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ORIGINAL ARTICLE

The subpopulation of CD44-positive cells promoted tumorigenicity and metastatic ability in lung adenocarcinoma

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Abstract

Background: Lung cancer is one of the major causes of carcinoma-related deaths in the world. Importantly, lung adenocarcinoma (LAC) is the most common type with poor outcome. However, the progressive clinical phenotype and biomolecular signature of lung cancer presenting the cancer stem-like and metastatic characteristics are still unclear.

Methods: In this study, we identified CD44 marker in lung cancers. The capabilities, including tumorigenic and migration assays, were analyzed in $CD44^{high}$ expression and $CD44^{low}$ expression subgroups. Meanwhile, the potential bio-signature and properties of lung tumor stem-like cells were further studied.

Results: The high expression of CD44 subpopulation (CD44-positive) in isolated lung cancer cells showed significantly higher abilities of tumorigenic colonies, tumor-sphere formation, and migratory properties when compared with the $CD44^{low}$ expression group. These subgroups of CD44-positive lung cancer cells further demonstrated the metastatic potential with epithelial–mesenchymal transition (EMT), as well as the high expression of *Twist* and *Snail* gene profile. Importantly, the overexpression of *Snail* with gene vector in $CD44^{low}$ expression cells further significantly promoted the properties of lung tumor stem-like cells.

Conclusion: The results of this study highlighted the role of CD44-posivite subpopulation in modulating tumor initiation and EMT-based metastatic ability of lung malignancy.

Keywords: CD44-positive cells; Epithelial–mesenchymal transition; Lung adenocarcinoma; Metastatic ability; Tumorigenicity

1. INTRODUCTION

Lung cancer is one of the major leading causes of cancer death in both men and women worldwide.[1] Based on the clinical and histological analysis, lung cancers can be divided into two major types: nonsmall cell lung cancers (NSCLC) and small cell lung cancer.[1–2] Lung adenocarcinoma (LAC) is one of the major NSCLC and carries a very dismal prognosis with a progressive relapse with complete resection followed by adjuvant chemoradiation.[3–7] Recent reports had indicated that the aggressiveness and recurrence of LAC could be produced from the persistence of cancer stem cells (CSCs) known as tumor initiating cells.[8–11] Further researches have exhibited that miRNAs are involved in regulating CSC properties in several types of malignant cancers.[12–14] However, it is still unclear whether the biomarkers or target-gene profiles may play the key function in regulating or maintaining CSCs in LAC, conferring LAC on metastasis, progression, recurrence, and resistance to clinical-oriented therapeutics.

Some studies showed the evidence that CD44, known as a multifunctional cell surface adhesion receptor, is a potential target regulating the tumorigenic properties in the malignant cancer.[15–17] Notably, the recent studies showed that CD44 may play a key role in initiating the tumorigenic stem cell properties, and promoting the metastatic characteristics with epithelial–mesenchymal transition (EMT).[18–20] Especially CD44v isoforms are CSCs markers and play a vital role in modulating the capabilities of CSCs, including tumor initiation, self-renewal, metastasis, and immunotherapy resistance.[18–22] Moreover, there are some clinical studies and observation to show that CD44 especially CD44v isoforms are key prognostic markers in various types of tumors.[20–24] Therefore, the main target of therapies is focusing on CD44 that it may destroy the CSCs population. However, there are many obstacles that remain to determine how to target CD44 as a biomarker and therapeutic treatment hubs.

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Transcriptomic and *in vivo* evidences supported that cancers possessing CSC-like properties were found to have worse prognosis.[25–27] Integration of network biology and signal pathways and axis with processing mechanics are dramatically used in cancer research community.[27] Here, we identified CD44, known as a multifunctional cell surface adhesion receptor, as a potential target modulating the stem-like characteristics of lung tumor stem-like cells (TSLCs) as well as the progressive propensity in LACs. The identification process was based on a network-signal-processing framework to translate information in the isolated CD44^{high} expression subpopulation, as well as lung-TSLCs. In addition, a series of TSLCs was developed and compared with the parental and normal counterparts to come up with concordant signatures for networks interplays. We developed scalable lung-TSLCs networks-signal-processing type to define CD44 hosting a significant role in the tuning of lung cancer with CSC properties. Notably, CD44 was in correlated synchronization with the markers of EMT, Twist and Snail. The results of this study highlighted the function of CD44-positive subpopulation in promoting tumor CSC-like initiation and EMT-based metastatic ability of TSLCs with lung malignancy.

