

Doxorubicin induces drug resistance and expression of the novel CD44st via NF- κ B in human breast cancer MCF-7 cells

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Abstract. CD44, a major receptor for hyaluronan (HA), is a member of a class of adhesion molecules of unknown classification involved in cell proliferation, differentiation, migration, angiogenesis, and the presentation of specific cytokines to the corresponding receptors as well as in cell signaling transduction. It has recently been discovered that CD44, a marker of tumor stem cells, is involved in the drug resistance and invasion of multiple types of tumors. The 20 exons in the CD44 gene that are alternatively spliced, give rise to many CD44 isoforms, possibly including tumor-specific sequences. Dozens of CD44 isoforms have been found, to date, and the standard CD44 (CD44s) isoform is the most common. We recently showed that a novel short-tail isoform of CD44 (CD44st) was expressed in multidrug-resistant human breast cancer MCF-7/Adr cells. Moreover, the novel CD44st was able to interact with HA and regulate the expression of matrix metalloproteinase (MMP)-2 and MMP-9, which increased the invasive capability of MCF-7 cells through the Ras/MAPK signaling pathway. In the present study, we verified that MCF-7 cells subjected to drug pressure develop multidrug resistance to doxorubicin, and the expression levels of multidrug resistance protein 1 (*MDR1*), *CD44st* and nuclear factor- κ B (NF- κ B) mRNA and protein were gradually upregulated in a dose-dependent manner in MCF-7 cells treated with doxorubicin. HA increases the secretion of MMP-2 and MMP-9 in multidrug-resistant MCF-7 cells and affected the invasive ability of MCF-7 cells through the upregulation of CD44st expression, and such an effect was blocked by the NF- κ B-specific inhibitor BMS-345541.

Introduction

Tumor invasion and metastasis are the leading causes of death from cancer. For a tumor cell to metastasize and form distal metastases, it must initially penetrate the extracellular matrix (ECM) and invade the vascular system (1).

The CD44 gene, which is located on the short arm of human chromosome 11, spans ~50 kb of genomic DNA. Theoretically, over 800 CD44 variants can be expressed in tissues and cells. However, not all CD44 isoforms can be expressed virtually. Dozens of CD44 isoforms have been identified to date, and the standard CD44 (CD44s) isoform is the most common; in CD44s, exon 5 is directly connected to exon 16, and lacks the entire variant exon region. In our previous study, the multidrug-resistant human breast cancer MCF-7/Adr cells were used to clone the short-tail isoform of CD44 (CD44st), a novel short-tail isoform of CD44, which contains exons 1-4 and 16-17, and 1-205 base pairs of exon 18. The CD44 family members and their structures have been described previously (2).

Nuclear factor (NF)- κ B, a member of the Rel protein family, is composed of a group of homodimers or heterodimers of transcription factors. NF- κ B is found to be involved in tumor occurrence, invasion and metastasis, and it is closely associated with multidrug resistance (MDR) in tumors. Abnormal NF- κ B expression and activity are found to affect the regulation of multidrug resistance protein 1 (*MDR1*) gene expression in multidrug-resistant tumor cells. Exon 1 at the promoter region of the *MDR1* gene contains an NF- κ B-binding sequence (5'-CCTTTCGGGG-3'), and NF- κ B can activate the transcription of the reporter gene connected to the *MDR1* promoter. It is therefore speculated that the transcription of the *MDR1* gene, which may be a downstream gene of NF- κ B, is activated by NF- κ B and then transcribes into P-glycoprotein (P-gp) resulting in tumor cell multidrug resistance.

NF- κ B is reported to be involved in regulating the expression of *MDR1* and *CD44* genes in multiple cancer cells (3-7), and it has been found that the CD44-hyaluronan (HA) interaction induces ankyrin binding to P-gp, resulting in the efflux of chemotherapeutic drugs and development of MDR in cancer (8,9).

It has been found that both *CD44st* mRNA and CD44st protein are highly expressed in multidrug-resistant MCF-7/Adr, Lovo/Adr, K562/Adr and HL-60/Adr cell lines,

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while the sensitive MCF-7, Lovo, K562 and HL-60 cells do not contain either *CD44st* mRNA or CD44st protein. In addition, matrix metalloproteinase (MMP)-2 and MMP-9 expression can be upregulated by HA treatment, which can be blocked by pretreatment with CD44 neutralizing antibody. Such findings demonstrate that the HA-CD44st interaction activates the MAPK signaling pathway, thereby increasing MMP-2 and MMP-9 secretion (2,10,11). To investigate the role of the transcription factor NF- κ B in doxorubicin-induced drug resistance and the association with CD44st expression, we used low-dose doxorubicin to induce MDR in human breast cancer MCF-7 cells and determined the expression of *MDR1*, *CD44st* and *NF- κ B* mRNA in these multidrug-resistant MCF-7 cells. In addition, the effects of an NF- κ B inhibitor on MMP-2 and MMP-9 expression and the invasive ability of MCF-7 cells mediated through the HA-CD44st signaling pathway were evaluated.

Materials and methods

Cell lines and cell culture. The human breast cancer MCF-7 cell line and the doxorubicin-resistant human breast cancer MCF-7/Adr cell line were purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). MCF-7 cells were cultured as described previously (2). Cells were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China) at 37°C in a humidified atmosphere containing 5% CO₂, while MCF-7/Adr cells were maintained in a medium containing 0.2 μ g/ml doxorubicin, with drug withdrawal occurring 1 week prior to the experiment.

