Mechanistic Exploration of Cancer Stem Cell Marker Voltage-Dependent Calcium Channel \( \alpha_2\delta_1 \) Subunit-mediated Chemotherapy Resistance in Small-Cell Lung Cancer

Jiangyong Yu1,5, Shuhang Wang2, Wei Zhao3, Jianchun Duan1, Zhijie Wang1, Hanxiao Chen2, Yanhua Tian4, Di Wang1, Jun Zhao2, Tongtong An2, Hua Bai1, Meina Wu2, and Jie Wang1,2

Abstract

Purpose: Chemoresistance in small-cell lung cancer (SCLC) is reportedly attributed to the existence of resistant cancer stem cells (CSC). Studies involving CSC-specific markers and related mechanisms in SCLC remain limited. This study explored the role of the voltage-dependent calcium channel \( \alpha_2\delta_1 \) subunit as a CSC marker in chemoresistance of SCLC, and explored the potential mechanisms of \( \alpha_2\delta_1 \)-mediated chemoresistance and strategies of overcoming the resistance.

Experimental Design: \( \alpha_2\delta_1 \)-positive cells were identified and isolated from SCLC cell lines and patient-derived xenograft (PDX) models, and CSC-like properties were subsequently verified. Transcriptome sequencing and Western blotting were carried out to identify pathways involved in \( \alpha_2\delta_1 \)-mediated chemoresistance in SCLC. In addition, possible interventions to overcome \( \alpha_2\delta_1 \)-mediated chemoresistance were examined.

Results: Different proportions of \( \alpha_2\delta_1 \)-positive cells were identified in SCLC cell lines and PDX models. \( \alpha_2\delta_1 \)-positive cells exhibited CSC-like properties (self-renewal, tumorigenic, differentiation potential, and high expression of genes related to CSCs and drug resistance). Chemotherapy induced the enrichment of \( \alpha_2\delta_1 \)-positive cells instead of CD133\(^+\) cells in PDXs, and an increased proportion of \( \alpha_2\delta_1 \)-positive cells corresponded to increased chemoresistance. Activation and overexpression of ERK in the \( \alpha_2\delta_1\)-positive H1104 cell line was identified at the protein level. mAb 1B50-1 was observed to improve the efficacy of chemotherapy and delay relapse as maintenance therapy in PDX models.

Conclusions: SCLC cells expressing \( \alpha_2\delta_1 \) demonstrated CSC-like properties, and may contribute to chemoresistance. ERK may play a key role in \( \alpha_2\delta_1 \)-mediated chemoresistance. mAb 1B50-1 may serve as a potential anti-SCLC drug. Clin Cancer Res; 24(9): 2148–58. ©2018 AACR.

Introduction

Small-cell lung cancer (SCLC) is an aggressive cancer of neuroendocrine origin with poor prognosis, accounting for approximately 15% of all lung cancer cases. The majority of SCLC patients have extensive stage disease (ES) with metastasis beyond one hemithorax at the time of diagnosis (1). Despite the robust response to initial chemotherapy and radiotherapy, most patients progress within 3 months and develop resistance to the initial treatment. Less than 30% of patients with limited disease and 1%–2% of patients with extensive disease survive to 5 years (2).

Putative cancer stem cells (CSC) have been identified in many human cancer types, including SCLC (3). CSCs are a small subset of cells that sustain tumor growth and remain in patients after conventional antitumor therapy has been completed (4). CSCs are also reported to be responsible for tumor initiation, maintenance, and spreading and are characterized by capacity for self-renewal, asymmetric cell division, slow division kinetics, increased capacity for invasion, metastasis, tumor formation and proliferation, and resistance to conventional chemotherapy and radiotherapy. They can be identified by a variety of cell markers (5–8). CSC populations are commonly isolated and enriched using cell surface markers such as CD133, CD44, CD87, and ALDH1. CD133 is a transmembrane glycoprotein and is the most widely used stem cell markers to identify CSCs from various tissue sources such as neural or brain tumors, renal epithelial, or kidney cancer and lung cancer (3, 9–12). SCLC contains identifiable populations of stem cells (13), which are thought to be able to evade conventional therapy and are implicated in chemoresistance.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

J. Yu, S. Wang, and W. Zhao contributed equally to this article.

Corresponding Author: Jie Wang, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China. Phone: 8615-9107-04669; Fax: 8610-8819-6562; E-mail: zhxui@163.com.

