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Research article

CD44 AND CD24 CANNOT ACT AS CANCER STEM CELL MARKERS IN HUMAN LUNG ADENOCARCINOMA CELL LINE A549

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Abstract: Cancer stem cells (CSCs) are subpopulations of tumor cells that are responsible for tumor initiation, maintenance and metastasis. Recent studies suggested that lung cancer arises from CSCs. In this study, the expression of potential CSC markers in cell line A549 was evaluated. We applied flow cytometry to assess the expression of putative stem cell markers, including aldehyde dehydrogenase 1 (ALDH1), CD24, CD44, CD133 and ABCG2. Cells were then sorted according to the expression of CD44 and CD24 markers by fluorescence-activated cell sorting (FACS) Aria II and characterized using their

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Abbreviations used: ABC – ATP-binding cassette; ADC – adenocarcinoma; ALDH1 – aldehyde dehydrogenase 1; bFGF – basic fibroblast growth factor; CFU – colony-forming unit; CSCs – cancer stem cells; DEAB – diethylaminobenzaldehyde; EGF – epidermal growth factor; GFs – growth factors; LCC – large-cell carcinoma; NSCLC – non-small cell lung carcinoma; SCC – squamous-cell carcinoma; SP – side population

clonogenic and sphere-forming capacity. A549 cells expressed the CSC markers CD44 and CD24 at 68.16% and 54.46%, respectively. The expression of the putative CSC marker ALDH1 was 4.20%, whereas the expression of ABCG2 and CD133 was 0.93%. Double-positive CD44/133 populations were rare. $CD44^{+}/24^{+}$ and $CD44^{+}/CD24^{-/low}$ subpopulations respectively exhibited 64% and 27.92% expression. The colony-forming potentials in the $CD44^{+}/CD24^{+}$ and $CD44^{+}/CD24^{-/low}$ subpopulations were 84.37 ± 2.86% and 90 ± 3.06%, respectively, while the parental A549 cells yielded 56.65 ± 2.33% using the colony-formation assay. Both isolated subpopulations formed spheres in serum-free medium supplemented with basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). CD44 and CD24 cannot be considered potential markers for isolating lung CSCs in cell line A549, but further investigation using *in vivo* assays is required.

Key words: Cancer stem cells, Lung cancer, Cell line A549, Colony-formation assay, Sphere-formation assay, CD44, CD24, CD133, ALDH1, ABCG-2

INTRODUCTION

Non-small cell lung carcinoma (NSCLC), the worldwide leading cause of cancer-related death, comprises heterogeneous histological types, including squamous-cell carcinoma (SCC) and adenocarcinoma (ADC), which occur with similar frequencies (30-40% each), and large-cell carcinoma (LCC), which occurs with a lower frequency (< 10%) [1]. Increasing evidence indicates the existence of a subpopulation of tumor cells termed cancer stem cells (CSCs), which may be produced by transformation of normal stem cells or by multistep dedifferentiation of specialized progenitor cells through a progressive accumulation of genetic aberrations [2]. CSCs display the ability of self-renewal, multilineage differentiation, high telomerase and ABC transporter expression, and resistance to apoptosis and conventional chemotherapy [3, 4].

The high mortality and morbidity of lung cancer means that there is an urgent need to study the process of lung tumorigenesis. Understanding the biology of lung CSCs may help to identify the causes of failure in lung cancer treatment. Conventional chemotherapeutic drugs or radiotherapy for lung cancer eliminate the bulk of tumor cells, but they cannot eradicate CSCs. Residual lung CSCs can regenerate tumor cells leading to tumor recurrence after therapy [1, 5, 6].

The recent identification of characteristic markers or combinations of markers for lung CSCs has led to a deeper understanding of the biology of lung tumorigenesis. There is no consensus on a ubiquitous characteristic marker or markers for lung CSCs, mainly due to the heterogeneity of lung cancer. Isolation of lung CSCs has also proven to be a major challenge due to the lack of universal morphological characteristics and marker expression. Many studies have attempted to identify lung CSCs based on the expression of the CD133 antigen, which is a five-transmembrane glycoprotein with an unknown function.

