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ORIGINAL ARTICLE

Identification and expansion of cancer stem cells in tumor tissues and peripheral blood derived from gastric adenocarcinoma patients

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Gastric cancer is the fourth most common cancer worldwide, with a high rate of death and low 5-year survival rate. To date, there is a lack of efficient therapeutic protocols for gastric cancer. Recent studies suggest that cancer stem cells (CSCs) are responsible for tumor initiation, invasion, metastasis, and resistance to anticancer therapies. Thus, therapies that target gastric CSCs are attractive. However, CSCs in human gastric adenocarcinoma (GAC) have not been described. Here, we identify CSCs in tumor tissues and peripheral blood from GAC patients. CSCs of human GAC (GCSCs) that are isolated from tumor tissues and peripheral blood of patients carried CD44 and CD54 surface markers, generated tumors that highly resemble the original human tumors when injected into immuno-deficient mice, differentiated into gastric epithelial cells *in vitro*, and self-renewed *in vivo* and *in vitro*. Our findings suggest that effective therapeutic protocols must target GCSCs. The capture of GCSCs from the circulation of GAC patients also shows great potential for identification of a critical cell population potentially responsible for tumor metastasis, and provides an effective protocol for early diagnosis and longitudinal monitoring of gastric cancer.

Keywords: cancer stem cells; gastric adenocarcinoma; CD44; CD54; circulating tumor cells

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Introduction

Gastric adenocarcinoma (GAC) originates from malignant stomach mucosa. The disease incidence increases with age, and is highly associated with diet, bile reflux, and *helicobacter pylori* infection [1, 2]. GAC is the fourth most common cancer worldwide and has a high number of deaths per year [3]. The 5-year survival rates are very low (< 20%), and patients frequently die due to metastasis [3]. To date, surgery remains a key curative therapy for gastric cancer, with chemotherapy serving as

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an important adjuvant therapy [4, 5]. However, a lack of efficient therapeutic protocols persists, and as such, new strategies for GAC treatment are critically important.

The concept of cancer stem cells (CSCs) may provide a new approach for gastric cancer therapies. CSCs are a small subpopulation of cells that can give rise to tumor mass [6, 7]. CSCs can be viewed as the result of misdifferentiation and possess self-renewal and differentiation potential [8]. Recent studies demonstrated that CSCs are responsible for tumor initiation, invasion, distant metastasis, and resistance to anticancer drugs, so therapies that target CSCs are becoming increasingly appealing [9]. Currently, CSCs have been found in many types of solid tumors, such as breast cancer [10], glioblastoma [11], and colon cancer [12, 13]. However, CSCs of gastric adenocarcinoma (GCSCs) in primary human tumor tissues and in the peripheral blood of GAC patients have not yet been described. Here, we report the identification of CSCs in human GAC tumor tissues and the peripheral blood of GAC patients, and show that these cells can be

captured and expanded for further study.

Results

Tumorigenic spheres formed from gastric adenocarcinoma patient tumor tissues

We first examined the immunophenotypes of tumor cells obtained from 18 GAC patients and found that they did not carry most known stem-cell-related markers. CSCs are believed to be able to form spheres in culture that possess extensive similarities to endogenous CSCs in human tumor tissues [13-15]. Therefore, we cultured GAC cells to induce sphere formation and then examined the sphere cell surface proteins to determine the identity of GCSC markers. Isolated gastric cancer cells from human tumor tissues were cultured in a serum-free medium with EGF and FGF. After 3-4 weeks, some tumor cells grew to form spheres (Figure 1A). The tumor spheres were maintained in culture for at least 6 months and were passaged 12 times, indicating that the sphere
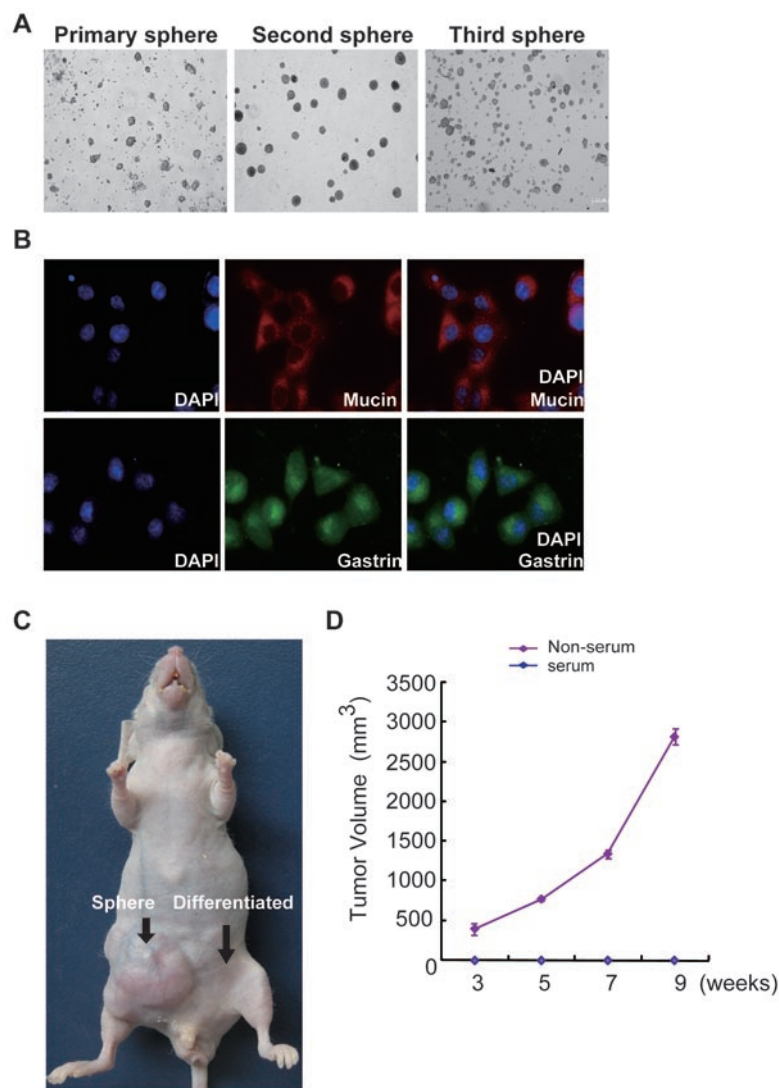

