

Voltage-dependent calcium channel $\alpha 2\delta 1$ subunit is a specific candidate marker for identifying gastric cancer stem cells

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Ziran Zhang¹
Wei Zhao²
Xiaoting Lin¹
Jing Gao¹
Zhiqian Zhang²
Lin Shen¹

¹Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education/Beijing), Department of Gastrointestinal Oncology, Peking University Cancer Hospital and Institute, Beijing, People's Republic of China; ²Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Cell Biology, Peking University Cancer Hospital and Institute, Beijing, People's Republic of China

Correspondence: Zhiqian Zhang
Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Cell Biology, Peking University Cancer Hospital and Institute, Fu-Cheng Road 52, Hai-Dian District, Beijing 100142, People's Republic of China
Tel +86 108 819 6792
Fax +86 108 819 6792
Email zlzqzhang@bjmu.edu.cn

Lin Shen
Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education/Beijing), Department of Gastrointestinal Oncology, Peking University Cancer Hospital and Institute, Fu-Cheng Road 52, Hai-Dian District, Beijing 100142, People's Republic of China
Tel +86 108 819 6561
Fax +86 108 819 6561
Email linshenpk@163.com

Purpose: Cancer stem cells (CSCs) are a subpopulation of cancer cells with self-renewal property and responsible for tumor malignancy, progression and drug resistance. Researches on CSC-specific markers in gastric cancer remain limited. Our current study explored the expression of voltage-dependent calcium channel $\alpha 2\delta 1$ subunit and the potential of using $\alpha 2\delta 1$ as a CSC marker in gastric cancer. We also compared the specificity of $\alpha 2\delta 1$ and CD44 in identifying gastric cancer stem cells (GCSCs).

Materials and methods: Expression of $\alpha 2\delta 1$ was analyzed in gastric cancer cell lines, patient-derived xenograft (PDX) models and clinical samples of malignant ascites of gastric cancer patients. $\alpha 2\delta 1^+$ gastric cancer cells were isolated from gastric cancer cell lines. CSC properties of $\alpha 2\delta 1^+$ gastric cancer cells were then verified by subsequent tests both in vitro and in vivo.

Results: The expression level of $\alpha 2\delta 1$ was found to differ drastically among gastric cancer cell lines, PDX models and clinical samples of malignant ascites. $\alpha 2\delta 1^+$ gastric cancer cells sorted from HGC-27 and SGC-7901 cell lines demonstrated significant self-renewal properties, including tumorigenic capacity, sphere-formation capacity and asymmetric differentiation potential. Knockdown of $\alpha 2\delta 1$ in $\alpha 2\delta 1^+$ HGC-27 significantly inhibited CSC properties and rendered HGC-27 more sensitive to chemotherapy. Flow cytometry showed that $\alpha 2\delta 1^+$ gastric cancer cells accounted for a small fraction of CD44⁺ gastric cancer cells. Isolated CD44⁺ $\alpha 2\delta 1^+$ HGC-27 cells displayed more significant tumorigenic capacity and sphere-forming capacity compared with their CD44⁺ $\alpha 2\delta 1^-$ counterparts.

Conclusion: $\alpha 2\delta 1^+$ gastric cancer cells possessed CSC properties. $\alpha 2\delta 1$ could be a proper marker in identifying GCSCs with superior specificity than CD44. The combination of $\alpha 2\delta 1$ and CD44 could be used to identify GCSCs with improved accuracy. Knockdown of $\alpha 2\delta 1$ combined with chemotherapy displayed higher therapeutic efficacy on gastric cancer cells, suggesting that $\alpha 2\delta 1$ could be a potential target for anticancer treatment.

Keywords: $\alpha 2\delta 1$, gastric cancer, cancer stem cell, HGC-27

Introduction

Gastric cancer is the fourth most common malignant tumor and the third leading cause of cancer-related mortality worldwide, with an estimated 1.3 million new cases in 2015.¹ Approximately 75% of all patients diagnosed with gastric cancer are at advanced stages. Up to now, conventional cytotoxic chemotherapy has been the major treatment for advanced gastric cancer. Unfortunately, the prognosis for patients with advanced gastric cancer remains gloomy, with median survival time

of <1 year, mainly due to tumor metastatic, relapse and drug resistance.¹⁻³ The cancer stem cell (CSC) theory has brought new hope to this situation.

CSC is defined as a small subset of cancer cells with self-renewal property and could differentiate asymmetrically into daughter CSC to maintain its population as well as into non-CSC to form the bulk of the tumor.⁴ CSCs are highly invasive and are responsible for metastasis, drug resistance and relapse. Therefore, novel treatment targeting CSCs has the potential to revolutionize cancer treatment. Numerous studies have succeeded in identifying gastric cancer stem cell (GCSC) with various molecular markers including CD44, aldehyde dehydrogenase (ALDH), CD90, Sca-1, EpCAM, ABCG-2, Lgr5, Villin and MIST-1.⁵⁻¹⁵ However, there are still limitations due to poor specificity of these markers. For example, ALDH, Lgr5 and EpCAM were also found to be expressed in normal gastric stem cells, while CD44 was reported sometimes to be expressed in cancer cells without significant CSC properties.^{16,17} Thus, it is necessary to seek for more specific GCSC markers in order to develop treatment safely targeting GCSC.

$\alpha 2\delta 1$ is a subunit of the voltage-dependent calcium channel complexes expressed on cell membrane. Zhao et al have reported the expression of $\alpha 2\delta 1$ in a population of primary hepatocellular carcinoma (HCC) cells with CSC properties. They generated a new monoclonal antibody 1B50-1mAb that could specifically bind to $\alpha 2\delta 1$ and suppress tumor growth by blocking the MAPK pathway through the inhibition of ERK functions. The target of 1B50-1mAb is the isoform 5 of $\alpha 2\delta 1$, which is found to be expressed exclusively in HCC stem cells, whereas none was expressed in normal hepatic stem cells. Moreover, combined treatment using 1B50-1mAb and chemotherapy led to more significant tumor decrease in HCC patient-derived xenograft (PDX) models than using chemotherapy alone.¹⁸ In another more recent study, Yu et al identified small cell lung cancer (SCLC) cells with CSC properties using $\alpha 2\delta 1$ as the marker and further confirmed that $\alpha 2\delta 1^+$ SCLC cells are closely related to chemoresistance. Also, combination of 1B50-1mAb and chemotherapy demonstrated significant therapeutic effect in originally chemoresistant SCLC PDX model.¹⁹ These previous studies provide evidence that $\alpha 2\delta 1$ may be a suitable biomarker for identifying CSCs in various solid tumors and a potential target for anticancer treatment. There have been no reports about $\alpha 2\delta 1$ expression in gastric cancer.

We hypothesized that $\alpha 2\delta 1$ might play a similar role in gastric cancer. To test our hypothesis, we analyzed the

expression of $\alpha 2\delta 1$ in different gastric cancer cell lines, cancer PDX models and clinical samples of malignant ascites collected directly from gastric cancer patients. We further tested the CSC properties of $\alpha 2\delta 1^+$ gastric cancer cells using a series of assays both in vitro and vivo. Also, we conducted initial tests about the potential therapeutic effect of $\alpha 2\delta 1$ inhibition.