It was reported as the first lung CSC marker in cell lines or samples from patients [7-9]. A recent attempt to identify lung CSCs has suggested that tumorogenic properties of lung cancer $CD133^+$ cells depend on *oct-4*, a protein that is also involved in embryonic stem cell development [10].

CD44, a multifunctional class 1 transmembrane glycoprotein, is another proposed marker for most solid tumor stem cells. It is used alone or in combination with other putative CSC markers such as CD24 [11-13] in identifying tumors. Previous studies have introduced $CD44^+/CD24^{-/low}$ as a CSC subpopulation in breast [11] and prostate [14] cancers and $CD44^+/CD24^+$ linage cells known as the CSC subpopulation in colorectal cancer [15]. Although previous studies have demonstrated that CD133 and CD44 are the most robust markers for lung CSCs, their co-expression has not yet been evaluated in lung cancer.

CSCs can also be identified or isolated using the activity of aldehyde dehydrogenase 1 (ALDH1), which is involved in the detoxification of intracellular aldehydes, oxidation of retinol to retinoic acid, and early stem cell differentiation [16]. Some recent studies demonstrated that $ALDH1^+$ lung cancer cells display *in vitro* and *in vivo* CSC features, so ALDH1is identified as a lung cancer stem cell-associated marker [17, 18].

Finally, CSCs have the ability to pump out a wide range of endogenous and exogenous compounds that depend on the expression of ABC (ATP-binding cassette) transporters, including ABCG2. Although the physiological functions of ABCG2 in stem cells have yet to be identified, it is widely distributed in normal tissues and is highly expressed in a subpopulation of cells that are enriched in the stem-like cells that are known as side populations (SP) [19]. In a recent study, SP isolated from lung cancer cell lines exhibited higher invasiveness, higher resistance to chemotherapeutic drugs and higher tumorigenicity *in vivo* than non-SP cells [20].

Despite the identification of a large number of risk factors involved in lung cancer, the incidence of the most common subtype of lung cancer, lung adenocarcinoma, is increasing, so the evaluation of critical aspects of cellular and molecular pathogenesis of adenocarcinoma is essential [21]. Previous studies showed the presence of CSCs in cell line A549, a human lung adenocarcinoma epithelial cell line [20, 22], but no definite data on putative CSC markers are available. Therefore, in this study, we used flow cytometry to examine the expression of potential CSC markers, including aldehyde dehydrogenase 1 (ALDH1), CD24, CD44, CD133 and ABCG2, in cell line A549. Based on these flow cytometric profiles, the importance of the $CD44^+/CD24^{-/low}$ and $CD44^+/CD24^+$ subpopulations in the most common epithelial cancers, and the lack of comprehensive data in lung cancer, we then focused on isolating $CD44^+/CD24^{-/low}$ and $CD44^+/CD24^+$ subpopulations from cell line A549 and investigating their potential for colony- and sphere-formation assays.

MATERIALS AND METHODS

Cell Culture

Lung cancer cell line A549 was obtained from the Iranian Biological Resource Center and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen) supplemented with 10% fetal calf serum (FCS; Gibco, Invitrogen), 2 mM L-glutamine, 2 mM non-essential amino acid, 100 U/ml penicillin, and 100 µg/ml streptomycin in a cell culture incubator at 37ºC with 5% CO_2.

Immunophenotyping of A549 cells

Adherent growing A549 cells were detached using 0.02% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS), counted, and washed in 0.05% bovine serum albumin (BSA) in PBS. At least 1×10^5 cells were incubated with 1 mg/ml of fluorescently labeled monoclonal antibodies or respective isotype controls at 4-8ºC for 20 min in the dark. After washing, the labeled cells were analyzed by flow cytometry using fluorescence-activated cell sorting (FACS) Aria II (Becton & Dickinson).

The following antibodies were used in flow cytometry: mouse anti-human CD133 PE-conjugated; mouse anti-human CD24 FITC-conjugated; mouse anti-human CD44 PE-conjugated; and mouse anti-human ABCG2 PE-conjugated (all from Dako). All data were analyzed using CellQuest software (Becton & Dickinson).