Detection of the 50% inhibitory concentration (IC₅₀) of doxorubicin against MCF-7 cells by MTT assays. Log phase MCF-7 cells were seeded onto 96-well cell culture plates at a density of 2x10⁴ cells/well, and doxorubicin treatment was administered after the cells were adherent to the plate wall. Doxorubicin was formulated into concentrations of 0, 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 μ g/ml, and 4 replicate wells were used for each concentration, with a final volume of 200 μ l. Following incubation at 37°C in a humidified atmosphere containing 5% CO₂, 20 μ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml) was added to each well, and the cells were cultured for another 4 h. The supernatant was then removed, 100 μ l of dimethyl sulfoxide (DMSO) was added to each well, and the plates were then shaken on a horizontal shaker for 15 min to allow for complete dissolution. The optical density (OD) was read at 492 nm on an enzyme-linked immunosorbent assay (ELISA) microplate reader. The inhibition of cell growth (IR) was calculated using the following formula: Inhibitory rate (%) = (1 - mean OD value in the experimental group/mean OD value in the control group) x 100% (Table I). The IC₅₀ value was estimated from the OD value and was applied to evaluate the cytostatic efficacy of doxorubicin on MCF-7 cells. Each experiment was repeated in triplicate.

Induction of doxorubicin resistance in MCF-7 cells. MCF-7 cells were seeded into 96-well cell culture plates at a density of 5x10³ cells/well, and after the cells were adherent to the plate

Table I. The 50% inhibitory concentration (IC₅₀) of doxorubicin against MCF-7 cells (mean \pm SD, n=3).

Concentration of DOX (μ g/ml)	OD value	IR (%)
0	1.25 \pm 0.02	
0.05	1.10 \pm 0.03	12.0
0.1	0.95 \pm 0.02	24.0
0.2	0.82 \pm 0.03	34.4
0.4	0.71 \pm 0.01	43.2
0.8	0.60 \pm 0.01	52.0
1.6	0.48 \pm 0.02	61.2

DOX, doxorubicin; OD, optical density; IR, inhibitory rate.

wall, doxorubicin was added to each well at concentrations of 0, 0.01, 0.03, 0.06 and 0.12 μ g/ml. Four replicate wells were used for each concentration, with a final volume of 200 μ l, and the plates were placed at 37°C in a humidified atmosphere containing 5% CO₂. The medium was not replaced, and the OD value was read at 492 nm on an ELISA microplate reader 7 days following the doxorubicin treatment. The inhibitory rate of MCF-7 cells following doxorubicin treatment for 7 days was estimated according to the OD value, and cell morphology was observed to aid in the determination of the appropriate doxorubicin concentration for the induction of drug resistance. Each experiment was repeated in triplicate.

MCF-7 cells were seeded onto cell culture flasks at a density of 1x10⁶ cells/well, and drug resistance was induced starting at a doxorubicin concentration of 0.01 μ g/ml after the cells were adherent to the flask wall. Following incubation for 24 h, the supernatant was removed and the medium was replaced with complete RPMI-1640 medium until cell recovery. Following trypsin digestion, cells were harvested and seeded onto cell culture flasks at a density of 1x10⁶ cells/well. Following incubation with doxorubicin at a final concentration of 0.02 μ g/ml for 24 h, the supernatant was removed and the medium was replaced with fresh complete RPMI-1640 medium for the further induction of drug resistance. The doxorubicin concentration was gradually increased to 0.06 μ g/ml. The MCF-7 cells treated with doxorubicin at final concentrations of 0.01, 0.03 and 0.06 μ g/ml were harvested for the subsequent experiments.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), sequencing and quantitative real-time PCR (qRT-PCR). The MCF-7 cells treated with doxorubicin at concentrations of 0.01, 0.03 and 0.06 μ g/ml were digested, centrifuged and harvested. The total RNA was extracted from the cells using the reverse-transcription PCR reagent (Fermentas, Glen Burnie, MD, USA) according to the manufacturer's instructions and reverse-transcribed into cDNA. The primer sequences containing *Eco*RI and *Kpn*I cleavage sites were designed using the software Primer version 5.0 (Premier Corporation, Canada).

The primers for *CD44st-1* (GenBank accession no: FJ216964; amplification fragment size, 1023 bp, used for