Materials and methods

Cell lines, animals and clinical samples

The expression of $\alpha 2\delta 1$ and CD44 was analyzed in human gastric cancer cell lines HGC-27, SGC-7901, BGC-823, MGC-803, MKN-45, MKN-74, MKN-28, AGS and NCI-N87. HGC-27, SGC-7901, BGC-823, MGC-803, MKN-45, MKN-74, MKN-28 and AGS were purchased from Peking Union Medical College, Beijing, China. NCI-N87 was provided by You-yong Lv, PhD (Peking University Cancer Hospital and Institute). HGC-27, SGC-7901, BGC-823, MGC-803, MKN-45, MKN-74, MKN-28 and AGS cells were cultured in the RPMI-1640 medium supplemented with 10% FBS (Gibco-BRL, Gaithersburg, MD, USA) and 100 U/mL penicillin (Gibco-BRL) and 100 mg/mL streptomycin (Gibco-BRL). NCI-N87 were cultured in the DMEM (Gibco-BRL) supplemented with 10% FBS (Gibco-BRL), 100 U/mL penicillin (Gibco-BRL) and 100 mg/mL streptomycin (Gibco-BRL). Cells were incubated in a humidified incubator (37°C) supplemented with 5% CO₂.

The gastric PDX models were previously established in our laboratory through inoculation of fresh fragments of gastroscopy biopsies in NOD/SCID mice.²¹ The NOD/SCID female mice (aged 4–6 weeks, weighing 18–21 g) involved in our current research were purchased from Beijing HFK Bio-Technology Co. Ltd. (Beijing, China). All mice were raised in the specific pathogen free animal laboratory of Peking University Clinical Cancer Hospital Animal Center. All animal experiments were performed under sterile conditions and 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 of Peking University Cancer Hospital.

The ascites samples were collected directly from gastric cancer patients receiving treatment at our department. All patients had been pathologically diagnosed with malignant ascites due to gastric cancer. Fresh ascites were collected in sterile test tubes and centrifuged within 2 hrs at 1,000 rpm for 10 mins at 4°C. The cell pellet was

resuspended in 4 mL PBS and carefully transferred to a 15 mL test tube containing 3 mL Ficoll–Paque™ PLUS isolation media (Amersham Biosciences, Piscataway, NJ, USA). After centrifugation at 1,500 rpm for 30 mins at 18–20°C, the upper layer was removed and the second layer containing tumor cells was transferred to another test tube. The tumor cells were washed through the addition of 6 mL PBS and centrifuged at 800 rpm for 10 mins at 18–20°C. Finally, the upper layer was removed and the tumor cell pellet was used in further experiments. All patients had signed written informed consent form before we collected the clinical samples. Acquisition and use of these samples were approved by the Ethics Committee of Beijing Cancer Hospital.

Antibody labeling, flow cytometry analysis and flow cytometry sorting

The cells were collected and suspended at 1×10^6 cells/mL in PBS. Fluorescent-labeled monoclonal antibodies were added to the samples at the concentrations recommended by the manufacturers. After washed using PBS, the labeled cells were analyzed using flow cytometry BD Accuri™ C6 (BD Biosciences, Bedford, MA, USA) and sorted using fluorescence-activated cell sorting (FACS) BD Aria II (BD Biosciences). The antibodies included allophycocyanin-conjugated mouse anti-human CD44 (Miltenyi Biotec, Bergisch Gladbach, Germany), phycoerythrin-conjugated mouse anti-human CD326 (EpCAM) (Miltenyi Biotec) and their corresponding IgG isotypes (Miltenyi Biotec). Detailed information of antibodies is listed in Table 1. The 1B50-1 monoclonal antibody and the corresponding IgG isotype were provided by Zhiqian Zhang, PhD (Laboratory of Carcinogenesis and Translational Research, Department of Cell Biology, Peking University Cancer Hospital and Institute). Both of the 1B50-1 monoclonal antibody and the corresponding IgG isotype were labeled with fluorescein using Lightning Conjugation Kits (Innova Biosciences Ltd, Cambridge, UK) following the manufacturer's protocol.

Sphere-formation assays

The sorted cells from HGC-27 and SGC-7901 cell lines were counted and mixed with the DMEM/F12 (Invitrogen, Grand Island, NY, USA) supplemented with B27 (Invitrogen), 20 ng/mL epidermal growth factor, 20 ng/mL basic fibroblast growth factor (Peprotech, Rocky Hill,

Table 1 Antibody information used in this study is summarized

Antibody	Manufacture	Catalog No.
CD44-APC	Miltenyi Biotec	130-098-110
CD326 (EpCAM)-PE	Miltenyi Biotec	130-098-115
Anti-IgG antibodies-APC	Miltenyi Biotec	130-099-126
Anti-IgG antibodies-PE	Miltenyi Biotec	130-099-201
CD44 (156-3C11) mouse mAb	Cell Signaling Technology	#3570
ALDH1A1 (D9Q8E) XP® rabbit mAb	Cell Signaling Technology	#54135
Monoclonal anti-β-actin	Sigma-Aldrich	A5441
Anti-mouse IgG, HRP-linked antibody	Cell Signaling Technology	#7076
Anti-rabbit IgG, HRP-linked antibody	Cell Signaling Technology	#7074

Abbreviation: APC, allophycocyanin; PE, phycoerythrin; FITC, fluorescein isothiocyanate; IgG, Immunoglobulin G; mAb, monoclonal antibody; ALDH, aldehyde dehydrogenase; HRP, horseradish peroxidase.

NY, USA), 10 ng/mL hepatocyte growth factor (Peprotech) and 1% methylcellulose (Sigma-Aldrich, St. Louis, MO, USA). Then, the mixture was seeded in semi-suspension and was cultured in Ultra Low Attachment 96-well plates (Corning Incorporated Life Sciences, Acton, MA, USA) with 100 cells/well. After incubation of 2–3 weeks, the number of spheres was counted and recorded under a stereomicroscope (Olympus, Tokyo, Japan). The frequency of sphere formation was calculated.

Tumorigenicity assay

The sorted cells from HGC-27 and SGC-7901 cell lines were suspended in PBS and mixed with Matrigel (BD Biosciences, Bedford, MA, USA) at a ratio of 1:1. The mixture was transplanted subcutaneously into the back of NOD/SCID mice (aged 4–6 weeks, weighing 18–21 g) bilaterally, with $\alpha 2\delta 1^+$ and $\alpha 2\delta 1^-$ cells at each side. Tumor formation was observed weekly, and the frequency of tumor formation was calculated.

Differentiation assays

The sorted cells from HGC-27 and SGC-7901 cell lines were reanalyzed by flow cytometry BD Accuri™ C6 (BD Biosciences) to reassure the accurate measurement of the frequency of $\alpha 2\delta 1$ expression. Then, the sorted cells were counted and cultured separately in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C under 5% CO₂. After 2 weeks, the cells were collected and the frequency of $\alpha 2\delta 1$ expression was reanalyzed by flow cytometry.