2. METHODS

2.1. Lung cancer cell lines

The human LAC cell line A549 and H1299 were obtained from the American Type Culture Collection and grown in DMEM (Gibco, Grand Island, NY,) containing 100 units/ml penicillin, 100 μg/ml streptomycin, 4 mmol/l glutamine, and 10% fetal bovine serum (FBS; Gibco). Other primary cultivation from original patients was approved by the IRB, reviewed by Taipei Veterans General Hospital.

2.2. Sorting of CSC-like cells using flow cytometry

Cells were dissociated and washed by cooled 1× PBS, and suspended in incubation buffer (containing PBS of pH 7.2, 0.5% BSA, and 2 mmol/l of EDTA) on ice for 10 minutes for blocking. Cells were resuspended in 1 ml incubation buffer to reach a final concentration of 2 × 10^5 cells/ml before being stained with 0.5 g/ml of the following primary antibodies on ice for 30 minutes: CD44-FITC (BJ18; Biolegend). Aldefluor assay kit (Aldagen, Durham, NC) was used for the detection of aldehyde dehydrogenase (ALDH) activity according to the manufacturer's instructions. Data were collected by the FACSCalibur flow

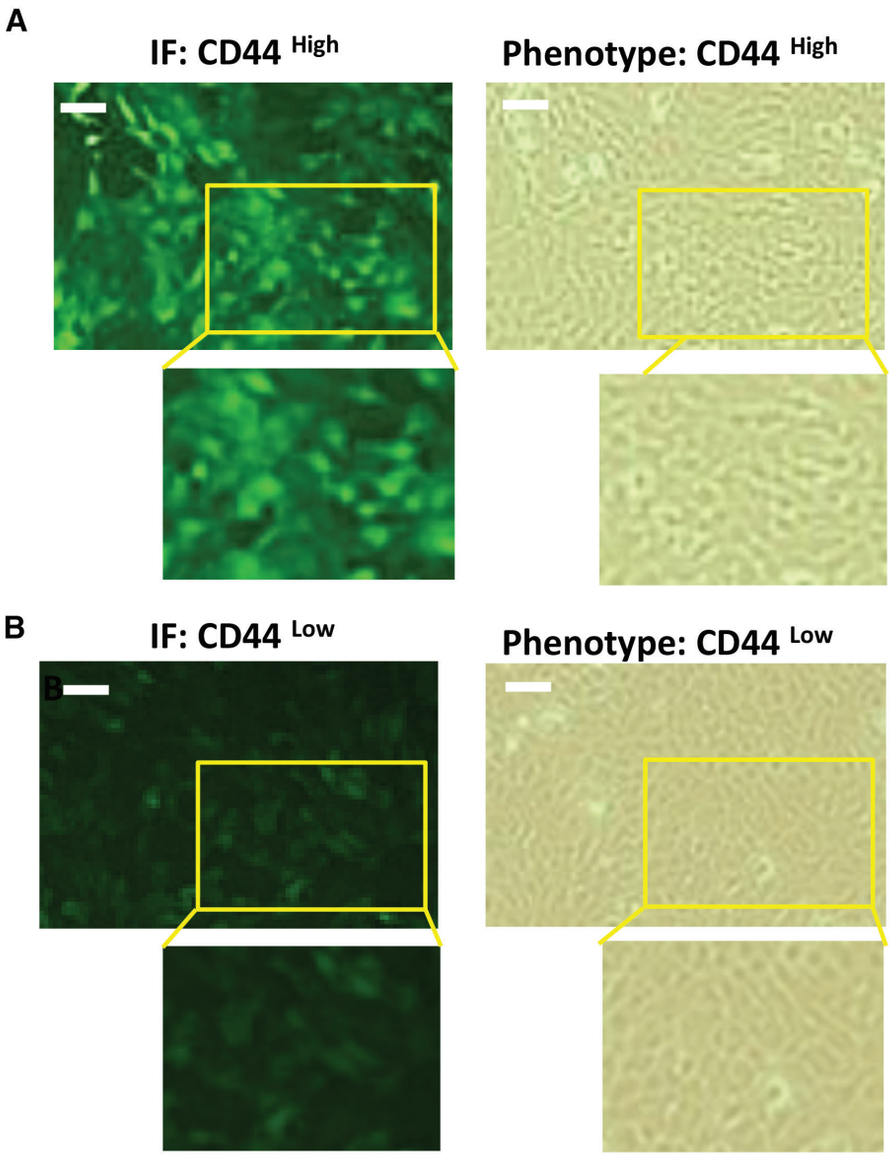

Fig. 1 Isolation and characterization of CD44^{high} expression cells from lung cancer cells. (A)The results showed the phenotype of CD44 expression pattern. By using the analysis of immunofluorescent assay, our result showed that the isolated CD44^{high} expression subgroup (CD44^{high}) from lung cancer cells are detected with the higher signals of CD44 surface protein compared (B) with the CD44^{low} expression of lung cancer subgroup (Bar: 100 μm).

