



ALDH1+ stem cells demonstrate more stem cell-like characteristics than CD44⁺/CD24^{-/low} stem cells in different molecular subtypes of breast cancer

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Background: CD44⁺/CD24^{-/low} and ALDH1+ are both markers of breast cancer stem cell (BCSC), we compared the biological characteristics of CD44⁺/CD24^{-/low} and ALDH1+ BCSCs in the four molecular subtypes of breast cancer.

Methods: Four fresh blocks of breast cancer tissue were obtained from patients with Luminal A, Luminal B, human epidermal receptor-2 (HER2)-overexpression and triple-negative breast cancer, respectively. Flow cytometry was used to sort CD44⁺/CD24^{-/low} and ALDH1+ BCSCs. The mammosphere (MS) formation experiment and NOD/SCID mouse xenograft experiment were performed to compare the self-renewal and tumorigenic abilities of CD44⁺/CD24^{-/low} with those of ALDH1+ BCSCs.

Results: The proportions of CD44⁺/CD24^{-/low} BCSC and ALDH1+ BCSC in Luminal A, Luminal B, HER2 overexpression and triple-negative subtypes were 12.1% and 8.7%, 2.7% and 5.7%, 0.8% and 8.7%, 0.7% and 4.5%, respectively. The MS formation experiment demonstrated that ALDH1+ BCSC formed significantly more MSs than CD44⁺/CD24^{-/low} BCSC in the four molecular subtypes (P<0.001). The NOD/SCID mouse xenograft experiment demonstrated that the sizes of grafted tumors formed by ALDH1+ BCSC were larger than those formed by CD44⁺/CD24^{-/low} BCSC in the four molecular subtypes (P<0.05).

Conclusions: In the four molecular subtypes of breast cancer, ALDH1+ BCSC demonstrated better self-renewal and tumorigenic abilities than CD44⁺/CD24^{-/low} BCSC.

Keywords: Breast cancer; breast cancer stem cell (BCSC); CD44⁺/CD24^{-/low}; ALDH1+; molecular subtype

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Introduction

Breast cancer is the most common malignancy among women in the United States, accounting for 29% of new cases (1). In China, the incidence of breast cancer also ranks first among female malignant tumors, and has been increasing annually (2). With the popularization of breast cancer screening and improvement of treatment methods, the overall survival of breast cancer patients has been greatly improved. However, approximately one-third of the patients still die of recurrence and distant metastasis after treatment. A recent theory on cancer stem cell (CSC) suggested that recurrence and distant metastasis of breast cancer are related to the residual occult CSC.

Breast cancer stem cells (BCSCs) are a small group of cells having the characteristics of stem cells. They have the potential of self-renewal and differentiation as well as are resistant to traditional adjuvant treatment, thereby playing a crucial role in the occurrence, development, and distant metastasis of breast cancer. In 2003, Al-Hajj *et al.* confirmed for the first time that the CD44⁺/CD24^{-low} breast cancer cells presented with properties of CSC. These cells could renew themselves, and were able to form tumors with phenotypic heterogeneity in the subcutaneous fat pads of NOD/SCID mice, whereas the non-CD44⁺/CD24^{-low} cells could not (3). In 2005, Ponti *et al.* confirmed the stem cell-like characteristics of CD44⁺/CD24^{-low} breast cancer cells via Mammosphere (MS) assay (4). Subsequently, CD44⁺/CD24^{-low} cells were widely used as BCSC in several studies (5-9). Other biomarkers have also been used to identify BCSCs. Acetaldehyde dehydrogenase 1 (ALDH1) is a member of the acetaldehyde dehydrogenase families, which catalyzes oxidation of acetic acid to acetaldehyde in the cell and participates in stem cell differentiation as well as in the transformation of retinol and retinoic acid (10). In 2007, Ginestier *et al.* suggested that even ALDH1⁺ breast cancer cells presented with characteristics of BCSCs and could form tumors with phenotypic heterogeneity in the subcutaneous fat pads of NOD/SCID mice, whereas ALDH1⁻ cells could not (11). Other authors further confirmed that the ALDH1⁺ breast cancer cells presented with stem cell-like characteristics, and that ALDH1⁺ phenotype was a reliable marker for BCSC (7,8,12-19). CD44⁺/CD24^{-low} and ALDH1⁺ were both widely used biomarkers for identifying BCSCs in several studies.

