Effect of CD44 knockdown on the biological characteristics of breast cancer stem cells

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Abstract

Background: CD44 is an important surface marker of breast cancer stem cells (BCSCs), but it is unclear whether it is involved in the stemness of BCSCs. This has limited the development of new therapeutic strategies for breast cancer. Previous studies have shown that many CD44 variants generated through alternative splicing are involved in the development of breast cancer, but their exact role in BCSCs remains unclear. Therefore, we analyzed the CD44 transcript variants in BCSCs derived from the MDA-MB-435 cell line, and aimed to investigate whether CD44s knockdown could affect the biological characteristics of BCSCs.

Methods: CD44⁺/CD24⁻ cells were isolated among the MDA-MB-435 cells by flow cytometry, and the CD44 transcript variants were detected by RT-PCR in CD44⁺/CD24⁻ cells. Due to the high expression of CD44 standard splice isoform (CD44s) in CD44⁺/CD24⁻ cells, CD44s knockdown was generated using small hairpin RNA (shRNA). The effects of CD44s knockdown on the biological characteristics of BCSCs was detected using cell proliferation assay, colony formation assay, cell cycle and apoptosis assay, tumor sphere formation assay, wound-healing assay, and Matrigel invasion assay. Tumorigenesis of the CD44⁺/CD24⁻ cells with CD44s knockdown was investigated in vivo with NOD/SCID mice. The expression of cancer stem cell stemness-related genes, such as Bcl-2, CCNE2, EGFR, MMP7, Muc1, and Myc was also detected by qPCR.

Results: Our results revealed that the mRNA expression of CD44 transcript variants was heterogeneous, and CD44s is highly expressed in BCSCs. CD44s depletion inhibited the proliferation, made cell cycle stay in G0/G1 phase, promoted the apoptosis and necrosis of BCSCs, inhibited the ability of self-renewal and invasion along with the expression of cancer stem cell-related genes in BCSCs. Moreover, CD44s knockdown inhibited the tumorigenesis ability in vivo.

Conclusion: Our findings revealed that CD44s is the predominant isoform expressed in BCSCs, and is an important molecule for maintaining the properties of BCSCs. Targeting CD44s in BCSCs may be a potential new direction for breast cancer treatment.

Background

The cancer stem cells (CSCs) have the potential of self-renewal and differentiate into multiple types of cancer cells; they are involved in tumorigenesis, metastasis, and resistance to anticancer drugs [1]. An increasing body of evidence has implicated CSCs in a variety of malignancies, thereby suggesting that human cancers are stem cell diseases [2].

Knowledge of CSC-specific biomarkers has facilitated the identification, isolation, and study of CSCs. In 2003, Al-Hajj et al. found that a minority of breast cancer cells with CD44⁺/CD24^{-/low} phenotype possessed the ability to form new tumors in NOD/SCID mice. Only 100 cells with this phenotype could form tumors in mice, while tens of thousands of cells with other phenotypes could not form tumors.

This population of cells that promotes tumor formation as well as generates tumor cell heterogeneity, contained tumorigenic and non-tumorigenic cells [3]. Dario Ponti isolated and expanded these cells in vitro by mammosphere culture from breast tumor specimens. They obtained CD44⁺/CD24⁻ cells with the properties of stem/progenitor cells, which were able to form new tumors when little more than 10³ cells were subjected into SCID mice [4]. Clinically, we previously detected CD44⁺/CD24⁻ cells in benign hyperplasia of breast, atypical hyperplasia of breast, carcinoma in situ of breast, and invasive breast cancer tissues, which led us to the finding that CD44⁺/CD24⁻ cells increased with the development of the lesions, and the number of these cells in patients with lymph node metastasis were higher than that in patients without lymph node metastasis. CD44⁺/CD24⁻ has been widely used as a breast cancer stem cell (BCSC) biomarker [5–8]. However, whether CD44 is a critically functional molecule in maintaining the characteristics of BCSCs remains controversial [9–12]. This has limited the development of new therapeutic strategies for breast cancer. Through alternative splicing, CD44 can generate different isoforms, such as the CD44 standard isoform (CD44s) and CD44 variant isoform (CD44v). Many studies have shown that both CD44s and CD44v are involved in the development of breast cancer [13–17]. However, the exact function and transformational mechanism of each isoform in BCSCs is still unclear.

