Mechanistic exploration of cancer stem cell marker voltage-dependent calcium channel α2δ1 subunit-mediated chemotherapy resistance in small cell lung cancer

Jiangyong Yu\textsuperscript{a}, Shuhang Wang\textsuperscript{b}, Wei Zhao\textsuperscript{c}, Jianchun Duan\textsuperscript{a}, Zhijie Wang\textsuperscript{a}, Hanxiao Chen\textsuperscript{b}, Yanhua Tian\textsuperscript{d}, Di Wang\textsuperscript{d}, Jun Zhao\textsuperscript{b}, Tongtong An\textsuperscript{b}, Hua Bai\textsuperscript{a}, Meina Wu\textsuperscript{b}, Jie Wang\textsuperscript{a,b,*}

\textsuperscript{a} State Key Laboratory of Molecular Oncology, Department of Medical Oncology, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China

\textsuperscript{b} Department of Thoracic Medical Oncology, Key laboratory of Carcinogenesis and Translational Research, Peking University Cancer Hospital & Institute, Beijing, China

\textsuperscript{c} Department of Cell Biology, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education/Beijing), Peking University Cancer Hospital and Institute, Beijing 100142, China.

\textsuperscript{d} Peking-Tsinghua Center for Life Sciences, Academy for Advanced Interdisciplinary Studies, Peking University, Beijing, China.

* Corresponding author: Jie Wang, MD, PhD, \textsuperscript{a} National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Chaoyang District, Panjiayuan Nanli No.17, Beijing 100021, China; \textsuperscript{b} Previous address: Key laboratory of Carcinogenesis and Translational Research, Peking University Cancer Hospital & Institute, Beijing 100142, China; Phone: 86-13910704669. E-mail: wangjie_cc@yahoo.com.

Conflicts of Interest and Source of Funding: The authors declare no potential conflicts of interest.

Note: Jiangyong Yu, Shuhang Wang and Wei Zhao contributed equally to this work.
Statement of 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 chemo-resistant 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 chemo resistance 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.
Abstract

Purpose: Chemo-resistance in small cell lung cancer (SCLC) is reportedly attributed to the existence of resistant cancer stem cells (CSCs). Studies involving CSC-specific markers and related mechanisms in SCLC remain limited. The current study explored the role of the voltage-dependent calcium channel $\alpha_2\delta_1$ subunit as a CSC marker in chemo-resistant SCLC, and explored the potential mechanisms of $\alpha_2\delta_1$-mediated chemo-resistance 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 xenografts (PDXs) 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 chemo-resistance in SCLC. Additionally, possible interventions to overcome $\alpha_2\delta_1$ mediated chemo-resistance were examined.

Results: Different proportions of $\alpha_2\delta_1+$ 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 $\alpha_2\delta_1+$ cells instead of CD133+ cells in PDXs, and an increased proportion of $\alpha_2\delta_1+$ cells corresponded to increased chemo-resistance. Activation and over-expression of Erk in the $\alpha_2\delta_1$ positive H1048 cell line was identified at the protein level. 1B50-1 mAb 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 chemo-resistance. Erk may play a key role in $\alpha_2\delta_1$ mediated chemo-resistance. 1B50-1 inhibitors may serve as potential anti-SCLC drugs.
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 (CSCs) 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 anti-tumor 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 trans-membrane 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 chemotherapy 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 express CD133 and possess stem-cells 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). Sarvi et al 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 his colleagues studied CD133 and CD87 as CSC markers in a panel of six SCLC cell line and concluded that CD133 and CD87 were inadequate CSC markers in SCLC (17). Meng et al 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 cancer stem cells 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. Wei Zhao et al. (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 HCC recurrence. The functional part was identified and validated as isoform 5 of α2δ1, and its monoclonal antibody 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 chemo-resistance and seeking novel strategies to overcome chemo-resistance 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 RPMI-1640 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 to 6 weeks, weighing 18 to 21 g) used in vivo experiments were purchased from Huafu Kang Experimental Animal Co., Ltd (Beijing, China). All animals were fed in the SPF class room by the Peking University Clinical Cancer Hospital Animal Center.