Figure 1 Tumor spheres derived from human gastric adenocarcinoma samples are of gastric origin, can self-renew, and can produce tumors in mice. **(A)** Example of tumor spheres generated from a human gastric adenocarcinoma sample that were passaged two times. Primary spheres: spheres directly generated from human tumor tissues. Second and third spheres: spheres represent the second and the third passages of primary spheres. **(B)** Spheres were cultured with serum and differentiated to gastric epithelial cells detected by specific antibodies against gastrin (green) and mucin (red) antigens. **(C)** Example showing that spheres cultured in serum-free media but not in serum-supplemented media produce tumors in mice. **(D)** Tumorigenic potential of tumor spheres after subcutaneous injection. Tumor volumes generated by spheres were derived from three independent experiments in duplicate, referring to those tumors in which 10^3 sphere cells were able to engraft.

cells were able to self-renew. The cells in the spheres at all passages were negative for gastric mucosa markers (Supplementary information, Figure S1). After addition of serum-containing media, these cells differentiated into gastric epithelial cells, as detected by specific antibodies (Figure 1B). Tumor spheres from the first three passages
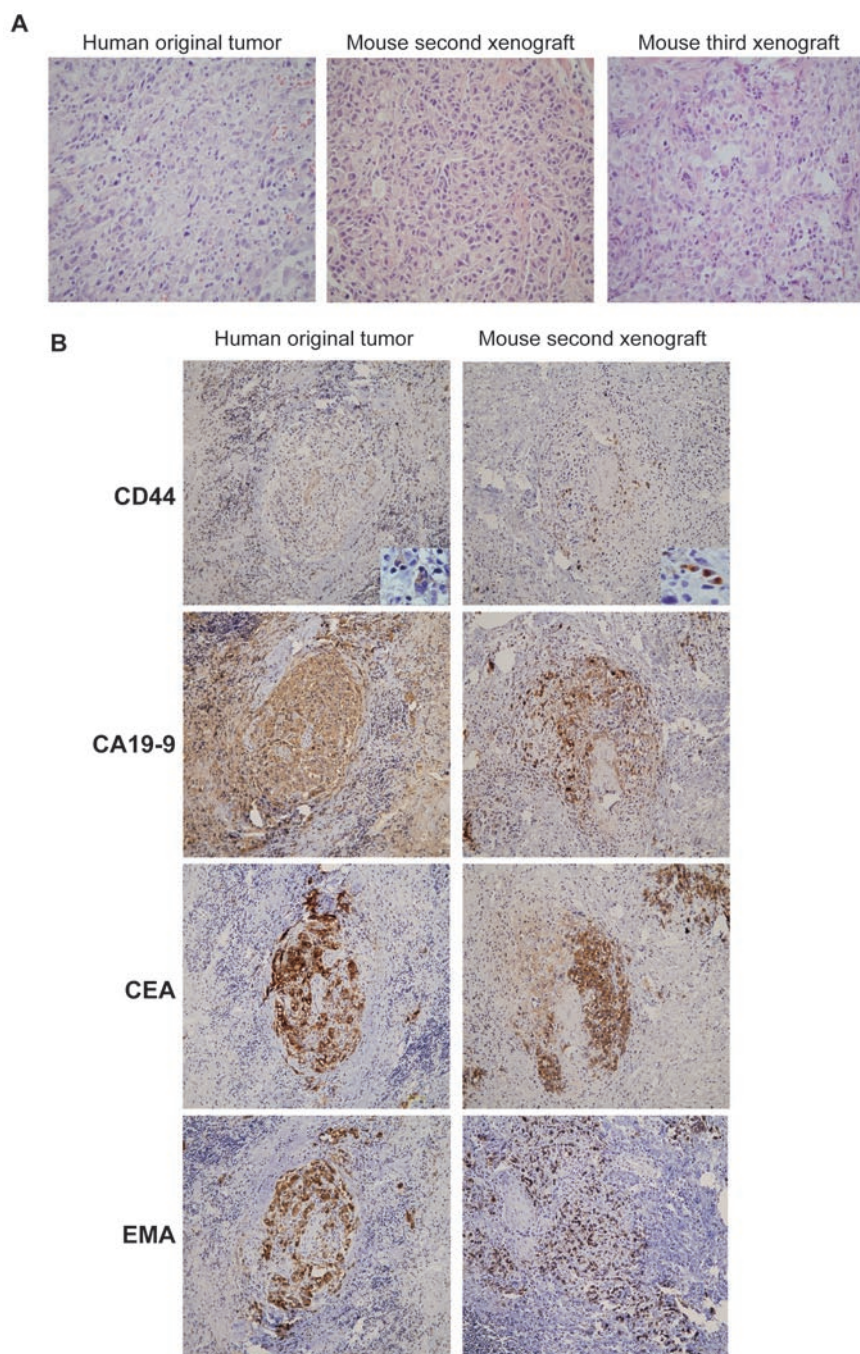