Transwell assays

The transwell assays were conducted using BD BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences). The sorted $\alpha 2\delta 1^+$ and $\alpha 2\delta 1^-$ HGC-27 cells were suspended in serum-free RPMI-1640 and placed separately on the upper layer of the inserts with permeable membrane. The lower chambers contained RPMI-1640 supplemented with 10% FBS. The cells were then incubated at 37°C under 5% CO₂ for 24 hrs. After the incubation period, the cells that had migrated through the membrane were fixed and stained and then counted under a stereomicroscope (Olympus).

Western blot analysis

Gastric cancer cells were collected and lysed using cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) and quantified using a bicinchoninic acid assay (Applygen, Beijing, China). Lysates were resolved on 12% SDS-PAGE gels and electroblotted onto nitrocellulose membranes. Primary antibodies including 1B50-1 mAb, CD44 mAb (Cell Signaling Technology), ALDH1A1 mAb (Cell Signaling Technology) and anti- β -actin (Sigma-Aldrich, St. Louis, MO, USA) were added and incubated overnight at 4°C. Then, secondary antibodies (Cell Signaling Technology) including horseradish peroxidase-labeled anti-rabbit IgG or anti-mouse IgG were added and incubated for 2 hrs at room temperature. The bands were visualized using enhanced chemoluminescence and photographed with a Fujifilm Dark Box.

The 1B50-1 mAb was provided by Zhiqian Zhang, PhD (Laboratory of Carcinogenesis and Translational Research, Department of Cell Biology, Peking University Cancer Hospital and Institute).

Antibodies used in this study are summarized in Table 1.

Statistical analysis

Statistical analysis was performed with SPSS 21.0 software (IBM, Armonk, NY, USA). The significance of differences was determined with Student's *t* test or a χ^2 test. The tumorigenic frequency and the comparison between different groups were calculated based on extreme limiting dilution analysis using the web tool at <http://bioinf.wehi.edu.au/software/elda/> (Hu and Smyth, 2009).³⁶ *p*-value <0.05 was considered statistically significant.

Results

Expression levels of $\alpha 2\delta 1$ in gastric cancer cell lines, PDX models and clinical samples of malignant ascites from gastric cancer patients

We first analyzed the expression of $\alpha 2\delta 1$ in nine gastric cancer cell lines (HGC-27, SGC-7901, MKN-45, MKN-74, MKN-28, AGS, BGC-823, MGC-803 and NCI-N87) using flow cytometry. As shown in Figure 1A, B and Table 2, $\alpha 2\delta 1$ was found to be expressed at different levels in the tested gastric cancer cell lines. Of the tested cell lines, expression level of $\alpha 2\delta 1$ was found to be very low or undetectable in six of the cell lines, whereas it was found to be very high in cell line HGC-27, which is the only undifferentiated cell line among all the nine tested cell lines.²⁰ We also investigate the expression of $\alpha 2\delta 1$ in the nine gastric cancer cell lines by Western blotting. As shown in Figure 1C, $\alpha 2\delta 1$ was found to be highly expressed in HGC-27 cell line and very lowly expressed or not expressed in the other tested gastric cancer cell lines.

To investigate the expression of $\alpha 2\delta 1$ in gastric cancer tumors, we then analyzed the tumor samples of six different gastric cancer PDX models previously established in our laboratory by flow cytometry.²¹ Similarly, $\alpha 2\delta 1$ were found to be expressed at different levels ranging from 4.4% to 31.4% in the tested PDX models, as shown in Figure 1D.

To further investigate the expression of $\alpha 2\delta 1$ in clinical sample of gastric cancer patients, we collected samples of malignant ascites from ten different gastric cancer patients with widespread metastasis and isolated the tumor cells from each clinical sample following the procedure described above, and then we analyzed the expression of $\alpha 2\delta 1$ in the isolated tumor cells by flow cytometry. The results showed $\alpha 2\delta 1$ expressed at different levels in clinical samples ranging from 0.1% to 30% among the ten ascites samples, as shown in Figure 1E and Table 3.

$\alpha 2\delta 1^+$ gastric cancer cells possess highly self-renewal property

GCSC is known closely related to various malignant characteristics including poor differentiation, metastasis and chemoresistance.^{12,22} The results that $\alpha 2\delta 1$ was especially highly expressed in the undifferentiated cell line HGC-27 and was present in clinical samples of patients with

