Int J Clin Exp Pathol 2016;9(10):10128-10138
www.ijcep.com /ISSN:1936-2625/IJCEP0036628

Original Article

Enrichment and characterization of ovarian cancer stem cells and its potential clinical application

Wenxia Wang[1], Zhenbo Zhang[2], Yin Zhao[1], Zeng Yuan[1], Xingsheng Yang[1], Beihua Kong[1], Wenxin Zheng[3,4,5,6]

[1]Department of Obstetrics and Gynecology, Qilu Hospital, Shandong, China; [2]Department of Obstetrics and Gynecology, Shanghai General Hospital, Shanghai, China; [3]Department of Pathology, University of Arizona, Tucson, AZ, USA; Departments of [4]Pathology, [5]Obstetrics and Gynecology, [6]Simmmon Comprehensive Cancer Center, University of Texas Southwestern Medical Center, Dallas, TX, USA

Received June 27, 2016; Accepted August 28, 2016; Epub October 1, 2016; Published October 15, 2016

Abstract: The cancer stem cell (CSC) theory proposes that a minor population in tumor cells with specific features, such as self-renewal and reproducible tumor phenotype could contribute to tumor relapse and chemotherapy resistance. Several studies have convincingly documented the existence of ovarian CSC, but questions related to the biologic behavior and specific biomarkers of ovarian CSC remain to be clarified. In the present study, we firstly established a tumor cell line with capability of regenerating tumors through serial transplantation of ovarian tumor tissue in non-obese/severe combined immunodeficient (SCID) mice. After separation of CD133+ cells with magnetic beads, we compared the phenotype and biologic behavior of CD133+ versus CD133- cells. It was found that the CD133+ cells were much more potent to produce colonies in semi-solid agar culture than CD133- cells. The proportion of the cells in G0/1 cell cycle is much higher in CD133+ cells than in CD133- cells. Furthermore, in vivo experiments demonstrated that the CD133+ cells were capable of repeatedly regenerate tumors in NOD/SCID mice, while the CD133- cells were not. Compared with CD133- cells, the CD133+ cells expressed much higher levels of the stem cell markers Oct4, Sox2, Nanog and Mcl-1. Clinically, among a total of 290 ovarian epithelial cancers, increased level of CD133 expression was positively correlated with a high cancer stage and had a worse 5-year survival rate. Taken together, the results suggest that the CD133+ cells from human ovarian cancer have the characteristics of CSC, which may contribute to ovarian cancer relapse and anti-apoptotic activity. The method of ovarian CSC enrichment we established provides a feasible and practical way of ovarian cancer research in a molecular level. In addition, CD133 may be used as a prognostic marker for ovarian epithelial cancer, which may have a role for future therapeutic effect.

Keywords: Ovarian epithelial cancer, cancer stem cells, CD133

Introduction

In recent years, fast accumulating data demonstrate that there exists a small subset of cells within a tumor, termed cancer stem cells (CSC), responsible for cancer development and recurrence [1]. Characterization of CSC in leukemia and several other malignancies showed that these cells are relatively quiescent and highly express molecules mediating multi-drug resistance [2-4]. That is the main reason that the conventional chemotherapeutic agents are usually effective in reducing the bulk of tumor but unable to clear the stem cells. The residual CSCs after chemotherapy will eventually result in disease recurrence. Thus, the finding CSC opened a new path to explore novel therapies aiming to understand the molecular mechanisms of chemoresistance and cancer recurrence in order to significantly improve clinical management and cure patients with cancers [5, 6].

Ovarian epithelial cancer (OEC) is a common malignancy and a leading cause of death in women, with epithelial ovarian cancer being the most frequent and aggressive subtype. Among the tumors originated from female reproductive system, ovarian epithelial cancer is the third commonest cancer and sits at the first place in terms of cancer mortality rate. Several research groups have convincingly documented the exis-

tence of CSC in ovarian cancers [7-11]. Ovarian CSCs have been defined and isolated from ovarian cancer cell lines or peritoneal fluid from ovarian cancer patients using different markers, including CD44, CD117, Myd88 and ABCG2. Among these biomarkers, the CD44+/CD117+ immunophenotype holds promise as a defining characteristic of ovarian CSCs since these isolated cells exhibit stem cell functionality well. Recently, CD133 has been documented as a superb CSC marker in several non-ovarian cancers including cancers of the colon, the lung, and the brain [7-11]. CD133, a biomarker, is selectively expressed by neural stem cells, hematopoietic stem cells and epithelial progenitor cells. Singh et al. isolated CD133+ cells from brain tumors and compared the capabilities of tumor formation after subcutaneous inoculation of CD133+ or CD133- cells. While inoculation of 105 CD133- cells failed to regenerate tumors in NOD/SCID mice, inoculation of only 100 CD133+ cells generated tumors easily [11]. O'Brien et al. reported that all the colon cancer-initiating cells are CD133+. In contrast, the CD133- cells accounting for the bulk of the tumors were unable regenerate tumor [9]. Therefore, it is reasonable to speculate that the CD133+ cells may play a role in cancer development, recurrence, metastasis, and patient survival. However, it is unknown if ovarian epithelial cancers have CD133+ cells and whether they can be enriched and isolated efficiently for future applications in research as well as in clinical management.