cytometer (Becton-Dickinson, Franklin Lakes, NJ) and analyzed with CellQuest software (BD Biosciences) or FlowJo software (TreeStar, San Carlos, CA). Cell debris was excluded from the analysis based on scatter signals. For fluorescent-activated cell sorting in cells, cells were suspended in incubation buffer, stained with indicated primary antibodies, and subjected to the FACSAria (Becton-Dickinson) for CD44 sorting. Positive populations were defined and compared with isotype control staining groups.

2.3. Reverse transcription polymerase chain reaction, quantitative reverse transcription polymerase chain reaction and gene profiling study

The detail of reverse transcription polymerase chain reaction (RT-PCR) and quantitative reverse transcription polymerase chain reaction (qRT-PCR) were conducted. Total RNA was extracted from cells using Trizol reagent (Life Technologies, Bethesda, MD, USA) and the QiagenRNAeasy (Qiagen, Valencia, CA, USA) column for purification, according to the manufacturer's instructions. The RNA was quantized by UltroSpec 3100 Pro (Amersham), and 1 μg of RNA was reversely transcribed with a SuperScript III reverse transcriptase kit (Invitrogen). qRT-PCR was performed in real time using an ABI 7900 Fast System and SYBR Green Master Mix. The real-time PCR cycling condition used was as follows: 94°C for 3 minutes; 35 thermal cycles (denaturing the DNA at 94°C for 30 seconds, annealing with primers at 60°C for 5 seconds, extending the length of the product at 72°C for 1 minute); and a final extension of the product at 72°C for 10 minutes. The reactions were carried out by specific primers and are listed below TWIST1-forward: GTCCGCAGTCTTACGAGGAG, TWIST1-reverse: GCTTGAGGGTCTGAATCTTGCT, SNAIL-forward: ACTGCAACAAGGAATACCTCAG, SNAIL-reverse: GCACTGGTACTTCTTGACATCTG, GAPDH-forward: ACAA CTTTGGTATCGTGGAAGG, SNAIL-reverse: GCCATCACGC CACAGTTTC. Moreover, glyceraldehyde 3-phosphate dehydrogenase was used to normalize the raw data of qPCR, and the error bar indicated the standard deviation of triplicate measurements.

