Specific inhibition of Notch1 signaling suppresses properties of lung cancer stem cells

ABSTRACT

Objective: Lung cancer is the leading cause of cancer-related death worldwide with a relatively low 5-year relative survival rate of 16%. Novel and efficient therapeutic approach for lung cancer is desperately needed.

Materials and Methods: Targeting cancer stem cells (CSCs) provides a promising strategy to eradicate malignancies. The Notch signaling pathway plays a crucial role in the control of cell fates and developmental processes including CSCs. The function of Notch1 in the regulation of CSCs and whether targeting Notch1 could be a potential therapy for lung cancer were explored in this study. Lung CSCs (LCSCs) were isolated from A549 cells and identified as CD44+/CD24− cells by magnetic-assisted cell sorting, then the putative LCSCs were treated with Notch1 inhibitor and Notch1 small interfering RNAs (siRNAs); the growth and proliferation of LCSCs were investigated to test the effect of Notch1 blocking on the growth and viability of LCSCs.

Results: CD44+/CD24− cells isolated from A549 cells possessed stem cell-like properties with high expression of Notch1. Blocking Notch1 by inhibitor DAPT or siRNA both inhibited the growth capacity of LCSCs.

Conclusion: Our discovery demonstrated a depression of growth in CD44+/CD24− and A549 cells caused by blockade of Notch signaling pathway. Further studies are needed to demonstrate the detailed effects of Notch1 blocking on the LCSCs. Nevertheless, targeting the Notch pathway has exhibited great potential to be an improved lung cancer treatment.

KEY WORDS: CD24, CD44, lung cancer stem cells, Notch1, siRNA

INTRODUCTION

Lung cancer remains to be a major cause of cancer-related deaths worldwide. In China, 432,400 males and 177,800 females died of lung cancer in 2015.[3] The current treatments of lung cancer are mostly surgery, chemotherapy, and radiotherapy.[6] However, the 5-year relative survival rate for lung cancer remains only 16%[6] mainly on account of tumor metastasis and resistance. A novel and efficient therapeutic approach for lung cancer is desperately needed.

Recent studies found that a subpopulation of cancer cells were more origin of cancer relapse and these cells also played a critical role in cancer progression, metastasis, and resistance which were named cancer stem cells (CSCs). The first CSCs, CD44+/CD24− breast CSCs, were first isolated by Al-Hajj et al.[4] in solid tumor. Now, it was reported that CSCs were isolated in brain cancer,[5] colon cancer,[6] prostate cancer,[6] lung cancer,[11] and other cancers.[8,9] The CSC hypothesis has attracted much attention due to the potential for discovery and development of CSC-related therapies and the identification of key molecules involved in controlling the unique properties of CSC populations.[10] Targeting CSCs provided a promising strategy to eradicate malignancies, and identification of the key molecules in CSC growth is critically important in cancer therapy. To target CSCs in cancer therapy, it will be the first step to isolate and identify CSCs. There are three types of methods to isolate CSCs by now: sphere formation, isolation by flow cytometry based on surface markers, or isolating side population.[11] Surface markers are the most popular method for CSCs isolation and identification. Previous studies have reported some lung CSCs (LCSCs) markers such as CD44, CD24, ABCG2, CD133, and aldehyde dehydrogenases (ALDH).[12] In this study, we used CD44 and CD24 as markers to isolating LCSCs from...
lungs cancer cell line A549, and the isolated CSCs were identified by sphere formation assay and the expression of another LCSCs marker ALDH1A.

Based on the hypothesis that the tumor stem cells originate from common stem cells, the signaling pathway regulating the normal stem cell self-renewal and differentiation may also play a role in tumor stem cells. The Notch signaling pathway is considered to play an important role in the control of cell fates and developmental processes. Notch1 is a member of the Notch family and serves as receptors for Notch ligands. Recent studies have shown that Notch1 is implicated in carcinogenesis in a variety of human malignancies by regulating many basic processes essential for cancer development and progression including the cell growth, survival, apoptosis, migration, and invasion, suggesting a potential role for Notch1 as a therapeutic target of cancer. In lung cancer, previous studies have shown that Notch1 acts as a driver of lung tumor initiation, growth, and metastasis. To explore the function of the Notch1 in the regulation of CSCs and whether targeting Notch1 could be a potential therapy for lung cancer, the isolated LCSCs were treated with Notch1 inhibitor and Notch1 small interfering RNA (siRNA) to investigate the effect of Notch1 blocking in LCSCs.