Cell sorting of $CD44^+/CD24^+$ and $CD44^+/CD24^{-/low}$ A549 populations

Single-cell suspensions from cell line A549 were prepared as described above. Cells were double stained with PE-conjugated anti-CD44 antibody and FITC-conjugated anti-CD24 antibody, and sorted with FACS Aria II. The cut-off point was the co-expression of CD44 and CD24 at high levels and the weak or absent expression of CD24 in conjunction with high expression of CD44. A small amount of cells were reanalyzed for $CD44^+/CD24^+$ and $CD44^+/CD24^{-/low}$ purity (Fig. 1).

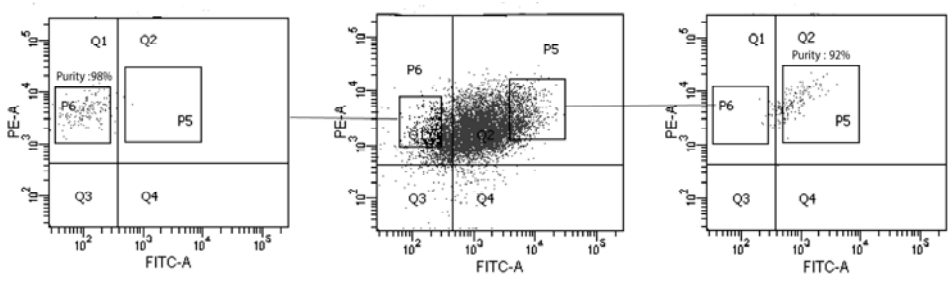

Fig. 1. Lung cancer cell line A549 was sorted by expression of CD44/CD24 using FACS Aria II (middle panel). The purity of $CD44^+/CD24^+$ (right panel) and $CD44^+/CD24^{-/low}$ (left panel) was assessed post sorting.

ALDH1 labeling

ALDH1 activity was evaluated using the Aldefluor kit (Stem Cell Technologies, Vancouver). Cells were suspended in assay buffer, incubated with the ALDH substrate Aldefluor or with diethylaminobenzaldehyde (DEAB) as the ALDH inhibitor for 30 min at 37ºC, and washed with assay buffer. Cells incubated with DEAB were used to establish the baseline fluorescence (R1) and to determine the $ALDH1^+$ region (R2). Incubation of cells with the substrate in the absence of DEAB induced a shift in the ALDH substrate Aldefluor, defining the Aldefluor-positive population.

Colony-formation assay

Based on the biological and morphological features of colonies, defined in an earlier study [23], three types of colony were found here: holoclone, meroclone and paraclone. Holoclones are large colonies with small compact cells and homogenous patterns of cell morphology, while paraclones are fragmented structures with fully differentiated and aborted cells. Meroclones contain heterogeneous cell populations and have an intermediate size and structure between holoclones and paraclones. Holoclones have higher growth potential than meroclones and paraclones [23]. Similarly to stem cells in keratinocyte cell lines, the CSCs of prostate cancer [24], glioma [25] and pancreatic cancer [26] displayed all three types of colony, with the holoclone fraction being enriched in CSCs.

The colony-formation assay was performed to determine the clonogenicity and regeneration ability of single cells. An equal number of viable cells from A549 parental cells and each population ($CD44^+/CD24^+$, $CD44^+/CD24^{-/low}$) were seeded in DMEM supplemented with 10% FCS at a density of 80 single cells per well in six-well culture plates. After 10 days, the number of colonies with more than 50 cells adhering to the bottoms of the plates was counted using an Olympus-IX71 fluorescent microscope (Olympus Inc.). Colony-forming efficiency was calculated for 10 days of culture by dividing the number of colonies by the cell number plated and multiplying by 100 [27].