RT-PCR and gene sequencing) were as follows: forward primer, 5'-GGGAATTCATGGACAAGTTTGGTGGCACG-3' and reverse primer, 5'-GGGGTACCTTACACCCAATCTTCATGTCC-3'. PCR amplification for *CD44st-1* was performed under the following conditions: incubation at 94°C for 5 min; 30 cycles at 94°C for 30 sec, at 65°C for 30 sec and at 72°C for 1 min; and extension at 72°C for 10 min. The *MDR1* gene primers (amplification fragment size, 173 bp) were as follows: forward primer, 5'-GTTGCCATTGACTGAAAGAAC-3' and reverse primer, 5'-ACAGGAGATAGGCTGGTTTGA-3'. PCR amplification for *MDR1* was performed under the following conditions: incubation at 94°C for 5 min; 30 cycles at 94°C for 30 sec, at 56°C for 30 sec and at 72°C for 1 min; and extension at 72°C for 10 min. The *NF-κB* gene primers (amplification fragment size, 340 bp) were as follows: forward primer, 5'-AGCACAGATACCACCAAGACCC-3' and reverse primer, 5'-CCCACGCTGCTCTTCTATAGGAAC-3'. PCR amplification for *NF-κB* was performed under the following conditions: incubation at 94°C for 5 min; 30 cycles at 94°C for 30 sec, at 61°C for 30 sec and at 72°C for 1 min; and extension at 72°C for 10 min. The *MMP-2* gene primers (amplification fragment size, 162 bp) were as follows: forward primer, 5'-CGGTGCCAAGAATAGATG-3' and reverse primer, 5'-AAAGGAAGAGAGCTGAAGTG-3'. PCR amplification for *MMP-2* was performed under the following conditions: incubation at 94°C for 5 min, 30 cycles at 94°C for 30 sec and at 59°C for 30 sec, incubation at 72°C for 30 sec, and extension at 72°C for 10 min. The *MMP-9* gene primers (amplification fragment size, 682 bp) were as follows: forward primer, 5'-CGGAGCACGGGATAT-3' and reverse primer, 5'-GCCGCCACGAGGAAACT-3'. PCR amplification for *MMP-9* was performed under the following conditions: incubation at 94°C for 5 min; 30 cycles at 94°C for 30 sec, at 62°C for 30 sec and at 72°C for 45 sec; and extension at 72°C for 10 min. The β -actin gene primers (amplification fragment size, 330 bp), which were used as a control, were as follows: forward primer, 5'-CTCGCGCTACTCTCTCTTTC-3' and reverse primer, 5'-CATGTCTCGATCCCACTTAAC-3'. PCR amplification was performed under the following conditions: incubation at 94°C for 5 min, followed by 30 cycles at 94°C for 30 sec, at 58°C for 30 sec, at 72°C for 30 sec and at 72°C for 10 min. The PCR amplification product of *CD44st* was extracted and purified using the gel extraction and PCR purification kit. Following T/A cloning, the purified product was transformed into *Escherichia coli*, and the plasmid DNA was extracted and validated using double digestion with *EcoRI* and *KpnI* (New England Biolabs, Inc., Ipswich, MA, USA). The positive plasmid was sequenced by Sangon Biotech Co., Ltd. (Shanghai, China).

The *CD44st-2* gene primers used for qRT-PCR amplification (amplification fragment size, 682 bp) were as follows: forward primer, 5'-CCCTGCTACCAGACACTCA-3' and reverse primer, 5'-TGTTACCAAATGCACCAT-3'. The sequences of the *MDR1*, *NF-κB* and β -actin gene primers were the same as described above. qRT-PCR amplification was performed with a total volume of 50 μ l containing 32 μ l of ddH₂O, 5 μ l of 10X buffer, 1 μ l of dNTP, 4 μ l of MgCl₂ (25 mmol/l), 2 μ l of the forward and reverse primers (10 pmol/l), 2 μ l of cDNA template and 2 μ l of *Taq* DNA polymerase (Takara Bio, Co., Ltd., Dalian, China) under the

following conditions: incubation at 94°C for 5 min; 40 cycles at 94°C for 30 sec, at 60°C for 1 min and at 72°C for 45 sec; and incubation at 72°C for 10 min. The relative level of mRNA expression was normalized to β -actin, and the difference in mRNA expression was estimated using the $2^{-\Delta\Delta C_t}$ method.

Western blot analysis. CD44 and NF- κ B protein expression was determined using western blot analysis. Briefly, 1x10⁶ MCF-7 cells treated with doxorubicin at various concentrations were transferred to Eppendorf tubes, and 100 μ l of boiling 2X loading buffer was added to each tube. The cell lysates were collected and boiled at 100°C for 5 min for protein denaturation. The NF- κ B protein was extracted using the Nuclear/Cytosol Fractionation kit following the manufacturer's instructions, electrophoresed, transferred to membranes, incubated with antibodies, and stained with 3,3'-diaminobenzidine (DAB; Invitrogen).

P-gp expression as detected by flow cytometry. An estimated 5x10⁵ MCF-7 cells treated with doxorubicin at various concentrations were harvested and marked. After being washed three times with phosphate-buffered saline (PBS), the harvested cells were blown uniformly in PBS buffer to prepare a single-cell suspension. A volume of 20 μ l of the cell suspension was collected, and 1 μ l of FITC-conjugated mouse anti-human CD44 monoclonal antibody was added (eBioscience, San Diego, CA, USA). Additionally, 20 μ l of PE-labeled P-gp monoclonal antibody (BD, San Jose, CA, USA) was transferred to the cell suspension for the determination of P-gp expression. The cells were incubated in darkness at room temperature for 30 min and centrifuged at 1,000 rpm for 5 min. The supernatant was then discarded, and the cells were washed twice with PBS solution and re-suspended with 500 μ l of PBS solution. The relative fluorescence intensity was detected with a flow cytometer (BD), and data analyses were performed using the CellQuest software. The experiment was repeated in triplicate.

P-gp activity as detected by Rhodamine-123 (Rho-123) retention assay. An estimated 2x10⁵ MCF-7 cells treated with doxorubicin at various concentrations were harvested, washed once with aseptic PBS solution at 37°C, and added to fresh medium. Fluorescent dye Rho-123 was added to each flask to achieve a final concentration of 1 μ g/ml, and the flasks were incubated for another 2 h. Cells were harvested using the conventional method and washed twice with PBS. After removal of the extracellular Rho-123, 5 μ g (50 μ l) of propidium iodide (PI) was added to the cells. The solution was incubated in darkness at room temperature for 30 min and centrifuged at 1,000 rpm for 5 min. The supernatant was then discarded, and the cells were washed twice with PBS solution and blown gently with PBS solution. The P-gp activity was detected with a flow cytometer (BD), and data analyses were performed using the CellQuest software. The experiment was repeated in triplicate.