Figure 2 Xenografts generated from tumor spheres highly resemble original human tumor tissues. **(A)** Hematoxylin & Eosin analysis with 40× original magnification of human gastric cancer sections from the original human tumor and corresponding xenografts obtained after injection of sphere cells. Second xenograft and third xenograft: tumors were obtained from the second and third transplantations, respectively. **(B)** Immunohistochemical analysis of the original human tumor and sphere-derived second xenografts. EMA: epithelial membrane antigen; CEA: carcinoma embryonic antigen. The inserts show the high-magnification images.
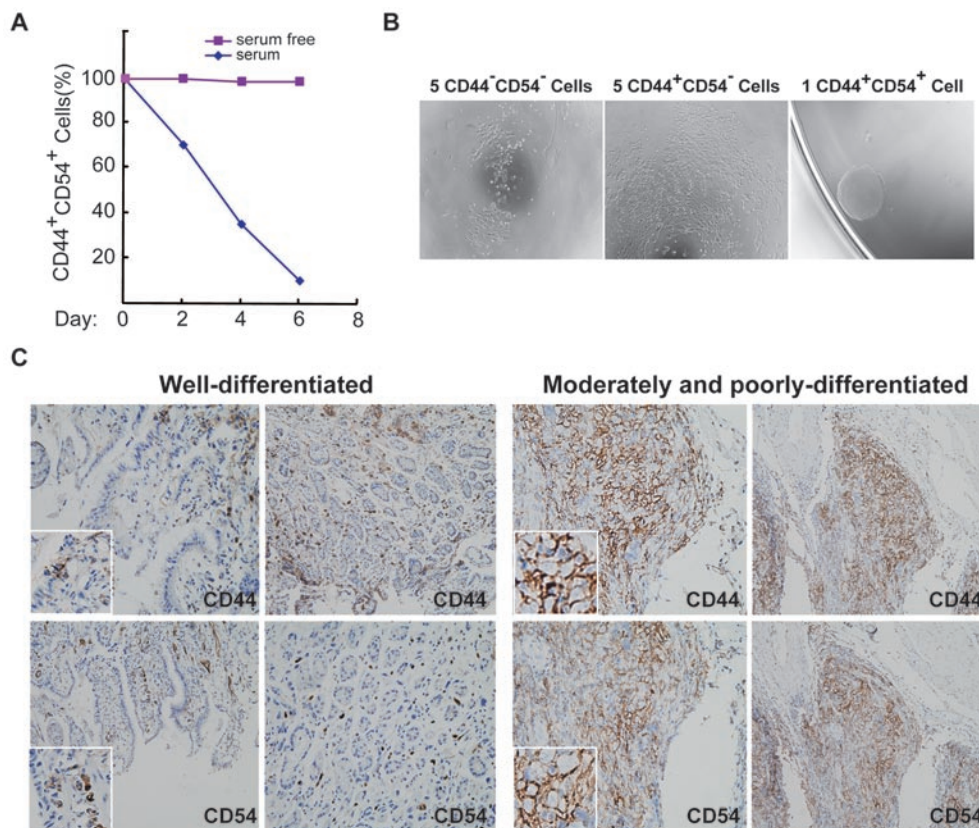
Figure 3 CD44 and CD54 double-positive cells are detected in tumor spheres and original human tumor tissues. **(A)** Percentage of sphere cells carrying CD44 and CD54 antigen with and without serum in the culture media. **(B)** A single CD44 and CD54 double-positive cell generated one sphere in culture. **(C)** Immunohistochemical analysis revealed that CD44 and CD54 are expressed in original human tumors of different pathological grades. The inserts show the high-magnification images.

injected into immunodeficient mice formed tumors after 3 weeks, while sphere cells cultured in serum-containing media did not generate any detectable tumors in mice (Figure 1C and 1D). Thus, these data showed that cells in the tumor spheres are of gastric origin, have self-renewal capability *in vitro*, and are able to form tumors in mice, suggesting that GCSCs are enriched in tumor spheres.

Tumor spheres contain GAC stem cells

CSCs are characterized by their self-renewal *in vivo* [6, 7]. Therefore, we performed serial transplantation in mice to test the self-renewal capacity of the sphere cells (Figure 2). The sphere cells were injected into immunodeficient mice subcutaneously. When the tumor diameter reached ~1 cm, tumor mass was resected and dissociated to form tumor spheres in the culture for transplantation. Second and third transplantations were performed accordingly and showed that these cells also generated tumor mass in mice. These results demonstrate that the sphere cells possess self-renewal capability. Since another feature of CSCs is their ability to produce grafts in

mice that are highly similar to the original human tumors [7, 9], we performed a pathology examination of murine xenografts. Hematoxylin & Eosin (H&E) and immunostaining analysis of original human GAC tumors and mouse xenografts showed that the first, second, and third xenografts were highly similar to the original human GAC tumors (Figure 2A and 2B, data not shown). Thus, our data demonstrate that sphere cells from GAC tissues are able to generate human GAC in mice and have self-renewal capability, indicating that the tumor cell population in the spheres contains GCSCs.

