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Corresponding Author:	Xin Sui, Ph.D. Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education/Beijing), Peking University Cancer Hospital and Institute Beijing, Beijing CHINA
Corresponding Author's Institution:	Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education/Beijing), Peking University Cancer Hospital and Institute
Corresponding Author E-Mail:	suixin@pku.edu.cn
Order of Authors:	Xin Sui, Ph.D. Jian-Hao Geng Hui-Ming Yu Yong-Heng Li Wei-Hu Wang
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TITLE:

Radiosensitivity of Cancer Stem Cells in Lung Cancer Cell Lines

AUTHORS & AFFILIATIONS:

Xin Sui¹, Jian-Hao Geng¹, Hui-Ming Yu¹, Yong-Heng Li¹, Wei-Hu Wang¹

¹Key Laboratory of Carcinogenesis and Translational Research, Ministry of Education, Department of Radiation Oncology, Peking University Cancer Hospital and Institute, Beijing, China

Corresponding Author:

Wei-Hu Wang (wangwei88@163.com)

Email Addresses of Co-Authors:

Xin Sui (suixin@pku.edu.cn)

Jian-Hao Geng (gengjh3@sina.com)

Hui-Ming Yu (huiming740512@126.com)

Yong-Heng Li (yonghenglee@163.com)

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SUMMARY:

The presence of cancer stem cells have been associated with relapse or poor outcomes after radiotherapy. This manuscript describes the methods to study the radiosensitivity of cancer stem cells in lung cancer cell lines.

ABSTRACT:

The presence of cancer stem cells (CSCs) has been associated with relapse or poor outcomes after radiotherapy. Studying radioresistant CSCs may provide clues to overcoming radioresistance. Voltage-gated calcium channel $\alpha 2\delta 1$ subunit isoform 5 has been reported as a marker for radioresistant CSCs in non-small cell lung cancer (NSCLC) cell lines. Using calcium channel $\alpha 2\delta 1$ subunit as an example of a CSC marker, methods to study the radiosensitivity of CSCs in NSCLC cell lines are presented. CSCs are sorted with putative markers by flow cytometry, and the self-renewal capacity of sorted cells is evaluated by sphere formation assay. Colony formation assay, which determines how many cells lose the ability to generate descendants forming the colony after a certain dose of radiation, is then performed to assess the radiosensitivity of sorted cells. This manuscript provides initial steps for studying the radiosensitivity of CSCs, which establishes the basis for further understanding of the underlying mechanisms.

INTRODUCTION:

Radiotherapy plays an important role in cancer treatment. However, existence of radioresistant

cancer stem cells (CSCs) may lead to relapse or poor outcomes after radiotherapy^{1,2}. CSCs are characterized by their self-renewal capacity and ability to generate heterogenous cancer cells³. Armed with a more efficient DNA damage repair capacity or higher levels of free-radical scavenging systems or other mechanisms, CSCs are relatively resistant to radiotherapy⁴⁻⁸. Identifying CSC markers and exploring their mechanisms will facilitate the development of drugs that will overcome radioresistance without increasing normal tissue damage.

Voltage-gated calcium channel $\alpha 2\delta 1$ subunit isoform 5 has been reported as a marker for radioresistant CSCs in NSCLC cell lines⁹. $\alpha 2\delta 1$ was originally identified as a CSC marker for hepatocellular carcinoma (HCC)¹⁰. Using subtractive immunization with a pair of HCC cell lines derived from the primary and recurrent tumors in the same patient, an antibody named 1B50-1 was identified to target recurrent HCC cells specifically. 1B50-1-positive cells showed high sphere formation efficiency in vitro and high tumorigenicity in vivo. Its antigen was identified by mass spectrometry, as calcium channel $\alpha 2\delta 1$ subunit isoform 5. $\alpha 2\delta 1$ specifically expresses in CSCs and is undetectable in most normal tissues, making it a potential candidate for targeting CSCs¹⁰. $\alpha 2\delta 1$ can also serve as a CSC marker for NSCLC cell lines, and it has been shown to impart radioresistance to NSCLC cells partially by enhancing the efficiency of DNA damage repair in response to radiation⁹.

Studying radioresistant CSCs may provide clues to overcoming radioresistance. Using $\alpha 2\delta 1$ in NSCLC as an example, major methods to study the radiosensitivity of CSCs are presented. Usually, CSCs are isolated with a putative surface marker, and the stem cell characteristics and radiosensitivity of the positive and negative cell populations are compared. Sphere formation in a serum-free medium supplemented with growth factors that support self-renewal is a useful assay to evaluate the stemness of cells in vitro. Cells with high sphere formation capacity are likely to show high tumorigenicity when injected into immunodeficient mice¹⁰⁻¹². Colony formation assay is then used to assess the radiosensitivity of cells, which determines how many have lost the ability to generate descendants forming the colony after a dose of radiation¹³.

PROTOCOL:

NOTE: Steps are performed under the indicated temperature. For steps in which the temperature is not mentioned, perform under room temperature (18–25 °C). Cell culture medium should be stored at 4 °C, and other reagents should be stored according to the manufacturer's guides. Medium should be pre-warmed to 37 °C before being added to cells.

1. Cell sorting

1.1. Antibody conjugation

NOTE: Considering the shorter incubation time, direct-labeled antibody is preferred for cell sorting. If no commercial direct-labeled antibody is available, use fluorescein-conjugating reagents to conjugate the antibody to a fluorescent dye according to the manufacturer's guide (see **Table of Materials**). As cells will be cultured after sorting, all steps should be performed in

a biological safety cabinet or laminar clean bench. To avoid contamination, antibiotics (penicillin and streptomycin) are recommended to be added to the culture medium after sorting.