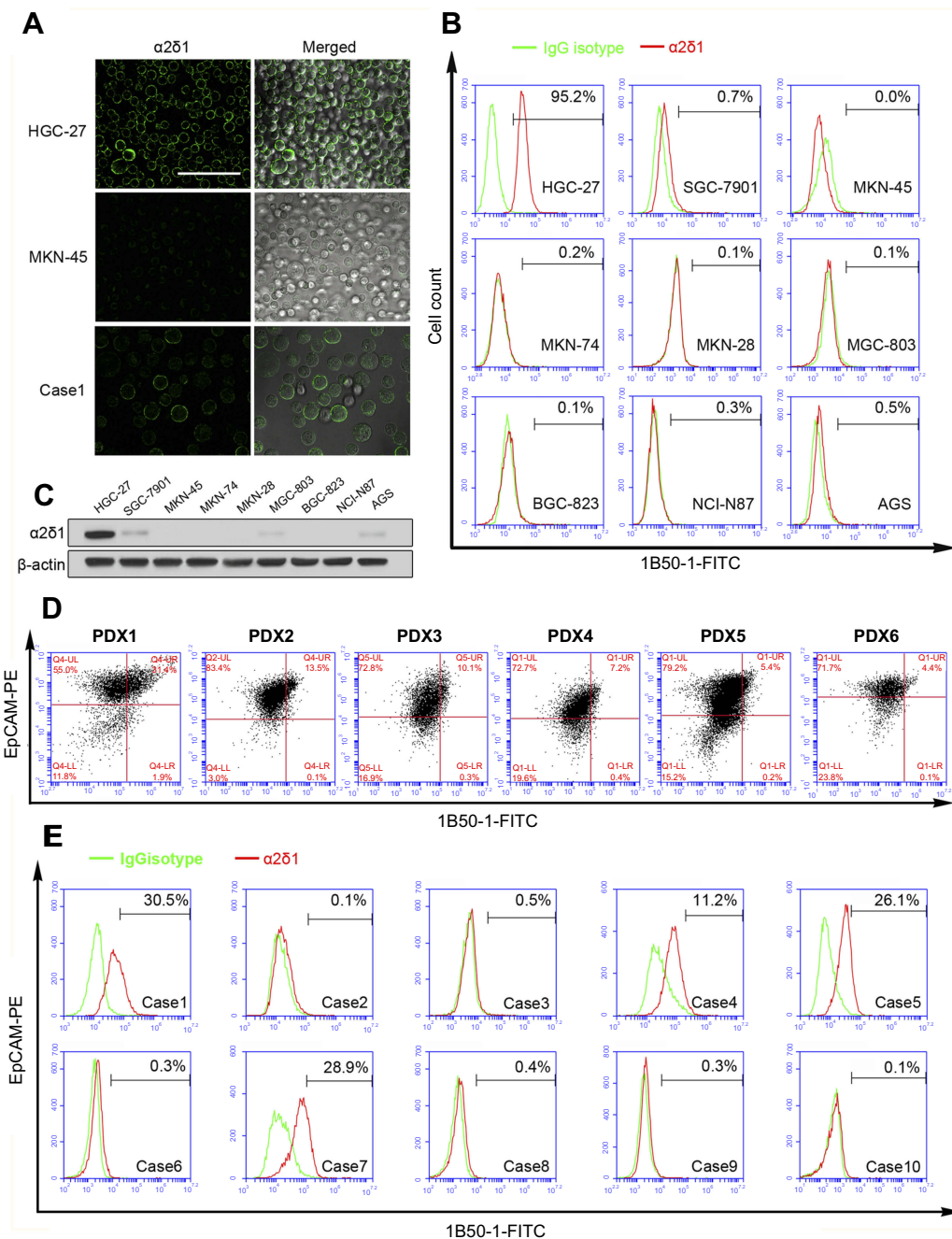


Figure 1 Expression levels of $\alpha 2\delta 1$ were different in gastric cancer cell lines, PDX models and ascites samples. **(A)** Representative images illustrating the expression of $\alpha 2\delta 1$ in HGC-27 cell line, MKN45 cell line and ascites sample (Case 1) detected using confocal microscope. Merged images showed $\alpha 2\delta 1$ expressed on the cell membranes of cells. **(B)** Flow cytometry analysis showed $\alpha 2\delta 1$ expressed differently in nine gastric cancer cell lines. **(C)** Western blotting analysis was conducted to investigate the expression of $\alpha 2\delta 1$ in gastric cancer cell lines. Results showed $\alpha 2\delta 1$ expressed at different levels in the nine tested cell lines. **(D)** Flow cytometry analysis showed $\alpha 2\delta 1$ expressed differently in six gastric cancer cell PDX models. **(E)** Flow cytometry analysis showed $\alpha 2\delta 1$ expressed differently in 10 clinical samples of ascites of gastric cancer patients (Cases 1–10). Scale bar: 100 μ m.

Abbreviations: PDX, patient-derived xenograft; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

metastatic and chemoresisted gastric cancer led us to speculate whether $\alpha 2\delta 1$ is associated with GCSC. Therefore, we performed a series of assays to evaluate the correlation between $\alpha 2\delta 1$ expression and the self-renewal property in gastric cancer cells.

We first tested the tumorigenic capacity of $\alpha 2\delta 1^+$ gastric cancer cells in vivo. The purified $\alpha 2\delta 1^+$ and $\alpha 2\delta 1^-$ cells from HGC-27 and SGC-7901 cell lines were inoculated subcutaneously into NOD/SCID mice with limiting dilution (Figure 2A, Table 4). Results

Table 2 Expression levels of $\alpha 2\delta 1$ and CD44 are analyzed in gastric cancer cell lines

Cell lines	Percentage of positive cells	
	$\alpha 2\delta 1$	CD44
AGS	0.1–0.5	90.0–92.5
BGC-823	0.1–0.3	91.2–95.0
HGC-27	89.7–95.2	90.2–98.4
MGC-803	0.1–0.4	87.4–8–96.9
MKN-28	0.3–0.6	93.1–93.3
MKN-45	0.0–0.3	96.9–97.0
MKN-74	0.1–0.5	0.1–0.4
NCI-N87	0.1–0.3	87.0–93.2
SGC-7901	0.7–1.1	91.8–95.7

Table 3 Ratio of MFI between $\alpha 2\delta 1$ versus control in ascites samples of gastric cancer patients

Sample	Percentage of $\alpha 2\delta 1$	MFI		Ratio
		$\alpha 2\delta 1$	Control	
Case 1	30.5	6,7128.53	14,267.03	4.72
Case 2	0.1	21,379.60	16,869.24	1.27
Case 3	0.5	5,531.14	5,012.46	1.10
Case 4	11.2	94,299.57	37,966.42	2.48
Case 5	26.1	33,638.29	9,631.68	3.49
Case 6	0.3	1,826.10	2,693.33	1.48
Case 7	28.9	79,353.95	19,983.89	3.97
Case 8	0.4	1,936.05	1,463.52	1.32
Case 9	0.3	2,455.17	2,103.87	1.17
Case 10	0.1	610.14	523.52	1.16

Notes: Ratio is calculated as MFI of $\alpha 2\delta 1$ /MFI of control.

Abbreviation: MFI, mean fluorescence intensity.

showed that as few as 100 $\alpha 2\delta 1^+$ HGC-27 cells could trigger tumor formation in five out of eight of the inoculated mice, whereas the $\alpha 2\delta 1^-$ HGC-27 cells formed tumor only occasionally (two of eight) within the same observation period. When inoculated with 1,000 cells, $\alpha 2\delta 1^+$ cells triggered tumor formation in seven of eight inoculated mice for HGC-27 and in six out of nine inoculated mice for SGC-7901, whereas their $\alpha 2\delta 1^-$ counterparts only triggered tumor formation in two of eight mice for HGC-27 and two of nine mice for SGC-7901. These observations suggested that $\alpha 2\delta 1$ defined a subpopulation of gastric cancer cells with highly tumorigenic capacity in vivo. We then sorted $\alpha 2\delta 1^+$ and $\alpha 2\delta 1^-$ cells from the tumors formed previously by the $\alpha 2\delta 1^+$ cells in the first generation and performed subsequent transplantation

assays. The results showed that the $\alpha 2\delta 1^+$ cells successfully initiated tumor formation in the subsequently transplanted mice with high frequency, whereas the $\alpha 2\delta 1^-$ cells remained extremely lowly tumorigenic (Figure S1, Table S1). These results indicated that $\alpha 2\delta 1^+$ cells possessed self-renewal property in vivo.

We also performed the sphere-formation assays in vitro with the purified $\alpha 2\delta 1^+$ cells from HGC-27 and SGC-7901 cell lines. As shown in Figure 2B, $\alpha 2\delta 1^+$ cells displayed significant sphere-forming capacity, whereas their corresponding $\alpha 2\delta 1^-$ counterparts displayed extremely low sphere-forming capacity. It was important to note that the $\alpha 2\delta 1^+$ cells sorted from the sphere formed previously continued to display similarly high sphere-forming capacity after subsequent propagation, demonstrating self-renewal capacity in vitro. Again, their sorted $\alpha 2\delta 1^-$ counterparts formed spheres at extremely low frequency after subsequent propagation.

These results suggested that $\alpha 2\delta 1^+$ subpopulations displayed self-renewal capacity both in vitro and in vivo.

$\alpha 2\delta 1^+$ gastric cancer cells possess differentiation potential and invasiveness

Another important characteristic of CSC is the differentiation potential. We purified $\alpha 2\delta 1^+$ cells from HGC-27 and SGC-7901 cell lines. After cultured in vitro for 2 weeks, the percentage of $\alpha 2\delta 1^+$ cells decreased to 95.2% for HGC-27 and 0.8% for SGC-7901, similar to that of the unsorted parental cells. We also purified $\alpha 2\delta 1^-$ cells from HGC-27 and SGC-7901 cell lines and cultured the sorted cells under the same condition for 2 weeks. It is notable that the percentage of $\alpha 2\delta 1^+$ remained extremely low in the postcultured $\alpha 2\delta 1^-$ cells for both cell lines. These results are shown in Figure 2C. This indicated that the $\alpha 2\delta 1^+$ gastric cancer cells could differentiate into $\alpha 2\delta 1^-$ cells but not *vice versa*.