A single CD44⁺CD54⁺ cell can form gastric tumorigenic spheres

We next examined the cell surface markers of the tumor spheres. Two adhesion molecules, CD44 and CD54, were positively expressed by the majority of tumor sphere cells (Figure 3A, Supplementary information, Figure S2). Interestingly, the expression of both molecules decreased gradually after tumor spheres were cultured in a serum-containing medium (Figure 3A). No-

Table 1 Case description, sphere formation, and tumorigenic activity of CD44$^+$ and CD54$^+$ gastric cancer cells

Case	Sex/age	Site	Grade	CD44$^+$CD54$^+$ expression (%)	Sphere formation	Tumor formation			Secondary tumor formation
						CD44$^+$CD54$^{+\,1}$	Slice (cm^3) ^{2}	Total cell	
1	F/71	Pyloric	Moderate	0.4	No	10^3 (0/3)	0.05 (2/3)	3×10^6 (0/3)	N/A
2	M/60	Cardial	Moderate	1.8	Yes	10^3 (3/3)	0.06 (3/3)	3×10^6 (2/3)	Yes
3	F/61	Corpus	Moderate	0.6	No	10^3 (0/3)	0.07 (1/3)	3×10^6 (0/3)	N/A
4	M/41	Pyloric	Poor	1.3	Yes	10^3 (3/3)	0.05 (2/3)	3×10^6 (2/3)	Yes
5	F/62	Cardial	Moderate	7.0	Yes	10^3 (3/3)	0.05 (3/3)	3×10^6 (3/3)	Yes
6	M/55	Pyloric	Moderate	0.2	No	10^3 (0/3)	0.07 (2/3)	3×10^6 (0/3)	N/A
7	M/53	Cardial	Moderate with signet-ring cell	0.8	Yes	10^3 (3/3)	0.075 (2/3)	3×10^6 (2/3)	Yes
8	M/62	Corpus	Poor	19.7	Yes	10^3 (3/3)	0.06 (3/3)	3×10^6 (1/3)	Yes
9	M/76	Fundus	Moderate	6.1	Yes	10^3 (3/3)	0.07 (3/3)	2×10^6 (3/3)	Yes
10	F/72	Pyloric	Moderate	1.2	No	10^3 (0/3)	0.075 (3/3)	3×10^6 (0/3)	N/A
11	F/59	Pyloric	Moderate with signet-ring cell	2.2	Yes	10^3 (3/3)	0.068 (2/3)	2×10^6 (2/3)	Yes
12	F/56	Pyloric	Moderate with signet-ring cell	1.5	Yes	10^3 (3/3)	0.056 (3/3)	2×10^6 (1/3)	Yes
13	M/58	Cardial	Moderate	0.1	Yes	10^3 (3/3)	0.07 (3/3)	2×10^6 (1/3)	Yes
14	M/55	Cardial	Moderate	0.1	No	10^3 (0/3)	0.06 (2/3)	3×10^6 (0/3)	N/A
15	M/77	Angular notch	Moderate with signet-ring cell	2.3	Yes	10^3 (3/3)	0.05 (2/3)	2×10^6 (2/3)	Yes
16	M/72	Angular notch	Moderate	0.2	No	10^3 (0/3)	0.07 (3/3)	3×10^6 (0/3)	N/A
17	M/41	Pyloric	Moderate with signet-ring cell	0.7	Yes	10^3 (3/3)	0.075 (3/3)	2×10^6 (2/3)	Yes
18	M/71	Angular notch	Moderate with signet-ring cell	0.6	Yes	10^3 (3/3)	0.05 (2/3)	2×10^6 (2/3)	Yes

[1] Number of CD44$^+$CD54$^+$ cells injected into mice (number of mice that have tumor formation in three mice).

[2] Volume of tissue directly cut from tumor (number of mice that have tumor formation in three mice). N/A: not available.

tably, high expression levels of CD10, CD29, and EGFR were also observed on the surface of many sphere cells (Supplementary information, Figure S2). Consistent with the result of the tumor tissues, most known stem-cell-related markers such as CD133, CD34, CD117, CD15, and especially Lgr5, which is associated with gastric stem cells in mice [16], were not detected. Next, we sorted the cell population by different surface markers expressed on the spheres and cultured the sorted cells. Five cells were seeded in each well of a 96-well plates and more than 300 wells were seeded for each cellular population. After 2 weeks, the CD44$^+$CD54$^+$ cells in each well formed spheres in a serum-free medium. These sphere cells were also able to generate tumors in mice. The other cellular populations, including CD44$^+$CD54$^-$, CD44$^-$CD54$^+$, and CD44$^-$CD54$^-$ cells were unable to produce any spheres in culture and could not form tumors in mice (Figure 3B

and data not shown). The same results were obtained from the tumor spheres derived from three tumor samples. Furthermore, a single CD44$^+$CD54$^+$ cell was sorted and formed spheres (Figure 3B) in serum-free media and subsequently generated tumors in mice. This single CD44$^+$CD54$^+$ tumor cell also had self-renewal ability *in vivo*, as tested by serial transplantation in mice. Taken together, these results indicate that the CD44$^+$CD54$^+$ subpopulation contains the GCSCs in the spheres derived from gastric cancers.

