully developed HA-liposomes that deliver CD44-specific siRNA selectively to CD44-overexpressing chemoresistant NSCLC cells to effectively downregulate CD44 in an in vitro model. We found that the HA-mediated liposomal delivery of siRNA to cisplatin-resistant NSCLC cells significantly reduced CD44 expression and thus improved their chemosensitivity. Our results provide an effective strategy for treating chemoresistant cancer cells whereby cell surface protein overexpression in chemoresistant cells is exploited to achieve self-targeted knockdown prior to chemotherapy.

Author contributions Conception and design: YHQ, J-HP, and HKK. Development of methodology: YHQ. Acquisition of data (provided materials, provided facilities, etc.): YHn, JL, and YHC. Analysis and interpretation of data (e.g. statistical analysis, biostatistics, computational analysis): YHQ. Writing, review, and/or revision of the manuscript: YHQ, J-HP, and HKK. Administrative, technical, or material support (i.e. reporting or organizing data, constructing databases): JL, YC, and YHC. Study supervision: J-HP and HKK.

Funding This work was supported by a Korea University Grant and the National Research Foundation of Korea (NRF) grant funded by the Ministry of Education, Science, and Technology (NRF-2017R1E1A1A01074847) and Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare (Grant no. HI17C0654).

Data Availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest All authors read and approved the final version of the manuscript, and the authors declare no conflicts of interest.

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