Therefore, in this study we analyzed the CD44 transcript variants in BCSCs derived from the MDA-MB-435 cell line, and aimed to investigate whether CD44s knockdown could affect the biological characteristics of BCSCs. The results will help shed some light on whether CD44 is just a biomarker of BCSCs or an important molecule that maintains CSC properties, thereby promoting targeted treatment for breast cancer by BCSC-targeting therapy.

Methods

Cells and cell cultures

This study received the approved of the Ethics Board of Kunming General Hospital and was also conducted in accordance with the Helsinki Declaration institute of 1975. The MDA-MB-435 human breast cancer cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and were grown in RPMI-1640 Medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biological Industries, Inc., Israel, USA), 100 units/mL penicillin and 100 µg/mL streptomycin in humidified conditions with a 5% CO₂ at 37 °C.

RNA preparation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Total RNA of cells was extracted using the TRIzol reagent (mrcgene, OH, USA) and reverse transcribed to cDNA using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Waltham, USA). The iTaq™ Universal SYBR Green Supermix kit (Bio-Rad, Hercules, USA) was used to perform PCR

amplification on the CFX96™ Real-Time PCR Detection System. The expression of RNA was normalized to the level of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Data were analyzed using the Bio-Rad CFX96 Manager software. The sequences for qRT-PCR are listed in Table 1.

Table 1
Primers used in RT-qPCR

Primer NO.	Primer sequence	Tm(°C)	Product
CD44 transcript variants1 F	GAGCAGCACTTCAGGAGGTTA	60	119 bp
CD44 transcript variants1 R	TCATCAAAGTGGTAGCAGGGA		
CD44 transcript variants2 F	CAGGAGGTTACATCTTTTACACC	60	112 bp
CD44 transcript variants2 R	TTTGAAGACGTACTGGTAGCAG		
CD44 transcript variants3 F	TTCAGGAGGTTACATCTT	60	78 bp
CD44 transcript variants3 R	ATATTGGTAGCAGGGATT		
CD44 transcript variants4 F	CAGCACTTCAGGAGGTTACATC	60	120 bp
CD44 transcript variants4 R	GTGTCTTGGTCTCTGGTAGCA		
CD44 transcript variants5 F	GCTGACCTCTGCAAGGCTTTC	60	99 bp
CD44 transcript variants5 R	ACTGCA ATGCAA ACTGCAGGT		
CD44 transcript variants6 F	TTCAGGAGGTTACATCTT	60	111 bp
CD44 transcript variants6 R	TCATTCCTATTGGTAGCA		
CD44 transcript variants7 F	GCAGCACTTCAGGAGGTTAC	60	119 bp
CD44 transcript variants7 R	ATGTGAGTGTCTGGTAGCAGG		
CD44 transcript variants8 F	AGAATTATGGACTCCTTAC	60	187 bp
CD44 transcript variants8 R	CTCAATGGTATAGATAGCA		
GAPDHF	TGACAACAGCCTCAAGAT	58	103 bp
GAPDHR	GAGTCCTTCCACGATACC		
CD44F	CAGCACTTCAGGAGGTTACATC	58	120 bp
CD44R	GTGTCTTGGTCTCTGGTAGCA		
Bcl-2F	GAGTGCTGAAGATTGATG	58	110 bp
Bcl-2R	TCCTCTGTGATGTTGTATT		
Cyclin E2F	GTTCTTCTACCTCAGTATTCTC	58	114 bp
Cyclin E2R	AGCAGCAGTCAGTATTCT		
EGFRF	CTGGTTATGTCCTCATTG	58	120 bp
EGFRR	CATAGTTAGATAAGACTGCTA		
MMP7F	CAGTGATGTATCCAACCTAT	58	157 bp

Primer NO.	Primer sequence	Tm(°C)	Product
MMP7R	CAATCCAATGAATGAATGAATG		
Muc1F	ACTTCTGCCAACTTGTAG	58	79 bp
Muc1R	AAGAACCTGAGTGGAGTG		
MycF	CTCAAGTCATAACAATGCTAA	58	100 bp
MycR	AATCAACAGTATCTCCTTCA		