2.4. Sphere formation assay

All cells were trypsinized and centrifuged at 1500 rpm at 4°C for 5 minutes, followed by wash with PBS. Cell were plated in 24-well plates (BD, falcon) at a density of 5000 viable cells/ml and grown in a serum-free DMEM (Gibco, Grand Island, NY) with 1× N2 supplement (Invitrogen), 20 ng/ml EGF, 20 ng/ml bFGF (Invitrogen), and 4 μg/ml heparin (Sigma). Cells were further cultured for 12 days, and the number of tumor spheres were counted by using a microscope.

2.5. Statistical analysis

The results are reported as mean ± SD. Statistical analysis was performed using Student's t test or an one-way or two-way analysis of variance (ANOVA) test followed by Turkey's test, as appropriate; $p < 0.05$ was considered to be statistically significant.

3. RESULTS

CD44 has been reported as cell surface structural molecule and as highlight biomarker of CSCs.[20–26] However, it is unclear whether the involvement of CD44 plays a role in regulating CSCs or lung tumor stem-like cells (TSLCs) in lung cancer. To explore the role of CD44 in lung cancer, we isolated and purified the CD44-positive cells by using flow-cytometry and cell sorters. As shown in Fig. 1, the data exhibited the ratio of CD44-positive cells primary tumor cells from lung cancer cell line originally was 31.2%. Furthermore, we validated that the ratio of sorted