Sphere-formation assay

Due to the self-renewal and differentiation properties of CSCs, these cells are cultured as spheres *in vitro* under non-adherent and serum-free medium in the presence of certain amount of growth factors [28]. To evaluate sphere-formation potential, single cells at a density of 3×10^4 from A549 parental cells and each population ($CD44^+/CD24^+$ and $CD44^+/CD24^{-/low}$) were plated in DMEM serum-free medium supplemented with human epidermal growth factor (EGF; 20 ng/ml) and human basic fibroblast growth factor (bFGF; 10 ng/ml) in six-well ultra-low attachment plates (Corning Inc.). Moreover, sphere-formation was examined in A549 parental cells in the absence of human EGF and bFGF. Cells were incubated in a humidified atmosphere at 37ºC with 5% CO_2. After 48-72 h,

sphere structures from 150 μm to nearly 1000 μm in size [28, 29] were counted using an Olympus-IX71 fluorescent microscope.

Statistical analysis

The data are presented as the means ± SD of at least three different experiments. Student's t-test was performed to evaluate the difference between the mean values. A value of $p \leq 0.05$ was considered statistically significant.

RESULTS

Analysis of putative CSC markers in cell line A549 using flow cytometry

In order to investigate the CSC populations, the expressions of ALDH1, CD24, CD44, CD133 and ABCG2 were evaluated using single-cell suspensions of A549 cells. As shown in Table 1, ALDH1 was assumed to be a potential CSC marker in cell line A549, but DEAB (an inhibitor of ALDH1 activity) could not be considered an efficient inhibitor of the expression of ALDH1 in the control tube. We failed to improve the results by increasing the amount of the inhibitor 2-fold (Fig. 2). Therefore, we suggest that DEAB cannot completely block the expression of ALDH1 in A549 cells, making it difficult to sort the cells. The brightly fluorescence of the ALDH1-expressing cells ($ALDH1^+$ cells) faded quickly, meaning it was impossible to detect using FACS Aria II.

The expression of the proposed CSC markers CD44, CD24, ABCG2 and CD133 varied from 1% to 70% (Table 1). Our findings showed that the expressions of CD44 and CD24 were 68.16 ± 0.8% and 54.46 ± 1%, respectively. Furthermore, $ABCG2^+$ and $CD133^+$ cells were always less than 1% of the total population (Table 1).

Table 1. The expression of putative cancer stem cell markers in cell line A549.

Putative marker	Mean percentage of positive cells (range)
CD24	54.46 ± 1% (53.8-55.6)
CD44	68.16 ± 0.8% (67.5-69)
CD133	0.94 ± 0.05% (0.9-1.01)
ABCG2	0.91 ± 0.04% (0.86-0.95)
ALDH1	4.20 ± 0.7% (3.53-4.9)
$CD44^+/CD24^+$	64.1 ± 2.02 (62.6-66.4)
$CD44^+/CD24^{-/low}$	27.92 ± 3.63% (24.1-31.9)
$CD44^-/CD24^{-/low}$	1.68 ± 0.69% (1-2.51)
$CD44^-/CD24^+$	0.2 ± 0.3% (0-0.7)
$CD44^+/CD133^+$	0.4 ± 0.1% (0.3-0.5)
$CD44^+/CD133^-$	0.07 ± 0.1% (0-0.2)
$CD44^-/CD133^-$	26.14 ± 0.9% (25.12-27)
$CD44^-/CD133^+$	72.23 ± 0.68% (71.7-73)

In the next step, the dual expressions of CD44/CD24 and CD44/CD133 were analyzed in cell line A549. Our results revealed that $CD44^{+}/CD24^{+}$ expressed in 64 ± 2.02% and $CD44^{+}/CD24^{-/low}$ in 27.92 ± 3.63% of the cells. The dual expression of CD133 with CD44 was not frequently found and was under the limitation of the cell sorter (Table 1). Therefore, the $CD44^{+}/CD24^{+}$ and $CD44^{+}/CD24^{-/low}$ subpopulations were chosen for further analysis.