Electrophoretic mobility shift assay (EMSA). An estimated 6x10⁶ MCF-7 cells treated with doxorubicin at various concentrations were harvested, and 300 μ l of plasmosin extraction solution buffer A was added. Then, the solution was still on ice for 15 min and centrifuged at 2,000 rpm for 10 min at 4°C. The

supernatant was discarded, and the sediment was washed with 100 μ l of buffer A and then centrifuged at 5,000 rpm for 10 min at 4°C. After the supernatant was discarded, the sediment was mixed evenly with 100 μ l of buffer B, still on ice for 10 min, mixed evenly, and still on ice for 10 min again. Following centrifugation at 15,000 rpm for 20 min at 4°C, the supernatant was collected, which served as the nuclear protein extraction solution. The nuclear protein concentration was determined using the Bradford method, and 2 μ l of the nuclear protein was stored at -70°C. The sequence of the NF- κ B DNA probe was as follows: 5'-AGTTGAGGGGACTTTCCAGGC-3' and 5'-GCCTGGGAAAGTCCCCTACT-3'. Probe labeling, the binding reaction, electrophoresis and membrane transferring were performed according to the manufacturer's instructions (Promega, Madison, WI, USA), and the DNA was labeled with chemiluminescent biotin. The membrane was placed in the film cassette and visualized using X-rays. The exposure time was adjusted to obtain the appropriate result.

Gelatin zymography. Cells were divided into five groups. MCF-7 cells in group 1 did not receive any treatment; MCF-7 cells in group 2 were treated with doxorubicin at a concentration of 0.06 μ g/ml; MCF-7 cells in group 3 were treated with 0.06 μ g/ml doxorubicin and HA following pretreatment with BMS-345541 (Merck, Whitehouse Station, NJ, USA); MCF-7 cells in group 4 were treated with 0.06 μ g/ml doxorubicin and HA; and MCF-7/Adr cells in group 5 were treated with HA. An estimated 1.5×10^6 cells in each group were seeded into cell culture flasks, and 1.5 ml of serum-free RPMI-1640 medium was transferred to the flasks. HA (Sigma-Aldrich Corporation, St. Louis, MO, USA) was boiled at 100°C for 5 min prior to use to eliminate the potential contamination by other growth factors. MCF-7 cells in the BMS-345541 pretreatment group were pretreated with BMS-345541 at a concentration of 5 μ mol/l for 3 h, while cells in groups 3, 4 and 5 were treated with HA at a concentration of 100 μ g/ml, and the cells following incubation in HA for 24 h were harvested for RT-PCR amplification and the Transwell invasion assay. Following centrifugation, the supernatant of the cell culture was used for gelatin zymography following the manufacturer's protocol (Applygen Technologies Inc., Beijing, China). A 1:1 ratio of whole human blood and 2X non-denaturing SDS-PAGE loading buffers (both 100 μ l) served as the positive control.

Invasive ability of MCF-7 cells as detected by the Transwell invasion assay. Matrigel (BD) was diluted with serum-free RPMI-1640 medium at a ratio of 1:1 and then transferred to a Transwell (Corning Inc., Corning, NY, USA) at a low temperature at a concentration of 150 μ l/chamber. The Transwell was then placed at 37°C for 1 h. After the Matrigel polymerized, 1 ml of serum-free RPMI-1640 medium containing 0.1% FBS was transferred to the lower compartment of the Transwell for a 1-h incubation period. Cells in each of the aforementioned 5 groups were prepared as cell suspensions at a concentration of 1×10^5 cells/ml, and 200 μ l of the cell suspension was transferred to the upper compartment of the Transwell and incubated for 24 h. The Matrigel on the surface of the polycarbonate membrane was lightly wiped with cotton buds, and the upper and lower sides of the polycarbonate membrane were gently flushed with PBS solution. The polycarbonate

Table II. Inhibitory effect of doxorubicin on the growth of MCF-7 cells (mean \pm SD, n=3).

Concentration of DOX (μ g/ml)	OD value	IR (%)
0	1.50 \pm 0.02	-
0.01	1.44 \pm 0.03 ^a	4.0
0.03	1.37 \pm 0.02 ^a	8.7
0.06	1.31 \pm 0.01 ^a	12.7
0.12	1.18 \pm 0.03 ^a	21.3

^aP<0.05, comparison between the group treated with doxorubicin and the untreated group. DOX, doxorubicin; OD, optical density. IR, inhibitory rate.

membrane was dried in a shady environment, stained with 0.1% crystal violet for 10 min, destained and visualized under an optical microscope. The cells that had invaded the polycarbonate membrane were counted in each of five random fields using optical microscopy (x200). The invasive capability of the tumor cells was determined by averaging the number of positively stained cells in each of the microscopic fields. The experiment was repeated in triplicate.

Statistical analysis. All data are presented as the means \pm standard deviation (SD), and all statistical analyses were performed using the statistical software SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). To test for statistical significance, experimental data were analyzed by one-way analysis of variance (ANOVA) and the SNK-*q* test. A P-value of <0.05 was considered to indicate a statistically significant result.