Five SCLC patients derived xenografts (PDXs) 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 (HE staining), IHC staining (common SCLC markers, CD56, TTF1, CgA and Syn) or therapy response to standard regimens (Table S1). Histological 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 National Institutes of Health 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 labelling and Flow cytometry analysis

Cells were collected and re-suspended at 1x10⁶ cells/mL of PBS buffer. Fluorescent-labeled monoclonal antibodies 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, Mountain View, CA, USA), respectively. The antibodies used were APC/PE-conjugated mouse antihuman CD133 and corresponding isotype IgG. Detailed information of antibodies is listed in supplement data table S2. The 1B50-1 antibody and isotype IgG were supplied friendly by Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Cell Biology, Peking University Cancer Hospital and Institute, Beijing. 1B50-1 was directly labeled with PE-Cy5 or fluorescein using the respective Lightning conjugation kits following the vendor’s Protocol (Innova Biosciences Ltd., Cambridge, UK) [20].

Properties of CD133 and a2δ1 marked stem cells of H1048 cell line
**Sphere Formation Assay:** After counting, cells were mixed with a medium of Dulbecco’s modified Eagle’s medium/F12 (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, Rocky Hill, NJ, USA), and 1% methylcellulose (Sigma), and were cultured in semi-suspension in Ultra Low Attachment 96-well plates (Corning Incorporated Life Sciences, Acton, MA, USA) [20]. The number of spheres was counted and recorded under a stereomicroscope (Olympus, Tokyo, Japan) after 2-3 weeks’ incubation.

**Tumorigenicity assay:** Cells purified by fluorescence-activated cell sorting (FACS) were suspended in PBS and mixed 1:1 with Matrigel (BD Biosciences, Bedford, MA, USA). Suspensions of $10^2$-$10^3$ cells were transplanted s.c. bilaterally in the back of NOD/SCID mice to investigate the capacity of tumorigenicity. The tumor was observed and measured twice weekly.

**Reverse transcription-polymerase chain reaction (RT-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 α2δ1/CD133 positive and negative cells. The expression of CACNA2D1 (α2δ1 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 lysed into a single cell suspension and RNA was extracted using Trizol and chloroform. cDNA was synthesized using EasyScript First Strand cDNA Synthesis SuperMix (TransGen, Beijing, China). 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 supplement data table S3.

**Western Blot Analysis**

Cells were lysed using cell lysis buffer (Cell Signaling Technology) and quantified by a bicinchoninic acid assay (Applygen). 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 2h 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, pS6, SOX2, and beta-actin et al. Detailed information of antibodies applied are listed in supplement data 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 etopside (the standard chemotherapy regimen for SCLC) for 72 hours. Following exposure to the drug, we analyzed expression of α2δ1 and CD133 by flow cytometry. We implanted Balb/c mice with H1048 cells. After the tumors grow to a volume of about 200 mm$^3$, we administered standard chemotherapy for SCLC (etopside and cis-platinum, EP) for 3 cycles. We obtained the residual tumors and analyzed α2δ1 and CD133 expression in EP-treated mice as compared to 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 to the supplementary documents.*)

Animals were treated by intraperitoneal injection with etopside and cisplatin (EP) [4 mg kg$^{-1}$ cisplatin dissolved in 0.9% saline solution on day 1, and 12 mg kg$^{-1}$ etopside (Sigma) dissolved in 0.9% saline solution on days 1-3], or the corresponding vehicle only, each week 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 was ulcerated, mice were euthanized using carbon dioxide.

Chemotherapeutic response was evaluated as the percentage of tumor growth inhibition (% TGI), calculated using the equation “100 - (the mean RTV of the treated tumor / the mean RTV in the control group at the time of sacrifice×100)”, 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 standard deviation were calculated for each group (19).

To test the therapeutic effect of 1B50-1, we treated PDX models Bclu80 and Bclu62 with 3 cycles of EP regimes combined or sequenced with 1B50-1 antibody (800 µg/mouse, qod, intraperitoneal injection, until disease progression). PDXs and corresponding clinical data refer to table S1.