Flow Cytometric Analysis

To isolate the CD44⁺/CD24⁻ cells from MDA-MB-435 cells, flow cytometric analysis was performed as previously described [18]. Briefly, 2×10^6 cells were harvested and washed with 1X PBS. PE-conjugated mouse anti-human CD44 monoclonal antibody and fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD24 monoclonal antibody (all from BD Biosciences, Franklin Lakes, USA) were added to the cells, respectively. After 20 min incubation on ice and in the dark, cells were washed twice with chilled 1X PBS containing 2% FBS followed by cell sorting using the flow cytometer (BD FACSVantage SE) [19].

For cell cycle assessment, cells were harvested and washed twice by 1X PBS, and fixed in 80% ethanol for 30 min at 4 °C. After washing the cells twice with 1X PBS, they were suspended in 200 µL propidium iodide (PI, 0.1 mg/mL, BD Biosciences) and 200 µL RNase A (1 mg/mL). After 30 min incubation on ice and in dark [20], cells were scanned by flow cytometer and data were analyzed using the FlowJo software V7.6.

To detect apoptosis, cells were washed gently with PBS to make a cell suspension followed by centrifugation at 500 g for 5 min. Using 200 µL Binding Buffer, the cells were resuspended at a cell density of $2-5 \times 10^5$ cells/mL. Then, 5 µL Annexin V- FITC (BD Biosciences) was added to the 195 µL cell suspension followed by incubation at room temperature in dark for 10 min. A total of 190 µL binding buffer and 10 µL of PI was added to the cells, and incubated in dark at 4 °C for 30 min. The cell suspension was filtered with 300 mesh nylon mesh. Apoptosis was measured using flow cytometry.

Plasmids And Transduction

The CD44s-shRNA (Target to CD44 standard isoform transcript variant 4, CD44s, target sequence was GGAAGAAGATAAAGACCATCCTTCAAGAGAGGA

TGGTCTTTATCTTCTTCCTT) and NC-shRNA (Negative Control) were generated by Shanghai GenePharma Co. (GenePharma Co,Ltd, Shanghai, China). The sequences were inserted into the *Bbs*I and *Bam*HI positions of plasmid pGPU6/GFP/Neo with GFP reporter gene. With this approach, the target sequences were placed between the hU6 promoter and the SV40 polyA signal (Fig. 3).

Plasmids, pGPU6-CD44s and pGPU6-NC, were transfected into the BCSCs by mixing with Lipofectamine™ 2000 reagent (Invitrogen, Beijing, China) according to the instructions of the manufacturer. Subsequently, 24 h after transfection, cells were passaged and were cultured in RPMI-1640 medium containing 10% FBS and 400 µg/mL G418. After 10 days, 200 µg/mL G418 was added to the culture in order to maintain the screening pressure. G418-resistant clones were visible approximately 14 days after the selection pressure was applied. After subsequent culture of these clones, we obtained two cell lines with stable expression of CD44s-shRNA (CD44s-shRNA group) and NC-shRNA (NC-shRNA group) [21]. CD44s knockdown was confirmed using qRT-PCR.

Cell Proliferation And Colony Formation Assay

The CD44s-shRNA, NC-shRNA, and untransfected BCSCs were plated in 24-well plates with 1×10^4 cells per well and incubated at 37 °C. Cell proliferation was assessed on day 1, 2, 3, 4, 5, 6, and 7 after transfection by counting the average of three random wells. After observing for seven days, the proliferation curves were drawn. For the colony formation assay, 1000 cells from each group were plated in 6-well plates. After about 4 days, when the cell number of one clone was about 50, the cells were fixed with formaldehyde and stained using Giemsa staining. The number of clones was determined using images.