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Translational Relevance

Despite the robust response to initial chemotherapy and radiotherapy, most of small-cell lung cancer (SCLC) patients progress within three months and develop resistance to the initial treatment. The current research found that α2δ1 subunit positive SCLC cells have CSC properties, and are related to chemotherapy resistance. This clinically relevant biomarker of CSCs may contribute to select chemoresistant SCLC before treatment. A specific antibody of 1B50-1 to α2δ1 subunit could improve response to chemotherapy and delay preclinical relapse, whether combined with chemotherapy or as sequential therapy. Further clinical trials are guaranteed using 1B50-1 to reverse chemoresistance for SCLC patients. The ERK protein in MAPK pathway might be the critical molecular for resistance mediated by the α2δ1 subunit. ERK inhibitors alone or combined with conventional therapy might play a role in treatment of SCLC.

resistance and high recurrence rate of SCLC, both of which are major challenges in SCLC. In a preliminary study, it was demonstrated that a small subset of cells in SCLC expresses CD133 and possess stem cell-like properties. Freshly dissociated human SCLC contains CD133-positive cells, which can generate long-term lung tumor spheres in vitro that can both differentiate and preferentially form tumors in vivo (3). Seri and colleagues reported that level of CD133 was elevated in SCLC tumors following chemotherapy (14). However, the evidence for CD133 as an effective CSC marker in lung cancer remains contradictory (15, 16). Kubo and colleagues studied CD133 in a panel of six SCLC cell lines and concluded that CD133 and CD87 were inadequate CSC markers (17). Meng and colleagues found that CD133 status in A549 and H446 cell lines was not significantly related to proliferative capacity, invasiveness, drug resistance, or tumorigenic ability in xenograft models (16). Because CSCs are phenotypically heterogeneous, no marker or set of markers have been found to identify CSCs in solid tumors in general nor for specific tumor entities. Therefore, new biomarkers are necessary for further identification of CSCs.

Recent research has identified the voltage-dependent calcium channel α2δ1 subunit, which is part of a voltage-gated calcium channel complex, as a promising potential marker for CSCs. Zhao and colleagues (18) studied resistant primary hepatocellular carcinoma (HCC) and found the α2δ1 + cells presented as CSCs. They also found that α2δ1 was a functional marker for prediction of HICC recurrence. The functional part was identified and validated as isoform 5 of α2δ1, and its monoclonal antibody (mAb) 1B50-1, is a potential anti-HCC agent. Whether α2δ1 subunit plays the same role in SCLC CSCs warrants further exploration.

We hypothesized that α2δ1 might be an excellent marker of CSCs, comparable with CD133, in SCLC. To test this hypothesis, we investigated the expression levels of CD133 and α2δ1 using three SCLC cell lines and five PDX models. Meanwhile, we examined whether α2δ1 might be effective for selecting cancer stem-like cells from SCLC. We also explored the relationship between α2δ1 + CSCs and mechanisms of chemotherapy resistance in SCLC. Deep understanding of this population may be helpful in addressing chemoresistance and seeking novel strategies to overcome chemoresistance in SCLC patients.

Methods

Cell lines and animals

Expression of stem cell-like markers was analyzed in NCI-H1048, NCI-H69, and NCI-H209 SCLC cell lines. All the cell lines were from BeiGene (Beijing) Co. Ltd., and were cultured in RPMI1640 supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C under 5% CO2. The NOD/SCID female rats and Balb/c female rats (aged 4–6 weeks, weighing 18–21 g) used in vivo experiments were purchased from Huafu Kang Experimental Animal Co., Ltd. All animals were fed in the SPF class room by the Peking University Clinical Cancer Hospital Animal Center.

Five SCLC patient-derived xenografts (PDX) including Bclu80, Bclu62, Bclu82, Bclu44, and Bclu77 were used to explore the expression of CSC marker and role of drugs. Five PDX models kept high fidelity with the primary patients' tumors whether histopathology (hematoxylin and eosin (H&E) staining), immunohistochemistry (IHC) staining (common SCLC markers, CD56, TTF1, CgA, and Syn) or therapy response to standard regimens (Supplementary Table S1). Histologic analysis was performed by two experienced pathologists (with more than 5 years of experience in lung cancer) who were blinded to the clinical data. Histopathology and IHC staining of five PDX models both showed typical characteristics of SCLC.

All animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals with protocols approved by the Animal Care and Use Committee at Peking University Cancer Hospital.

Antibody labeling and flow cytometry analysis

Cells were collected and resuspended at 1 × 106 cells/mL of PBS buffer. Fluorescence-labeled mAbs were added at the concentrations recommended by the manufacturer. After washing, the labeled cells were analyzed and sorted using BD Accuri C6 and FACS Aria flow cytometer (Becton Dickinson), respectively. The antibodies used were APC/PE-conjugated mouse anti-human CD133 and corresponding isotype IgG. Detailed information of antibodies is listed in Supplementary Table S2. The 1B50-1 antibody and isotype IgG were supplied by Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Cell Biology, Peking University Cancer Hospital and Institute (Beijing, China). 1B50-1 was directly labeled with PE-Cy5 or fluorescein using the respective Lightning conjugation kits following the manufacturer’s protocol (Innova Biosciences Ltd.; ref. 20).