RNA-Sequencing

Total RNA was extracted from α2δ1+ and α2δ1- 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, USA) 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 (5X). 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, Beverly, USA). 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, San Diego, CA, USA) 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 based on 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 DEGSeq R package (1.12.0) (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., Armonk, NY, USA). Comparisons between groups were performed by paired Student’s \( t \)-test, independent \( t \)-test or one-way analysis of variance (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 α2δ1 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 α2δ1 expression was 1.40 ± 0.26%, 12.08 ± 2.23% and 4.20 ± 1.35% respectively (Figure 1A). A relationship between α2δ1+ and CD133+ was observed in H1048. The expression of CD133 in H1048 cells was significantly higher than α2δ1 (P < 0.001) (Figure 1B), and CD133+ population is nearly 100% positive in α2δ1+ cells, while only 2.67% α2δ1+ cells occur in CD133+ cells. (Figure 1C). Total α2δ1+ cells are a particular sub-population in CD133+ cells, which regards as CD133+/α2δ1+ cells in H1048. Confocal laser scanning microscopy showed that CD133 was expressed on the membrane, and α2δ1+ demonstrated granula membrane expression (Figure 1D).

α2δ1+ positive and CD133 positive cells exhibited stem-cell properties

α2δ1+ and CD133+ cells presented high self-renewal capacity

α2δ1+/− and CD133+/− populations of H1048 cell line were identified and sorted by FACS and cultivated in semi-solid media without serum to evaluate self-renewal capacity. With FBS-free medium, we performed the clone formation ability of α2δ1+ and CD133+ cells compared with control cells. Results suggested both α2δ1+ and CD133+ cells demonstrated higher sphere-forming efficiency compared with α2δ1- and CD133- populations as well as parental H1048 cells, respectively (Figure 2A, 2B). The sphere-forming ability of α2δ1+ cells were even stronger than CD133+ cells (P = 0.013). What’s more, cells separated from α2δ1+ or CD133+ spheres could be passaged and the efficiency of sphere formation elevated with passage (Figure 2C). For demonstrating in vivo, FACS-sorted α2δ1+/− and CD133+/− cells of H1048 were bilaterally inoculated into the backs of NOD/SCID mice with different magnitude (10^2 or 10^3) to observe the tumorigenic ability (Figure 2D). Double positive (α2δ1+CD133+) cells exhibited higher frequency of tumorigenity compared with α2δ1-CD133+ and α2δ1-CD133- cells respectively (Table S5). Moreover, α2δ1+ and CD133+ cells had higher tumor-formation rates compared with α2δ1- and CD133- population respectively in H1048, H69, Belu80 PDX (Table S6). Serial transplantation assay was performed into mice for validating self-renewal in vivo. Both α2δ1+ and CD133+ cells constantly obtained the ability of self-renew when generating. However, α2δ1+ cell exhibited no significantly higher growth rate than only CD133+ cells at the same concentration (Tables S7), and continuously feed for 80 days, tumor volumes were measured as shown in Figure 2E. A series of passaged paraffin-embedded sections were examined by HE examination. The morphology of cells remained consistent between different passages and also with parent sample (Figure 2F).
ɑ2δ1+ cells also presented an elevated self-renewal capability in H69 cell lines both in vitro and in vivo (Figure S1). These data indicated ɑ2δ1+/CD133+ cell contributes higher tumorigenic ability in SCLC.

ɑ2δ1+ and CD133+ cells exhibited differentiation properties

When cultured in medium with complete serum (10% FBS), the purified ɑ2δ1+ and CD133+ cells of H1048 cell line showed differentiation ability. After two weeks of culture, flow cytometry analysis showed that the proportion of ɑ2δ1+ and CD133+ cells returned from 98.2% and 97.8% to baseline level 1.1% and 43.2%, respectively (Figure 3A). Moreover, FACS-sorted purified ɑ2δ1+/CD133+ cells from the first-generation tumors formed by ɑ2δ1+/CD133+ cells were transplanted into NOD/SCID mice again to examine the serial transplantation capacity in vivo. The proportion of ɑ2δ1+ 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% (Figure 3B). This phenomenon was also observed in serial passage of ɑ2δ1+ Bclu80 PDX models (Figure S2). It demonstrated the differentiation properties of ɑ2δ1+ and CD133+ cells were conserved both in vitro and in vivo.