We also compared the invasive capacity of purified $\alpha 2\delta 1^+$ cells and $\alpha 2\delta 1^-$ cells with the transwell assay. The isolated $\alpha 2\delta 1^+$ and $\alpha 2\delta 1^-$ cells from HGC-27 were cultured separately in the transwell chambers for 24 hrs. After incubation, the cells that had migrated through the membrane were stained and counted. As shown in Figure 3, the isolated $\alpha 2\delta 1^+$ HGC-27 cells presented more significant invasiveness than their $\alpha 2\delta 1^-$ counterparts.

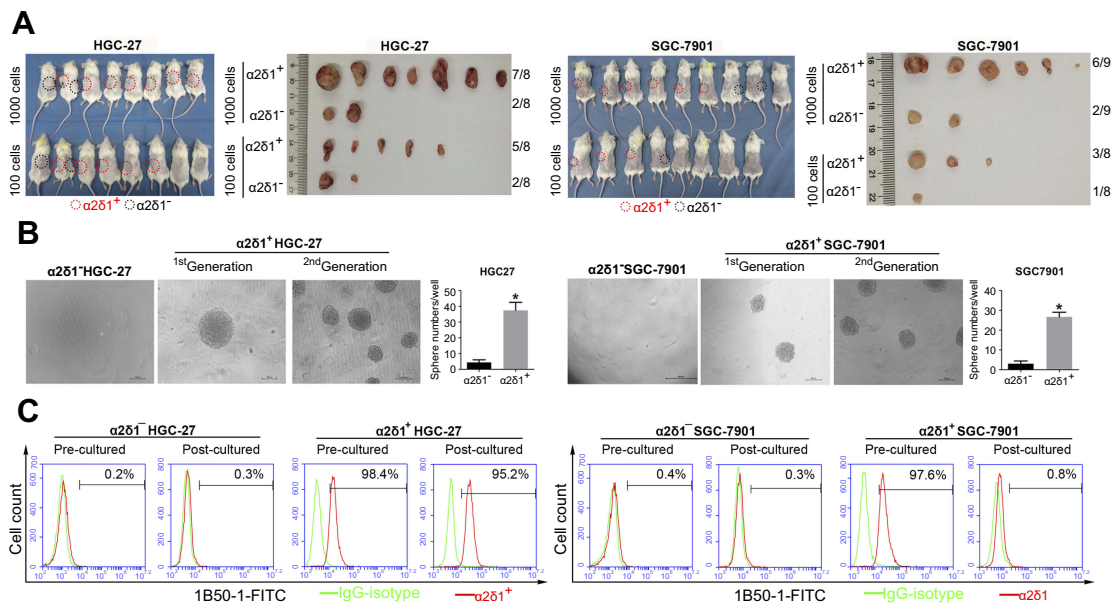


Figure 2 $\alpha 2\delta 1^+$ HGC-27 and $\alpha 2\delta 1^+$ SGC-7901 cells displayed cancer stem cell properties. **(A)** Comparison of tumor formation frequency of purified $\alpha 2\delta 1^+$ and $\alpha 2\delta 1^-$ cells in NOD/SCID mice. $\alpha 2\delta 1^+$ cells displayed significant tumorigenic capacity. **(B)** Comparison of sphere-formation frequency of purified $\alpha 2\delta 1^+$ and $\alpha 2\delta 1^-$ cells in vitro. $\alpha 2\delta 1^+$ cells displayed significant sphere-forming capacity. $\alpha 2\delta 1^+$ cells sorted from the sphere formed previously continued to display high sphere-forming capacity after subsequent propagation. **(C)** Comparison of differentiation potential of $\alpha 2\delta 1^+$ and $\alpha 2\delta 1^-$ cells in vitro. Purified $\alpha 2\delta 1^+$ cells were cultured in vitro for 2 weeks, and the percentage of $\alpha 2\delta 1^+$ cells decreased to the value similar to that of the unsorted parental cells. Purified $\alpha 2\delta 1^-$ cells were cultured in vitro under the same condition for the same period, and the percentage of $\alpha 2\delta 1^+$ cells remained extremely low in the post-cultured $\alpha 2\delta 1^-$ cells. * $p < 0.05$. Scale bar: 100 μ m.

Abbreviation: FITC, fluorescein isothiocyanate.

Table 4 $\alpha 2\delta 1^+$ HGC-27 and $\alpha 2\delta 1^+$ SGC-7901 cells possess highly tumorigenic capacity

Group	Tumor formation (tumor formed/mice injected)		Frequency of tumorigenic cells	(95% CI)	p-Value
	Cells inoculated				
	10 ²	10 ³			
$\alpha 2\delta 1^+$ HGC-27(A)	5/8	7/8	1/271	(1/639–1/115)	0.0006 (A vs B)
$\alpha 2\delta 1^-$ HGC-27(B)	2/8	2/8	1/1,903	(1/5,342–1/678)	
$\alpha 2\delta 1^+$ SGC-7901(C)	3/8	6/9	1/657	(1/1,382–1/312)	0.0177 (C vs D)
$\alpha 2\delta 1^-$ SGC-7901(D)	1/8	2/9	1/2897	(1/9,210–1/912)	

Notes: The tumorigenic frequency and the comparison of the frequencies between different groups was calculated based on extreme limiting dilution analysis using the web tool at <http://bioinf.wehi.edu.au/software/elda/> (Hu and Smyth, 2009).³⁶ p-value <0.05 was considered statistically significant.

$\alpha 2\delta 1$ is more specific than CD44 in defining gastric CSCs

In order to find out the possible relationship between $\alpha 2\delta 1$ and the conventional GCSC marker CD44, we analyzed the expression of $\alpha 2\delta 1$ and CD44 in the nine gastric cancer cell lines mentioned above using flow cytometry. As shown in Figure 4A and B, unlike the differential expression of $\alpha 2\delta 1$, CD44 was found highly expressed in eight of the nine tested cell lines, and the percentage of CD44⁺ fraction of those cell lines accounts for >90%. Interestingly, further analysis showed the $\alpha 2\delta 1^+$ fraction

of those cell lines accounts for a small part of the CD44⁺ fraction. This led us to propose that $\alpha 2\delta 1$ might be more specific than CD44 in defining GCSC population.