Some studies pertaining to the different characteristics of CD44⁺/CD24^{-low} and ALDH1⁺ BCSCs. Through a

NOD/SCID mouse xenograft experiment, Ginestier *et al.* suggested that ALDH1⁺ BCSC had better tumorigenic ability than CD44⁺/CD24^{-low} BCSC (11). Breast cancer is a heterogeneous disease and can be divided into four subtypes [Luminal A, Luminal B, human epidermal receptor-2 (HER2) overexpression, and triple-negative] based on intrinsic molecular portraits. Each subtype presents with different clinicopathological characteristics, treatment response and prognosis (20,21). However, few studies have compared the biological characteristics of CD44⁺/CD24^{-low} and ALDH1⁺ BCSCs in different molecular subtypes of breast cancer.

Here, we compared the self-renewal and tumorigenic abilities of CD44⁺/CD24^{-low} with those of ALDH1⁺ BCSCs in four molecular subtypes of breast cancer using fresh surgical specimens.

Methods

This study was approved by the Ethics Committee of Sichuan Provincial People's Hospital in China. Human samples were collected after obtaining informed written consents from all participants.

Patients and tumors

We included 4 female patients with primary breast cancer in the Department of Breast Surgery of Sichuan Provincial People's Hospital from June 2017 to September 2017, with a median age of 48 years old. All patients were diagnosed with infiltrating ductal carcinoma by preoperative core needle biopsy, there was one case in each molecular subtype. Tumor samples were obtained within 30 minutes after surgery and washed by the D-Hanks solution containing streptomycin for three times. The necrotic tissue, fat tissue and blood clots were carefully removed with the ophthalmic scissors and the tumor samples were cut into 1 mm × 1 mm × 1 mm blocks.

The digestion was performed using trypsinase and type I collagenase for 5 min in a constant-temperature bath at 37 °C, and terminated by fetal bovine serum (FBS). Cell filtrate was filtered and collected using a 200-mesh stainless steel filter. After low-speed centrifugation (69.4 ×g) for 5 min, the supernatant was discarded and the cells were collected. The primary breast cancer cell suspension was prepared with serum-free DMEM-F12 medium (1:50 B27, 20 ng/mL EGF, 10 ng/mL bFGF, 5 µg/mL insulin, GIBICO, US) and cultured in a constant temperature box at 37 °C with 5% CO₂ and 95% humidity.

Flow cytometry

The above primary breast cancer cell suspension was stained with trypan blue, and the number of living cells was counted to be over 90%. CD44⁺/CD24^{-low} cells were collected with reference to the method of Al-Hajj *et al.* (3). Anti-CD44-PE/CY7 (Anti-CD44 antibody, Abcam, English) and CD24-FITC (Anti-CD24 antibody, Abcam, English) antibodies were added into the test tube, and homologous control antibodies were added into the control tube, which were mixed well and incubated at room temperature for 30 min in the dark. The mixtures were washed twice with PBS and centrifuged at a low speed (69.4 ×g) for 5 min. Then, the supernatant was discarded, and the cells were re-suspended by adding PBS solution with cell concentration adjusted to 1×10⁶/mL, and sorted immediately on the machine. The main steps of ALDH1+ cell sorting were as follows: 5 μL/mL activated ALDELUOR™ (ALDELUOR™ Kit, STEMCELL, Canada) substrates were added, mixed well and incubated at 37 °C for 30 min. After low-speed centrifugation (69.4 ×g) for 5 min, the supernatant was discarded, ALDELUOR™ buffer was added for re-suspension with cell concentration adjusted to 1×10⁶/mL, and the cells were immediately sorted on the machine. The selected cells were cultured in serum-free DMEM-F12 medium in a constant temperature box at 37 °C with 5% CO₂ and 95% humidity.