Properties of CD133 and α2δ1 marked stem cells of H1048 cell line

Sphere formation assay: After counting, cells were mixed with DMEM/F12 medium (Invitrogen) supplemented with B27 (Invitrogen), 20 ng/mL epidermal growth factor, 20 ng/mL basic fibroblast growth factor (PeproTech), 10 ng/mL hepatocyte growth factor (PeproTech), and 1% methylcellulose (Sigma), and were cultured in semisuspension in Ultra Low Attachment 96-well plates (Corning Incorporated Life Sciences; ref. 20). The number of spheres was counted and recorded under a stereomicroscope (Olympus) after 2- to 3-week incubation.
Tumorigenicity assay. Cells purified by fluorescence-activated cell sorting (FACS) were suspended in PBS and mixed 1:1 with Matrigel (BD Biosciences). Suspensions of $10^2$–$10^3$ cells were transplanted subcutaneously bilaterally in the back of nude/SCID mice to investigate the capacity of tumorigenicity. The tumor was observed and measured twice weekly.

Reverse transcription-PCR
Reverse transcription-PCR (RT-PCR) was applied to detect the expression of stem cell–related transcription factors (SOX2, OCT4, and Nanog) and drug resistance–related genes (MDR, ABCG2) in $\alpha$281–related gene in PDX model was also detected by RT-PCR. The FACS-purified cells were lysed by QIAzol Lysis Reagent and total RNA was extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. Tumor tissue from PDX model was lyzed into a single-cell suspension and RNA was extracted using TRIzol and chloroform. cDNA was synthesized using EasyScript First Strand cDNA Synthesis SuperMix (TransGen). The quantitative PCR reaction was carried out in a Stratagene Mx3000P system and data were analyzed using the System detection software (MxPro-Mx3000P software, Agilent Technologies). The primers of each gene are listed in Supplementary Table S3.

Western blot analysis
Cells were lysed using cell lysis buffer (Cell Signaling Technology) and quantified by a bicinchoninic acid assay (Appygen). Lysates were resolved on 12% SDS-PAGE gels and electroblotted onto nitrocellulose membranes. Primary antibodies were added and incubated overnight at 4°C followed by horseradish peroxidase–labeled anti-rabbit or anti-mouse secondary antibodies (Cell Signaling Technology) for 2 hours at room temperature. Bands were visualized using enhanced chemiluminescence and photographed using a Fujifilm Dark Box II. Primary antibodies included ERK1/2, pERK1/2, pAKT, p56, SOX2, and β-actin, etc. Detailed information of antibodies applied are listed in Supplementary Table S4.

Treatment of H1048 cell lines and transplanted mice
To examine the sensitivity of the different cell populations to chemotherapy, we treated H1048 cells with different concentrations of etoposide (the standard chemotherapy regimen for SCLC) for 72 hours. Following exposure to the drug, we analyzed expression of $\alpha$281 and CD133 by flow cytometry. We implanted Balb/c mice with H1048 cells. After the tumors grew to a volume of about 200 mm$^3$, we administered standard chemotherapy for SCLC (etoposide and cis-platinum, EP) for 3 cycles. We obtained the residual tumors and analyzed $\alpha$281 and CD133 expression in EP-treated mice as compared with untreated mice by flow cytometry.

Treatment of PDX models with chemotherapy and 1B50-1 antibody
For detailed methods regarding the establishment of PDX models, please refer Supplementary Data.

Animals were treated by intraperitoneal injection with etoposide and cisplatin (EP) [4 mg/kg cisplatin dissolved in 0.9% saline solution on day 1, and 12 mg/kg etoposide (Sigma) dissolved in 0.9% saline solution on days 1–3], or the corresponding vehicle only, 10 days/cycle for a total of 3 cycles.

Individual body weight and tumor volume were recorded twice weekly, with mice monitored daily for clinical signs of toxicity for the duration of the study. When the tumor volume reached 2,000 mm$^3$, or when the tumor ulcerated, mice were euthanized using carbon dioxide.

Chemotherapeutic response was evaluated as the percentage of tumor growth inhibition (% TGI), calculated using the equation “$100 – \left(\text{mean RTV of the treated tumor/mean RTV in the control group at the time of sacrifice } \times 100\right)$”, where RTV (relative tumor volume) was defined as $Vx/V1$, in which $Vx$ was the volume in mm$^3$ at a given time and $V1$ was the volume at the start of treatment. Mean RTV and SD were calculated for each group (19).

To test the therapeutic effect of 1B50-1, we treated PDX models Bchu80 and Bchu62 with 3 cycles of EP regimes combined or sequenced with 1B50-1 antibody (800 μg/mouse, once daily, i.p., until disease progression). For PDXs and corresponding clinical data, refer to Supplementary Table S1.