In an initial attempt to test our hypothesis, we performed sphere-formation assays in vitro and compared the sphere-forming capacity of purified CD44⁺ $\alpha 2\delta 1^+$ fraction and CD44⁺ $\alpha 2\delta 1^-$ fraction of HGC-27 (Figure 4C). Sphere-formation assays showed CD44⁺ $\alpha 2\delta 1^+$ cells displayed significantly higher sphere-forming capacity compared to their CD44⁺ $\alpha 2\delta 1^-$ counterparts. Furthermore, the descendant generations of

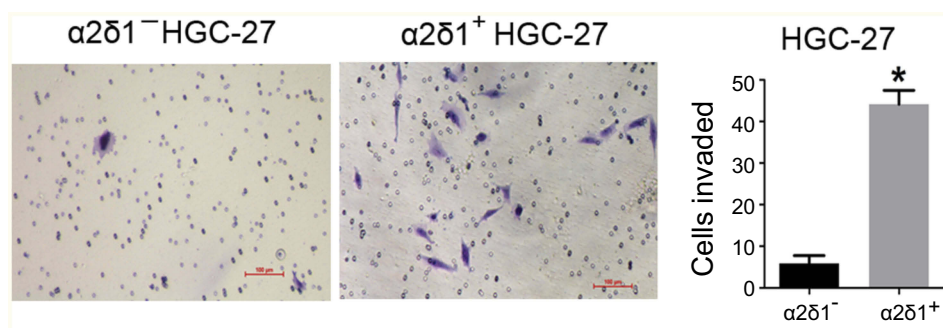


Figure 3 $\alpha 2\delta 1^+$ HGC-27 cells presented more significant invasiveness than $\alpha 2\delta 1^-$ HGC-27 cells. Comparison of invasiveness of $\alpha 2\delta 1^+$ and $\alpha 2\delta 1^-$ HGC-27 cells using the transwell assays. The purified $\alpha 2\delta 1^+$ and $\alpha 2\delta 1^-$ HGC-27 cells were cultured separately in the transwell chambers for 24 hrs. The cells that had migrated through the membrane were stained and counted. $\alpha 2\delta 1^+$ HGC-27 cells displayed more invasiveness than their $\alpha 2\delta 1^-$ counterparts. * $p < 0.05$.

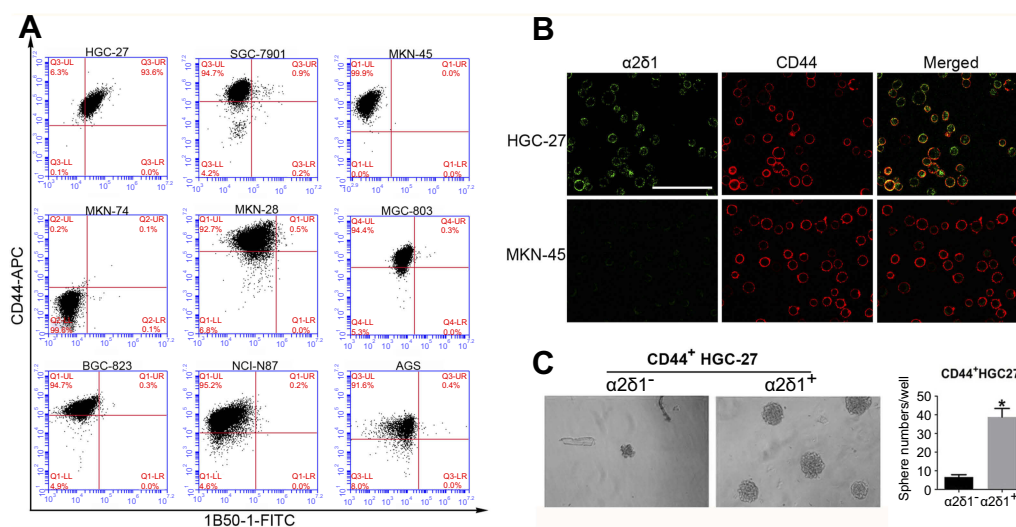


Figure 4 $\alpha 2\delta 1$ is more specific than CD44 in identifying gastric cancer stem cells. (A) Coexpression analysis of $\alpha 2\delta 1$ and CD44 in gastric cancer cell lines by flow cytometry. $\alpha 2\delta 1^+$ cells account for a fraction of CD44⁺ cells. (B) Coexpression analysis of $\alpha 2\delta 1$ and CD44 in HGC-27 and MKN-45 cell lines by confocal microscope. (C) Comparison of sphere-formation frequency of purified $\alpha 2\delta 1^+$ CD44⁺ and $\alpha 2\delta 1^-$ CD44⁺ HGC-27 cells in vitro. $\alpha 2\delta 1^+$ CD44⁺ cells displayed significantly higher sphere-forming capacity compared with their $\alpha 2\delta 1^-$ CD44⁺ counterparts. * $p < 0.05$.

Abbreviations: FITC, fluorescein isothiocyanate; APC, allophycocyanin.

CD44⁺ $\alpha 2\delta 1^+$ cells showed similarly high sphere-forming capacity after subsequent serial propagations.

Knockdown of $\alpha 2\delta 1$ in $\alpha 2\delta 1^+$ gastric cancer cells inhibits the CSC capacity

To further explore the correlation between $\alpha 2\delta 1$ and CSC properties in gastric cancer, we sorted $\alpha 2\delta 1^+$ cells from HGC-27 cell line by FACS. Then, we performed RNAi experiments using the purified $\alpha 2\delta 1^+$ HGC-27 cells to knock down $\alpha 2\delta 1$ and evaluated the subsequent effects. Western blot analysis was used to validate that $\alpha 2\delta 1$ expression was significantly reduced while CD44 expression remained unchanged at high level, as shown in Figure 5A. Then, we compared the sphere-formation frequency

and the tumor-formation frequency of $\alpha 2\delta 1$ knocked-down HGC-27 cells and $\alpha 2\delta 1^+$ HGC-27 cells. The results showed that both of the sphere-formation capacity in vitro and the tumorigenic capacity in vivo were significantly inhibited in HGC-27 cells after $\alpha 2\delta 1$ knockdown, indicating the decreased self-renewal property, as shown in Figure 5B, C, and Table 5.

In addition, we treated $\alpha 2\delta 1$ knockdown HGC-27 cells and $\alpha 2\delta 1^+$ HGC-27 cells with cytotoxic drug cisplatin separately and evaluated their viability with apoptosis assays. As a result, enhanced apoptosis was observed in $\alpha 2\delta 1$ knockdown HGC-27 cells compared with $\alpha 2\delta 1^+$ HGC-27 cells when treated with cisplatin at the same concentration, indicating that $\alpha 2\delta 1$ knockdown rendered $\alpha 2\delta 1^+$ HGC-27 cells more sensitive to cisplatin, as shown in Figure 5D.