1.1.1. Add 1 μL of modifier reagent for each 10 μL of antibody to be labeled. Mix gently.

1.1.2. Add the mixture of antibody and modifier directly onto the lyophilized fluorescein. Pipet gently. Leave the vial standing for 3 h or overnight at room temperature (RT) in the dark.

1.1.3. Add 1 μL of quencher reagent for every 10 μL of antibody. The conjugate can be used after 30 min. The fluorescein conjugate can be stored at 4 $^{\circ}\text{C}$ for up to 18 months.

1.1.4. Before sorting, titration of conjugated antibody is recommended. Prepare cells that express (e.g., H1299 or A549 for $\alpha\delta 1$) and do not express (e.g., H1975) the marker. Aliquot the cells and incubate them with antibody at different concentrations (e.g., 15 $\mu\text{g}/\text{mL}$, 7.5 $\mu\text{g}/\text{mL}$, 1.5 $\mu\text{g}/\text{mL}$, and 0.75 $\mu\text{g}/\text{mL}$, respectively).

1.1.5. Perform flow cytometric analysis (with histogram or dot plot) and choose the concentration in which positive cells of H1299 or A549 can be separated from the isotype control and H1975 cells which have little unspecific signals.

1.2. Cell labeling

1.2.1. Culture A549 cells in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) in 10 cm dishes at 37 $^{\circ}\text{C}$ and 5% CO_2 in a cell culture incubator. Digest cells as follows for sorting when they reach 80%–90% confluence (about $5\text{--}10 \times 10^6$ cells per dish).

1.2.2. Remove the culture medium. Wash cells with 3 mL of phosphate buffered saline (PBS) briefly. Add 3 mL of 0.05% trypsin to each dish. Remove the trypsin and leave the residuary trypsin to digest cells at 37 $^{\circ}\text{C}$ for 1–3 min. Check the detachment of the cells frequently to avoid overdigestion.

NOTE: Some cell lines may be relatively difficult to digest, such as NSCLC cell lines H520 and PC9. In such situation, 3 mL of trypsin can be left in the dish, rather than digestion with residuary trypsin. Moreover, the digestion time can be extended.

1.2.3. When cells become loose and begin to detach from the dishes, add 3 mL of RPMI 1640 medium supplemented with 10% FBS and pipette the cell suspension into a 50 mL tube.

1.2.4. Centrifuge at 300 x g at 4 $^{\circ}\text{C}$ for 5 min. Discard the supernatant. Add 10 mL of PBS to resuspend the cells. Transfer 0.5 mL (or at least 5×10^5 cells) of cell suspension into a new tube as an isotype control.

1.2.5. The remaining cells are for sorting. Centrifuge both the control and experimental tubes at 300 x g at 4 $^{\circ}\text{C}$ for 5 min. Discard the supernatant.

1.2.6. Prepare the working solution for staining by diluting the fluorescent dye-conjugated isotype control or antibody in PBS to the optimal concentration titrated as mentioned above.

NOTE: In this experiment, FITC-conjugated 1B50-1 (the antibody of $\alpha 2\delta 1$) was diluted into 7.5 $\mu\text{g}/\text{mL}$. The volume of working solution is dependent on the cell amount.

1.2.7. Resuspend the control and experimental cells with each working solution at about $2\text{--}5 \times 10^7$ cells/mL and mix gently. Incubate the samples at RT for 30–40 min in the dark.

1.2.8. Wash the cells by adding 10 mL of PBS to samples, mix gently and centrifuge at $300 \times g$ at 4°C for 5 min. Discard the supernatant. Resuspend cells with 10 mL of PBS.

1.2.9. Put 40 μm cell strainers on new 50 mL tubes for control and experimental cells respectively. Apply cell suspension onto the strainer and collect the flow-through.

NOTE: This step removes the cell clumps that may block the capillary of the cell sorter.

1.2.10. Centrifuge the flow-through at $300 \times g$ at 4°C for 5 min. Discard the supernatant. Resuspend the cells with 0.2–1.0 mL PBS then transfer into a 5 mL tube for flow cytometry (section 1.3).

1.3. Cell sorting by flow cytometry

1.3.1. Prepare two 5 mL tubes to collect the positive and negative cell populations. Add 1 mL of RPMI 1640 medium in each tube. Place the tubes at the collection platform of the cell sorter.

1.3.2. Analyze the control cells and experimental cells respectively with the dot plot. Gate the live cells with the parameters forward scatter (FSC) and side scatter (SSC). Gate the positive and negative population of live cells according to the FL-1 intensity.

1.3.3. Collect the $\alpha 2\delta 1$ -positive cells and $\alpha 2\delta 1$ -negative cells. Record the number of cells obtained.

1.3.4. To confirm that the harvested cell populations have different level of $\alpha 2\delta 1$ expression, a quantitative polymerase chain reaction (PCR) can be performed.

1.3.4.1. Extract RNA of positive and negative cells with guanidinium thiocyanate reagent and reverse transcript the RNA into cDNA according to the manufacturer's guide. Perform QPCR with SYBR green reagent. The sequences of primers are as follows: $\alpha 2\delta 1$ -F, CAGTTGAGATGGAGGATGATG; $\alpha 2\delta 1$ -R, TTGTATGAGCAGTCGTGTGTC; *GAPDH*-F, GTCGGAGTCAACGGATTTGG; and *GAPDH*-R, AAAAGCAGCCCTGGTGACC.

2. Sphere formation assay

NOTE: Sphere formation assay is applied to determine the self-renewal capacity of cells. Cells with self-renewal capacity could form spheres in this serum-free semisolid medium supplemented with growth factors. All steps should be performed in a biological safety cabinet or laminar clean bench. To avoid contamination, antibiotics (penicillin and streptomycin) are recommended to be added to the culture medium after sorting.