CD44$^+$CD54$^+$ gastric cancer stem cells in human GAC tissues

We next directly examined tumor cells from 18 human patients to confirm the presence of CD44$^+$CD54$^+$ gastric CSCs. CD44$^+$CD54$^+$ cells were identified and accounted for 0.1%-19.7% of the cells in the patient samples (Table

Table 2 Case description and frequency of CD44^{+} and CD54^{+} gastric cancer cells in circulation

Case	Sex/age	Grade	Stage	Chemotherapy	Surgery	CD44^{+}CD54^{+} expression (%)[1]
1	M/38	Poor	T3N1M0	FOLFOX,CF	Yes	3
2	M/58	Poor	T3N1M0		Yes	0.1
3	M/53	Poor	T1N2M0	FOLFOX	Yes	12.9
4	M/51	Poor	T2N1M0		Yes	2.4
5	F/52	Poor	T3N2M0	FOLFOX	Yes	1.7
6	M/42	Signet-ring cell	T2N1M0		Yes	1.3
7	M/56	Moderate	T3N3M0		Yes	0.3
8	F/62	Poor	T4aN1M0		Yes	0.2
9	F/36	Poor	T1bN3M0		Yes	5
10	F/80	Poor	T2N0M0		Yes	3.1
11	M/64	Moderate to Poor	T4aN3M0		Yes	1.2
12	F/37	N/A	IV		No	0.4
13	M/79	N/A	IV		No	3.5
14	M/62	Poor	T2N3M0		Yes	2.4
15	F/54	Moderate	T2N0M0		Yes	0
16	F/33	Poor	T4aN3M0		Yes	0.8
17	F/79	Moderate	T4aN3M0		Yes	0.5
18	M/69	Poor	T4aN1M0		Yes	4.2

[1]Ratio of CD44 and CD54 double-positive cells is derived from non-hematopoietic and non-endothelial cells in the peripheral blood of patients. Non-hematopoietic and non-endothelial cells were obtained by first depleting hematopoietic and endothelial cells by specific antibodies before FACS analysis and gating out the hematopoietic and endothelial-specific antibody labeling during FACS analysis.

Table 3 Case description and sphere formation and tumorigenic activity of CD44^{+} and CD54^{+} gastric cancer cells in circulation

Case	Sex/age	Grade	Stage	Metastasis	Chemotherapy/ cycles	Surgery	CD44^{+}CD54^{+} expression (%)[1]	Sphere formation	Tumor formation[2]
1	M/57	Poor	TXNXM1	Liver	Lapatinib (1)	No	5.1	Yes	9×10^3 (3/3)
2	M/74	Poor with signet-ring cell	T2N1M0	No	FOLFOX (3)	Yes	0.7	Yes	9×10^3 (3/3)
3	M/61	Poor	TXNXM0	No	FOLFOX (1)	Yes	0.9	Yes	9×10^3 (3/3)
4	M/59	Poor	T2N2M1	Bone	DCF (2)	Yes	2.1	Yes	9×10^3 (3/3)
5	M/66	Poor	TXNXM1	Liver	Mfolfox7 (4)	Yes	0.9	Yes	9×10^3 (3/3)
6	M/59	Poor with signet-ring cell	T2N1MO	No	FOLFOX (2)	Yes	0.5	No	No
7	M/62	Poor with signet-ring cell	T2N1M0	No	FOLFOX (2)	Yes	0.4	Yes	9×10^3 (3/3)

[1]Ratio of CD44 and CD54 double-positive cells is derived from non-hematopoietic and non-endothelial cells in the peripheral blood of patients. Non-hematopoietic and non-endothelial cells were obtained by first depleting hematopoietic and endothelial cells by specific antibodies before FACS analysis and gating out the hematopoietic and endothelial-specific antibody labeling during FACS analysis.
[2]Number of CD44^{+}CD54^{+} cells from spheres injected into mice (number of mice which have tumor formation in three mice).

1). We also examined normal gastric mucosa and found that CD44^{+}CD54^{+} cells were barely detectable. Immunohistochemical staining of normal tissues showed that CD44^{+} or CD54^{+} cells were sparingly distributed in the gastric mucosa. In contrast, poorly differentiated gastric cancer had a high frequency of CD44^{+} and CD54^{+} cells (Figure 3C). A few cells were CD44 or CD54 positive in a well-differentiated gastric cancer (Figure 3C). In these

tumors, CD44 was also found to be expressed by mesenchymal cells of the tumor tissues. The CD44$^+$CD54$^+$ cells were sorted from the tumor tissues and injected into mice to test their capability to form xenografts or cultured to form tumor spheres in serum-free media (Table 1). Among the 18 samples, as few as 10^3 CD44$^+$CD54$^+$ cells derived from 12 samples were able to form tumors in mice and formed tumor spheres *in vitro*. The murine xenografts had self-renewal capacity, as tested by serial transplantation (Table 1). Up to 3 × 10^3 CD44$^+$CD54$^-$ and CD44$^-$CD54$^+$ cells could also form tumor nodules in mice. In contrast, the injection of up to 1 × 10^4 CD44$^-$CD54$^-$ cells failed to induce tumor formation (Supplementary information, Table S1). In addition, up to 2 × 10^6 of the total tumor cells from these samples were able to form tumors in mice (Table 1), suggesting that CD44$^+$CD54$^+$ cells are enriched by at least 10^3-fold for GCSCs from tumor tissues. Tumor cells sorted from the remaining six samples did not produce tumors in mice and did not form tumor spheres in culture (Table 1). Furthermore, 3 × 10^6 of the total tumor cells from these six samples were also unable to produce tumors in mice or spheres in culture. When the cell population expressing CD44 and CD54 in a single-cell suspensions of these six tumor samples was stained with DAPI, only a few living cells were observed (Supplementary information, Table S2), suggesting that tumor cells from these samples had no viable CD44$^+$CD54$^+$ GCSCs after *in vitro* procedures were performed to prepare the single-cell suspensions. In agreement with these findings, tissue slices from the same tumors transplanted subcutaneously were able to produce tumors in mice (Table 1). Thus, these data indicate that the CD44$^+$CD54$^+$ subpopulation from human GAC tissues contains GCSCs.