RNA sequencing
Total RNA was extracted from $\alpha$281– and $\alpha$281+ cells sorted by FACS using All Prep DNA/RNA Mini Kit (Qiagen) according to the manufacturer’s instructions. Sequencing libraries were generated using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB) following manufacturer’s recommendations. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer ($5\times$). First-strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNaseH-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. The library fragments were purified with AMPure XP system (Beckman Coulter). Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The cDNA library was sequenced on a HiSeq 2500 platform (Illumina) using PE125 sequencing strategy.

Raw data of fastq format were firstly processed through in-house perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing poly-N and low quality reads from raw data. Reference genome and gene model annotation files were downloaded from UCSC (genome.ucsc.edu/) directly. Index of the reference genome was built using Bowtie v2.0.6 (20) and paired-end clean reads were aligned to the reference genome using TopHat v2.0.9 (21). HTSeq v0.6.1 (22), which considers the effect of sequencing depth and gene length for the reads count at the same time, was used to count the reads numbers mapped to each gene. And then RPKM (Reads Per Kilobase of exon model per Million mapped reads) of each gene was calculated on the basis of the length of the gene and reads count mapped to this gene. Prior to differential gene expression analysis, for each sequenced library, the read counts were adjusted by edgeR program package through one scaling normalized factor. Differential expression analysis of two conditions was performed using the DESeq R package (1.12.0 ref. 23, 24). The $P$ values were adjusted using the Benjamini–Hochberg method. Corrected $P$ value of 0.05 and log₂ (fold change) of 1.5 were set as the threshold for significantly differential expression.
Statistical Analysis

All statistical analyses were performed with the SPSS statistical software, version 19.0 (IBM Corp.). Comparisons between groups were performed by paired Student t test, independent t test, or one-way ANOVA. Bonferroni correction or Dunnett T3 method was used for the multiple comparisons. The statistical significance level was defined as two-sided P < 0.05.

Results

Expression of stem cell-like markers in SCLC cell lines

The expression of CD133 and α281 by flow cytometry analysis were different in several SCLC cell lines. The expression of CD133 in NCI-H1048, NCI-H69, and NCI-H209 was 41.10% ± 4.63%, 93.22% ± 3.42%, and 91.05% ± 3.68%, respectively, while the α281 expression was 1.40% ± 0.26%, 12.08% ± 2.23%, and 4.20% ± 1.85%, respectively (Fig. 1A). A relationship between α281+/− and CD133−/− was observed in H1048. The expression of CD133 in H1048 cells was significantly higher than α281+ (P < 0.01; Fig. 1B), and CD133+ population is nearly 100% positive in α281+ cells, while only 2.67% α281+ cells occur in CD133+ cells (Fig. 1C).

The expression of CD133 and α281 in CD133+ cells was significantly higher than CD133− (P < 0.013). What’s more, cells separated from α281+ or CD133+ spheres could be passaged and the efficiency of sphere formation elevated with passage (Fig. 2C). For demonstrating in vivo, FACS-sorted α281+ and CD133+ cells were bilaterally inoculated into the backs of NOD/SCID mice with different magnitude (102 or 103) to observe the tumorigenic ability (Fig. 2D). Double positive (α281+CD133+) cells exhibited higher frequency of tumorigenicity compared with α281−CD133− and α281−CD133+ cells, respectively (Supplementary Table S5). Moreover, α281+ and CD133+ cells had
These data indicated different magnitude $10^2, 10^3$.

Using ANOVA analysis, $P < 0.01$. Cells of CD133$^+$ and a2b1$^+$ sphere could be serially passaged and sphere formation efficiency elevated with passage in vitro. Using ANOVA analysis, $P < 0.01$.

Figure 2. a2b1$^+$ and CD133$^+$ marked cells presented high self-renewal capacity in vitro and in vivo. A, The first-generation sphere of CD133$^+$ and a2b1$^+$ sorted H048 cells in semisolid media without serum. B, Comparison of sphere formation efficiency of parental H048 cells with first-generation CD133$^+$ and a2b1$^+$ sorted H048 cells. Using ANOVA analysis, $P < 0.01$. C, Cells of CD133$^+$ and a2b1$^+$ sphere could be serially passaged and sphere formation efficiency elevated with passage in vitro. Using ANOVA analysis, $P < 0.01$. D, Tumor formation of a2b1$^+$ and CD133$^+$ sorted H048 cells with bilateral transplantation (positive and negative were noted with red and black arrow, respectively) in NOD/SCID mice. E, Growth curves of FACS-sorted a2b1$^+$ and CD133$^+$ H048 cells with different magnitude ($10^2$, $10^3$). F, Representative H&E staining images of parent tumor and serially passaged CD133$^+$ and a2b1$^+$ tumor specimens.}