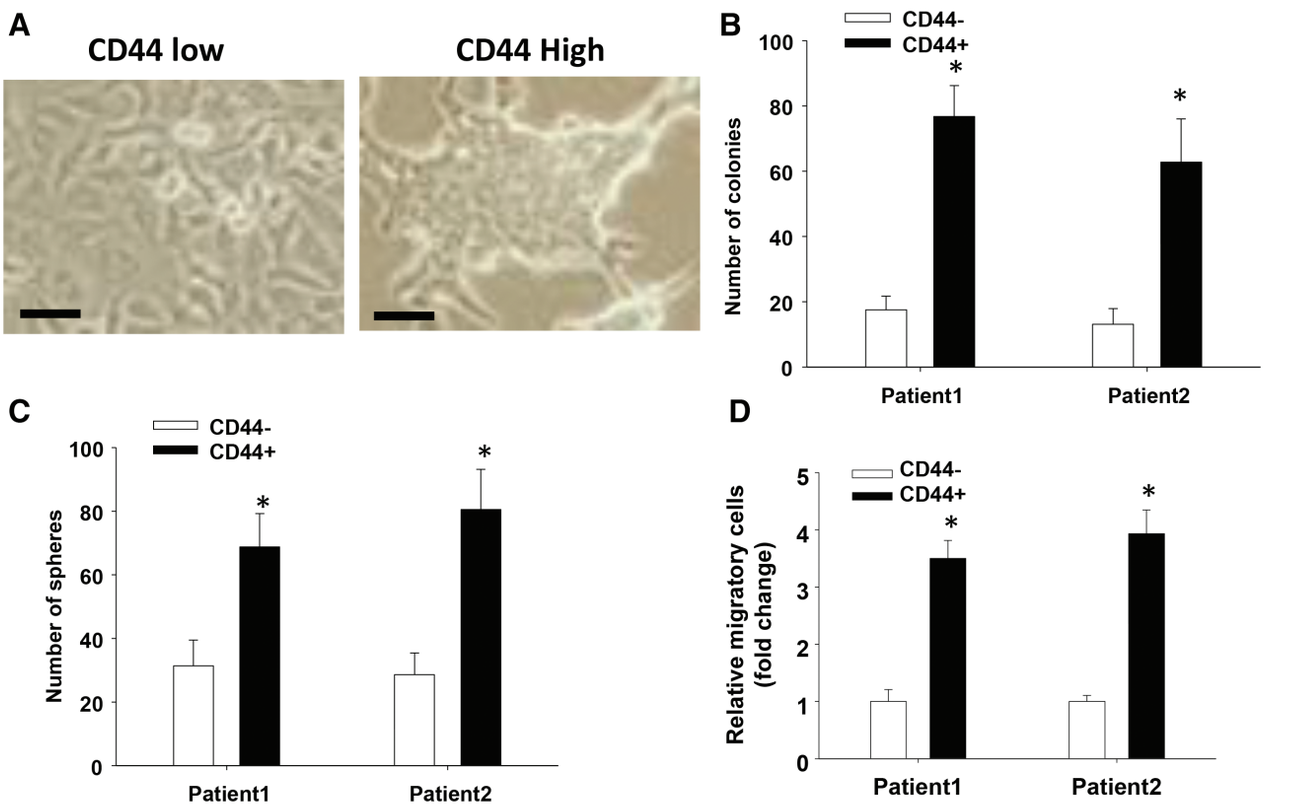

Fig. 2 CD44^{high} expression cells are tumorigenic with self-renewal property. (A) The results showed that the CD44^{high} subpopulation of TSLCs presented the significant higher capabilities of (B) tumorigenic colonies, (C) tumor-sphere formation, and (D) migratory compared to the CD44^{low} subpopulation of lung cancer cells (Bar: 100 μm); *$p < 0.05$.

CD44-positive cells by flow-cytometry was up to 95% (data not shown). Notably, we further evaluated the phenotype of CD44 expression pattern. By using the analysis of immunofluorescent assay, our result showed that the isolated CD44^{high} expression subgroup (CD44^{high}) from lung cancer cells (Fig. 1A) are detected with higher signals of CD44 surface protein when compared with the CD44^{low} expression of lung cancer subgroup (Fig. 1B).

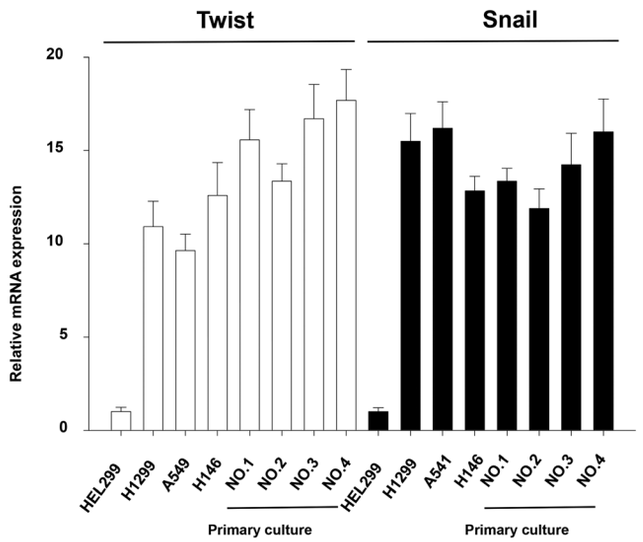

Fig. 3 The *EMT* gene profiling of Twist and Snail in CD44-hgih and CD44^{low} expression subgroups of lung cancer cells. We examined the expression level of CD44 and EMT transcription markers (Twist and Snail) in lung cancer cell lines and LAC primary cultivated cells. We compared with normal lung fibroblasts HEL-299 and MRC-5 cells by qRT-PCR, our result showed the higher CD44 corresponded with elevated Twist and Snail expressions in A549 CD44-positive cell lines and five primary LAC cell types; *$p < 0.05$.

To evaluate the tumorigenicity of the CD44^{high} subpopulation of lung cancer cells, we performed the colony formation assay, tumor-spheres formation, invasive/metastatic abilities (Fig. 2). Importantly, our studies and results clearly showed that the CD44^{high} subpopulation of TSLCs (Fig. 2A) presented the significant higher capabilities of tumorigenic colonies (Fig. 2B), tumor-sphere formation (Fig. 2C), and migratory properties (Fig. 2D) compared with the CD44^{low} subpopulation of lung cancer cells.