2.1. Medium preparation

2.1.1. Prepare 2x DMEM/F-12 medium by reconstituting a 1 L package of DMEM/F-12 powder into 475 mL of distilled water. Add 2.438 g of NaHCO_3 . Adjust the pH to 7.0–7.2 by slowly adding 1 N NaOH or 1 N HCl with stirring. Add distilled water to a final volume of 500 mL. Sterilize the medium by filtering through a 0.2 μm filter.

2.1.2. Prepare a 2% methylcellulose solution by adding 2 g of methylcellulose into 100 mL of distilled water. Autoclave the solution. The methylcellulose may show a cotton-like state after autoclaving. Mix it by reversing the bottle up and down gently for few times and leave it for natural cooling. It will become a transparent semisolid overnight.

2.1.3. To obtain 20 mL of self-renewal medium, add 10 mL of 2x DMEM/F-12 medium to a 50 mL tube. Add 400 μL of B27 to the medium. Add recombinant human epidermal growth factor (EGF) to a final concentration of 25 ng/mL. Add recombinant human basic fibroblast growth factor (bFGF) to a final concentration of 25 ng/mL. Add 2% methylcellulose to reach a final volume of 20 mL.

2.1.4. Mix the medium by vortex so that the self-renewal medium is obtained.

2.2. Cell seeding

2.2.1. Transfer the harvested cells into the self-renewal medium to reach 2000 cells/mL and mix them with a brief vortex. Add 100 μL of cell suspension to each well of an ultra-low attachment 96 well plate. Do not use the wells at the rim of the plate, as the medium is more likely to evaporate in these wells. Add sterilized water to these wells.

2.2.2. Culture at 37 °C with 5% CO_2 in cell culture incubator for 10–14 days. Add 50 μL of self-renewal medium at day 5–7.

2.3. Sphere counting and calculation

2.3.1. 10–14 days after seeding, count the number of spheres with more than 50 cells (approximately) or with a diameter more than 100 μm under a microscope (4x objective lens, 10x eyepiece).

NOTE: Starting from the upper left of the well, count the spheres while moving the objective

table horizontally towards the right with the joystick of the microscope. Then, move the objective table to the middle row of the visual field and count the spheres from right to left. Then, move to the lower row and count from left to right. A well of 96 well plate can be counted within three rows.

2.3.2. Calculate the sphere formation efficiency as the number of spheres divided by the number of cells seeded. Compare the sphere formation efficiency between the positive and negative cell populations.

3. Colony formation assay

NOTE: Colony formation assay is applied to determine the radiosensitivity of cells. Radiation can be delivered by a linear accelerator used for radiotherapy. Cell irradiation system for laboratory use can also be used if the equipment is available. Steps 3.1, 3.2.3, and 3.2.6 should be performed in a biological safety cabinet or laminar clean bench. To avoid contamination, antibiotics (penicillin and streptomycin) are recommended to be added to the culture medium after sorting.

3.1. Cell seeding

3.1.1. Prepare one 6 well plate for each radiation dose including the 0 Gy group. Add 1.5 mL of medium to each well of 6 well plate. Culture medium for colony formation assay is the conventional RPMI 1640 medium supplemented with 10% FBS (named as “completed 1640”).

3.1.2. Dilute harvested cells with completed 1640 media to 1000 cells/mL.

3.1.3. Add cell suspension to the wells. Shake the plates horizontally to make cells distribute evenly in the wells. Record the volume added in each dose group.

NOTE: Generally, seed 200 cells per well for 0 Gy, 200 cells for 2 Gy, 400 cells for 4 Gy, 800 cells for 6 Gy, and 1600 cells for 8 Gy, respectively. It is better to adjust the cell numbers in unsorted cells in pre-experiments.

3.1.4. Culture at 37 °C with 5% CO₂ in cell culture incubator.

3.2. Radiation

3.2.1. After cells have attached to the bottom of the wells (usually one day after seeding, and longer time is not recommended considering cell proliferation), proceed to perform cell radiation.

3.2.2. Calculate the number of monitor units (MU) for each dose at a depth of 1 cm in a 20 cm x 20 cm radiation field.

NOTE: Number of MU = dose (cGy)/output factor for the radiation field/percentage depth dose (PDD) for the depth. For example, the output factor for a 20 cm x 20 cm field is 1.066, PDD for 1.0 cm is 0.987, and number of MU for 8 Gy (800 cGy) is 760 ($800/1.066/0.987 = 760.35$). If multiple plates need to be radiated for each dose, a larger field can also be used, and the numbers of MU need to be recalculated according to the size of the field. Output factor and PDD differ in different accelerators. Refer to radiation physicists for these parameters.

3.2.3. Add completed 1640 to each well to reach 1 cm for the height of the medium.

NOTE: Cell growth area of 6 well plate can be found on the manufacturer's guide. For example, if the growth area is 9.5 cm² for each well of 6 well plate, and 1.5 mL of medium and 0.4 mL of cell suspension has been added on the day before, then add 7.6 mL medium to the well. The height is needed for dose build-up. A medium height of less than 0.8 cm is not recommended.

3.2.3.1. Keep the plates covered during radiation. When the plates are transferred between cell culture room and radiation therapy room, wrap the plates with aluminum foil or put them in a clean box to avoid contamination. Hold the plates flat to avoid medium spilling out.

3.2.4. Set a 20 cm x 20 cm radiation field. Place a tissue-equivalent bolus of 1.0 cm thickness on the treatment couch, and place the 6 well plate on the bolus to keep them in contact. Make sure the whole plate is within the radiation field. Set the source skin distance as 100 cm. Align the medium surface level to the laser level by adjusting the height of the treatment couch.

NOTE: Plates with the same radiation dose can be radiated at the same time. For this case, a larger radiation field is needed, and the number of MU should be recalculated according to the corresponding output factor. Make sure all the plates are within the radiation field.

3.2.5. Deliver the each assigned dose to each plate in sequence.

CAUTION: The linear accelerator can only be operated by qualified technicians. Keep away from radiation.