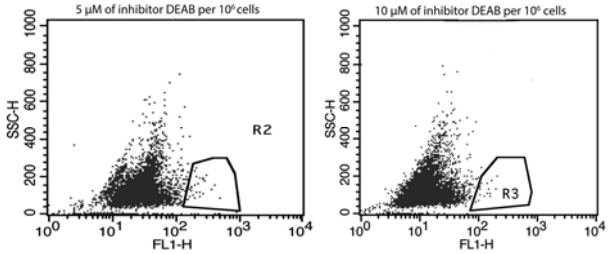

Fig. 2. A549 cells expressed the ALDH1 marker in the range of 3.53 to 4.9%. Increasing the content of the inhibitor DEAB 2-fold did not block the expression of ALDH1 in the control tube.

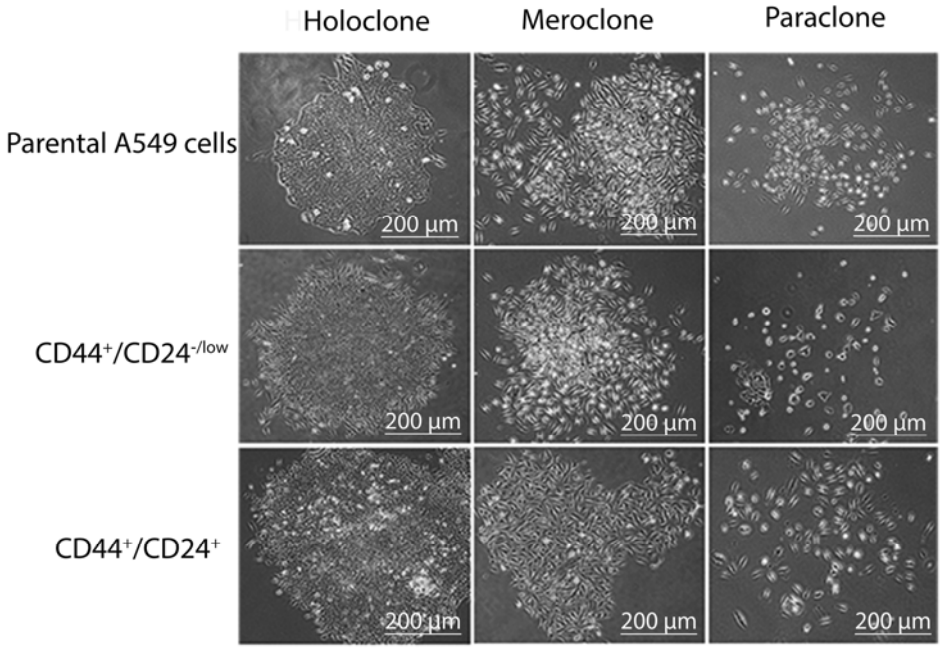

Fig. 3. The morphology of the three colony types identified during the colony-formation assay of parental A549 cells and $CD44^{+}/CD24^{-/low}$ and $CD44^{+}/CD24^{+}$ subpopulations.

Characterization of $CD44^+/CD24^+$ and $CD44^+/CD24^{-/low}$ subpopulations

Colony- and sphere-formation assays were performed to characterize the sorted cells. After plating the parental A549 cells and $CD44^+/CD24^+$ and $CD44^+/CD24^{-/low}$ subpopulations at clonal densities similar to those for keratinocyte cell lines [23], three types of colony formed: holoclone, meroclone and paraclone (Fig. 3). Although the formation rates of the meroclone and paraclone colonies were significantly lower in the case of the $CD44^+/CD24^{-/low}$ and $CD44^+/CD24^+$ cells than in the case of the parental A549 cells (Table 2A; $p = 0.03$), the parental cells generated more meroclone and paraclone than holoclone colonies and more than the $CD44^+/CD24^+$ and $CD44^+/CD24^{-/low}$ cells (Table 2A; $p = 0.04$).

The colony-forming potentials for parental A549 cells and $CD44^+/CD24^+$ and $CD44^+/CD24^{-/low}$ subpopulations were 56 ± 2.33%, 84 ± 2.86% and 90 ± 3.06%, respectively (Table 2), but there was no significant difference in the colony-forming potential between the three cell types ($p > 0.05$). Our findings indicated that the sphere-formation efficiencies for the two isolated $CD44^+/CD24^+$ and $CD44^+/CD24^{-/low}$ subpopulations were 0.05 ± 0.01% and 0.05 ± 0.02%, respectively, and they had similar size (Table 2B, Fig. 4). The A549 cell sphere-formation efficiencies in the presence and absence of human EGF and bFGF were 0.5 ± 0.04% and 0.9 ± 0.04%, respectively (Table 2B, Fig. 4).