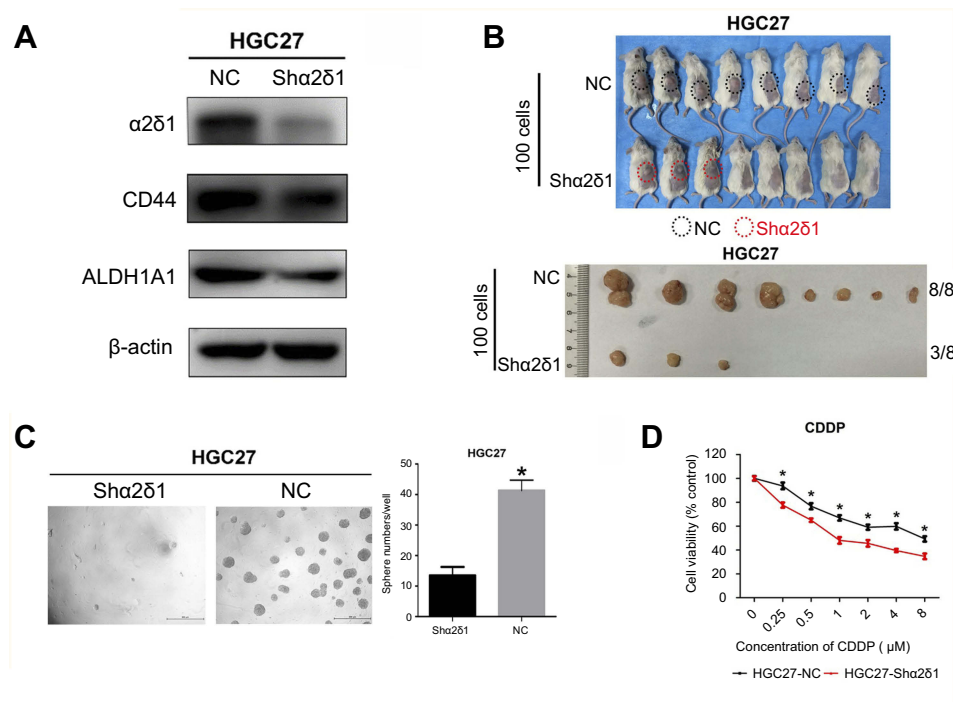


Figure 5 Knockdown of $\alpha 2\delta 1$ inhibited cancer stem cell properties of $\alpha 2\delta 1^+$ HGC-27 cells. **(A)** Western blotting showed the difference of $\alpha 2\delta 1$, CD44 and ALDH expression between unmediated $\alpha 2\delta 1^+$ HGC-27 cells (NC) and $\text{sha}2\delta 1$ HGC-27 cells. $\alpha 2\delta 1$ was confirmed downregulated in $\text{sha}2\delta 1$ HGC-27 cells, while CD44 and ALDH remained at the same level. **(B)** Comparison of tumor-formation frequency of unmediated $\alpha 2\delta 1^+$ HGC-27 cells (NC) and $\text{sha}2\delta 1$ HGC-27 cells in NOD/SCID mice. The tumorigenic capacity was significantly inhibited after $\alpha 2\delta 1$ was knocked down. **(C)** Comparison of sphere-formation frequency of unmediated $\alpha 2\delta 1^+$ HGC-27 cells (NC) and $\text{sha}2\delta 1$ HGC-27 cells in vitro. The sphere-formation capacity was significantly inhibited after $\alpha 2\delta 1$ was knocked down. **(D)** Comparison of IC₅₀ between unmediated $\alpha 2\delta 1^+$ HGC-27 cells (black) and $\text{sha}2\delta 1$ HGC-27 cells (red) when treated with cisplatin. * $p < 0.05$.

Abbreviation: NC, negative control; CDDP, cisplatin.

Table 5 The tumorigenic capacity of $\alpha 2\delta 1^+$ HGC-27 cells are inhibited after $\alpha 2\delta 1$ knockdown

Group	Tumor formation (tumor formed/mice injected)	Frequency of tumorigenic cells	(95% CI)	p-Value
$\alpha 2\delta 1^+$ HGC-27	8/8	1/1	(1/86–1/1)	0.0023
Sha2 $\delta 1$ HGC-27	3/8	1/213	(1/667–1/67.9)	

Notes: The tumorigenic frequency and the comparison of the frequencies between different groups was calculated based on extreme limiting dilution analysis using the web tool at <http://bioinf.wehi.edu.au/software/elda/> (Hu and Smyth, 2009).³⁶ p -value < 0.05 was considered statistically significant.

Discussion

There is now accumulating evidence supporting the existence of CSC in various types of tumors including gastric cancer.^{5,23–29} In our current study, we are the first to analyze the expression of $\alpha 2\delta 1$ in gastric cancer cell lines, PDX models and clinical samples of malignant ascites from gastric cancer patients, and further investigate the relationship between $\alpha 2\delta 1$ and GCSC. We find out for the first time that the expression level of $\alpha 2\delta 1$ varies drastically in gastric cancer and that the $\alpha 2\delta 1^+$ subpopulation of gastric cancer cells possessed CSC properties. Moreover, through comparison of the different biological characteristics of CD44⁺ $\alpha 2\delta 1^+$ and

CD44⁺ $\alpha 2\delta 1^-$ cells, we find that $\alpha 2\delta 1$ is more specific than CD44 in identifying GCSC.

$\alpha 2\delta 1$ is a subunit of the voltage-dependent calcium channel complex expressed on cell membrane. It has been verified in other studies that $\alpha 2\delta 1$ could be used to identify subpopulations of cancer cells with CSC properties in HCC and SCLC.^{18,19} In our current research, we sorted $\alpha 2\delta 1^+$ and $\alpha 2\delta 1^-$ cells from HGC-27 and SGC-7901 cell lines using FACS and compared their sphere-formation capacity in vitro and tumorigenic capacities in vivo. Our results showed that $\alpha 2\delta 1^+$ subpopulation of gastric cancer cells demonstrated both significantly high efficiency of sphere formation in vitro and high efficiency of

tumor formation *in vivo*, whereas $\alpha 2\delta 1^{-}$ cells formed sphere or tumors at extremely low efficiency under the same condition. In addition, the transwell assay results showed that $\alpha 2\delta 1^{+}$ HGC-27 cells were much more invasive than $\alpha 2\delta 1^{-}$ HGC-27 cells. These results indicate the level of $\alpha 2\delta 1$ expression defines a subpopulation of gastric cancer cells demonstrating both highly tumorigenic capacity and invasiveness, which are two of the reported key properties of CSC.^{30,31} It is notable that the sphere-formation capacity and tumorigenic capacity of $\alpha 2\delta 1^{+}$ cells expanded in subsequent propagation *in vitro* and serial transplantation *in vivo*, demonstrating self-renewal property both *in vitro* and *in vivo*, which has been established as a golden standard in identifying CSC.^{29,32,33} Moreover, knockdown of $\alpha 2\delta 1$ in $\alpha 2\delta 1^{+}$ HGC-27 cells led to drastically decreased efficiency of sphere formation or tumor formation, further indicating that the expression of $\alpha 2\delta 1$ is closely correlated with CSC properties.

Another important property of CSC is the differentiation capacity. Previous studies have demonstrated that CSCs undergo symmetric division to maintain the CSC population while at the same time undergo asymmetric division into non-CSC to expand the bulk of tumor, but non-CSC seldom differentiate into CSC.^{29,34,35} Our results showed that after being cultured *in vitro* in RPMI-1640 supplemented with 10% FBS, purified $\alpha 2\delta 1^{+}$ cells from HGC-27 and SGC-7901 cell lines gave rise to both $\alpha 2\delta 1^{+}$ and $\alpha 2\delta 1^{-}$ descendant cells, but not vice versa. This is consistent with the differentiation capacity of CSC.