Capture of cancer stem cells from peripheral blood of GAC patients

Tumor-derived cells have been reported in peripheral blood from cancer patients and are considered to be the origin of metastatic tumors [14]. Current opinion also suggests that CSCs in the blood stream are the root of metastatic tumors [14]. So far, strategies for isolating circulating tumor cells (CTCs) have been unable to identify CSCs in the circulation [17]. Therefore, the identification of the GCSC markers prompted us to determine whether there are GCSCs in the peripheral blood of GAC patients. Analysis of CD44 and CD54 expression was performed in 10 normal humans, and FACS assays showed that CD44$^+$ cells existed in all peripheral blood samples. In contrast, the CD44$^+$CD54$^+$ subpopulation was not detected in these blood samples (data not shown), indicating that the frequency of the CD44$^+$CD54$^+$ subpopulation, if present in normal peripheral blood, is too low to be detected. We then examined blood samples from 25 patients with gastric cancers and detected CD44$^+$CD54$^+$ epithelial-like cells in 24 samples (Tables 2 and 3, Supplementary information, Figure S3). The cells were sorted and verified by cytological examination (Figure 4A). Using the GCSCs markers, we performed serial dilution assays and were able to detect tumor cells in 10^7 blood cells (Supplementary information, Table S2).

CD44$^+$CD54$^+$ cells were sorted from the blood samples, but cell survival after sorting was low, possibly due to the low frequency of CD44$^+$CD54$^+$ epithelial-like cells
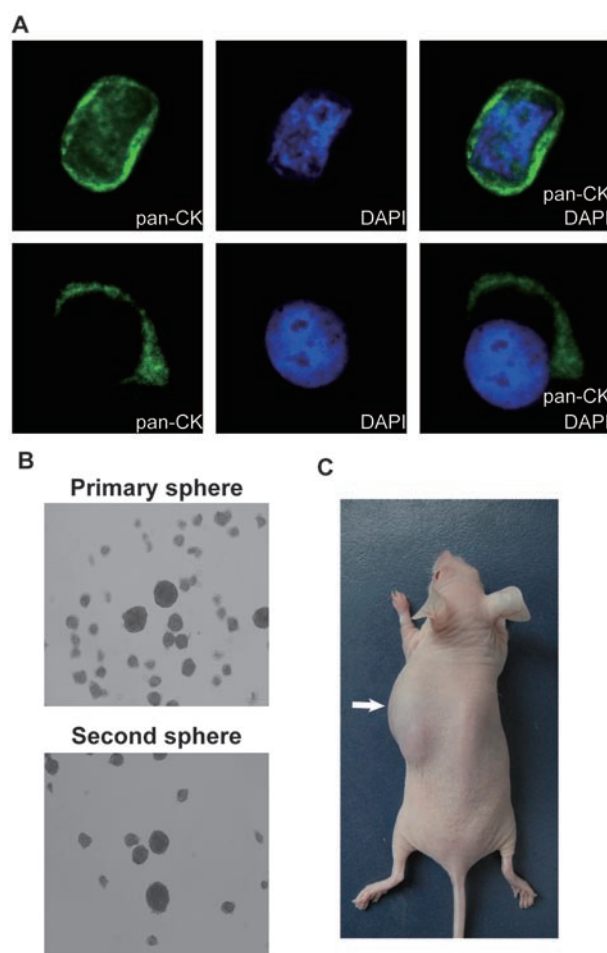

Figure 4 Tumorigenic spheres are derived from the peripheral blood of gastric cancer patients. **(A)** CD44- and CD54-positive cells were sorted, stained with anti-pan-cytokeratin (PAN-CK) antibody and DAPI, and visualized by fluorescence microscopy. **(B)** Spheres were generated from fewer than 10^3 CD44-positive cells. Primary sphere: original spheres formed from blood cells. Second sphere: second passage of spheres. **(C)** An example of a mouse bearing a tumor (arrow) produced by blood-borne spheres.
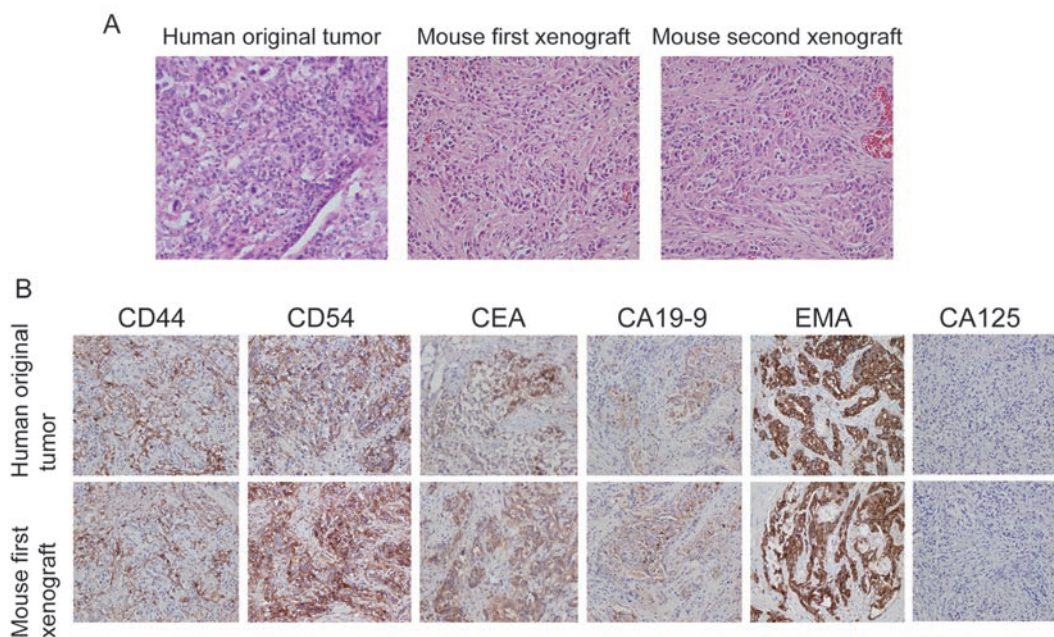
Figure 5 Xenografts generated from tumor spheres derived from blood samples highly resemble original human tumor tissues. **(A)** Hematoxylin & Eosin analysis with 40× original magnification of a human gastric cancer section from the original human tumor and corresponding xenografts obtained after injection of blood-borne sphere cells. First and second xenografts: tumors were obtained from the first and second transplantation, respectively. **(B)** Immunohistochemical analysis of original human tumor and sphere-derived first xenografts.