Mammosphere culture

Single-cell suspensions after flow sorting of four molecular subtypes of breast cancer were collected, including CD44⁺/CD24^{-low}, ALDH1+ and non-BCSC (ALDH1-) cells, which were re-suspended in DMEM-F12 medium, with the cell concentration adjusted to 1×10³/mL. Cells in each group were cultured on a 96-well plate with 5 duplicated wells and 100 μL per well. Then, each well was incubated in DMEM-F12 medium with a total volume of 200 μL in a constant temperature box at 37 °C with 5% CO₂ and 95% humidity.

Animal model

All NOD/SCID mice were divided into Luminal A group, Luminal B group, HER2 overexpression group and Triple-negative group, with 3 subgroups in each group, namely CD44⁺/CD24^{-low} subgroup, ALDH1+ subgroup and non-BCSCs (ALDH1-) subgroup, with five NOD/SCID mice in each subgroup. The CD44⁺/CD24^{-low} subgroup, ALDH1+ subgroup and non-BCSCs (ALDH1-) subgroup were inoculated with selected CD44⁺/CD24^{-low}

cells, ALDH1+ cells and non-BCSCs (ALDH1-) cells, respectively. The NOD/SCID mouse xenografted experiment was performed with reference to Al-Hajj (3) and Ginestier (11). After collecting well-growing cells from each group, single-cell suspensions were prepared with PBS, with the cell concentration adjusted to 1×10⁶/mL. The cells of each group were mixed with Matrigel matrix at a volume ratio of 1:1, with the final cell concentration of 0.5×10⁶/mL. The number of cells inoculated in each group was set at 0.5×10⁵. Each NOD/SCID mouse was inoculated with 0.1 mL mixture into the subcutaneous fat pad of the forechest using a 1-mL medical syringe for 8 weeks under standard conditions. Once a week, the inoculated sites of mice were examined by touching, and the longest diameter of the tumors was measured and recorded. The mice were sacrificed by dislocation of the cervical spine 8 weeks later.

Statistical procedures

All data in this study was expressed as mean ± SD. T test was used for the comparison of two independent samples. All statistical analyses were performed by SPSS (IBM SPSS Statistics, Chicago, IL, US). All statistical analyses were 2-sided and P<0.05 was defined as statistically significant.

Results

Proportion of CD44⁺/CD24^{-low} and ALDH1+ BCSCs in four molecular subtypes of breast cancer after flow cytometry

Primary cells of Luminal A, Luminal B, HER2 overexpression and triple-negative subtypes were successfully cultured, and CD44⁺/CD24^{-low} and ALDH1+ BCSCs were sorted using flow cytometry. The proportion of CD44⁺/CD24^{-low} and ALDH1+ BCSCs in four molecular subtypes of breast cancer is shown in *Figures 1,2* and *Table 1*.

Comparison between MS formation ability of CD44⁺/CD24^{-low} and that of ALDH1+ BCSCs in different molecular subtypes of breast cancer

Primary breast cancer cells of each molecular subtype were successfully obtained after mechanical and chemical treatments. After 2–4 days of culture in a serum-free medium, the cells began to divide and proliferate, and a small amount of MSs appeared within 5–6 days. After 7–10 days, typical mature MSs could be observed, consisting of hundreds of cells. On continuing the serum-free suspension culture, it was observed that the MSs no longer significantly