**MATERIALS AND METHODS**

**Cell culture**

The human lung adenocarcinoma cell line A549 was obtained from China Center for Type Culture Collection, Wuhan, China. Cells were cultured in Dulbecco’s Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% fetal calf serum (HyClone, USA) and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin). Cultures were maintained in incubator at 37°C in 5% CO2 atmosphere.

**Isolation of CD44+CD24- cells**

Magnetic-assisted cell sorting (MACS) was used for the sequential separation of CD24+ followed by CD44+ population. The cells were labeled with CD24+ biotin antibody (Miltenyi Biotec), incubated at 4°C for 15 min, washed, and further labeled with Anti-Biotin MicroBeads of MultiSort kit. After incubation, cells were washed and re-suspended. Magnetic separation was carried out with the help of LS columns arranged in the magnetic field of a magnetic separator. The CD24 depleted fraction was collected in flow-through and further subjected to CD44 magnetic labeling and purification to isolate the putative stem cells (CD44+/CD24+). The labeled cells were treated with the MicroBead release reagent followed by stop release agent provided in the kit. The MACS sorted cell populations were tested for percentage purification using flow cytometry.

**Inhibition of Notch1 by DAPT and small interfering RNA**

Isolated CD44+/CD24- cells were treated with γ-secretase inhibitor - DAPT in different concentrations (0, 25, 50, and 75 µM) for 48 h to block the Notch1 signaling pathway. siRNAs were also constructed to block Notch1. siRNA vectors targeting to Notch1 gene were synthesized by Santa Cruz Biotechnology. Commercially available siRNA to random noncoding sequences (scrambled siRNA) was used for control transductions. Cells were plated at a density of 5 × 10^6 cells per well in 6-well dishes and settled overnight. Cells were transfected with constructs and/or siRNA using Lipofectamine 2000 (Invitrogen, USA) next morning.

**Immunocytochemistry**

After sorting, isolated CD44+/CD24- cells and unsorted A549 cells were washed with PBS, centrifuged under 800 × g centrifugal force for 3 min, and then resuspended in 200 mL of PBS. An aliquot was taken from each sample and placed on glass slides coated with poly-L-lysine followed by 15 min incubation in a cell culture incubator. Then, cells were fixed with 4% paraformaldehyde for 30 min, blocked with BSA 100 mg/mL for 1 h at room temperature, and incubated with anti-ALDH1A-alkyphycocyanin (1:100) overnight at 4°C. Glass slides were then mounted using VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA, USA). Finally, images were collected under Zeiss confocal microscope.

**Sphere forming assay**

Cells were cultured at a density of 5000 viable cells/mL in low adherent 35 mm dishes (Costar) under serum-free condition and supplemented with 20 mg/mL of insulin, 20 mg/mL epidermal growth factor, and 10 mg/mL of basic fibroblast growth factor (Invitrogen) for 21 days. Formation of sphere-like structures was visible at 4–7 days, and the photographs of comparable groups were captured under phase-contrast microscope (Leica CTR4000) at ×20 magnification at 7–14 days.

**Colony formation assay**

The CD44+/CD24- cells with different treatment were split into 6-well dishes at 1 × 10^5 cells/well and cultured for 7–10 days with medium change every 2–3 days. Eight days later, the colonies were observed under microscope and taken pictures at day 7. Colonies for three independent experiments were counted.

**Cell proliferation assays**

The Cell Counting Kit-8 (CCK-8) (Beyotime, China) was used to quantify the proliferation of CD44+/CD24- cells with different treatments. The major constituent of the kit is 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8). Cells were dispersed at a density of 3 × 10^5 cells/mL in medium and seeded at 100 µL/well in a 96-well plate. The plate was incubated for the appropriate length of time (24, 48, 72, and 96 h) in the incubator, and 10 µL of CCK-8 solution was added to each well of the plate. The absorbance was measured at 490 nm using a microplate reader after an hour.
**Real-time polymerase chain reaction analysis**

Total RNA was extracted from the cells using TRIzol Reagent (Invitrogen, Carlsbad, CA) and reverse transcribed to cDNA using Verso cDNA synthesis kit (Thermo Fisher Scientific Inc., MD). Real-time polymerase chain reaction (RT-PCR) was carried out using start universal SYBR Green master mix (Roche, Basel, Switzerland) on the 7500 Fast RT-PCR system (Applied Biosystems, Foster City, CA). Data were normalized with β-actin. The primer sequences of Notch1 are listed as below: forward 5'-CAGTGAGGGCCGGGTCC-3' and reverse 5'-GTTGTATTGTTCCGACCAT-3'.