3.2.6. Take the plates back to the cell culture room. Change the medium with 2 mL of completed medium for each well. Culture at 37 °C with 5% CO₂ in cell culture incubator. Change the medium every 3–5 days.

3.3. Staining

3.3.1. 7–10 days after radiation, when many colonies form and before they grow too large and merge together, then perform staining and counting as follows. Remove the medium and wash briefly with PBS. Add 1 mL of 4% formaldehyde to each well to fix the cells.

Caution: Formaldehyde is volatile. Use formaldehyde in a fume hood.

3.3.2. After 10 min of fixation, remove the formaldehyde and wash with 2 mL of distilled water each well twice.

3.3.3. Add 1 mL of 1% crystal violet stain solution to each well. Stain for 10 min. Remove the crystal violet stain solution, and wash with distilled water 3x. Remove the water and dry the plates.

3.4. Colony counting and calculation

3.4.1. Count the number of colonies with more than 50 cells. Do not include small colonies with less than 50 cells. Check the colonies under a microscope to get an impression of a colony constituted by 50 cells and mark it on the bottom of the plate with a marker pen.

NOTE: Photographing the wells and using the software ImageJ will facilitate the counting process.

3.4.2. Calculate the colony formation efficiency as the number of colonies divided by the number of cells seeded. Calculate the survival fraction as the colony formation efficiency at a certain irradiation dose divided by the colony formation efficiency at 0 Gy.

3.4.3. Using the dose as the x-axis and survival fraction as the y-axis, a survival curve can be obtained. Compare the survival curve between the positive and negative cell populations.

REPRESENTATIVE RESULTS:

$\alpha 2\delta 1$ -high and $\alpha 2\delta 1$ -low A549 cells were sorted (**Figure 1A**). Some markers may show distinct populations and are easy to gate. However, some markers just show high and low expression patterns, rather than distinct positive and negative populations. In this situation, an isotype control is very important for gating. The expression of $\alpha 2\delta 1$ in sorted cells is validated by qPCR. The expression of *CACNA2D1*, the gene that encode $\alpha 2\delta 1$, is higher in sorted $\alpha 2\delta 1$ -high cells compared with $\alpha 2\delta 1$ -low cells (**Figure 1B**).

Typical morphology of spheres is shown in **Figure 2A**. The sphere formation efficiency is calculated in $\alpha 2\delta 1$ -high and $\alpha 2\delta 1$ -low cells (**Figure 2B**). $\alpha 2\delta 1$ -high cells showed higher sphere formation efficiency, suggesting a higher self-renewal capacity. Typical images of cell colonies are shown in **Figure 3A**. A colony with about 50 cells can be examined under a microscope and marked as a reference. A survival fraction at each dose can be calculated, and the survival curve is presented in **Figure 3B**. $\alpha 2\delta 1$ -high cells are relatively resistant to radiation compared to $\alpha 2\delta 1$ -low cells.

Unpaired two-sided Student's *t*-test was performed to evaluate the significance between groups. A value of $p < 0.05$ was considered to be statistically significant. Data are represented with the mean and standard deviation (SD). Representative data from at least three biologically independent experiments with similar results are presented.

FIGURE AND TABLE LEGENDS:

Figure 1: Sorting $\alpha 2\delta 1$ -high and $\alpha 2\delta 1$ -low cells in A549. (A) Representative flow cytometry analysis of $\alpha 2\delta 1$ expression. Cells are gated based on the isotype control. **(B)** Confirmation of $\alpha 2\delta 1$ expression in high and low populations by quantitative PCR. The error bars indicate SD.

Figure 2: Sphere formation assay of $\alpha 2\delta 1$ -high and $\alpha 2\delta 1$ -low A549 cells. (A) Representative morphology of the spheres formed by the sorted $\alpha 2\delta 1$ -high and $\alpha 2\delta 1$ -low cells (bar = 200 μ m). **(B)** Sphere formation efficiency of $\alpha 2\delta 1$ -high and $\alpha 2\delta 1$ -low cells. The error bars indicate SD (** $p < 0.001$).

Figure 3: Colony formation assay of $\alpha 2\delta 1$ -high and $\alpha 2\delta 1$ -low A549 cells. (A) Representative images of the colonies formed by the sorted $\alpha 2\delta 1$ -high and $\alpha 2\delta 1$ -low cells. **(B)** Survival curves of $\alpha 2\delta 1$ -high and $\alpha 2\delta 1$ -low cells. The numbers of seeded cells were 200 cells per well for 0 Gy, 400 cells per well for 4 Gy, and 800 cells for 8 Gy. The error bars indicate SD (** $p < 0.05$, *** $p < 0.001$).

DISCUSSION:

This protocol describes methods to study the radiosensitivity of CSCs in cancer cell lines in vitro. In this study, the expression of $\alpha 2\delta 1$ is continuous in NSCLC cell lines. Therefore, gating is based on an isotype control. Before sorting, $\alpha 2\delta 1$ expression should be examined in multiple cell lines by flow cytometry and validated by QPCR or western blot. It is recommended to re-analyze $\alpha 2\delta 1$ expression of the sorted $\alpha 2\delta 1$ -high and $\alpha 2\delta 1$ -low cells by flow cytometry, by observing fluorescence under a fluorescent microscope, or by QPCR (after sorting).