and the long time needed for cell capture. Therefore, to maintain cell survival, a single CD44 antibody bound to magnetic beads was used to isolate CD44$^+$ cells from non-hematopoietic and non-endothelial cells of the blood samples. Up to 10^3 CD44 positive non-hematopoietic and non-endothelial cells were sorted from 2-4 ml of blood from seven patients who were treated with one to four cycles of chemotherapy (Table 3). The cells obtained from six samples formed tumor spheres and were successfully passaged in the culture (Figure 4B, Table 3). The resulting CD44$^+$CD54$^+$ cells from the tumor spheres were injected into nude mice and quickly generated growing tumors that were highly similar to the original cancers from human stomach (Figures 4C, 5A and 5B, Table 3). Serial transplantation also demonstrated that the CD44$^+$CD54$^+$ cells carried self-renewal ability (Figure 5A and 5B). The remaining cellular population from the spheres had no ability to generate tumors in mice. These results indicate that the CD44$^+$CD54$^+$ subpopulation derived from the blood samples also displays CSC properties. After depleting CD44-positive cells, more than 10^6 non-hematopoietic and non-endothelial cells derived from patient blood samples were unable to form tumor spheres in culture and to generate tumors in mice. Taken together, our results indicate that the tumor cells

in patient peripheral blood contain a GCSC population, and suggest that the GCSC markers are able to detect and capture GCSCs in the circulation of GAC patients.

Discussion

In this study, we identified a CD44$^+$CD54$^+$ population from GAC tissues and the peripheral blood of GAC patients that possesses the capacity for sphere formation, self-renewal, gastric differentiation, and tumor initiation. The murine tumors formed by this CD44$^+$CD54$^+$ population were highly similar to the original human GAC tumors. All of these features are hallmarks of CSCs. In addition, we also showed that a single CD44$^+$CD54$^+$ tumor cell exhibits all of the CSC properties, whereas other cell populations in tumor tissues and the peripheral blood of patients did not have these properties. Thus, the CD44$^+$CD54$^+$ subpopulation in GAC tumor tissues and in peripheral blood from GAC patients contains GCSCs, and CD44 and CD54 are potential GCSC markers.

CD44 is a transmembrane glycoprotein that is well known as a CSC marker in several cancers, including breast, prostate, and colon cancer [18, 19]. CD54 (also called intercellular adhesion molecule-1; ICAM-1) is a 90-kDa member of the immunoglobulin superfamily

and is widely expressed in tumor, stromal, and immune cells [20]. Both CD44 and CD54 are strongly associated with metastasis in gastric adenocarcinoma, and CD44 is positively and significantly associated with tumor recurrence and mortality in gastric cancer [21, 22]. Here, we show that CD44+CD54+ cells exhibit CSC capabilities in GAC tissues, which is consistent with previous reports. In addition, we demonstrate that CD44+CD54+ cells from patient peripheral blood also display CSC features. CSCs are reportedly responsible for tumor initiation, invasion, and distant metastasis. Therefore, our data may explain previous observations that both CD44 and CD54 are associated with metastasis, tumor recurrence, and mortality in gastric cancer, and could be used as prognostic factors for gastric cancer and as cellular markers for disease surveillance and therapeutic response.

Cells expressing Lgr5 in the stomachs of mice have been reported to possess stem cell properties and the capacity for tumor initiation [16]. However, we found no Lgr5 expression in gastric tumor cells from human samples. Instead, Lgr5 was stained in the stromal cells of tumor tissues. Therefore, differences in Lgr5 expression patterns may be species dependent.

Accumulated evidence suggests that CSCs may be a source for CTCs [23-25]. For example, the subpopulation of CD45−CD90+ cells, which is a marker of liver CSCs, in peripheral blood from liver carcinoma patients showed tumor initiation capacity [17]. In our study, we found that the CD44+CD54+ tumor cell subpopulation in peripheral blood from GAC patients is able to form tumor spheres in a serum-free medium, generates tumors where the histopathology resembles human GAC, and has self-renewal capability both in cell culture and in mouse models. These results indicate that the GCSCs are indeed in the circulation and support the hypothesis that the CTC population contains CSCs. Currently, CTCs are examined by RT-PCR, immunohistochemistry, immunofluorescence, and flow cytometry [14, 23, 26]. However, these protocols have limitations in that they cannot directly confirm whether the examined CTCs are indeed cancer cells. Using our working protocol, CTCs with cancer characteristics can be defined. The identification of cancer-initiating cells in peripheral blood will allow for novel advances in therapeutic protocols for GAC, as well as prompt modifications of therapeutic strategies for patients according to their therapeutic response.