Tumor Sphere Formation Assay

The CD44s-shRNA, NC-shRNA, and untransfected BCSCs were seeded in ultralow attachment plates (Corning, USA) at a density of 3×10^4 viable cells/mL in primary culture, respectively. They were then cultured in a sphere culture medium containing serum-free DMEM-F12 (HyClone, Utah, USA), B27 (1:50, Gibco, USA), 10 ng/mL EGF (Peprotech, Rocky Hill, USA), 20 ng/mL bFGF (Peprotech, Rocky Hill, USA), and 5 µg/mL insulin (Sigma, USA). After 5 days, the primary sphere was collected and blown mechanically to a single cell by enzyme digestion (10 min in 0.05% trypsin, 0.53 mM EDTA-4Na; Invitrogen, USA). The single-cell were cultured to the next generation. The size and percentage of microspheres were calculated after two weeks[22].

The Matrigel Invasion Assay

The invasion assay was cultured in a transwell chamber (24-well, 8 mM pore size, Corning). The three groups of cells were plated in the upper chamber with 100 µL serum-free RPMI containing 0.2% BSA (1×10^5 cells), respectively. A total of 400 µL RPMI containing 20% FBS was placed in the lower chamber as chemoattractant. After 48 h, the cells at the lower surface of the chamber membranes (migrated) were fixed with 90% ethanol and stained with hematoxylin. The number of migrated cells was counted under the microscope in ten random high power fields (400) per membrane [23].

Tumor Growth And Morphologic Analysis In Vivo

All animals were handled in strict accordance with good animal practices as deemed by the relevant national and local animal welfare bodies, and in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. A total of 12 six-week old female NOD/SCID specific pathogen-free (SPF) mice (Vital River Laboratories, Beijing, China) were fed under SPF conditions, and randomly divided into four groups. A total of 10^6 , 10^5 , 10^4 , and 10^3 BCSCs were subcutaneously injected into the left back of the four groups of mice, respectively, while each group of mice was subcutaneously injected with BCSCs stably expressing CD44s shRNA into the right back. The subcutaneous tumor formation as well as tumor size and weight of nude mice were observed every two days about 60 days. When the tumor diameters reached 2000 mm, the mice were anaesthetized by intraperitoneal administration of 2% pentobarbital sodium (45 mg/kg), and then euthanized by rapid cervical dislocation. The dynamic observation of tumor growth was monitored with calipers using the following formula: $\text{Volume} = 1/2 (\text{width}^2 \times \text{length})$ [24].

Histopathology, Immunohistochemistry, And Immunofluorescence

The cells growing on glass slips and tissue sections of the paraffin-embedded tumor blocks were subjected to hematoxylin and eosin (H&E) staining [25]. Immunohistochemistry staining was carried out as follows: peroxide was used to block the activity of endogenous peroxidase after PBS flushing followed by addition of CD44 antibody (clone 156-3C11, Newmarkers; 1:200) and CD24 antibody (clone SN3b, Newmarkers; 1:50) at room temperature, and overnight incubation at 4 °C. Next, biotin-labeled secondary antibody was added followed by DAB staining and hematoxylin restaining. The sections were photographed with microscope scan and analyzed by two pathologists. The Allred immunostaining scoring system was used for semiquantitative evaluation in the three groups of cells [26]. A total score was calculated by the sum of the proportion and intensity score, and the staining intensity was scored as 0 (none), 1 (mild), 2 (moderate), or 3 (strong); the proportion of stained cells was scored as 0 (none), 1 (> 0–1%), 2 (≥ 1 –10%), 3 (> 10–33%), 4 (> 33–66%), and 5 (> 66–100%).

For immunofluorescence, the cells growing on glass slips and the frozen sections of tissues [27] were fixed with acetone for 15 min, followed by three washes with 1X PBS. Then, the tissue sections were incubated with PE-conjugated anti-human CD44 antibody and FITC-conjugated anti-human CD24 antibody (BD Biosciences) [28]. After washing twice with 1X PBS, a drop of glycerol was added on a glass slide, and the slide was analyzed under a fluorescence microscope (Nikon, Tokyo, Japan). The images were acquired using the accompanying software package.

Statistical Analysis

Each result is expressed as the mean $\pm$ standard deviation. All experiments were performed in triplicates. All statistical analyses were performed using the SPSS software version 11.0. Comparisons among all groups were performed using one-way analysis of variance (ANOVA) and the Student–Newman–Keuls method. Statistical significance is indicated by a *P* value less than 0.05.