Sphere formation assay is a simple way to evaluate self-renewal capacity, which can be used to preliminarily characterize putative CSCs. This method has been used to culture neurospheres or mammospheres to characterize stem cells in glioma or breast cancer cells, and the formula is modified to culture other types of cancer^{4,6,10}. EGF and bFGF can support the self-renewal of stem cells in serum-free medium; however, basic medium and growth factors may differ for culturing different cell types. The semisolid medium is used to immobilize cells so that spheres are formed by cell proliferation rather than clustering together. Ultralow attachment plate is used in the experiment to maintain sphere morphology. Moreover, as CSCs are enriched after sphere culture, when the spheres are collected, digested, and seeded for secondary sphere formation, sphere formation efficiency is likely to increase in subsequent serial propagation^{9,10}. A more rigorous criteria for characterizing CSCs is in vivo limiting dilution assay, in which a series of numbers of sorted positive and negative cells are injected subcutaneously into immunodeficient mice, then the frequencies of tumorigenic cells in positive and negative cell populations are calculated^{10,14}. If a putative cancer stem marker is identified by sphere formation assay, further characterization by in vivo limiting dilution assay is recommended.

In this study the radiosensitivity of CSCs is evaluated. Colony formation assay is a classical way to assess the radiosensitivity. Seeding same number of cells in parallel wells is important. Adjust the cell number per mL to a suitable range to ensure the volume of cell suspension

added to each well is more than 100 μ L. If the volume is too small, the error is likely to increased. For cell radiation, medium with a 1 cm height is added for dose build-up, and a tissue-equivalent bolus is placed under the plate for dose backscatter. Researchers are recommended to refer to radiation physicists and technicians to set up the cell radiation model and calculate the dose.

This manuscript provides the initial few steps for the radiosensitivity study of CSCs. Further mechanism studies may involve proteins related to DNA damage repair, clearance of reactive oxygen species, cell cycle arrest, etc.¹⁵. These experiments need a large amount of cells; therefore, many dishes of cells are needed to be prepared. Overexpression or knockout/knockdown of CSC genes also provide important insights into how CSCs gain radioresistance.

These methods can potentially be applied to identify CSCs in cancer tissues. Zhang et al. described isolating CD166-positive Lin-negative cells from NSCLC surgical samples¹². Tissues were chopped with a sterile blade and digested with an enzyme cocktail. Cells collected by passing through cell strainers were labeled for sorting, and then characterized by sphere formation assay and in vivo limiting dilution assay. For α 2 δ 1 as a CSC marker, it has been reported to isolate CSC in hepatocellular carcinoma surgical tissues and small cell lung cancer patient-derived xenograft tissues^{10,16}. Considering a high number of cells are required for analysis, it is relatively difficult to isolate CSCs in biopsies or circulating tumor cells. Alternatively, staining the sections of biopsies may potentially provide clues for the existence of CSC in the tumors. However, this presumptive application needs to be evaluated in patient tissues and clinical data.

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DISCLOSURES:

The authors declare no conflict of interest.

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Figure 1 isotype

$\alpha 2 \delta 1$ [Click here to B](#)



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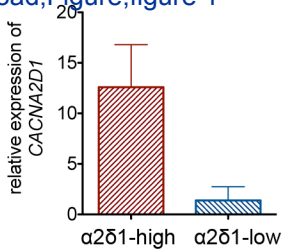
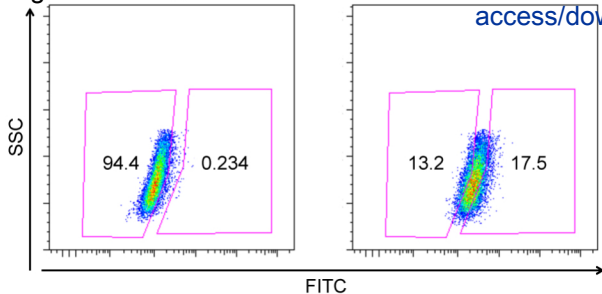
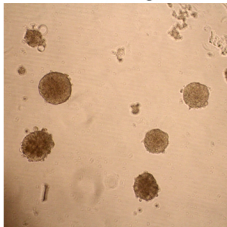
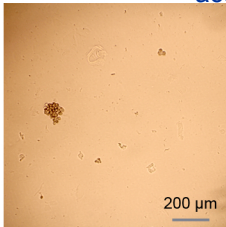


Figure 2

$\alpha 2\delta 1$ -high



$\alpha 2\delta 1$ -low



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sphere formation efficiency

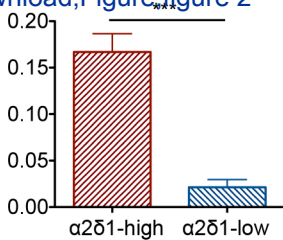
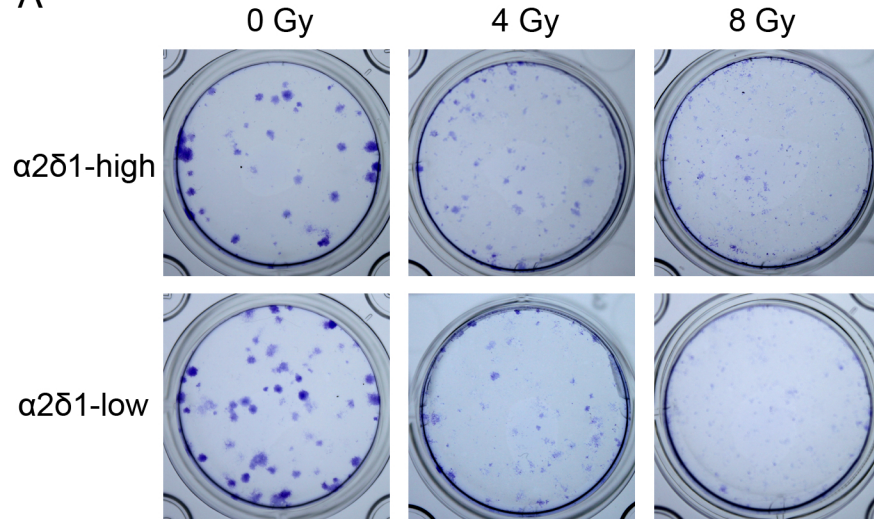