To further close dissect the association and characteristics of CD44^{high} subpopulation of TSLCs, the isolated CD44^{high} expression subgroup was determined by microarray and quantitative real-time PCR. The correlation of cancer stemness pathways/axis and EMT process has explored the malignant progression, tumor metastasis, and clinical replase.[26–29] To investigate the relationship between the expression levels of CD44 and *EMT* genes in lung cancer and TSLCs, we examined the expression level of CD44 and EMT transcription markers (Twist and Snail) in lung cancer cell lines, LAC primary cultivated cells, and LAC tissue specimens were determined. We compared with normal lung fibroblasts HEL-299 and MRC-5 cells by qRT-PCR, our result showed the higher CD44 corresponded with elevated Twist and Snail expressions in A549 CD44-positive cell lines and five primary LAC cell types (Fig. 3). We further demonstrated that the elevated CD44 expression compared with the normal counterparts in LAC tissue specimens derived from LAC patients (Fig. 3) and Twist and Snail expression is also higher in LAC tissue compared to normal tissue (Fig. 3). In summary, our results highly suggested that CD44 expression is correlated with EMT

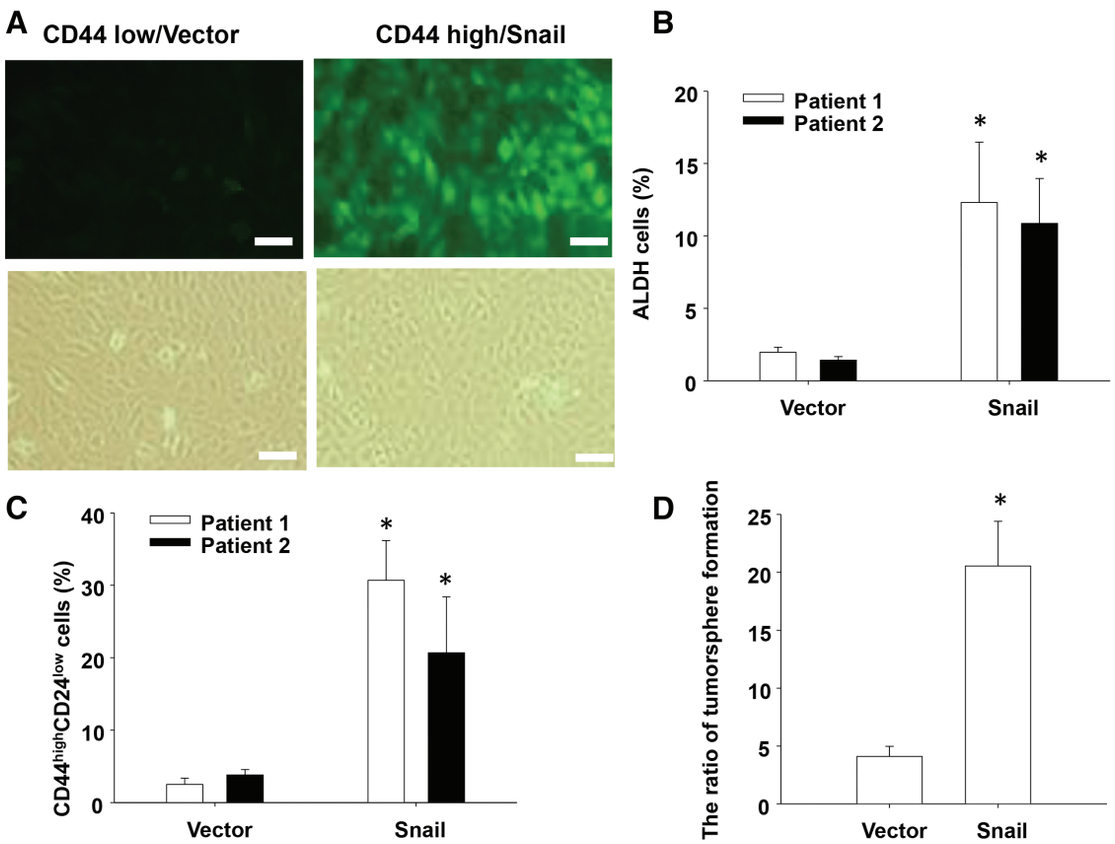

Fig. 4 Overexpressing Snail in CD44^{low} expression cells promoted the CSC-like properties. We therefore test the role of Snail, one of a key transcriptional factor and biomarker of EMT in lung cancer. (A) The immunofluorescent data showed that overexpression of Snail with gene vector in CD44^{low} expression cells significantly promoted the properties of lung tumor stem-like cells, including promoting the percentage of (B) ALDH1 subpopulation and (C) CD44^{high}/CD24^{low} groups, and (D) the generating abilities of tumor-sphere formation (Bar: 100 μm); *$p < 0.05$.

transcription factor (Twist and Snail) expression during LAC tumorigenesis.