Results

Expression of different CD44 isoforms in BCSC subpopulation among MDA-MB-435 cells

Firstly, we isolated and identified BCSCs in the human breast cancer cell line, MDA-MB-435. The CD44⁺/CD24⁻ cells were isolated from MDA-MB-435 cells using flow cytometer. The sub-population of CD44⁺/CD24⁻ cells in MDA-MB-435 cells was 91.2% when determined for the first time (Fig. 1A). We continued the culture of CD44⁺/CD24⁻ cells and detected their numbers again. The population of CD44⁺/CD24⁻ cells was 100% when detected the second time (Fig. 1B). The CD44 and CD24 protein expression of CD44⁺/CD24⁻ cells were confirmed by H&E staining as well as immunohistochemical analysis and immunofluorescence staining (Fig. 1C). Using immunohistochemistry, the cells were shown to be positive for CD44 protein and negative for CD24 protein. Immunofluorescence analysis revealed cells that showed clear red fluorescent membrane indicating CD44 protein positivity, while being negative for CD24. According to the previous studies that considered CD44⁺/CD24⁻ cells as BCSCs, we used the CD44⁺/CD24⁻ cells as BCSCs for the subsequent studies.

The CD44 transcript variants were detected by RT-PCR in BCSCs. The relative expression levels of CD44 transcript variant mRNA in BCSCs, CD44V1, CD44V2, CD44V3, CD44V4, CD44V5, CD44V6, CD44V8, were 0.14 ± 0.01 , 0.32 ± 0.02 , 0.43 ± 0.01 , 1.6 ± 0.01 , 0.93 ± 0.04 , 0.28 ± 0.02 , 0, 0.32 ± 0.01 , respectively. The expression of the CD44 transcript variant 4 (standard form, CD44s) mRNA is the highest in BCSCs (Fig. 1D). The data showed that the mRNA expression of CD44 transcript variants was heterogeneous, and CD44s is a suitable siRNA interference target on account of its highest mRNA expression among the CD44 transcript variants.

CD44s depletion inhibits the proliferation, regulates the cell cycle, and promotes the apoptosis and necrosis of BCSCs in vitro

To determine the proliferation status of CD44s knockdown in BCSCs, CD44s knockdown in BCSCs was achieved using short hairpin RNAs (shRNAs) (Fig. 2A); the stable expression of shRNA targeting CD44 was established in BCSCs (Fig. 2B). The CD44s knockdown efficiency was verified by qRT-PCR and immunohistochemistry both at the mRNA and protein level. The results showed that CD44s knockdown was successfully achieved by CD44shRNA in BCSCs (Fig. 2C, 2D).

The cell proliferative curve showed that CD44s knockdown significantly reduced BCSC proliferation and the ability of clone formation as compared to that in the negative control (NC-shRNA) and untransfected BCSCs (Fig. 3A, 3B).

In addition, we evaluated the cell cycle by flow cytometry and found that CD44s knockdown resulted in an raising number of cells in the G0/G1-phase and decreased the number of cells in the S-phase (Fig. 3C). Similarly, the apoptosis assay showed that CD44 knockdown can promote the apoptosis and necrosis of BCSCs (Fig. 3D).

These results suggest that CD44s knockdown can induce proliferation of BCSCs by regulating the cell cycle, and promoting apoptosis and necrosis in vitro.

CD44s knockdown inhibits the ability of self-renewal and invasion as well as the expression of cancer stem cell-related genes in BCSCs

In the tumor sphere formation assay, the number of tumor spheres reflects the quantity of CSCs capable of self-renewal in vitro, and the size of spheres reflects the self-renewal capacity of each sphere-generating cell. The CD44s knockdown inhibited the tumor sphere number and the size of tumor spheres (Fig. 4A). Thus, CD44s knockdown can inhibit the ability of self-renewal.

We next performed the transwell invasion assay to explore the activity of BCSCs with or without CD44s. The data showed that CD44s knockdown could down-regulate BCSC invasion ability (Fig. 4B).

BCSCs are important for maintaining the proliferation and invasion capacities in breast cancer. Hence, we investigated the expression of genes related to proliferation (EGFR and Myc), cell cycle (CCNE2), metastasis (MMP7 and Muc1) and apoptosis (Bcl-2) in BCSCs with or without CD44s. CD44s knockdown was found to significantly reduce the mRNA expression of these genes when compared with the control group in BCSCs (Fig. 4D).