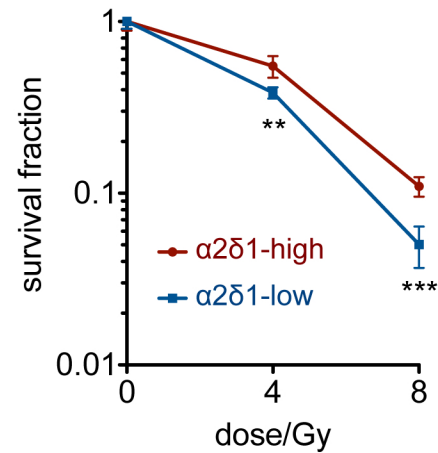
Figure 3

[Click here to access/download;Figure;figure 3 20190325.pdf](#) 

A



B



Name of Material/ Equipment	Company
0.5% Trypsin-EDTA (10X), no phenol re	Thermo Fisher
1B50-1	
4% formaldehyde solution	Solarbio
A549	ATCC
B27	Thermo Fisher
Biological Safety Cabinet	Thermo Fisher
Centrifuge	Eppendorf
DMEM/F-12	Thermo Fisher
EGF Recombinant Human Protein	Thermo Fisher
Fetal bovine serum	Thermo Fisher
FGF-Basic (AA 1-155) Recombinant Hu	Thermo Fisher
Flow cytometer/cell sorter	BD
H1299	ATCC
H1975	ATCC
Lightning-Link Fluorescein Kit	Innova Biosciences
linear accelerator	VARIAN
Methyl cellulose	Sigma Aldrich
Penicillin-Streptomycin, Liquid	Thermo Fisher
Phosphate buffered saline	Solarbio
RPMI-1640	Thermo Fisher
SYBRGREEN	TOYOBO

TRIzol

Thermo Fisher

Violet crystal staining solution

Solarbio

Catalog Number	Comments/Description
15400054	Dilute in to 0.05% (1X) with autoclaved d
	This antibody is produced and friendly su
G2160	
	RRID: CVCL_0023
17504044	
1336	
5910R	
12500062	
PHG0311	
16140071	
PHG0261	
FACSARIA III	
	RRID: CVCL_0060
	RRID: CVCL_1511
310-0010	
CLINAC 600C/D	
M7027	
15140122	
P1020	
11875093	
QPK-201	

15596026

G1062

distilled water

provided by Laboratory of Carcinogenesis and Translational Research (Ministry of Education/Beijing), Department of

of Cell Biology, Peking University Cancer Hospital and Institute. See reference 10. Alternatively, commercial antil

body of calcium channel $\alpha 2\delta 1$ subunit can be used (ABCAM, ab2864) (Yu, et al., Am J Cancer Res, 2016; 6(9): 201

88-2097)

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Radiosensitivity study of cancer stem cells in lung cancer cell lines

Author(s):

Xin Sui, Jian-Hao Geng, Hui-Ming Yu, Yong-Heng Li, Wei-Hu Wang

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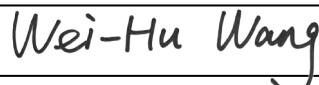
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CORRESPONDING AUTHOR

Name:	Wei-Hu Wang	
Department:	Department of Radiation Oncology	
Institution:	Key Laboratory of Carcinogenesis and Translational Research, Peking University Cancer Hospital and Institute, Beijing, China	
Title:	Professor	
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Dear Editors and Reviewers,

Thank you so much for reviewing our manuscript entitled "Radiosensitivity study of cancer stem cells in lung cancer cell lines". The comments are very helpful for improving our manuscript.

The manuscript is revised according to the editors and reviewers' suggestions. Replies to comments point-by-point are as follows:

Editorial Comments:

- Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We proofread the manuscript and correct the spelling and grammatical errors in the new version.

- **Protocol Language:**

- 1) Please split long steps into 2 or more steps. E.g. 1.1.4.

Thanks for the editor's suggestion. Long steps are split into 2 or more steps in the new version.

- 2) Add a single line space between each of your steps.

A single line space is added between steps in the new version.

- **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:

- 1) 1.1.4: please describe flow cytometry in greater detail. Unclear what kind of analysis is to be performed.

Thanks for the editor's suggestion. We provide more details of 1.1.4 in the new version.

- 2) 1.2.1: what is the cell density? How much (mL) trypsin should be added?

Digest cells for sorting when they reach 80-90% confluence (about $5-10 \times 10^6$ cells per dish). Add 3 ml 0.05% trypsin to each dish (1.2.1 & 1.2.2 in new version).

- 3) 1.2.3: mention antibodies and concentrations.

FITC-conjugated 1B50-1 (the antibody of $\alpha\delta 1$) was diluted into 7.5 $\mu\text{g/ml}$ (1.2.5 in new version).

- 4) 1.3: greater detail is needed if this section is to be filmed.

Details are added in the new version (1.3).

5) 2.2.2: how and when are the cells counted?

The number of cells harvested after sorting were counted by the cell sorter automatically (1.3.3).

6) 2.3.1: mention magnification.

Magnification is added: 4 × objective lens, 10 × eyepiece.

7) 3.2.3: RPMI 1640?

In this context, completed 1640 refers to conventional RPMI 1640 medium supplemented with 10% FBS. It is mentioned in 3.1.1.

- **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

- 1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.

- 2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

- 3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

- 4) Please bear in mind that calculations cannot be filmed.

Steps are highlighted in the new version (1.2; 2.2; 3.2.3-6; 3.3).

- **Results:** Mention statistical tests performed and sample sizes.

The following description is added at the end of the Results part: Unpaired two-sided Student's t-test was performed to evaluate the significance between groups. $P < 0.05$ was considered to be statistically significant. Data are represented with the mean and standard deviation (SD). Representative data from at least 3 biologically independent experiments with similar results are presented.

- **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

The Discussion part is modified in the new version according to the editors and reviewers' suggestions.