Table 2. Comparison of the *in vitro* tumorigenicity of parental A549 cells and $CD44^+/CD24^{-/low}$ and $CD44^+/CD24^+$ subpopulations. A – Percentages of colonies formed from parental A549 cells and $CD44^+/CD24^+$ and $CD44^+/CD24^{-/low}$ subpopulations shown as the mean percentage of colonies ± SD. CFU: Colony-forming unit. B – Percentages of spheres formed from parental A549 cells with or without growth factors (GFs), and from $CD44^+/CD24^+$ and $CD44^+/CD24^{-/low}$ subpopulations shown as the mean percentage of spheres ± SD.

A

Populations	CFU	Holoclone	Meroclone	Paraclone
Parental A549	56.65 ± 2.33%	13.75 ± 2.44%	50.15 ± 3.78%	35.79 ± 4.3%
$CD44^+/CD24^{-/low}$	90 ± 3.06%	60.35 ± 4.91%	33.66 ± 3.95%	5.98 ± 1.16%
$CD44^+/CD24^+$	84.37 ± 2.86%	78.83 ± 6.05%	18.62 ± 5.03%	2.53 ± 2.69%

B

Populations	Number of spheres	Mean Size
Parental A549 with GFs	0.5 ± 0.03%	300 ± 4 μm
Parental A549 without GFs	0.9 ± 0.02%	375 ± 6 μm
$CD44^+/CD24^{-/low}$	0.055 ± 0.01%	250 ± 5 μm
$CD44^+/CD24^+$	0.05 ± 0.01%	245 ± 3 μm

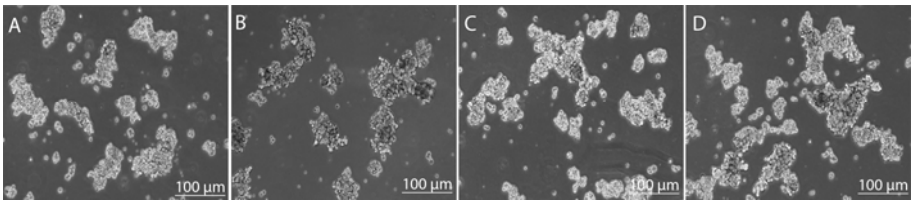

Fig. 4. Tumor spheres were formed by A549 lung cancer cells with (A) and without (B) growth factors (GFs), and by $CD44^{+}/CD24^{-/low}$ (C) and $CD44^{+}/CD24^{+}$ (D) subpopulations isolated from A549 cells.

DISCUSSION

Increasing evidence supports the notion that the initiation, metastasis and recurrence of tumors originate from a subpopulation of tumor cells known as CSCs [2, 3]. These cells are defined with unique characteristics, including self-renewal, differentiation, and resistance to apoptosis and conventional radio- and chemotherapy [2, 3]. Currently, the most widely applied methods for isolating CSCs are flow cytometric analysis and fluorescence-activated cell sorting (FACS), which separate labeled versus unlabeled populations of cells [2-4, 30]. Using cell surface markers, CSCs have been identified and characterized in various malignancies, including breast [11], prostate [14], colorectal [15] and brain [31] cancers.