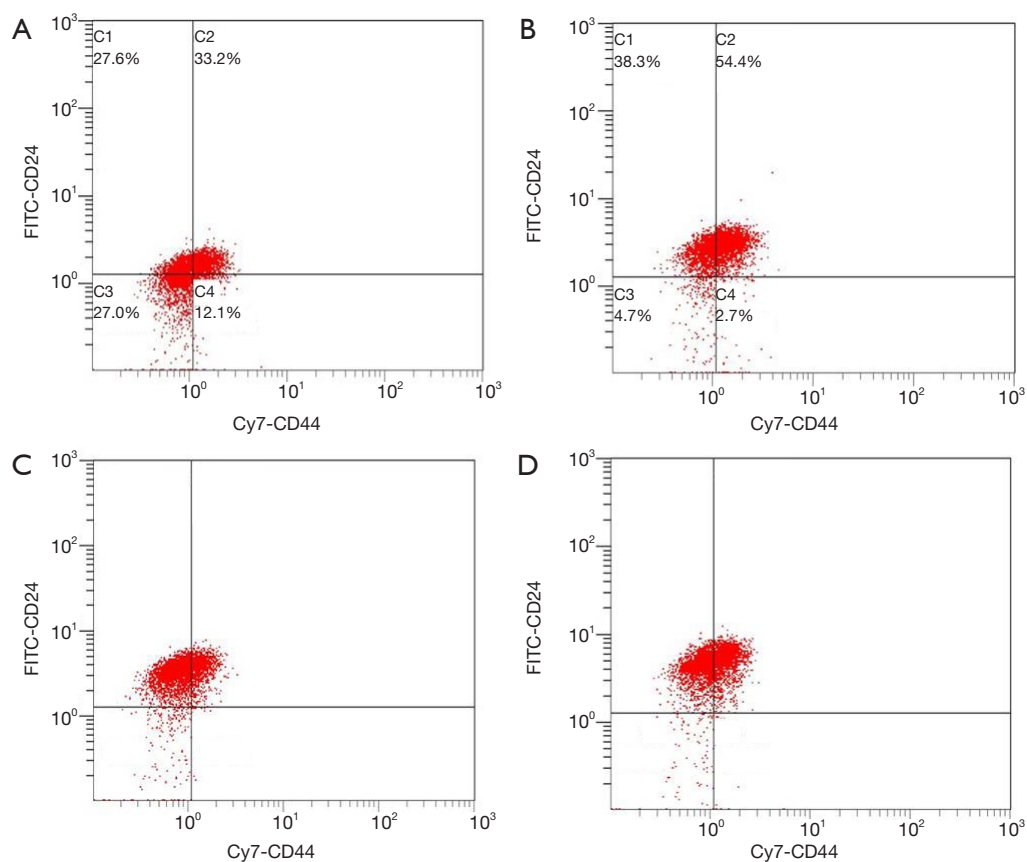


Figure 1 Subpopulations of breast cancer cells defined by the expression of the stem cell markers, CD44 and CD24, using flow cytometry. (A) Luminal A subtype; (B) Luminal B subtype; (C) HER2 overexpression subtype; (D) triple-negative subtype. HER2, human epidermal receptor-2.

increased, and most MSs underwent disintegration after 2 weeks. The CD44⁺/CD24^{-low} and ALDH1⁺ BCSCs of four molecular subtypes formed MSs within 2–3 days of serum-free culture, with a higher number of cells and larger MS diameters. Typically, MSs could be observed within 6–7 days, and the morphology remained stable after 2 weeks of culture. The non-BCSCs from four molecular subtypes could not form MS in the serum-free culture medium. The number of MSs was calculated after 7 days of culture. As shown in *Figure 3*, in the four molecular subtypes, the ALDH1⁺ BCSCs formed more MSs than those formed by CD44⁺/CD24^{-low} BCSCs.

Comparison between the tumorigenic ability of CD44⁺/CD24^{-low} and that of ALDH1⁺ BCSCs in different molecular subtypes of breast cancer

The growth curves of grafted tumors in the three groups

of cells of each molecular subtype were shown in *Figure 4*. After 8 weeks of observation, the non-BCSC groups did not form grafted tumor in the four subtypes. On the eighth week, in the four subtypes of breast cancer, ALDH1⁺ BCSC showed larger tumor diameters than CD44⁺/CD24^{-low} BCSC. The diameters of the grafted tumors of ALDH1⁺ and CD44⁺/CD24^{-low} BCSCs were 0.67±0.04 vs. 0.56±0.04 cm, P=0.002 (Luminal A subtype); 0.68±0.05 vs. 0.50±0.04 cm, P<0.001 (Luminal B subtype); 1.18±0.03 vs. 0.85±0.05 cm, P=0.001 (HER2 overexpression subtype); 1.28±0.03 vs. 0.95±0.05 cm, P=0.001 (triple-negative subtype).