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higher tumor formation rates compared with a2b1$^+$ and CD133$^+$ population, respectively, in H1048, H69, Bclu80 PDX (Supplementary Table S6). Serial transplantation assay was performed into mice for validating self-renewal in vivo. Both a2b1$^+$ and CD133$^+$ cells constantly obtained the ability of self-renewal when generating. However, a2b1$^+$ cells exhibited no significantly higher growth rate than only CD133$^+$ cells at the same concentration (Supplementary Table S7), and continuously feed for 80 days, tumor volumes were measured as shown in Fig. 2E. A series of passaged paraffin-embedded sections were examined by H&E examination. The morphology of cells remained consistent between different passages and also with parent sample (Fig. 2F). a2b1$^+$ cells also presented an elevated self-renewal capability in H69 cell lines both in vitro and in vivo (Supplementary Fig. S1). These data indicated a2b1$^+$/CD133$^+$ cells contribute to higher tumorigenic ability in SCLC.

a2b1$^+$ and CD133$^+$ cells exhibited differentiation properties. When cultured in medium with complete serum (10% FBS), the purified a2b1$^+$ and CD133$^+$ cells of H1048 cell line showed differentiation ability. After two weeks of culture, flow cytometry analysis showed that the proportion of a2b1$^+$ and CD133$^+$ cells returned from 98.2% and 97.8% to baseline level 1.1% and 43.2%, respectively (Fig. 3A). Moreover, FACS-sorted purified a2b1$^+$/CD133$^+$ cells from the first-generation tumors formed by a2b1$^+$/CD133$^+$ cells were transplanted into NOD/SCID mice again to examine the serial transplantation capacity in vivo. The proportion of a2b1$^+$ and CD133$^+$ cells in second-generation PDXs returned from 95.74% ± 1.44% and 97.49% ± 1.24% to the parental level 1.22% ± 0.27% and 44.9% ± 2.2% (Fig. 3B). This phenomenon was also observed in serial passage of a2b1$^+$ Bclu80 PDX models (Supplementary Fig. S2). It demonstrated the differentiation properties of a2b1$^+$ and CD133$^+$ were conserved both in vitro and in vivo.

**Stem cell and drug-resistant related gene expressions in stem cell–like cells.** To verify the stem cell–like properties of a2b1$^+$ and CD133$^+$ cells and investigate the relationship with drug resistance, RT-PCR was applied to detect the expression of stem cell–related transcription factor (SOX2, OCT4, and Nanog) and drug resistance–related genes (MDR, ABCG2) in FACS-sorted a2b1$^+/−$ and CD133$^+/−$ H1048 cells. Results showed that all of these genes were more highly expressed in a2b1$^+$ and CD133$^+$ cells compared with their corresponding negative cells. Notably, the expression levels were even higher in a2b1$^+$ cells than in CD133$^+$ cells, especially for OCT4, MDR1, and ABCG2 (Fig. 3C).

**Stem cell–like marker expression in PDXs.** Next, we evaluated the response of corresponding PDXs with different expression of a2b1 and CD133 to chemotherapy. Tissue clumps of SCLC patients from bronchoscopy biopsy were transplanted into NOD/SCID mice to establish PDXs. Preliminary experiments...
showed that the PDXs were highly consistent with primary tumor of patients with respect to morphologic, pathologic, genomic, and clinical characteristics. Pathologic and genetic characteristics were highly conserved throughout passaging (25).

After successful PDX establishment, the PDX tumors were disaggregated into single-cell suspension and analyzed for α2δ1 and CD133 expression by flow cytometry. Results showed that α2δ1 and CD133 expression varied between individual PDXs. Of note, Bclu80 model presented significantly higher expression of α2δ1 compared with Bclu62, Bclu82, Bclu44, and Bclu77, while expression of CD133 was relatively lower (Fig. 3D). The expression ratio of α2δ1/CD133 showed similar trend with the expression of α2δ1 in corresponding PDXs (Fig. 3E). Moreover, the expression of α2δ1-related genes (CACNA2D1) examined by RT-PCR was also consistent with the expression of α2δ1 protein determined by flow cytometry (Fig. 3F). We also explored the stem cell properties of α2δ1+ cells in PDX models. α2δ1+ populations showed high efficiency of sphere-forming and tumorigenicity compared with α2δ1− cells in the Bclu80 model. (Supplementary Fig. S4; Supplementary Table S6).