Stem cell and drug resistant related genes expressions in stem cell-like cells

To verify the stem cell-like properties of ɑ2δ1+ 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 ɑ2δ1+/- and CD133+/- H1048 cells. Results showed that all of these genes were more highly expressed in ɑ2δ1+ and CD133+ cells compared with their corresponding negative cells. Notably, the expression levels were even higher in ɑ2δ1+ cells than in CD133+ cells, especially for OCT4, MDR1 and ABCG2 (Figure 3C).

Stem cell-like marker expression in patient-derived xenografts (PDXs)

Next, we evaluated the response of corresponding PDXs with different expression of ɑ2δ1 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 morphological, pathological, 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 ɑ2δ1 and CD133 expression by flow cytometry. Results showed that ɑ2δ1 and CD133 expression varied between individual PDXs. Of note, Bclu80 model presented significantly higher expression of ɑ2δ1 compared with Bclu62, Bclu82, Bclu44 and
Bclu77, while expression of CD133 was relatively lower (Figure 3D). The expression ratio of a2δ1/CD133 showed similar trend with the expression of a2δ1 in corresponding PDXs (Figure 3E). Moreover, the expression of a2δ1 related genes (CACNA2D1) examined by RT-PCR was also consistent with the expression of a2δ1 protein determined by flow cytometry (Figure 3F). We also explored the stem-cell properties of a2δ1+ cells in PDX models. a2δ1+ populations showed high efficiency of sphere-forming and tumorigenicity compared with a2δ1- cells in the Bclu80 model. (Figure S4, Table S6)

In addition, PDXs with different expressions of stem-cell markers showed diverse responses to chemotherapy. The models with relatively low a2δ1 expression or a low a2δ1/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 a2δ1 and a high a2δ1/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 a2δ1 expression and a low a2δ1/CD133 ratio, showed only SD or limited remission (minimal PR) in PDXs. However, the corresponding patient acquired a clinical response of PR to chemotherapy (Table S1 and S8; Figure 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**

In order to investigate the effects of chemotherapy on expression of stem-like cells, H1048 cells were treated with different concentrations of etoposide for more than 2 weeks, and a2δ1 and CD133 were evaluated by flow cytometry. The proportion of a2δ1+ and CD133+ cells increased after treated with etoposide and the a2δ1+ population was enriched even more after treatment (Figure 4A). Similarly, the proportion of a2δ1+ and CD133+ cells significantly increased in tumors treated after 3 cycles of EP chemotherapy as compared to those in control group in H1048 transplanted mice (Figure 4B). Analogous with the in vitro results, the a2δ1+ population showed a more significant enrichment after treatment than the CD133+ population.

**Enrichment of stem like cells in PDX models**

Tumors from Bclu62 and Bclu82 PDX models were sensitive to chemotherapy with a best response of PR. The proportion of a2δ1+ cells in the Bclu62 and Bclu82 PDX tumors after 3 cycles chemotherapy of EP regimes increased significantly. However, CD133 expression
remained stable (Figure 4C-D). The Bclu80 PDX showed a response of SD with minimal shrinkage, and the proportion of α2δ1+ and CD133+ population did not significantly change before and after chemotherapy (Figure 4E). Similarly, non-sensitive Bclu44 PDX showed the same trend as Bclu80 (Figure 4F). Chemotherapy induced the enrichment of α2δ1+ population rather than CD133+ cells.