Cd44s Knockdown Inhibits Tumorigenesis Ability In Vivo

In order to test whether CD44s knockdown would affect tumorigenesis in vivo, xenograft models of human breast cancer were established. In the untransfected BCSC control group, the rate of tumor formation was 100% (3/3), and 66.7% (2/3) when injected with 10^6 cells and 10^5 cell per mice, respectively. However, in the CD44s knockdown BCSC group, the rate of tumor formation was 33.3% (1/3) and 0% (0/3) when injected with 10^6 cells and 10^5 cell per mice, respectively. Tumor-initiating capacity with different numbers of injected cells are shown in Table S1. At 60 days, in the group injected with 10^6 cells, tumors were smaller in size in CD44s-shRNA cells than those in the untransfected BCSCs (Fig. 5A). The tumor growth was slower in the CD44s-shRNA group than that in the control (Fig. 5B).

In order to observe the morphology of xenografts, we made tissue sections for H&E staining. It was found that the main difference between the untransfected group and the CD44 knockdown group was the heteromorphism of tumor cells. In the untransfected group, the tumor tissue was arranged in a cord without obvious ductal differentiation with large necrosis in the center, large cell volume, eosinophilic or bright cytoplasm, obvious enlargement and deep staining of nucleus, obvious nucleoli in most cells, strange giant cells in some parts, and pathological mitosis. In the CD44sh RNA group, most cells were of medium size, without large necrosis in the center (Fig. 5C)

To determine the expression of CD44 in vivo, the CD44 protein level was measured by immunofluorescence analysis. It showed that the CD44 protein (red fluorescence) was almost not present in the CD44s-shRNA cells of the tumor tissue, and only the GFP (green) was detected (Fig. 5C).

These findings indicated that CD44s knockdown could inhibit BCSC growth in xenograft tumors.

Discussion

In the last few decades, numerous cancer therapies have been developed. However, malignancies are still a threat because of relapse as well as resistance to drugs and radiation therapy [1]. The major cause of this resistance is CSCs, which can escape the therapy, leading to relapse and death [29–31]. Recently, targeting CSC surface biomarkers or key molecular signaling pathways of CSCs are emerging as important therapeutic strategies to effectively eradicate cancer [2, 32–34]. Breast cancer cells with CD44⁺/CD24[−] phenotype are regarded as BCSCs [3, 31]. W Li et al. found that high CD44⁺/CD24[−] ratio is related to cell proliferation and tumorigenesis in a xenograft tumor model [35]. In primary breast cancer tissues, CD44⁺/CD24^{low} cells demonstrated the strongest stemlike properties [36]. We previously found that the expression of CD44⁺/CD24[−] cells increased with the development of the lesions and were involved in lymph node metastasis in paraffin embedded breast cancer tissues [37]. These evidences suggest the importance of CD44⁺/CD24[−] cancer cells in the progression of breast cancer. The breast cancer cell line, MDA-MB-435, is considered to be the more aggressive cell line, and has the highest proportion of CD44⁺/CD24[−] cells (96.6 ± 1.2%) [38]. Our results also showed that the proportion of CD44⁺/CD24[−] cells was 91.2%; therefore, we chose the MDA-MB-435 cells to isolate the CD44⁺/CD24[−] cells for studying the properties of BCSCs.

Many studies imply that CD44 is an important biomarker on the surface of BCSCs, and this property was used to isolate BCSCs [39, 40]. Moreover, some researches showed that CD44 was involved in the tumorigenesis of CSCs in breast cancer [3, 41] and other cancers [42–46]. Nevertheless, the results are not conclusive, as some research data suggest that CD44 can maintain the properties of BCSCs. For example, knockdown of CD44 from BCSCs in primary cultures of malignant breast tumors led to differentiation of BCSCs into non-BCSCs with lower tumorigenic potential [47]. Suppression of CD44 by siRNA led to decreased tumorigenicity and cell migration capacity [35]. HA/CD44 signaling promotes protease-dependent invasion of breast cancer cells [48], while other studies showed that CD44 attenuates the features of CSCs. Jose I found that CD44 have a protective role against metastasis during breast

cancer progression through hyaluronan-CD44 dynamics [11]. Down-regulation of CD44 expression in breast cancer cells, head and neck squamous cell carcinoma cells, and leukemia cells have been shown to make cancer cells more sensitive to anticancer drugs [49–52]. A randomized investigation on breast conservation showed that CD44 is strongly associated with the increased overall survival with 25 years follow-up [53]. These data suggest that CD44 may be functionally critical for CSCs, but based on the dual nature of CD44 in the development of breast cancer, the exact function and mechanism of CD44 needs further exploration.