Discussion

BCSCs retain certain key properties of stem cells, including self-renewal that initiates and drives tumorigenesis as well as differentiation that contributes to tumor heterogeneity (22). BCSCs are also heterogeneous and are cells with different

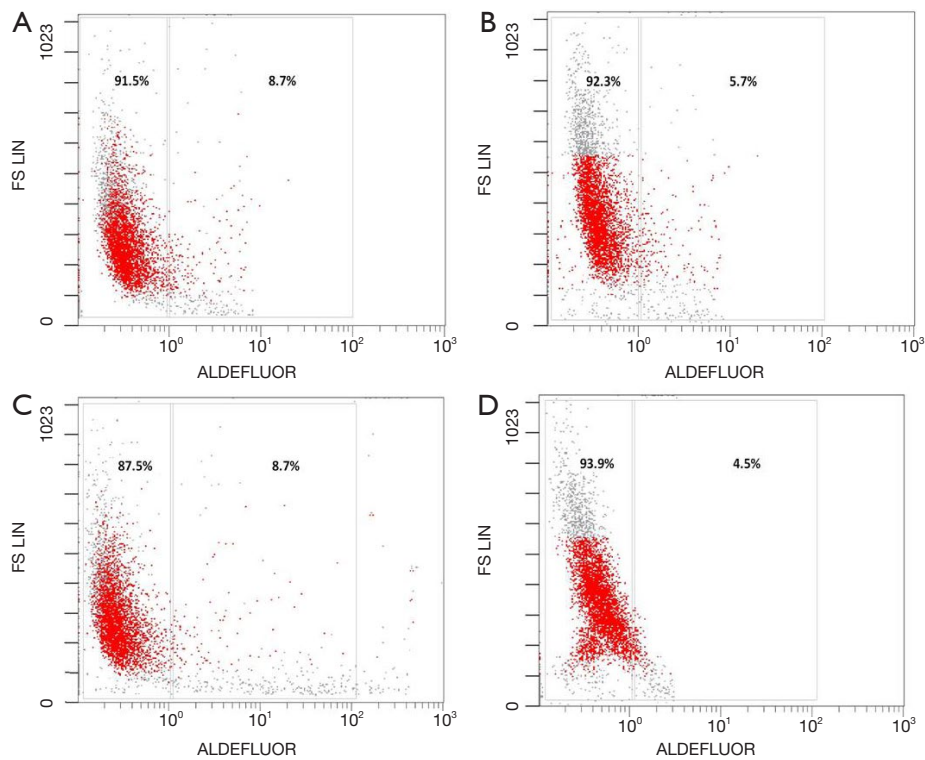


Figure 2 Subpopulations of breast cancer cells defined by the expression of the stem cell marker ALDH1 using flow cytometry. (A) Luminal A subtype; (B) Luminal B subtype; (C) HER2 overexpression subtype; (D) triple-negative subtype. ALDH1, Acetaldehyde dehydrogenase 1; HER2, human epidermal receptor-2.

Table 1 Proportion of CD44⁺/CD24^{-low} and ALDH1⁺ BCSCs in four molecular subtypes of breast cancer after flow cytometry

Subtypes	ALDH1 ⁺ (%)	CD44 ⁺ /CD24 ^{-low} (%)
Luminal A	8.7	12.1
Luminal B	5.7	2.7
HER2 overexpression	8.7	0.8
Triple-negative	4.5	0.7

BCSC, breast cancer stem cell.

phenotypes that share the properties of stem cells. Other studies (4,23) have corroborated the findings reported by Al-Hajj *et al.* (3) that CD44⁺/CD24^{-low} breast cancer cells had the properties of BCSC. In 2007, Ginestier *et al.* confirmed that ALDH1⁺ cells had the properties of BCSC (11). Both CD44⁺/CD24^{-low} and ALDH1⁺ phenotypes could be used as universal phenotypes of BCSC and have been used across several studies conducted on BCSC. However, the stem cell-like characteristics of the two phenotypes may be different, and there were few related studies.