**PDXs response to different treatment modality of 1B50-1 antibody**

1B50-1 antibody is a specific monoclonal antibody against the α2δ1 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 shrunk 75.5% compared to baseline) than the group treated with chemotherapy alone (tumor shrunk 49.7%). And the tumors in group of 1B50-1 antibody also exhibited slow growth compared with those in control group (Figure 5A). Similar results were observed in the Bclu62 PDX model (Figure 5B). In the alignment of sequential treatment of Bclu80 PDX, all animals received 3-cycles of EP regimes and then were grouped randomly in 1B50-1 antibody and control group. The antibody-treated group showed significantly slower tumor growth compared with control group (Figure 5C). Together, these data indicated that 1B50-1 antibody is effective in slowing down tumor growth whether combined with chemotherapy or alone, although the superiority of single-agent therapy was not obvious as compared to chemotherapy. We also explored the proportion of α2δ1+ population in different treatment groups. The groups treated with 1B50-1 antibody showed relatively low proportion of α2δ1+ cells compared with control and EP chemotherapy groups (Figure 5D).

**α2δ1+ population of H1048 were chemo-resistant in vitro**

To confirm the expression ratio of α2δ1 related to the chemo-sensitivity and chemotherapy induced the enrichment of α2δ1+ cells in PDX models, we then evaluated the sensitivity of α2δ1+ cells to chemotherapy. The sensitivity of parental cells versus the passage cells digested from spheres of α2δ1+ 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 (0.062 μmol/L vs. 0.546 μmol/L) (Figure 6A Left) and cisplatin (1.8 μmol/L vs. 11.3 μmol/L) (Figure 6A Right) after 72 hours plate culturing, which suggested sphere cells exhibited robust resistance to conventional chemotherapy. Additionally, the sphere formation ability of parental and α2δ1+/cells with cisplatin treatment was tested, and α2δ1+ cells showed stronger sphere formation ability and high risk of resistance (Figure 6B).

**α2δ1+ population presented different gene expression profiles.**
α2δ1+ cells exhibited the properties of cancer stem-cell and chemo-resistance. RNA-seq was used to explore the different gene expression profile of α2δ1+ and α2δ1- populations FACS-sorted from parental H1048 cells (Table S9). There were 28 genes (with at least 3.0-fold changes) concurrently up-regulated and 8 genes down-regulated in α2δ1+ cells compared with α2δ1- population (Figure S5 A-B). By mapping all of these genes to the KEGG pathway database, the involved signaling pathways included the mitogen-activated protein kinases (MAPK), PI3K-AKT, Cell adhesion molecules (CAMs), and p53 pathways, et al (Figure S5 C).

**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 chemo-resistance. The crucial proteins involved in MAPK, PI3K-AKT pathways and SOX2 protein were detected in FACS-sorted α2δ1+/− H1048 cells using western-blotting. The proteins of pErk1/2 and SOX2 were both over expressed in α2δ1+ population compared with α2δ1- cells (Figure 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 figure 6D, after overexpressed, the upregulated proteins included BMI1, Nanog, OCT4, p-ERK, BCL2, combined with the downregulation of pro-apoptosis proteins BAX and cleaved Caspase3. 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) (Figure 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 Figure 6F, when α2δ1+ cells were treated with single neutralize 1B50-1 antibody, single agent ERK inhibitor (SCH772984) and combined 1B50-1 antibody/ERK inhibitor, the sphere formation rate was all decreased. What’s 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 positive cells, and to identify the difference of α2δ1 and CD133 expression between chemo-sensitive and resistant PDXs models. Our data suggest that α2δ1 positive SCLC cells demonstrate CSC properties, and might contribute to chemotherapy resistance. Further, we have demonstrated that use of a 1B50-1 antibody could help overcome chemo-resistance 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 α2δ1+ cells also had a high differentiation potential when cultured with complete serum, suggesting that α2δ1 marked stem cells from the H1048 cell line exhibited stem cell properties. Therefore, α2δ1 identifies a highly tumorigenic and invasive subpopulation of SCLC. OCT4, Nanog, and SOX2 are three basic transcription factors that are expressed in both cancer stem cell-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, ABCG2) were more highly expressed in α2δ1+ and CD133+ cells, especially in α2δ1+ cells. Not mention that, no matter sphere-forming ability or differentiation property α2δ1+/CD133+ cells are both higher than CD133+ cells in vitro; also, in subsequent in vivo study, α2δ1+ cells also exhibited higher growth rate and proliferation property than CD133+ cells at the same concentration. These indicated that α2δ1+ cells exhibit stem cell like properties in vitro and in vivo, and α2δ1 could be a used as a marker for CSCs screening in SCLC. At the same time, α2δ1 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 α2δ1 expression varied in individual PDXs. Further, we treated H1048 cells and PDXs with chemotherapy, and found out that α2δ1/CD133 positive cells in H1048 cells significantly increased after treatment compared with control group, and α2δ1 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 α2δ1 and stable CD133 expression was observed after
chemo in PDXs. Contrarily, α2δ1 and CD133 expression stay the same before and after chemo in non-responsive PDXs.