**Western blot**

Cells were lysed in NP-40 lysis buffer (Invitrogen, Carlsbad, CA) supplemented with 1× protease inhibitor cocktail. Clear cell lysates were measured with Bradford reagent (Sigma-Aldrich, St. Louis, MO). Equivalent amounts of proteins were separated using SDS-PAGE (8%-10%), transferred on to PVDF membrane (Millipore, Billerica,) and immunoblotted with primary antibodies: Notch1, ALDH1A, GAPDH, and β-actin (Santa Cruz Biotechnology, CA, USA) followed by horseradish peroxidase-conjugated secondary antibody (Sigma). Bands were visualized using enhanced chemiluminescence (Thermo Scientific, Rockford, IL, USA).

**Statistical analysis**

Each treatment had triplicates and at least three independent experiments were performed for statistics. All data were represented as mean ± standard error of the mean. Data were analyzed using Student’s t-test for comparison between two groups or one-way ANOVA test for more than two groups by SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). A P = 0.05 was considered statistically significant.

**RESULTS**

CD44+/CD24- cells possessed stem cell-like properties with highly expression of Notch1. The putative LCSCs were isolated in A549 cells by MACS and then the percentage of CD44+/CD24- cells was analyzed by flow cytometry. Results showed that the percentage of CD44+/CD24- cells in A549 cells was 7.45%. Since tumor sphere culture has been widely used to assess the self-renewal potential of stem cells and CSCs, tumor sphere culture was employed to explore the stem cell potential of CD44+/CD24- cells. Spheroid colonies can be observed after in vitro culture for 7–14 days in serum-free media under nonadherent conditions [Figure 1a]. Compared with unsorted A549 cells, sphere formation of CD44+/CD24- cells was more quickly, and the spheres were bigger. As one of the well-known CSC-related makers, the expression of ALDH1A was detected in the isolated CD44+/CD24- cells; results showed that ALDH1A was highly expressed in the CD44+/CD24- cells compared with A549 cells [Figure 1b]. These results suggested that the CD44+/CD24- cells possessed stem cell-like properties and could be regarded as LCSCs.

To explore the role of Notch pathway in LCSCs, first we detected the expression of Notch1 in the CD44+/CD24- cells by real-time fluorescent quantitative PCR and Western blot analyses. RT-PCR revealed that both A549 cells and the CD44+/CD24- cells expressed Notch1, but CD44+/CD24- cells expressed significantly higher levels than A549 cells (P < 0.05) [Figure 1c]. Western blotting analysis provided similar results to those obtained by RT-PCR (P < 0.05) [Figure 1d]. Treatment with DAPT could inhibit the growth of CD44+/CD24- cells.

To study the role of the Notch signaling pathway in the growth of LCSCs, N-[N-(3,5-difluorophenacetyl)-L-alanyl]–2-phenylglycine tert-butyler ester (DAPT, GSI-IX) was used as a γ-secretase inhibitor. DAPT has been shown to cause a reduction in Ab40 and Ab42 levels and inhibit Notch signaling in human cancer cells. CD44+/CD24- cells were treated with different concentrations of DAPT, and the effect of Notch1 inhibition was detected by RT-PCR and Western blot. Results showed that DAPT treatment decreased both the mRNA and protein expression of Notch1 [Figure 2a and b]. The inhibition of Notch1 expression was positively correlated with the concentration of DAPT and the lowest expression of Notch1 was observed when the cells were treated with 75 μM DAPT. Besides, we observed that DAPT could inhibit the growth of CD44+/CD24- cells, and the effect was also positively correlated with DAPT concentration. After being treated with 50 μM DAPT for 2 weeks, colony-forming assay showed that the growth of CD44+/CD24- cells was significantly slower compared with control (P < 0.05) [Figure 2c] suggesting that blocking Notch signaling could inhibit the growth of LCSCs.

Notch1 silencing by siRNAs could inhibit cell growth and cell cycle in CD44+/CD24- cells.
To further determine whether inhibition of Notch1 expression could induce growth arrest in CD44+/CD24− cells, we transfected Notch1 siRNA or the control scrambled siRNA into CD44+/CD24− cells and examined cell viability by colony-forming assay and CCK-8 assay, the cell cycle analysis was also conducted with FACS to invest the effect of Notch1 silencing to the cell cycle of the putative LCSCs. We obtained four Notch1 siRNAs, and the interfering effect of each siRNA was detected by PCR and Western blot and the most efficient siRNA (siRNA-4) was chosen [Figure 3a and b]. The interfering effect of siRNA-2 was further verified by Western blot and results showed that the protein expression of Notch1 could be reduced by half with siRNA-2. Besides, we observed that the expression of ALDH1A, one of the CSC markers, was also significantly decreased by Notch1 silencing [Figure 4a], suggesting Notch1 plays a role in stemness maintenance of LCSCs. The sphere forming assay showed that the silencing of Notch1 significantly inhibited the formation of spheres compared with control group [Figure 4b and c]. After being treated with siRNA, CCK-8 assay revealed that the number of viable cells was decreased by >50% in siRNA transfected as compared with that in the scrambled siRNA-transfected cells [Figure 4d]. These results suggest that Notch1 may be an important target for inhibiting LCSC growth.