CSCs derived directly from human tumors by surface marker sorting and loading into NOD/SCID [7] or severe combined immune deficiency (SCID) mice [27, 28] have been used to establish tumor models reflecting the tumor features and development in the human body. However, the care of these immunocompromised mice is very dif-ficult and expensive, which prevents the convenient development of models for tumor initiation by CSCs on a large scale. Traditionally, nude mice are used to establish tumor models for cell lines that are used on a large scale for cancer research and pharmaceutical screening. However, cell lines often do not accurately reflect the development and physiological properties of the original human tumors [15]. Therefore, we tried to establish tumor models that do not require the care of SCID and nude mice to generate CSCs. We show here that our protocol is able to obtain sufficient CSCs from human cancers that can give rise to xenografts in both SCID and nude mice that reflect the original human tumors. This procedure can be used to obtain CSCs from original human tumor tissues and patient blood samples at a large scale for cancer research and pharmaceutical screening.

In this study, we demonstrated that tumorigenic stem cells of GACs are included in the rare CD44+- and CD54+-undifferentiated population that exists in tumor tissues and the peripheral blood of GAC patients. Our data indicate that a significant proportion of these cells is tumorigenic, which are in agreement with the CSC hypothesis that primary, invasive, and metastatic tumor mass are generated and maintained by a small subset of tumor cells that are able to self-renew and produce the bulk of cells in a tumor. In gastric cancer, the GCSCs seem to be a target of oncogenic transformation, invasion, and metastasis, similar to other types of cancer, such as leukemia [7], breast, colon, and brain cancer [29]. Therefore, the molecular characterization of tumorigenic GCSCs is crucial in order to develop new therapeutic strategies.

Materials and Methods

Sample collection

Tumor tissues were obtained from patients who underwent gastrectomy for gastric tumors at the Department of Gastrointestinal Surgery, and blood samples were collected from patients who received chemotherapy at the Department of Oncology, West China Hospital, Sichuan University. Informed consent was obtained from all patients who provided samples and the relevant institutional Ethics Committees approved this study.

Cell culture

Tumor samples were subjected to mechanical and enzymatic dissociation. The resulting cancer cells were cultured in a serum-free medium supplemented with 20 ng/ml EGF and 10 ng/ml FGF-2. The tumor cells were subjected to DMEM medium containing 10% FBS for differentiation. To obtain primary tumor cell cultures, the cells were plated onto collagen-coated dishes in DMEM medium containing 10% FBS after enzymatic dissociation. Red blood cells were lysed from whole blood, to deplete the hematopoietic and endothelial cells, and the resulting cells were incubated with

the following magnetic microbeads: CD31, CD45, CD19, CD56, CD64, CD4, CD8, and CD11b. Before flow-cytometry analysis, the PE-cy7-CD45 antibody was added to the samples again to deplete the hematopoietic cells.

Magnetic and cytofluorometric cell separation

For magnetic separation, cells were labeled 1-3 h after enzymatic dissociation with CD44 magnetic microbeads (Miltenyi Biotec) and separated according to the manufacturer's instructions. Alternatively, cells were labeled with CD44/CD54, CD45, CD31, CD19, CD56, CD65, CD4, CD8, CD11b, CD34, or CD117 antibodies (BD Biosciences) and sorted with a FACS Aria (BD Biosciences). After magnetic or cytofluorometric sorting, cell purity was evaluated by flow cytometry using CD44/CD54 antibodies (BD Biosciences).

Transplantation of cancer cells

Various unseparated and purified cell populations were injected subcutaneously into the flanks of SCID mice and nude mice as indicated. After 8-10 weeks, mice were sacrificed by cervical dislocation, tumors were removed, fixed in 10% neutral buffered formalin solution (Sigma), and were paraffin embedded.

Immunostaining

Immunostaining was performed on a formalin-fixed paraffin-embedded tissue, cell blocks, or frozen tissue. For immunohistochemistry, paraffin sections were subjected to antigen retrieval for 30 min at 95 °C and then dewaxed in xylene and rehydrated with distilled water. The slides were subsequently incubated with the following antibodies overnight at 4 °C: CD44, CEA, CA19-9, EMA, and CD54 (BD Transduction Laboratories). The reaction was performed using ABC systems (DakoCytomation) and DAB substrate chromogen (DakoCytomation) followed by hematoxylin counterstaining. For immunofluorescent staining, the cells were incubated with anti-gastrin and anti-mucin antibodies overnight at 4 °C. For sphere immunostaining, glass slides were coated with matrix gel, and then gastric tumor spheres were picked and cultured on the pre-coated glass slides with a serum-free medium containing EGF and FGF. After 1 day, the gastric tumor spheres were adhered to the glass slides and then examined by immunostaining.

Flow-cytometry analysis

Cells from tumor tissues, spheres, or blood samples were incubated on ice for 30 min with the following antibodies: anti-CD44, -EGFR, -CD31, -CD15, -beta-integrin, -CD184, -CD133, -CD29, -CD54, -CD34, -CD10, -CD117, -CD45, -CD19, -CD56, -CD65, -CD4, -CD8, and -CD11b. The cells were then washed with PBS and centrifuged for 5 min at 1 000 rpm. Samples were analyzed on a BD flow cytometer.

Acknowledgments

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)