The enrichment of α2δ1 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 fails 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 these limitations in mind, our data from in vitro, in vivo and PDXs consistently showed that α2δ1+ subpopulation might contribute to resistance and recurrence. What’s more, higher α2δ1 expression or a higher ratio of α2δ1/CD133 were related to chemo-resistance. This suggests that α2δ1 overexpression should be an independent factor for chemo-resistance in vivo.

We also performed transcriptome sequencing to explore the possible mechanisms of α2δ1-mediated chemo-resistance (Figure S5). We found an enrichment of genes related to MAPK, PI3K-AKT and CAMs signaling pathways. Combined analysis with previous literature [20], we speculated MAPK signaling pathway might be more important. We explored the expression of crucial proteins involved in MAPK, PI3K-AKT pathways and SOX2 protein, and found overexpression of phosphorylated Erk from MAPK pathway in α2δ1+ H1048 cells. ERK1/2 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 Carmela Ciccarelli et al reported that the MEK/ERK pathway plays a prominent role in maintaining the stem-like phenotype of rhabdomyosarcoma cells, their survival and their innate radio-resistance (38). In combination with our data demonstrating overexpression of phosphorylated Erk in α2δ1+ SCLC cells, this suggests that the role of the MEK/ERK pathway in SCLC chemo-resistance should be further investigated.

Wei Zhao et al. generated the monoclonal antibody, 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 chemo-resistant 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 which 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 α2δ1+ 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.

Acknowledgements

We thank Zhiqian Zhang and Haibo Han from the Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Cell Biology for the supply of antibody, experiment assistance and instruction, and Huirong Ding, Zhihua Tian and Lina Wu from the Central Laboratory, Key Laboratory of Carcinogenesis and Translational Research, Ministry of Education, Beijing Cancer Hospital & Institute for their technical support and assistance. We thank Wendy Bindeman (Mayo Clinic, Immunology Department) for English editing assistance and Chengli Song, from Novogene Company, for her contribution to biology information processing.
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

References:


Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.


Figure legends

**Figure 1: Expression of stem cell-like markers.**

Figure 1: A. Flow cytometry analysis (FCM) showed different expression of α2δ1 and CD133 in H1048, H69 and H209. B. Expression difference of α2δ1 and CD133 in H1048 cell line detected by FCM. Using independent t-test. **P < 0.01. C. FCM showed the co-expression of CD133 and α2δ1 in H1048 cells. D. Representative pictures showed the expression of α2δ1 and CD133 in H1048 cells using a confocal microscope.

**Figure 2 stem-cell properties of α2δ1 and CD133 marked cell in H1048 cell line and drug resistance related genes**

A. The first-generation sphere of CD133 and α2δ1 sorted H1048 cell in semi-solid media without serum. B. Comparison of sphere formation efficiency of parental H1048 cells with first generation CD133 and α2δ1 sorted H1048 cells. Using ANOVA analysis. **P < 0.01. C. Cells of CD133+ and α2δ1+ sphere could be serially passaged and sphere-formation efficiency elevated with passage in vitro. Using ANOVA analysis. **P < 0.01. D: Tumor formation of α2δ1 (Left) and CD133 (Right) sorted H1048 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 α2δ1+ and CD133+ H1048 cells with different magnitude (10^3, 10^4). F. Representative HE staining images of parent tumor and serially passaged CD133+ and α2δ1+ tumor specimens (200×).