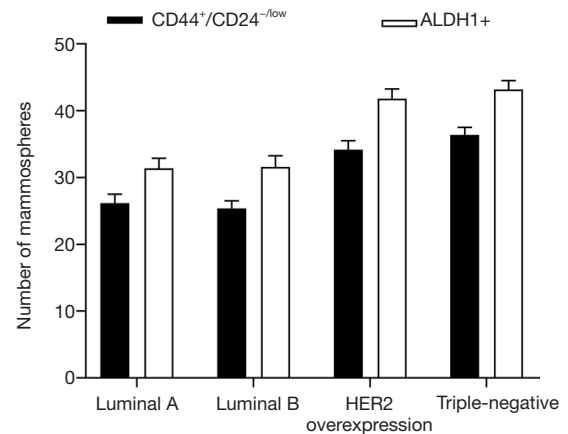


Figure 3 Mammospheres formed by ALDH1⁺ and CD44⁺/CD24^{-low} BCSCs in different molecular subtypes. Luminal A subtype, 31.40 ± 1.52 vs. 26.20 ± 1.30, P < 0.001; Luminal B subtype, 31.60 ± 1.67 vs. 25.40 ± 1.14, P < 0.001; HER2 overexpression subtype, 41.80 ± 1.48 vs. 34.20 ± 1.30, P < 0.001; Triple-negative subtype, 43.20 ± 1.30 vs. 36.40 ± 1.14, P < 0.001. Statistical analyses were performed using t-test, with P < 0.05 denoting statistical significance. ALDH1, Acetaldehyde dehydrogenase 1; BCSC, breast cancer stem cell; HER2, human epidermal receptor-2.