Results

IC₅₀ value of doxorubicin against MCF-7 cells and the doxorubicin concentration for the induction of drug resistance. Doxorubicin treatment for 48 h significantly inhibited the growth of the MCF-7 cells, and the IC₅₀ value of doxorubicin against MCF-7 cells was 0.68 \pm 0.04 μ g/ml. The OD values and inhibitory rates of MCF-7 cells following doxorubicin treatment are shown in Table I. Treatment with doxorubicin at concentrations of 0.01, 0.03, 0.06 and 0.12 μ g/ml for 7 days achieved significant inhibition of the growth of MCF-7 cells compared with the growth of cells that did not receive doxorubicin treatment (P<0.05). The MCF-7 cells treated with doxorubicin at concentrations of 0.03 and 0.06 μ g/ml for 7 days only developed mild swelling, with a good cell state, while the cells treated with 0.12 μ g/ml doxorubicin for 7 days exhibited a poor state with obvious swelling and apparent cell debris. Therefore, doxorubicin at a concentration of 0.06 μ g/ml was used for the subsequent induction of drug resistance (Table II).

Changes in MDRI, CD44st and NF- κ B mRNA expression in the MCF-7 cells following doxorubicin-induced drug resistance. Low expression of MDRI, CD44st and NF- κ B mRNA in MCF-7 cells was revealed by the qRT-PCR assay, and the mRNA expression of these genes gradually increased

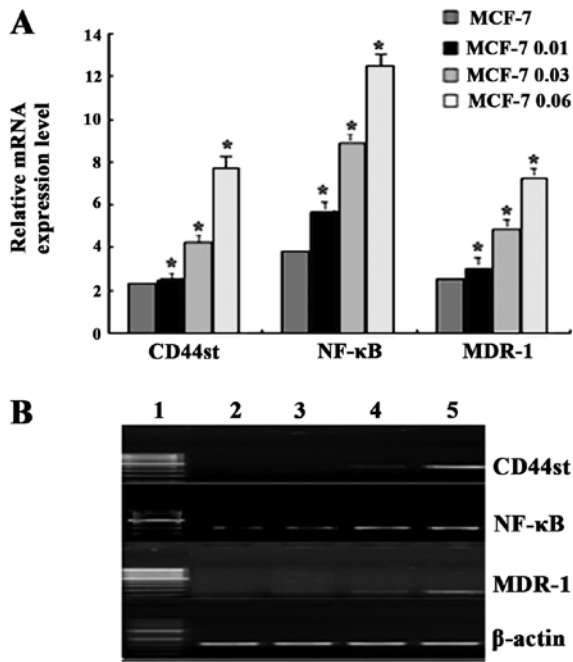


Figure 1. (A) Expression of *CD44st*, *NF-κB* and *MDR-1* mRNA in doxorubicin-treated MCF-7 cells as revealed by qRT-PCR assay. (B) Semi-quantitative reverse transcription-PCR amplification results obtained by qRT-PCR. Lane 1, marker; lane 2, MCF-7 cell group; lane 3, MCF-7/A 0.01 group; lane 4, MCF-7/A 0.03 group; lane 5, MCF-7/A 0.06 group. * $P < 0.05$ vs. the MCF-7 cell group. CD44st, short-tail isoform of CD44; NF-κB, nuclear factor-κB.

in the MCF-7 cells in a dose-dependent manner following treatment with doxorubicin at concentrations of 0.01, 0.03 and 0.06 $\mu\text{g/ml}$. There were significant differences in the expression of *MDR1*, *CD44st* and *NF-κB* mRNA between the doxorubicin-treated MCF-7 cells and the untreated MCF-7 cells (Fig. 1A) ($P < 0.05$). Semi-quantitative reverse transcrip-

tion-PCR amplification results were similar to the results obtained by qRT-PCR (Fig. 1B). Sequencing revealed that the CD44st gene sequence was consistent with the sequence of the gene we identified previously (GenBank accession no: FJ216964).

Changes in the percentage of MCF-7 cells positive for P-gp following doxorubicin treatment. Flow cytometry revealed that the percentages of P-gp-positive MCF-7 cells were 5.61 ± 0.52 , 11.53 ± 0.46 , 30.55 ± 1.62 and $61.12 \pm 2.05\%$ in the untreated MCF-7 cells and cells treated with doxorubicin at concentrations of 0.01, 0.03 and 0.06 $\mu\text{g/ml}$, respectively. Significant differences were detected in the percentage of P-gp-positive MCF-7 cells between the groups ($P < 0.05$) (Fig. 2).

Changes in Rho-123 efflux in MCF-7 cells following doxorubicin-induced drug resistance. The Rho-123 retention assay revealed that the fluorescence intensities of Rho-123 were 451.36 ± 15.43 , 326.07 ± 10.36 , 153.56 ± 7.83 and 78.84 ± 5.61 in the MCF-7 and MCF-7 cells treated with doxorubicin at concentrations of 0.01, 0.03 and 0.06 $\mu\text{g/ml}$, respectively. There were significant differences observed in the fluorescence intensity of Rho-123 between groups ($P < 0.05$). The fluorescence intensity of Rho-123 was gradually reduced in the MCF-7 cells, with or without doxorubicin treatment, with increased doxorubicin concentration, and the intracellular fluorescence intensity exhibited an inverse relationship with the percentage of P-gp-positive cells (Fig. 3).