**Figure 3: α2δ1+ and CD133+ cells of H1048 were highly tumorigenic in vivo**

A. FCM showed purified α2δ1+ and CD133+ H1048 cells rescued to their parental percentage after 2 weeks of culture in medium with 10% FBS. B. FCM showed the proportion of α2δ1+ and CD133+ cells in the first generation, purified cells and second generation PDX models of H1048. C. Expression difference of stem-cell and resistance related genes in α2δ1 and CD133 antibody FACS-sorted H1048 cells. D. Comparison of α2δ1 and CD133 expression in five patient derived xerograph (PDX) models, using ANOVA analysis, **P < 0.01. E. The expression ratio of α2δ1/CD133 in five PDX models, using ANOVA analysis, **P < 0.01. F. qRT-PCR analysis of the α2δ1 mRNA level in five PDX models.

**Figure 4: Chemotherapy induced CSC enrichment**

A: Comparison of CD133 and α2δ1 expression in H1048 cells treated with 300ng/ml,
600ng/ml etoposide or solution for 72 hours. B: Comparison of CD133 and α2δ1 expression in H1048 PDX models before and after 3 cycles of EP regimes. C-F. Comparison of CD133 and α2δ1 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.

**Figure 5:** PDXs response to different treatment modality of 1B50-1 antibody and chemotherapy.

A: Growth curves of Bclu80 PDX models treated with etoposide (12 mg kg⁻¹, d1-d3) and cisplatin (4 mg kg⁻¹) (E/P, 10 days/cycle), 1B50-1 antibody 800 mg/d qod, or combined with both. B. Growth curves of Bclu62 PDX models treated with E/P regimes, 1B50-1 antibody, or combined with both. C. Growth curves of Bclu80 PDX models treated with 3 cycles of E/P followed by 1B50-1 antibody or nonrelated IgG (control). The red and blue arrow indicated the initial time of E/P chemotherapy and 1B50-1 antibody, respectively. D: α2δ1 expression analyzed using flow cytometry in different treatment modality groups of Bclu80 PDX models.

**Figure 6:** α2δ1+ H1048 cells presented resistance property and MAPK pathway might be involved.

A: Comparison of IC50 between H1048 parental and sphere cells treated with etoposide (Left) and cisplatin (Right). B: The sphere formation analysis showed the different reaction of parental and FAC-sorted α2δ1+/− cells to different concentration cisplatin treatment. using ANOVA analysis. *, significant. C: Western blotting shows the representative molecules of MAPK, PI3K-AKT pathway and SOX2 using lysates of α2δ1+ and α2δ1- FACS-sorted H1048 cells. D: Western blotting shows the differences of representative molecules associated with cancer stem cell (CSC) related, MAPK, PI3K-AKT and apoptosis pathway after overexpression of α2δ1 in H1048 cells. E. RT-PCR indicated the fold changes of genes related to CSC and resistance after overexpression of α2δ1 in H1048 cells. F. Sphere formation was inhibited when cultured with Erk inhibitor and also combined with 1B50-1 antibody (Upper), and the inhibition was dose-dependent (Lower). *, compared with parental cells. #, compared with single ERK inhibitor. &, compared with single 1B50-1.
Figure 2

A

B

C

D

E

F
Figure 4

A. H1048 cell line

B. H1048 PDX

C. Bclu62 PDX

D. Bclu82 PDX

E. Bclu80 PDX

F. Bclu44 PDX
Figure 5

(A) Volume of tumor (Mean ± SEM mm$^3$) over days for different groups: E/P chemo, E/P+1B50-1, 1B50-1, and Control.

(B) Volume of tumor (Mean ± SEM mm$^3$) over days for E/P chemo, E/P+1B50-1, 1B50-1, and Control.

(C) Volume of tumor (Mean ± SEM mm$^3$) over days for Control and 1B50-1.

(D) Relative expression of α2β1 (%) for different conditions: Control, 1B50-1, E/P chemo, E/P+1B50-1, control seq, and 1B50-1 seq.
Figure 6

A

B

C

D

E

F

Research.
Clinical Cancer Research

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.

Clin Cancer Res  Published OnlineFirst February 6, 2018.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-17-1932

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2018/02/06/1078-0432.CCR-17-1932.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/early/2018/02/06/1078-0432.CCR-17-1932. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.