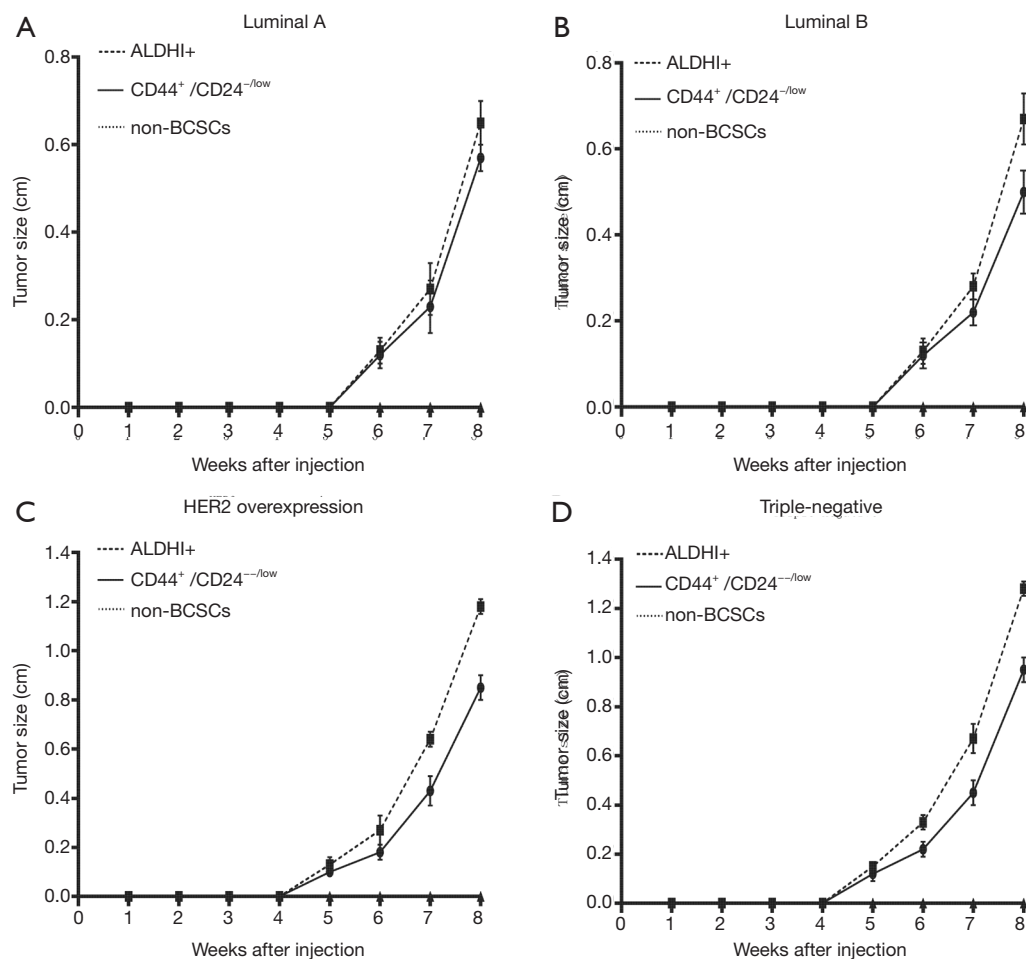


Figure 4 Comparison of the tumorigenic ability in NOD/SCID mice between ALDH1⁺ and CD44⁺/CD24^{-low} BCSCs. On the eighth week, the diameters of the grafted tumors of ALDH1⁺ and CD44⁺/CD24^{-low} BCSCs were 0.67±0.04 vs. 0.56±0.04 cm, P=0.002 (Luminal A subtype); 0.68±0.05 vs. 0.50±0.04 cm, P<0.001 (Luminal B subtype); 1.18±0.03 vs. 0.85±0.05 cm, P=0.001 (HER2 overexpression subtype); 1.28±0.03 vs. 0.95±0.05 cm, P=0.001 (triple-negative subtype). Statistical analyses were performed using *t*-test, with P<0.05 denoting statistical significance. ALDH1, Acetaldehyde dehydrogenase 1; BCSC, breast cancer stem cell; HER2, human epidermal receptor-2.

In this study, tumor tissues from Luminal A, Luminal B, HER2 overexpression, triple-negative subtypes of breast cancer were obtained; primary breast cancer cells were successfully cultured via serum-free suspension culture; and CD44⁺/CD24^{-low} BCSCs, ALDH1⁺ BCSCs and non-BCSCs were sorted using flow cytometry. This result suggested that BCSCs of different phenotypes exist in breast cancer, and CD44⁺/CD24^{-low} and ALDH1⁺ phenotypes could be used as reliable markers for identifying BCSC. In different molecular subtypes of breast cancer, the proportions of CD44⁺/CD24^{-low} and ALDH1⁺ BCSCs were 0.7% to 12.1% and 4.5% to 8.7%, respectively, which was consistent with those reported in other studies (0.6% to

12%) (4,11).

In this study, three phenotypes of cells were sorted, namely ALDH1⁺ BCSC, CD44⁺/CD24^{-low} BCSC and non-BCSC. The self-renewal and tumorigenic abilities of BCSCs were compared by performing MS formation and NOD/SCID mouse xenograft experiments. In the four molecular subtypes of breast cancer, non-BCSCs did not form MS in the MS formation experiment, and no grafted tumor was formed in the subcutaneous fat pad of NOD/SCID mice, which was consistent with the study conducted by Ginestier (11). In the MS formation experiment, the number of MS formed by ALDH1⁺ BCSCs in each molecular subtype was significantly higher than that formed

by CD44⁺/CD24^{-low} BCSCs (P<0.001). In the NOD/SCID mouse xenograft experiment, the diameters of tumors formed by ALDH1+ BCSCs in the four molecular subtypes were significantly larger than those formed by CD44⁺/CD24^{-low} BCSCs (P<0.05). The above results suggested that ALDH1+ BCSCs may be more important in the occurrence, development, and metastasis of breast cancer, and that it is crucial to study the underlying molecular mechanism and develop corresponding drugs to improve the survival of breast cancer patients

This study has some limitations. Only four fresh breast cancer tissue samples, including Luminal A, Luminal B, HER2 overexpression, and triple-negative subtypes, could be obtained. The self-renewal and tumorigenic abilities of BCSCs of different molecular subtypes were preliminarily explored; However, the results still need to be verified by conducting further studies with a larger sample size.

Conclusions

This study demonstrated that ALDH1+ BCSC had better self-renewal and tumorigenic abilities than CD44⁺/CD24^{-low} BCSC in the four molecular subtypes of breast cancer.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2020.01.53>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of Sichuan Provincial People's Hospital in China, human samples were collected after obtaining informed written consent from all participants.

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