Since different CD44 splicing variants may display different functions in breast cancer [12, 15, 16], we previously examined the expression of CD44 splice variants in breast cancer cell lines, MDA-MB-231 and MDA-MB-435, as well as primary breast cancer, and found that CD44 splicing variants were heterogeneously expressed and CD44s expression was higher in breast cancer cell lines and tissues, which correlated with a higher histology grade [54]. In this study, high level of CD44s mRNA was detected in CD44⁺/CD24⁻ breast cancer cells. Together with other data [15], we assume that CD44s is the predominant isoform expressed in BCSCs. Therefore, we further investigated the effects of CD44s knockdown on the stemness of BCSCs. The results show that CD44s knockdown significantly inhibits the proliferation of BCSCs. Our findings showed that the proliferation arrest may be through the regulation of cell cycle along with the promotion of apoptosis and necrosis in vitro. Furthermore, CD44s knockdown restrained the invasion, self-renewal, and tumorigenic ability of BCSCs in vivo.

There are plenty of CD44-related pathways, including PI3K/AKT, Rho GTPases, and the Ras-MAPK pathways and so on [55, 56]. CD44 is reported to interact with EGFR, CCND2, Myc, and Bcl-2, which are up-regulated by Hedgehog signals to promote cellular proliferation, epithelial-to-mesenchymal transition, and bone metastasis [57, 58]. Up-regulation of secreted-MMP7 through Wnt/ β -catenin pathway is associated with the loss of tumor suppressor gene, PTEN, in triple negative breast cancer [59]. Mucin 1 (Muc1) is reported to be aberrantly overexpressed in human breast cancer and contributes to the activation of PI3K/AKT, MEK/ERK, Wnt/ β -catenin, STAT, and NF- κ B pathways in breast cancer [60]. Our results also showed that CD44 knockdown can influence the mRNA expression of EGFR, Myc, CCNE2, MMP7, Muc-1, and Bcl-2; however, which among these is involved in CSC-specific signaling and the crosstalk between the complex pathway need to be identified in future studies. The functions of different CD44 splicing variants and the transport mechanism in BCSCs need to be explored.

Conclusions

In conclusion, our results indicated that CD44s was the predominant isoform expressed in BCSCs, thereby suggesting that CD44s is not only a biomarker of BCSCs, but also a breast cancer stemness-related molecule. CD44s is an important functional requirement for maintaining stemness in CSCs. Therefore, interfering with the CD44s and relative oncogenic pathway might provide a novel targeted-therapy based on its capability of BCSC population maintenance.

Abbreviations

CSCs: cancer stem cells; BCSC: breast cancer stem cell; FBS: fetal bovine serum; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; PI: propidium iodide; FITC: fluorescein isothiocyanate; ANOVA: analysis of variance; shRNAs: short hairpin RNAs.

Declarations

Ethics approval and consent to participate

This study received the approved of the Ethics Board of Kunming General Hospital and was also conducted in accordance with the Helsinki Declaration institute of 1975. All animals were handled in strict accordance with good animal practices as deemed by the relevant national and local animal welfare bodies, and in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ZL performed manuscript writing and part of the experiments. YLH carried out the most of the experiments. QF participated in the data organization and manuscript drafting. LW, XYP and LGY engaged in experimental guidance. JYL designed the project. All authors read and approved the final manuscript.