Changes in CD44 and NF-κB protein expression and NF-κB DNA-binding activity in MCF-7 cells following doxorubicin-induced drug resistance. Western blot analysis revealed that CD44 and NF-κB protein expression was correlated with *CD44* and *NF-κB* mRNA expression, and dose-dependent CD44 protein expression was gradually detected in the MCF-7 cells following treatment with doxorubicin at

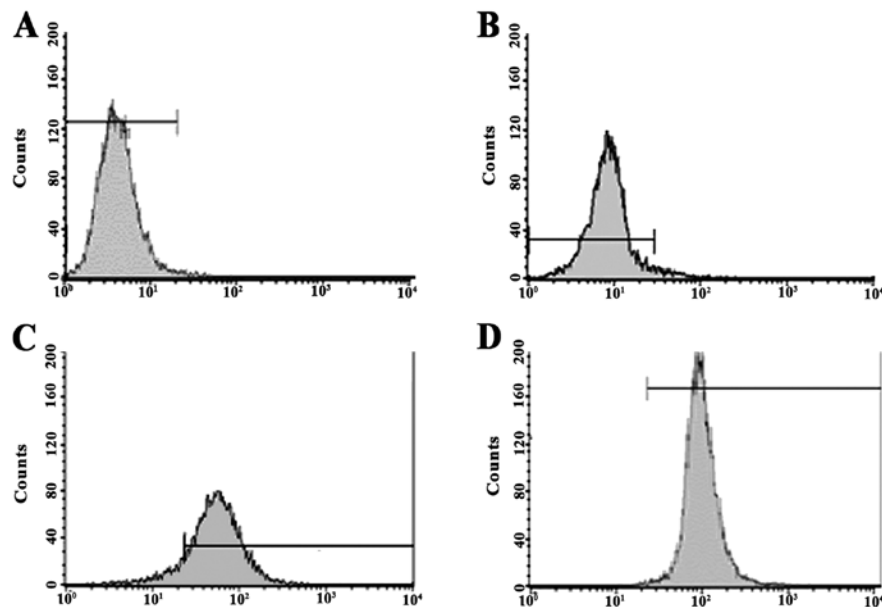


Figure 2. Expression of P-gp in doxorubicin-treated MCF-7 cells. (A) MCF-7 cell group; (B) MCF-7/A 0.01 cell group; (C) MCF-7/A 0.03 group; (D) MCF-7/A 0.06 group. P-gp, P-glycoprotein.

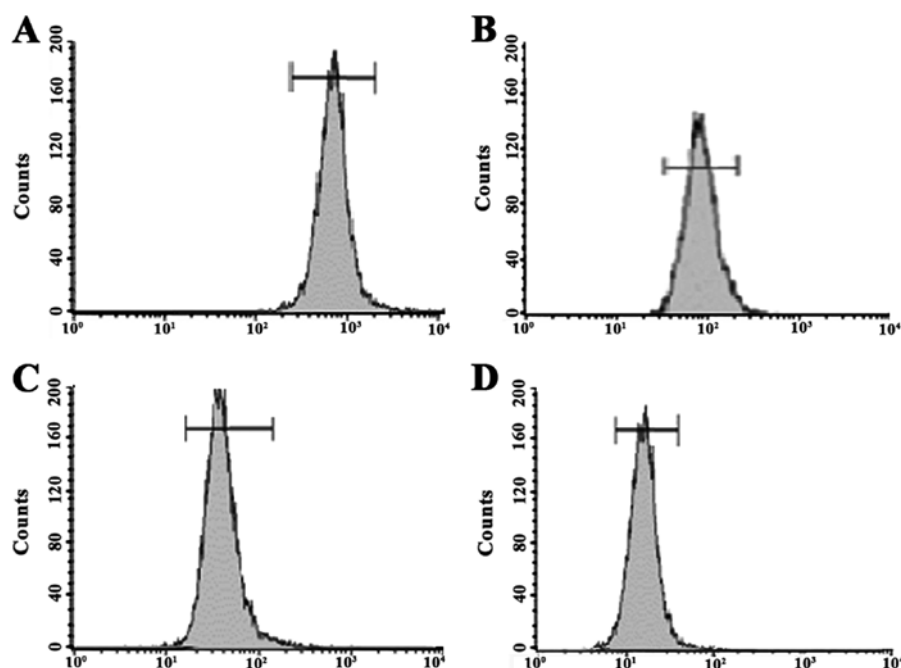


Figure 3. Intracellular accumulation and efflux of Rho-123 in doxorubicin-treated MCF-7 cells. (A) MCF-7 cell group; (B) MCF-7/A 0.01 group; (C) MCF-7/A 0.03 group; (D) MCF-7/A 0.06 group. Rho-123, Rhodamine-123.

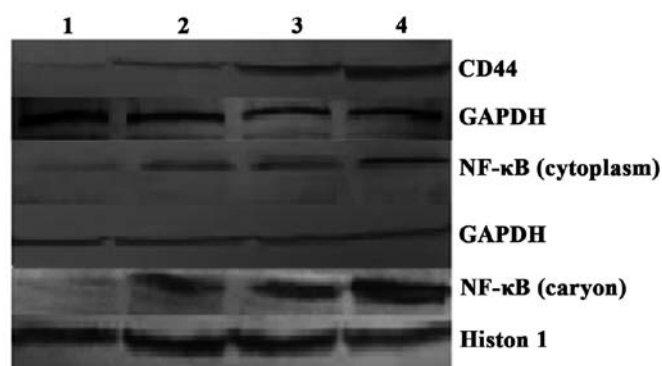


Figure 4. Expression of CD44 and NF- κ B protein in doxorubicin-treated MCF-7 cells. Lane 1, MCF-7 cell group; lane 2, MCF-7/A 0.01 group; lane 3, MCF-7/A 0.03 group; lane 4, MCF-7/A 0.06 group. NF- κ B, nuclear factor- κ B.

concentrations of 0.01, 0.03 and 0.06 μ g/ml. NF- κ B protein expression was found to gradually increase, and intranuclear transfer increased (Fig. 4). EMSA showed that the NF- κ B DNA-binding activity was enhanced in a dose-dependent manner with increasing concentrations of doxorubicin (Fig. 5).