In addition, PDXs with different expressions of stem cell markers showed diverse responses to chemotherapy. The models with relatively low α2δ1 expression or a low α2δ1/CD133 ratio (Bclu62, Bclu82, and Bclu77) showed good efficacy to EP chemotherapy with best response of partial response (PR) or even complete response (CR). However, Bclu80, which has high expression of α2δ1 and a high α2δ1/CD133 ratio, was not sensitive to EP chemotherapy with a best response of stable disease (SD). Accordingly, the response of PDXs to chemotherapy was consistent with clinical response of patients. Surprisingly, the Bclu44 model, which displayed low α2δ1 expression and a low α2δ1/CD133 ratio, showed only SD or limited remission (minimal PR) in PDXs. However, the corresponding patient acquired a clinical response of PR to chemotherapy (Supplementary Tables S1 and S8; Supplementary Fig. S3). CD133 showed different expression level in five PDXs. However, no correlation could be observed between CD133 expression and clinical response.

Chemotherapy induced CSC enrichment

Chemotherapy induced CSC enrichment in H1048 cell line and H1048-transplanted mice. To investigate the effects of chemotherapy on expression of stem-like cells, H1048 cells were treated with different concentrations of etoposide for more than 72 hours, and α2δ1 and CD133 were evaluated by flow cytometry. The proportion of α2δ1+ and CD133+ cells increased after treating with etoposide and the α2δ1+ population was enriched even more after treatment (Fig. 4A). Similarly, the proportion of α2δ1+ and CD133+ cells significantly increased in tumors treated after 3 cycles of EP chemotherapy as compared with those in control group in H1048-transplanted mice (Fig. 4B). Analogous with the in vitro results, the α2δ1+ population showed a more significant enrichment after treatment than the CD133+ population.
and Bclu82 PDX tumors after 3-cycle chemotherapy of EP regimes increased significantly. However, CD133 expression remained stable (Fig. 4C and D). The Bclu80 PDX showed a response of SD with minimal shrinkage, and the proportion of CD133+/CD133− population did not significantly change before and after chemotherapy (Fig. 4E). Similarly, nonsensitive Bclu44 PDX showed the same trend as Bclu80 (Fig. 4F). Chemotherapy induced the enrichment of CD133+ population rather than CD133− cells.

**PDX response to different treatment modality of 1B50-1 antibody.** 1B50-1 antibody is a specific mAb against the α281 subunit. We treated Bclu80 PDX with both concurrent therapy (chemo and 1B50-1 antibody) and subsequent therapy (chemo followed by 1B50-1 antibody). The group of combined treatment with EP regimes and 1B50-1 antibody in concurrent therapy showed an improved best response (tumor shrink 75.5% compared with baseline) than the group treated with both etoposide (0.546 μmol/L vs. 0.062 μmol/L; Fig. 6A, left) and cisplatin (11.3 μmol/L vs. 1.8 μmol/L; Fig. 6A, right) after 72-hour plate culturing, which suggested sphere cells exhibited robust resistance to conventional chemotherapy. In addition, the sphere formation ability of parental cells digested from spheres of α281+ cells to conventional chemotherapy regimen (etoposide and cisplatin) was examined in vitro. Results showed that the IC50 for sphere cells was higher compared with parental cells treated with both etoposide (0.546 μmol/L vs. 0.062 μmol/L; Fig. 6A, right) and cisplatin (11.3 μmol/L vs. 1.8 μmol/L; Fig. 6A, right) after 72-hour plate culturing, which suggested sphere cells exhibited robust resistance to conventional chemotherapy. In addition, the sphere formation ability of parental and α281+/− cells with cisplatin treatment was tested, and α281+− cells showed stronger sphere formation ability and high risk of resistance (Fig. 6B).

**α281+ population of H1048 were chemoresistant in vitro.** After confirmed the expression ratio of α281 related to the chemosensitivity and chemotherapy induced the enrichment of α281+ cells in PDX models, we then evaluated the sensitivity of α281+ cells to chemotherapy. The sensitivity of parental cells versus the passage cells digested from spheres of α281+ cells to conventional chemotherapy regimen (etoposide and cisplatin) was examined in vitro. Results showed that the IC50 for sphere cells was higher compared with parental cells treated with both etoposide (0.546 μmol/L vs. 0.062 μmol/L; Fig. 6A, left) and cisplatin (11.3 μmol/L vs. 1.8 μmol/L; Fig. 6A, right) after 72-hour plate culturing, which suggested sphere cells exhibited robust resistance to conventional chemotherapy. In addition, the sphere formation ability of parental and α281+/− cells with cisplatin treatment was tested, and α281+− cells showed stronger sphere formation ability and high risk of resistance (Fig. 6B).