- **Figures:** Define error bars in all figures.

The error bars refer to the standard deviation (SD). It is added in the figure legends.

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[Commercial information is removed in the new version \(1.1\).](#)

- **Table of Materials:** Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as A549 cells, flow cytometer, antibodies along with RRIDs,

[These items are added in the table of materials in the new version.](#)

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[Figures in this article are original.](#)

Comments from Peer-Reviewers:

Reviewer #1:

Manuscript Summary:

The manuscript describe a methodology that can be utilized to study the radioresistance of lung cancer stem cells. There are some issues that need to be resolved prior to publication.

Major Concerns:

1. Please discuss how this methodology can be applied to a real-life situation. Do you expect that lung CSC will be identified in lung cancer biopsies prior to treatment, or circulating tumour cells will be assessed for CSC properties? Given a high number of cells required for

analysis, can this assay have a clinical application, or it only can provide a basic scientific information?

Thanks for the reviewer's suggestion. Although this assay has a potential clinical application, it needs to be evaluated in patient tissues and clinical data. For now it only provide basic scientific information. We discussed this issue in the last paragraph of the Discussion part in the new version.

2. Please provide more information on why the voltage-gated calcium channel $\alpha 2\delta 1$ subunit isoform 5 is a good representative marker for lung CSC.

$\alpha 2\delta 1$ -positive cells show higher sphere formation capacity compared with $\alpha 2\delta 1$ -negative cells, and the sphere formation efficiency of $\alpha 2\delta 1$ -positive cells increased in subsequent serial propagation (Figure 2 in this manuscript; also in Figure 2 in reference 9). In small cell lung cancer cells, Yu et al reported $\alpha 2\delta 1$ -positive cells have higher tumorigenic capacity when injected subcutaneously into immunodeficient mice (reference 16). In non-small cell lung cancer cells, our colleagues obtained similar results in the mouse model (manuscript under preparation). In this manuscript, we mainly focus on the radiosensitivity study of CSC; therefore we didn't describe the methods of tumorigenicity experiment. We discussed this point in the second paragraph of the Discussion part.

3. Has the medium composition for sphere formation has been reported before, or this is a new development from the authors' laboratory?

As it is mentioned in the second paragraph of Discussion part, sphere formation assay has been used to culture neurosphere or mammosphere to characterize the stem cells in glioma or breast cancer cells, and the formula is modified to culture other types of cancer (see references 4,6 and 10). However, basic medium and growth factors may differ for culturing different cell types. In the paper that identified $\alpha 2\delta 1$ as a CSC marker for hepatocellular carcinoma, EGF, bFGF and HGF (hepatocyte growth factor) are used to support the sphere formation of hepatocellular carcinoma CSC (reference 10). In Yu et al's paper, same formula is used for sphere formation of small cell lung cancer CSC (reference 16). For non-small cell lung cancer cell lines, we found EGF and bFGF (without HGF) could also support sphere formation (reference 9). Therefore in this manuscript, we just use EGF and bFGF. In the new version, we mention that the formula of sphere formation medium may be different for diverse tissue or cell types.

4. If a mission of JOVE is to provide a video of a described methodology, a video has not been provided.

The video will be provided after the manuscript is revised.

Minor Concerns:

1. What is the origin of A549 cells?

A549 is a non-small cell lung cancer cell line purchased from American Type Culture Collection (ATCC). This information is added in the Table of Materials.

2. Please specify the temperature for various steps of the protocol.

Thanks for the reviewer's suggestion. We added the note for temperature in the first paragraph of Protocol part.

3. Please specify if the irradiation conditions described in the manuscript are particular for the VARIAN linear accelerator.

Thanks for the reviewer's suggestion. We emphasize this part in the note of 3.2.2: Output factor and PDD differ in different accelerators. Refer to radiation physicists for these parameters.

Reviewer #2:

Manuscript Summary:

This manuscript describes the methods to study the radiosensitivity of cancer stem cells in lung cancer cell lines. It will be of interest to other scientists. I suggest accepting this paper.

Major Concerns:

none

Minor Concerns:

none

Reviewer #3:

Manuscript Summary:

The manuscript described a detailed protocol for analyzing radioresistance of cancer stem cells (CSCs) in human lung cancer cell line A549 after FACS-based purification with Anti- $\alpha 2\delta 1$ antibody. The authors demonstrated that Anti- $\alpha 2\delta 1$ antibody could enrich CSCs by tumor-sphere formation assay and determined radiosensitivity of both $\alpha 2\delta 1$ -high CSCs and $\alpha 2\delta 1$ -low cancer cells by 2D colony formation assay.

Major Concerns:

1. The radiation procedure should be described more detailed;

Thanks for the reviewer's suggestion. More details are added in the new version (3.2).

2. For the cells should be cultured further, please describe how to avoid contaminant;

Thanks for the reviewer's suggestion. Notes were added to describe how to avoid contaminant in step 1, 2, 3 and specially emphasized in step 3.2.3.

3. For the sphere formation part, whole picture of the well should be provided.

Sphere formation assay is performed in 96-well plates. Therefore, a single well cannot be captured by a single picture or in a single visual field. The magnification in Figure 2A is 10 ×

objective lens and 10 × eyepiece. However when count the sphere number under a microscope, we recommend to use the magnification of 4 × objective lens and 10 × eyepiece. We describe how to count the number of spheres with more details in step 2.3.1 of the new version: Starting from the upper-left of the well, researchers can count the spheres while move the objective table horizontally towards the right with the joystick of the microscope. Then move the objective table to the middle row of the visual field and count the spheres from right to left. Then move to the lower row and count from left to right. A well of 96-well plate can be counted within 3 rows.

Minor Concerns:

1. For reagent setup, the word "make" should be replaced with "prepare".

Thanks for the reviewer's suggestion. It is corrected in the new version.