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Figures

Figure 1

Isolation of breast cancer stem cells (BCSCs) from human breast cancer cell line, MDA-MB-435, and expression of different CD44 isoforms in BCSC subpopulation of MDA-MB-435 cells. BCSCs were sorted by flow cytometry, the population of CD44⁺/CD24⁻ cells in MDA-MB-435 FCA was 91.2% (A). The population of CD44⁺/CD24⁻ cells in the isolated BCSCs was 100% (B). The expression and intracellular localization of CD44 and CD24 were determined by hematoxylin and eosin staining (HE) as well as

immunohistochemistry and immunofluorescence staining, respectively. Immunohistochemistry analysis showed a strong positive expression of CD44 in the cell membrane, while CD24 was negative; immunofluorescence staining displayed CD44 positivity as exhibited by the red fluorescent membrane, while CD24 was negative as expected by the green fluorescent membrane; HE exhibited cell atypia (C). The relative mRNA expression of CD44 transcript variants were tested by RT-PCR. The results showed that CD44 splice variants were heterogeneously expressed in BCSCs, and CD44s expression level was the highest (D). Results are expressed as mean $\pm$ S.D. n=3. *P $\leq$ 0.05; **P $\leq$ 0.01.

Figure 2

Knockdown of CD44s expression in breast cancer stem cells (BCSCs) CD44s-shRNA was cloned into pGPU6/GFP/Neo between the restriction enzyme site of Bam HI and Bbs I (A). A single clone formed from a CD44+CD24- cell transfected with CD44s-shRNA (B). CD44s mRNA and protein expression decreased significantly in the BCSCs with CD44s knockdown compared to that in NC-shRNA and untransfected shRNA groups by RT-PCR and immunohistochemistry, respectively. (C, D) Results are expressed as mean $\pm$ S.D. n=3. *P $\leq$ 0.05; **P $\leq$ 0.01; NS, not significant.

Figure 3

CD44s knockdown inhibited the proliferation and promoted the apoptosis of breast cancer stem cells (BCSCs). The cell proliferative curve showed that CD44s knockdown inhibited the proliferation of BCSCs by directly counting the cell number at different time points (A). CD44s knockdown is shown to reduce the clonal formation of BCSCs by colony formation assay (B); increased number of cells in the G0/G1-phase and decreased number of cells in the S-phase of cell cycle by flow cytometry (C), apoptosis and necrosis of BCSCs was shown to be promoted by using flow cytometric analysis (D). Q1-LL area: the cells in this area are living cells; Q1-LR area: the cells in this area are early apoptotic cells; Q1-UR area: cells in this area are advanced apoptotic cells; Q1-UL area: the cells in this area are necrotic cells. The typical images and quantitative analysis are shown in the left and right side. Results are expressed as mean $\pm$ S.D. n=3. *P $\leq$ 0.05; **P $\leq$ 0.01; NS, not significant.

Figure 4

CD44s knockdown inhibited self-renewal capacity and invasion, and down-regulated the expression of cancer stem cell-related genes in breast cancer stem cells (BCSCs) CD44s knockdown is shown to inhibit the sphere formation efficiency of BCSCs using the tumor sphere formation assay (A). Depletion of CD44s is shown to inhibit the invasion ability of BCSCs using the transwell invasion assay (C). Cancer stem cell-related genes of BCSCs are shown to be down-regulated with the CD44s knockdown using qRT-

PCR assay (D). The typical images and quantitative analysis are shown in left and right side (B,C,D). Results are expressed as mean $\pm$ S.D. n=3. *P $\leq$ 0.05; **P $\leq$ 0.01; NS, not significant.

Figure 5

CD44s knockdown inhibited tumorigenesis in vivo The xenograft growth in nude mice with CD44s knockdown cells was significantly inhibited as compared to that in the control group (A). The tumor growth curve showed that the tumor volume of CD44s interference group was significantly smaller than that of the control group (B). The effect of CD44 knockdown on cell morphology were detected by HE stain (C). The results showed that the tumor cells in the untransfected group were large in size, heteromorphic, and necrotic. The tumor cells in the CD44s-shRNA group were smaller in size and less heteromorphic than those in the untransfected group. The expression of CD44 and CD24 in the xenograft

were detected by immunofluorescence (C, right). The results showed that CD44 was highly expressed (CD44 was red fluorescence), while CD24 was not expressed (CD24 was green fluorescence) in the untransfected group, while the expression of CD44 was weak in the CD44s-shRNA group.

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