**α281+ population presented different gene expression profiles.** α281+ cells exhibited the properties of CSC and chemoresistance. RNA-seq was used to explore the different gene expression profile of α281+ and α281− populations. FACS-sorted from parental H1048 cells (Supplementary Table S9). There were 28 genes (with at least 3.0-fold changes) concurrently upregulated and 8 genes downregulated in α281+ cells compared with α281− population (Supplementary Fig. S5A and S5B). By mapping all of these genes to the KEGG pathway database, the involved signaling pathways included the MAPK, PI3K-AKT, cell adhesion molecules (CAM), and p53 pathways, etc. (Supplementary Fig. S5C).

Figure 4.
Chemotherapy-induced CSC enrichment. A, Comparison of CD133 and α281 expression in H1048 cells treated with 300 ng/mL 600 ng/mL etoposide or solution for 72 hours. B, Comparison of CD133 and α281 expression in H1048 PDX models before and after 3 cycles of EP regimes. C–F, Comparison of CD133 and α281 expression in PDX models Bclu62, Bclu82, Bclu80, and Bclu44 before and after 3 cycles of EP chemotherapy. Intergroup comparison using ANOVA analysis or independent t test. VP-16, etoposide; EP, etoposide and cis-platinum regimes; 3cs, 3 cycles of chemotherapy.
MAPK pathway involved in the stem cell properties of α2δ1⁺ cells.

Considering the transcription test results and previous literature, we hypothesized that the MAPK pathway activated in α2δ1⁺ population and that this may contribute to the stem cell properties and chemoresistance. The crucial proteins involved in MAPK, PI3K–AKT pathways, and SOX2 protein were detected in FACS-sorted α2δ1⁺/−/C0 H1048 cells using Western blotting. The proteins of pERK1/2 and SOX2 were both over expressed in α2δ1⁺ population compared with α2δ1⁺/C0 cells (Fig. 6C).

We further investigated the effect of overexpression of α2δ1 on the expression of stem cell and drug resistance–related proteins and genes in MAPK and apoptosis-related pathways. As shown in the Fig. 6D, after overexpression, the upregulated proteins included BMI1, Nanog, OCT4, p-ERK, BCL2, combined with the down-regulation of proapoptosis proteins BAX and cleaved caspase-3. For gene expression, compared with the vector, α2δ1 overexpression upregulated stem cell–related genes (BMI1, EPCAM, NANOG, SOX2, KLF4, and CTNNB) and drug resistance genes (ABCG2 and MDR1; Fig. 6E).

The role of an inhibitor of ERK alone or in combination with 1B50-1 antibody.

MAPK pathway involved the properties of stem cell and drug resistance of α2δ1⁺ cells. We further investigated the role of ERK inhibitor alone or in combination with 1B50-1 antibody in vitro. As shown in Fig. 6F, when α2δ1⁺ cells were treated with single neutralized 1B50-1 antibody, single-agent ERK inhibitor (SCH772984) and combined 1B50-1 antibody, the sphere formation rate was all decreased. What is more, combined SCH772984 and 1B50-1 antibody could inhibit the growth of α2δ1⁺ cells in the greatest extent. This suggested that combined ERK inhibitor and 1B50-1 treatment may conquer the α2δ1⁺ sphere formation ability.

Discussion

This study is the first that we are aware of to evaluate expression of α2δ1 in SCLC cells and PDXs models by FACS, to verify the stem cell–like properties of α2δ1⁺ cells, and to identify the difference of α2δ1 and CD133 expression between chemosensitive and resistant PDXs models. Our data suggest that α2δ1⁺ SCLC cells demonstrate CSC properties, and might contribute to chemotherapy resistance. Furthermore, we have demonstrated that use of a 1B50-1 antibody could help overcome chemoresistance and delay relapse of SCLC in PDX models.

The target of 1B50-1 has been previously identified and validated as isoform 5 of α2δ1, a subunit of voltage-gated calcium channel complexes (26). Currently, little is known about the expression pattern and specific function of isoform 5 of α2δ1. Our study found that α2δ1⁺ cells from H1048 had higher sphere-forming efficiency in vitro, and were highly tumorigenic in vivo. The


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Figure 6.

α281+ cells also had a high differentiation potential when cultured with complete serum, suggesting that α281 marked stem cells from the H1048 cell line exhibited stem cell properties. Therefore, α281 identifies a highly tumorigenic and invasive subpopulation of SCLC. OCT4, Nanog, and SOX2 are three basic transcription factors that are expressed in both CSC-like cells and embryonic stem cells (27). In our study, stem cell–related transcription factors (SOX2, OCT4, and Nanog) and drug resistance–related genes (MDR and ABCG2) were more highly expressed in α281− and CD133+ cells, especially in α281+ cells. Also, compared with CD133− cells, α281+CD133− population exhibited not only higher sphere forming ability in vitro, but also relatively higher tumorigenicity, growth rate, and proliferation property in vivo. These indicated that α281+ cells exhibit stem cell–like properties in vitro and in vivo, and α281 could be a used as a marker for CSC screening in SCLC. At the same time, α281 overexpression may attribute to the resistance of chemotherapy of SCLC.