Taken together, our results above confirmed that $\alpha 2\delta 1$ expression defines a subpopulation of cancer cells with CSC properties in gastric cancer and could be used as a biomarker for identifying GCSC.

A number of molecules have been reported by previous researches as suitable markers for identifying GCSC, including CD44/CD44v, CD133, ALDH, Sca-1, Lgr5, MIST-1 and Bmi-1.⁵⁻¹⁵ However, there have been concerns about the specificity of these reported GCSC markers. Therefore, it is necessary to seek additional marker or combined panels of markers that may improve the accuracy of identifying GCSC. In our current study, we analyzed the coexpression of $\alpha 2\delta 1$ and CD44 simultaneously and found that $\alpha 2\delta 1^{+}$ gastric cancer cells accounted for a subpopulation of CD44⁺ gastric cancer cells. Further results of sphere-formation assays showed that CD44⁺ $\alpha 2\delta 1^{+}$ HGC-27 cells formed sphere with significantly higher efficiency than their CD44⁺ $\alpha 2\delta 1^{-}$

counterpart, indicating that the $\alpha 2\delta 1^{+}$ subset of cells might be responsible for the sphere-formation capacity and CSC properties of the CD44⁺ gastric cancer cells. This suggests that $\alpha 2\delta 1$ might be a better and more specific marker than CD44 in identifying GCSCs, or $\alpha 2\delta 1$ in combination with CD44 could be used to improve the accuracy in identifying GCSC populations.

In previous studies, the therapeutic potential of a new monoclonal antibody 1B50-1 generated by Zhao et al, which recognized the isoform 5 of $\alpha 2\delta 1$ subunit, had been demonstrated on HCC.¹⁸ Zhao et al demonstrated that treatment of 1B50-1mAb or silencing of $\alpha 2\delta 1$ reduced the CSC properties of HCC cells via induction of cellular apoptosis. Moreover, compared with treatment of chemotherapy alone, treatment of 1B50-1mAb combined with doxorubicin led to more significant tumor reduction in HCC PDX models.¹⁸ In another study, Yu et al reported that the combined treatment of 1B50-1mAb and chemotherapy resulted in partial response in originally chemoresistant SCLC PDX model.¹⁹ These studies suggest $\alpha 2\delta 1$ could be a potential target for novel anticancer therapy targeting CSC. In our research, we evaluated the effect of $\alpha 2\delta 1$ knockdown in $\alpha 2\delta 1^{+}$ HGC-27 cells. The results showed that the $\alpha 2\delta 1$ knockdown in $\alpha 2\delta 1^{+}$ HGC-27 led to significantly decreased sphere-forming capacity and tumorigenic capacity, indicating an inhibition of CSC properties. Moreover, $\alpha 2\delta 1$ knockdown rendered HGC-27 cells more sensitive to cisplatin *in vitro*, suggesting that $\alpha 2\delta 1$ knockdown might mimic the effect of 1B50-1mAb binding and improve the therapeutic effect of chemotherapy in gastric cancer cells.

One of the limitations of our current study is that the analysis and assays mainly focused on gastric cancer cells. Further experiments with clinical samples may provide a more comprehensive view of the actual function of GCSC in patients. Moreover, further explorations into the molecular mechanism of $\alpha 2\delta 1$ in regulating CSC properties may provide a deeper understanding of how $\alpha 2\delta 1$ participate in tumor initiation, metastasis, chemoresistance and relapse of gastric cancer. Expansion of the size of clinical samples may also provide additional information about the possible clinical significance of $\alpha 2\delta 1$ in gastric cancer.

Conclusion

Our current study was the first to confirm that $\alpha 2\delta 1^{+}$ gastric cancer cells possessed CSC properties and that $\alpha 2\delta 1$ could be used as a marker for identifying GCSC

with higher specificity than CD44. The combination of $\alpha 2\delta 1$ and CD44 could be used to identify GCSC more accurately. Knockdown of $\alpha 2\delta 1$ sensitized HGC-27 cells to cisplatin, suggesting $\alpha 2\delta 1$ might be a potential target for anticancer treatment.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

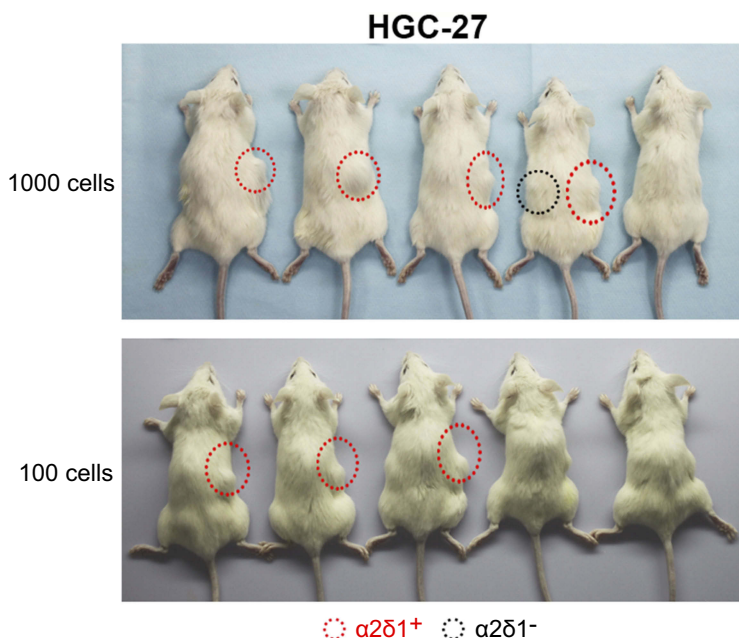


Figure S1 $\alpha 2\delta 1^+$ HGC-27 cells displayed significant tumorigenic capacity after subsequent transplantation. $\alpha 2\delta 1^+$ and $\alpha 2\delta 1^-$ HGC-27 cells were isolated from the tumors formed previously by the $\alpha 2\delta 1^+$ cells in the first generation and inoculated subcutaneously into NOD/SCID mice with limiting dilution. As few as 100 $\alpha 2\delta 1^+$ HGC-27 cells successfully initiated tumor formation in NOD/SCID mice, whereas their $\alpha 2\delta 1^-$ cell counterparts remained extremely lowly tumorigenic within the same observation period.

Abbreviation: NOD/SCID, nonobese diabetic-severe combined immune deficient.

Table S1 $\alpha 2\delta 1^+$ HGC-27 cells displayed significant tumorigenic capacity after subsequent transplantation

Group	Tumor formation (tumor formed/mice injected)		Frequency of tumorigenic cells	(95% CI)	p-Value
	Cells inoculated				
	10^2	10^3			
$\alpha 2\delta 1^+$ HGC-27(A)	3/5	4/5	1/360	(1/988–1/131)	0.0024 (A vs B)
$\alpha 2\delta 1^-$ HGC-27(B)	0/5	1/5	1/4,983	(1/35,191–1/706)	

Notes: The tumorigenic frequency and the comparison of the frequencies between different groups was calculated based on extreme limiting dilution analysis using the web tool at <http://bioinf.wehi.edu.au/software/elda/> (Hu and Smyth, 2009).³⁶ p-value <0.05 was considered statistically significant.

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