There is no consensus data on CSC markers in the lung adenocarcinoma cell line A549, so this study was performed to investigate CSC subpopulations in this cell line. We examined the expression profiles of putative CSC markers, including ALDH1, CD24, CD44, CD133 and ABCG-2 in cell line A549 using flow cytometry. $CD44^{+}/CD24^{+}$ and $CD44^{+}/CD24^{-/low}$ subpopulations derived from cell line A549 were characterized using their colony- and sphere-formation potential. CD133 is the most commonly reported cell surface marker that has been used to isolate CSCs from lung cancer cell lines and fresh tumor samples, but there is no consensus on the level of CD133 expression in cell line A549 [7, 12, 32-36]. Our data demonstrate that 0.94% of A549 cells expressed the CD133 marker. Although some recent studies reported expressions between 1.4% and 3.64% for A549 cells [34-36], other studies showed only 0.2% [7] or no expression of CD133 [12]. The differences in expression of CD133 suggest that this protein can be controlled at multiple steps, including transcriptional regulation, alternative transcription initiation sites, alternative splicing, and post-translational modifications [37, 38]. Changes in each of these processes could possibly influence the level of CD133 expression.

Here, we showed that ABCG2, another marker of CSCs, was expressed in 0.98% of cells, while no other study evaluated expression of ABCG2 in A549 cells. Moreover, 4.2% of A549 cells expressed ALDH1, which was higher than the value given other reports demonstrating its expression (about 2.55%) [34].

The Aldefluor reaction was performed in an intact cellular membrane; variations in viable cells, incubation time, and temperature could give rise to different expression levels.

We also assessed the expression of the CD44 and CD24 potential CSC markers in A549 lung cancer cells. Our results indicated that the average levels of expression of CD24 and CD44 markers were 56% and 69%, respectively. In accordance with our findings, in a study on NCI60 tumor cell lines panel, the expressions of CD44 and CD24 markers in A549 cells were high [34], while an independent study [12] indicated that cell line A549 did not express the CD44 antigen. We are unable to explain the wide variation in the level of CD44 expression in A549 cells, but a growing body of studies demonstrated that a CD44-high subpopulation may represent lung CSCs [12, 39].

CD44/CD24 expression has been identified as a CSC marker in many cancers including breast [11], prostate [14] and colorectal cancer [15]. In our study, the average expression of $CD44^{+}/CD24^{-/low}$ markers was 37.68%, whereas previous studies indicated that the expression of $CD44^{+}/CD24^{-/low}$ in A549 cells was much lower [33, 34]. No data on $CD44^{+}/CD24^{+}$ are available in the literature for comparison.

Our results showed that both $CD44^{+}/CD24^{+}$ and $CD44^{+}/CD24^{-/low}$ subpopulations were capable of forming holoclones, meroclones and paraclones in equal numbers. More importantly, the two subpopulations sustained cell proliferation in non-adherent, serum-free DMEM supplemented with bFGF and EGF, and formed spheres in similar amounts. We also found that A549 cells were even able to generate spheres in the absence of bFGF and EGF.

Recent studies demonstrated that the results of the sphere-formation assay do not correspond to *in vivo* stem cell frequency, and sphere size does not correspond to *in vivo* stem cell number. On the other hand, the cells have differentiation potential bias due to culture with exogenous growth factors [28]. There is currently no consensus on the use of growth factors in the sphere-formation assay [28, 40-42].

The results of our *in vitro* studies demonstrate that CD44 and CD24 may not suitable markers for cell line A549 because the sorted CD44/24 subpopulation showed no significant differences in terms of the colony- and sphere-formation potential compared to A549 cells. However, the ultimate test of tumorigenicity is transplantation *in vivo*. Therefore, these experiments need to be complemented with an *in vivo* study for tumorigenicity of these populations in NOD-SCID mice.

Although we did not examine the co-expression of CD44 and CD133 in cell line A549, the evaluation of pairs of the most common of lung CSC markers helps to identify new populations. The main reason for avoidance was the very low level of CD133 expression in the A549 cells.

Further characterization of lung CSCs, either in cell lines or tissue samples, is necessary to discover the definitive marker(s) to identify and understand the roles of CSCs in tumor biology and cancer-targeted therapies. Considerable progress has been made to identify lung CSCs, but there is still the need to fully

characterize CSCs in terms of cell surface markers. No universal cell surface antigen or combination of antigens for the purification of lung CSCs by antibody techniques has yet been identified. CD44 and CD24 surface markers may not play major roles as CSC markers in cell line A549, but further investigations with *in vivo* assays for the tumorigenicity of sorted populations in NOD-SCID mice are required.

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