Many studies have demonstrated that PDX models can be used as surrogates for clinical biopsies of lung cancer patients in research (28–31), including our former study (25). Therefore, we utilized PDX models and flow cytometry to analyze the two markers. We found that CD133 was overexpressed in most PDXs and that α281 expression varied in individual PDXs. Furthermore, we treated H1048 cells and PDXs with chemotherapy, and found out that α281+/CD133− positive cells in H1048 cells significantly increased after treatment compared with control group, and α281−positive cells increased even more. We observed the same trend in the tumors derived from H1048 cells in mice as well after they were treated with chemotherapy. However, in subgroup of PDXs with partial response (PR) to chemotherapy, significantly increased α281 and stable CD133 expression was observed after chemotherapy in PDXs. Contrarily, α281 and CD133 expression stay the same before and after chemotherapy in nonresponsive PDXs.

The enrichment of α281 and CD133 subpopulations after chemotherapy from H1048 cell line and PDXs were not exactly the same. The possible reasons are as follows: first, H1048 cell line–based xenografts grow primarily as homogeneous masses of cancer cells with minimal stromal infiltration; therefore, they might not recapitulate the human tumor architecture and interactions between stromal components and patient tumor cells (32). Second, PDXs should be better models for representing the condition of patient’s tumor. In vivo studies are always more convincing than in vitro studies because in vitro experiments fail to replicate the complex cellular conditions of an organism and sometimes may lead to results that do not correspond to the
circumstances occurring around a living organism (33). Keeping consistent showed that to resistance and recurrence. What is more, higher and metastasis in SCLC cells (34). Akt1 and Akt2 are viewed as canonical MAPKs with cytoplasmic and nuclear substrates involved in signal transduction and the regulation of transcription. These two kinases play redundant roles in proliferation and differentiation. Emerging evidence suggests that in addition to having a possible role in differentiation and proliferation, activation of the RAF/MEK/ERK pathway can promote cell invasion and metastasis in SCLC cells (34–37). There are no studies currently investigating the relationship between the voltage-dependent calcium channel α2δ1 and stem cells in SCLC. However, recently Ciccarelli and colleagues reported that the MEK/ERK pathway plays a prominent role in maintaining the stem-like phenotype of radhoblastosarcoma cells, their survival and their innate radioresistance (38). In combination with our data demonstrating overexpression of phosphorylated ERK in α2δ1þ SCLC cells, this suggests that the role of the MEK/ERK pathway in SCLC chemoresistance should be further investigated.

Zhao and colleagues generated the mAb, 1B50-1, which had a therapeutic effect on hepatic cell carcinoma (18). Our study is the first to treat SCLC PDXs with 1B50-1 antibody, and we found that originally chemoresistant PDX80 developed PR after combination treatment with 1B50-1 antibody and chemotherapy. However, no superiority of single-agent 1B50-1 treatment was seen compared with chemotherapy alone or combined therapy. This may suggest that stem cells only make up a small portion of tumor cells in SCLC that could be responsive to therapeutic agent targeted at stem cell markers such as α2δ1 instead of standard chemotherapy. The limitations of current study are that first this is a small sample size, and second it would be better if we also included the primary patients derived cell lines for in vitro study. These results should be further validated in additional samples and in the clinical setting.

In conclusion, α2δ1 subunit–positive SCLC cells have CSC properties, and are related to chemotherapy resistance. The ERK protein in MAPK pathway was highly expressed in the α2δ1þ H1048 cell line, and might be the critical molecular for resistance mediated by the α2δ1 subunit. Use of a specific antibody of 1B50-1 to α2δ1 subunit could improve response to chemotherapy and delay relapse, whether combined with chemotherapy or as sequential therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J. Yu, J. Wang
Development of methodology: J. Yu, S. Wang, Z. Wang, J. Wang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Yu, S. Wang, W. Zhao, H. Chen, Y. Tian, D. Wang, Z. Zhao, T. An, M. Wu, J. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Yu, S. Wang, W. Zhao, Z. Wang, Y. Tian, D. Wang, J. Wang
Writing, review, and/or revision of the manuscript: J. Yu, S. Wang, W. Zhao, H. Chen, T. An, J. Wang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Yu, S. Wang, W. Zhao, J. Duan, T. An, M. Wu, J. Wang
Study supervision: J. Yu, H. Bai, J. Wang
Other (submission of the manuscript): S. Wang

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Mechanistic Exploration of Cancer Stem Cell Marker Voltage-Dependent Calcium Channel $\alpha 2\delta 1$ Subunit-mediated Chemotherapy Resistance in Small-Cell Lung Cancer

Jiangyong Yu, Shuhang Wang, Wei Zhao, et al.

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