The main goals of this study are to enrich CD133+ cells through serial transplantation of ovarian epithelial cancer tissues into non-obese/severe combined immunodeficient (SCID) mice, to characterize if the CD133+ cells enriched from the ovarian cancer have the biologic function of CSC, and to correlate if CD133+ cancers have a negative impact in clinic.

Materials and methods

Source of human ovarian epithelial cancer stem cells

The study has been approved by Institutional Review Board of Qilu Hospital Ethic Committee in Shandong University, China. All primary human ovarian cancer samples were collected in accordance with the policies of Qilu Hospital of Shandong University. Tumor samples were obtained from the operating room and immediately taken to the laboratory for processing. All ovarian epithelial cancers were pathologically characterized based on WHO classification system. Only the high-grade serous carcinoma samples were used for the CSC enrichment study. The samples were minced with scalpels to 2 mm cubic pieces and implanted into 4- to 6-week-old female NOD/SCID mice (The Jackson Laboratory, Florida, USA). The mice were monitored biweekly for tumor formation. The mice bearing growing tumors were euthanized by CO_2 inhalation. Tumor explants were excised aseptically, processed as above, and implanted again into recipient female NOD/SCID mice. Tumor explant was processed and used as a source of human tumor derived cells in 9 subsequent serial transplantation experiments.

Cell culture and CD133+ cell isolation with magnetic beads

Cell suspensions were prepared from a portion of the tumor samples. Briefly, The specimen was mechanically dissected and filtered, red blood cells were lysed with ACK buffer, and cells were then washed with media containing serum and plated in RPMI 1640 supplemented with 10% heat-inactivated FBS, 20 ng/ml EGF, 1 ng/ml hydrocortisone, 5 μg/ml insulin, 100 μM β-mercaptoethanol, 10 ng/ml β-FGF, 1% penicillin/streptomycin, and 20 μg/ml gentamicin. The cell cones were allowed to grow until they were confluent. Then the clones were trypsinized and plated again. Cells were trypsinized and re-suspended in PBS for FACS analysis. The CD133+ cells were separated using magnetic beads. Briefly, the cells were harvested and single-cell suspension was prepared and cell number counted. The cell suspension was centrifuged at 1000 × g for 10 minutes and the cell pellet was re-suspended in 300 μl of buffer per 10^8 total cells. After adding FcR Blocking Reagent (100 μl per 10^8 total cells), the cells were incubated with CD133 MicroBeads (100 μl of per 10^8 total cells) for 30 minutes in the refrigerator (2-8°C). The cells were washed, centrifuged, re-suspended and applied onto a column. Both the labeled (CD133+ cells) and unlabeled (CD133- cells) were collected for further experiments.

Semi-solid agar culture to examine the clonogenic ability

The enriched CD133+ cells were assayed for their ability to form colonies in semisolid agar and compared with that of CD133- cells. Briefly, colony cultures were established in triplicate by plating 5.0×10^6 cells in per 35 mm cell culture dish in 1.0 ml of RPMI 1640 supplemented with 0.33% agar, 25% FCS and 2 mM L-glutamine. Colony growth was stimulated by the addition of 20 ng/ml EGF. Cultures were incubated in a humidified chamber at 37°C and 5% CO_2 for 2 weeks. Clones were allowed to grow until they were confluent and were then trypsinized, passaged to a 24-well plate, allowed to grow to confluence, and passaged a second time. Only the colonies that successfully passaged twice were deemed true clones. The colonies were counted under a light microscope.

Flow cytometry to analyze expression of CD133 and cell cycle distribution

Cultured cells were collected, washed once with PBS and stained with anti-CD133-PE for 30 minutes of incubation in the dark on ice. For cell cycle analysis, the harvested cells (~10^6 cells) were fixed with ice-cold 70% ethanol, treated with 500 μg/ml RNase A (Sigma), and subsequently stained with 25 μg/ml propidium iodide (Sigma). Then these cells were analyzed by using a flow cytometer FACS Calibur.

Quantitative real-time PCR to examine the expression of Oct-4, Sox2, Nanog and Mcl-1

The mRNA levels were determined by real-time quantitative PCR using the Applied Biosystems Taqman Gene Expression kit. Total RNA from cells was isolated with the RNeasy Mini Kit, accompanied by an on-column DNase digestion. The volume of each reaction was 10 μl per well, which consisted of 5 μl 2 × reaction buffer and 0.05 μl 200 × Euroscript RT (reverse transcriptase) enzyme and RNase inhibitor mix from the one-step RT-qPCR MasterMix Plus, 0.5 μl 20 × Taqman Gene Expression mix together with 2 μl of 50 ng RNA as amplification template. qRT-PCR was carried out on the ABI 7900HT Fast Real-Time PCR system. The reaction mixtures were incubated at 48°C for 30 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Samples were measured in triplicate. GAPDH also was amplified as a loading control for each batch of the test. Cycle threshold (Ct) values were used to determine the relative amounts of the targeted genes and GAPDH mRNA levels in the samples.