DISCUSSION

CSC is one of the hot spots in cancer research in recent years, which have been shown to possess the ability of self-renewal and differentiation in many studies. Compared with normal cancer cells, CSCs are considered more tumorigenic and can easily cause tumor relapse and metastasis by generating new tumors. In some in vivo experiments, small amounts of tumor stem cells can be injected into nude mice, and the rate of tumor growth is higher than that of common tumors. There are three major methods to isolate and identify CSCs mainly through their biological characteristics; surface marker is the most popular method for CSCs isolation and identification. For LCSCs, different studies have reported some different markers such as CD44, CD24, ABCG2, CD133, and ALDH. In a recent study, in the human lung adenocarcinoma cell line A549 cells, a subpopulation with high expression of CD44 and low expression of CD24 (CD44+/CD24−) showed stem cell characteristics and was considered as LCSCs. In our study, MACS was used for the sequential separation of CD44−/CD24− cells from the adenocarcinoma cell line A549 and the isolated CD44+/CD24− cells were identified. We observed that the population of CD44+/CD24− cells represented only a small proportion of all A549 cells (about 7.45%). Since tumor sphere culture has been widely used to assess the self-renewal potential of stem cells and CSCs, tumor sphere culture was employed to explore the stem cell potential of CD44+/CD24− cells. Spheroid colonies can be generated from CD44+/CD24− cells after in vitro culture for 7–14 days in
serum-free media under nonadherent conditions, proving the self-renewal potential of the CD44+/CD24− cells. Besides, as one of the well-known CSC-related makers, the expression of ALDH1A was also demonstrated in CD44+/CD24− cells. These results again confirmed that the CD44+/CD24− cells possessed stem cell-like properties and could be regarded as LCSCs.

The Notch signaling pathway has been known as a key pathway to regulate the differentiation of epithelial progenitors during lung development.19,20 The dysfunction of the Notch pathway has been found in many lung cancers and is a relatively frequent event in nonsmall cell lung cancers.21 The upregulation of Notch signaling contributes to lung cancer clonogenic capacity in vitro,22 and downregulation of Notch signaling inhibits the growth, migration, and invasiveness of cancer cells and induces cell apoptosis.23 However, there are also some controversial researches with opposite outcomes, and some studies reported that the overexpression of Notch signaling can cause cell cycle arrest and depress the growth of cancer cells,24 suggesting that the regulation of cell function by Notch signaling in cancer cells may highly depend on cellular contexts.

Notch1 is a transmembrane protein belonging to the NOTCH family. By binding with Notch ligands, Notch1 is cleaved by proteases such as γ-secretases, to release the intracellular domain NICD1 which translocates to the nucleus and acts as a transcription cofactor to regulate target gene expression.25,26 Recent studies showed that Notch1 is closely related with carcinogenesis in a variety of human malignancies by regulating many basic processes essential for cancer development and progression including the cell growth, survival, apoptosis, migration, and invasion, suggesting a potential role for Notch1 as a therapeutic target for cancer.27 Previous studies showed that Notch1 could act as a driver of lung tumor initiation, growth, and metastasis,28 however, completely opposite
findings were also reported, suggesting that the role of Notch1 in lung cancer may be associated with disease subtypes or specific genetic changes in a context-dependent manner. In this study, we found that Notch1 was highly expressed in the isolated CD44+/CD24− cells. When Notch1 signaling in the CD44+/CD24− cells was inhibited by DAPT, the growth and proliferation of CD44+/CD24− cells were also inhibited. The blocking of Notch1 by siRNA showed similar results.

CONCLUSION

Our discovery demonstrated a depression of growth in CD44+/CD24− and A549 cells caused by blockade of Notch signaling pathway. Further studies are needed to demonstrate the detailed effects of Notch1 blocking on the LCSCs. Nevertheless, targeting the Notch pathway has exhibited great potential to be an improved lung cancer treatment.

Financial support and sponsorship

The study was supported by funding from the National Natural Science Foundation of China (No.81403150), the Natural Science Foundation of Shandong Province (No. ZR2014HL064) and the Innovation Project of Shandong Academy of Medical Sciences.

Conflicts of interest

There are no conflicts of interest.

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