The current data showed the critical role of CD44 in regulation of cancer stemness and the research status of CD44 as biomarkers, and therapeutic targets in cancer are outlined. The current gage and future directions that may elicit the best use of CD44 or clinical applications are founded. However, the role of CD44 in lung cancer and TSLCs is not determined. As we have demonstrated that the subgroups of $CD44^{high}$ TSLCs revealed the cancer stem-like properties with the high expression of *EMT* gene profile, we, therefore, tested the role of Snail, one of a key transcriptional factor and biomarker of EMT in lung cancer. Notably, our data confirmed that the overexpression of Snail with gene vector in $CD44^{low}$ expression cells (Fig. 4A) further significantly promoted the properties of lung tumor stem-like cells, percentage of ALDH1 subpopulation (Fig. 4B) and $CD44^{high}/CD24^{low}$ groups (Fig. 4C), as well as the generating abilities of tumorigenic and tumor-sphere formation (Fig. 4D)

4. DISCUSSION

Recent studies indicated that CSCs are mainly responsible for cancer aggressiveness, immunotherapy resistance, and tumor relapse. CD44 has been identified as CSC surface markers for isolating and enriching CSCs in malignant cancers. The current findings showed the critical role of CD44 in regulation of cancer stemness and the research status of CD44 as biomarkers and therapeutic targets in recurrent tumors. The EMT plays a vital step during embryonic development, as well as in the initiation of cancer metastasis. A linkage of EMT and CSC has recently been demonstrated in breast cancer cell under transcriptome-based EMT-driven CSC characteristics. Snail, a member of the zinc-finger transcription factor family, is a transcription factor of EMT and facilitates to initiate and promote EMT by repressing E-cadherin. Herein, we demonstrated that overexpressing Snail in $CD44^{low}$ subgroup of noncancer stem-like cells further significantly regulates the ALDH1 subpopulation and $CD44^{high}/CD24^{low}$ groups. In turns, $CD44^{high}$ expression subgroups govern the CSC-like capabilities with the generating abilities of tumorigenic initiation and metastasis.

The ALDH, a family of cytosolic isoenzyme, plays a key role in responsively oxidizing intracellular aldehydes. This function will be further contributing to the oxidation of retinol to retinoic acid in cancer stem cell. The recent research has shown that up-regulated Snail expression significantly increased the subpopulation of $ALDH^{+}$ cells in malignant cancer and CSCs. In this study, we detected the significance of higher expression levels of Snail mRNA in $CD44^{high}$ subpopulation of TSLCs. Using the CSC platform of the serum-free medium culture with bFGF and EGF, the up-regulated expression of Snail can promote CSC generation and TSLCs to form 3D spheroid formation and enhanced self-renewal ability. Therefore, we aimed to develop a network-based model using the lung-TSLC panel to characterize or modulate the underlying biological perturbation leading to the variable survivals of LACs. It was expected that such network-based model derived from the lung-TSLCs could further elucidate the underlying regulatory mechanisms leading to invasion and metastasis. We hypothesized that the stemness marker of lung tumors modeled by the lung-TSLC networks could be utilized to estimate the prognostic survival times. Lastly, clinical CD44 expression levels associated with LACs metastasis and survivals were further verified by qRT-PCR and immunofluorescent staining in lung cancer cells, down-regulation of CD44 promotes EMT, while over-expression induces the opposite effect. In this study, we found that LAC-CSC-related CD44 could regulate the stemness properties in LAC-CSC and alter their metastatic potential.

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