Changes in MMP-2 and MMP-9 expression in MCF-7 cells following treatment with 0.06 μ g/ml doxorubicin and HA. RT-PCR and gelatin zymography revealed no MMP-2 or MMP-9 expression in the supernatants of the cultures of the MCF-7 cells, the MCF-7 cells treated with 0.06 μ g/ml doxorubicin, and the MCF-7 cells treated with 0.06 μ g/ml doxorubicin and HA following pretreatment with BMS-345541, while MMP-2 and MMP-9 were highly expressed in the MCF-7 cells treated with 0.06 μ g/ml doxorubicin and HA, as well as in the MCF-7/Adr cells treated with HA. These findings demon-

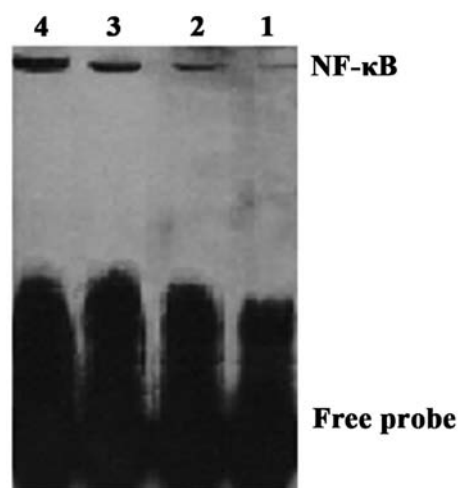


Figure 5. NF- κ B DNA binding activity as detected by EMSA. Lane 1, MCF-7 cell group; lane 2, MCF-7/A 0.01 group; lane 3, MCF-7/A 0.03 group; lane 4, MCF-7/A 0.06 group. NF- κ B, nuclear factor- κ B. EMSA, electrophoretic mobility shift assay.

strated that HA induced the secretion of MMP-2 and MMP-9 in the MCF-7 cells treated with 0.06 μ g/ml doxorubicin, which could be blocked by BMS-345541 treatment (Fig. 6).

Changes in the invasive ability of MCF-7 cells following treatment with 0.06 μ g/ml doxorubicin and HA. Transwell invasion assays revealed that the number of MCF-7 cells following treatment with 0.06 μ g/ml doxorubicin and HA (289 ± 23 cells/field) and the number of MCF-7/Adr cells treated with HA (391 ± 31 cells/field) that invaded the Transwell were significantly higher than those of the MCF-7 cells (153 ± 23 cells/field), the 0.06 μ g/ml doxorubicin-treated MCF-7 cells (162 ± 19 cells/field), and the MCF-7 cells treated

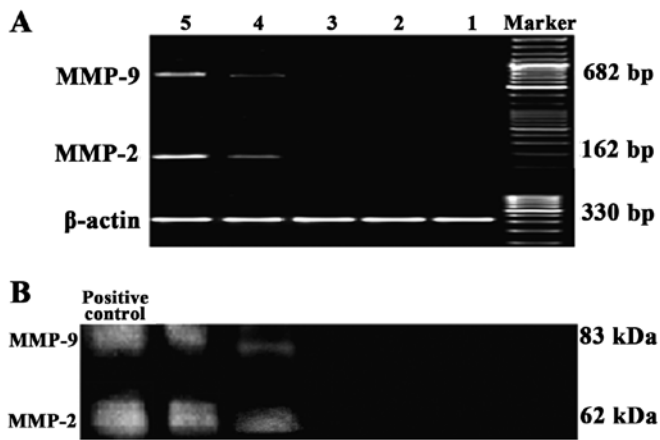


Figure 6. Expression of MMP-2 and MMP-9 protein in MCF-7/A 0.06 cells treated with HA. Lane 1, MCF-7 cell group; lane 2, MCF-7/A 0.06 group; lane 3, MCF-7/A 0.06 group pretreated with BMS-345541 + HA; lane 4, MCF-7/A 0.06 + HA group; lane 5, MCF-7/Adr + HA group. HA, hyaluronan; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9.

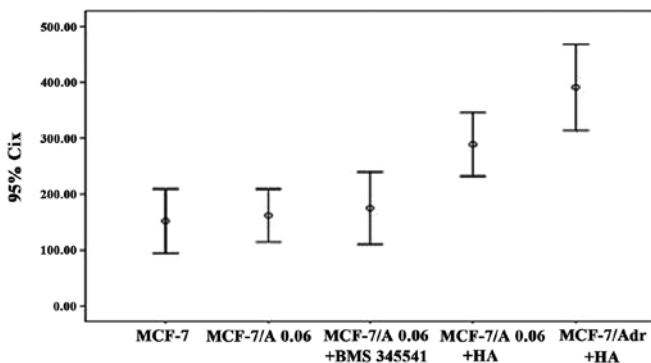


Figure 7. The invasive ability of MCF-7 and MCF-7/Adr cells as detected by Transwell invasion assay.

with 0.06 μ g/ml doxorubicin and HA following pretreatment with BMS-345541 (175 ± 26 cells/field) ($P < 0.05$). The number of MCF-7 cells that invaded the Transwell following treatment with 0.06 μ g/ml doxorubicin and HA was significantly lower than that of the MCF-7/Adr cells treated with HA in other groups ($P < 0.05$), while no significant differences were observed in the numbers of MCF-7 cells, the MCF-7 cells treated with 0.06 μ g/ml doxorubicin, and the MCF-7 cells treated with 0.06 μ g/ml doxorubicin and HA following pretreatment with BMS-345541 ($P > 0.05$). These findings showed that HA increased the invasive ability of the MCF-7 cells treated with 0.06 μ g/ml doxorubicin, which could be blocked by pretreatment with BMS-345541 (Fig. 7).