Reviewer #4:

General comment:

This method article explain the protocol for the isolation of radiotherapy resistant cancer stem cells from lung cancer cell lines, A549. The CSCs was isolated using α 2 δ 1 marker, which previously identified as marker for radio-resistant CSCs. The method article explained the protocol for the isolation of CSCs using the flow cytometry technique (including antibody conjugation with florescence and labeling the cells with the antibody). Only two assays, sphere forming and colony forming assay, were used to characterize the stemness characteristics of the isolated CSCs. Perhaps the author should include more characterization method such as checking the expression of stemness markers (eg SOX2, OCT4, KLF4, NANOG) in the isolated cells using PCR or qPCR methods, proliferation assay and differentiation assay. Comparison between α 2 δ 1-high and α 2 δ 1-low cells population can in those assays will confirm the CSCs characteristics of the isolated cells. Apart from that, the title of the article "Radiosensitivity study of cancer stem cells in lung cancer cell lines" is not fully represent the whole article. Only colony forming assay was performed after the cells being exposed to radiation. Perhaps the study should include testing on the cells before and after exposure to radiation. The result obtained could explain the effect of radiotherapy on the activation and CSCs behavior.

Thanks for the reviewer's suggestion. α 2 δ 1-positive cells show higher sphere formation capacity compared with α 2 δ 1-negative cells, and the sphere formation efficiency of α 2 δ 1-positive cells increased in subsequent serial propagation (Figure 2 in this manuscript and Figure 2 in reference 9). α 2 δ 1 overexpression upregulates the expression of NANOG (but not OCT4) and several DNA damage repair-related genes (reference 9). In small cell lung cancer cells, Yu et al reported α 2 δ 1-positive cells have higher tumorigenic capacity when injected subcutaneously into immunodeficient mice (reference 16). In non-small cell lung cancer cells, our colleagues obtained similar results in the mouse model (manuscript under preparation).

We had also tried to explore the mechanism of the radioresistance. Higher efficiency of γ H2AX clearance and higher level of phosphorylated ATM after radiation in α 2 δ 1-positive or α 2 δ 1-overexpression cells suggested that α 2 δ 1 imparted the radioresistance to NSCLC cells partially by enhancing the efficiency of DNA damage repair in response to radiation (reference 9).

QPCR, western blot and immunofluorescence were used to examine the stemness genes and the DNA damage repair-related proteins (reference 9). Considering these experiments were relatively routine, their methods were not included in this manuscript. As we mentioned in the second and the last paragraph of the Discussion part, more experiments are needed to characterise the CSC and to explore the mechanism of radioresistance. This manuscript just provides the initial few steps for the radiosensitivity study of CSCs.

Major revision:

1. In result section, Figure 1B, the author/s validated the expression of α 2 δ 1 marker in the cells by checking the expression of CACNA2D1 genes that encoded α 2 δ 1. However, the author did not include the detail protocol in method section.

Thanks for the reviewer's suggestion. The protocol is added in the new version (1.3.4): To confirm the harvested cell populations have different level of α 2 δ 1 expression, QPCR can be performed. Extract RNA of positive and negative cells with TRIzol reagent and reverse transcript the RNA into cDNA according to the manufacturer's guide. Perform QPCR with sybrgreen reagent. The sequences of primers are as follows: *α 2 δ 1-F*, CAGTTGAGATGGAGGATGATG; *α 2 δ 1-R*, TTGTATGAGCAGTCGTGTGTC; *GAPDH-F*, GTCGGAGTCAACGGATTTGG; and *GAPDH-R*, AAAAGCAGCCCTGGTGACC.

Minor revision:

1. Line 109: "Place the tubes at the collection platform of the....."

Please complete the sentence.

Sorry for the mistake. We complete the sentence: Place the tubes at the collection platform of the cell sorter.

2. Line 130: "Measure the volume of methylcellulose by the scale of the final tube rather than the scale of the tip or straw."

This sentence is confusing. Please re-write in simpler form.

Thanks for the reviewer's suggestion. We re-write these steps in the new version (2.1.3 and 2.1.4).

3. Sphere counting and calculation.

Is there any specific criteria to include the sphere in counting for example only sphere that reach certain size will be counted. The result only show the sphere number. Is the sphere size being measured as well? If yes, please include the sphere size as the sphere size is one of the criteria to describe the increase in the self-renewal characteristics.

There are no unified criteria for the size of the sphere. In our study, although it is hard to count the cells of a sphere accurately under a microscope, sphere with more than 50 cells (approximately) or with a diameter more than 100 μm are counted. We added this point in the new version (2.3.1).

4. If there any reason/s to use the different cells number for different radiation dosage? To see the effect of different radiation dosage on the two different cell type, the cell number should be constant.

The survival fraction is usually less 50% at 4 Gy and is less than 10% at 8 Gy. Therefore, if the same number of cells were seeded for different radiation dosage, the colonies may be too few to be counted in high dose groups.

5. Result Figure 3A

Based on the figure, there its look line there is no significant different between the colony forming capability of $\alpha\text{2}\delta\text{1}$ -high and the $\alpha\text{2}\delta\text{1}$ -low.

Experiments were repeated for at least 3 biologically independent experiments. Unpaired two-sided Student's t-test was performed to evaluate the significance between groups.

$P < 0.05$ was considered to be statistically significant. Similar results were shown in reference 9.

6. The author has include most of the updated and relevant references. However, the author should cite the references that being refer for each method performed in this study.

Thanks for the reviewer's suggestion. We added reference about clonogenic assay (reference 13) in the new version.

Thank you very much for your kind consideration. Look forward to hearing from you.

Sincerely Yours,

Wei-Hu Wang
Department of Radiation Oncology
Peking University Cancer Hospital and Institute
52 Fucheng Road, Haidian District
Beijing, China, 100142
(86)10-88196120
wangweihu88@163.com