Discussion

Our findings indicated that MCF-7 cells subjected to low-dose exposure to doxorubicin may develop multidrug resistance to doxorubicin, and the expression of *MDR1*, *CD44st* and *NF- κ B* mRNA and *MDR1*, *CD44* and *NF- κ B* protein was upregulated in a dose-dependent manner during the induction of

drug resistance in MCF-7 cells. In addition, HA pretreatment increased the secretion of MMP-2 and MMP-9 in the multi-drug-resistant MCF-7 cells and affected the invasive ability of MCF-7 cells, which could be blocked by the *NF- κ B*-specific inhibitor BMS-345541. The present study demonstrated that the transcription factor *NF- κ B* is involved in tumor MDR and invasion and plays a critical role in inducing *CD44st* expression. It is possible that the detection of this molecular target may play an important role in predicting tumor invasion and drug resistance and that the *NF- κ B*-specific inhibitor plays a critical role in inhibiting tumor invasion and drug resistance.

The major causes of tumor treatment failure involve tumor metastasis and MDR. It has been reported that MDR is closely associated with tumor metastasis and invasion. MMPs were found to be involved in ECM degradation and remodeling (12-15). It has been shown that MMP-2 and MMP-9 are involved in the invasion of breast cancer, and they may be potential cancer markers. In patients with basal-like breast cancer, the activity of precursor and active forms of MMP-2 and MMP-9 was found to significantly increase with each advancing clinical stage of disease, and the activity of the precursor and active forms of MMP-9 in tumor tissue showed a positive association with tumor size. In addition, patients with lymph node involvement had higher activity of precursor and active forms of MMP-2 and the active form of MMP-9 than node-negative patients, and patients with steroid receptor-negative tumors had enhanced MMP-2 and MMP-9 activity compared with patients with luminal A tumors (16).

CD44 is involved in multiple human pathophysiological processes. The binding of various abnormally expressed CD44 molecules to the receptor HA promotes the secretion of MMPs, resulting in ECM degradation, which is closely associated with axillary lymph node metastasis, increased angiogenesis, prognosis and drug resistance (17-22). It has been shown that HA binds to the CD44 variant that is widely distributed on the surface of the cell membrane during tumor progression, which induces the activation of ankyrin and small G-protein Rho, and the activation of the PI3K-AKT signaling pathway, thereby leading to enhanced tumor cell adhesion, growth, migration and invasion, and resultant tumor progression (23). HA is found to induce the elevation of MMP expression in breast cancer cell lines, which confers enhanced CD44 cleavage and cell migration during the invasion process (17).

NF- κ B was found to be involved in the regulation of the production of *MDR1* and *CD44* genes in multiple types of cancer cells. It was reported that the MAPK/*NF- κ B* pathway mediates doxorubicin-induced P-gp expression in MCF-7/Adr cells and subsequently alters the cellular pharmacokinetics of doxorubicin (3). Paracrine signaling through the receptor activator of the *NF- κ B* (RANK) pathway was reported to promote the development of mammary stem cells and breast cancer, and high levels of RANK were found in human primary breast adenocarcinomas that lack expression of the hormone receptors estrogen and progesterone (triple-negative breast adenocarcinomas) and in tumors with a high pathological grade and proliferation index; in addition, high RANK/RANKL expression was significantly associated with metastatic tumors. Together, the data indicate that RANK promotes tumor initiation, progression and metastasis in human mammary epithelial cells by increasing the population

of CD44⁺CD24⁻ cells, thereby affecting the prognosis of breast cancer patients (24).

Results recently indicate that CD44⁺CD24⁻ cells are a type of tumor stem cells in breast cancer tissues, which are usually termed triple-negative breast adenocarcinoma cells (25,26). The CD44⁺CD24⁻ phenotype was found to be significantly associated with lymph node involvement, progesterone receptor status in patients with recurrent or metastatic tumors, the phenotype of triple-negative breast cancer, and disease-free and overall survival of patients. It was therefore considered that the CD44⁺CD24⁻ phenotype may be an important factor associated with malignant relapse following surgical resection and chemotherapy in patients with invasive ductal carcinoma (22).

It has been shown in our clinical trials that the *CD44st* gene was highly expressed in breast cancer tissues, and it plays a critical role in breast cancer development and progression, which may be associated with breast cancer invasion, metastasis and prognosis (27). Taken together, these findings demonstrated that anti-CD44 treatment may inhibit cancer cell metastasis and improve the prognosis of breast cancer patients with high *CD44st* expression.

The present study revealed that expression levels of *MDR1*, *CD44st* and *NF-κB* mRNA and *MDR1*, CD44 and NF-κB protein were upregulated in a dose-dependent manner in MCF-7 cells with doxorubicin-induced drug resistance, and HA pretreatment increased MMP-2 and MMP-9 expression in the multidrug-resistant MCF-7 cells and affected the invasive ability of MCF-7 cells, which could be blocked by an NF-κB-specific inhibitor. In addition, the NF-κB-specific inhibitor was found to play a critical role in inhibiting tumor invasion and drug resistance. It is suggested that CD44st, a molecular marker, may play an important role in MDR and the invasive ability of breast cancer cells.

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