In vivo tumor formation experiment

The CD133+ and CD133- cells were collected, counted, washed and re-suspended in PBS. Then the cells were injected subcutaneously into NOD/SCID mice with 10^4-10^6 per mouse in 0.3 ml. The mice were monitored biweekly for tumor formation up to 8 weeks.

Tumor tissue immunohistochemical analyses

A cohort of 290 ovarian epithelial carcinoma patients hospitalized at the University of Arizona Medical Center and Arizona Cancer Center during 2000-2014 was studied after approval of Institutional Review Board (IRB). Tumor core samples (3 mm) were obtained at initial debulking surgery, scored according to the Federation International of Gynecology and Obstetrics (FIGO) guidelines, and were formalin-fixed and paraffin-embedded. Each new paraffin block contained 100 randomly distributed tumor cores, with at least 2 cores per patient, and were sectioned at 5 micron thickness as previously described [12]. Immunohistochemistry was performed on slides with tumor microarray (TMA) sections before being blocked using a solution of 1% bovine serum albumin (BSA) with 2% donkey serum in phosphate buffered saline (PBS) for 1 hour at room temperature, incubated with an antibody to CD133 at 1:200 dilutions overnight in a humidified chamber at 4C, washed with PBS, and incubated using a secondary goat anti-rat horseradish peroxidase (HRP) conjugated antibody (Santa Cruz biotechnology, Dallas TX) at 1:200 for 1.5 hours at room temperature. Slides were rinsed and stained with a diaminobenzidine (DAB) solution, counter stained with Hematoxylin (Dako, Carpinteria CA), dehydrated, and coverslipped before being scanned and digitized with a Scanscope (Leica, Buffalo Grove IL) and analyzed with the Aperio Imagescope software (Leica, Buffalo Grove IL). Positivity and Intensity (PI) scores were calculated as the product of stained area fraction (positivity) and its inten-

Table 1. Passage of ovarian epithelial cancer in NOD/SCID mice

Patient#	1st passage	2nd passage	3rd passage	4th passage	5th passage
1	0/6				
2	1/6	0/6			
3	2/8	0/8			
4	0/8				
5	5/8	6/8	8/8	8/8	8/8
6	3/8	3/8	0/8		
7	1/8	0/8			
8	5/8	2/8	3/8	2/8	
9	0/8				

Note: The fractions indicate the number of regenerated tumors among the total engrafted mice. #indicates tumor cells derived from individual patients.

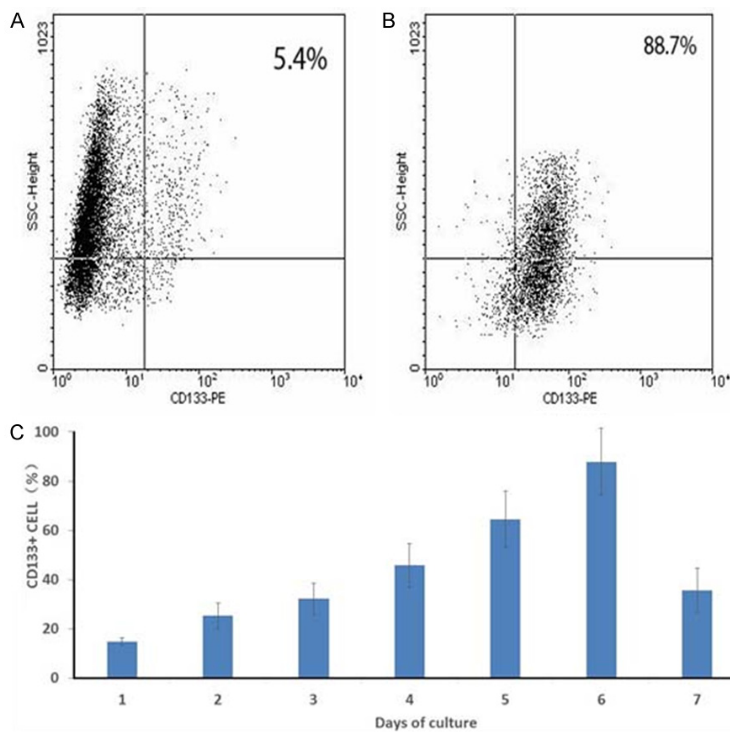

Figure 1. Isolation and enrichment of CD133+ cells from ovarian epithelial cancers. The CD133+ cells were isolated and accumulated with anti-CD133 coated magnetic beads. The proportions of CD133+ cells were examined by FACS. A, B. Represent the proportions before and after isolations, respectively. C. Represents the number of CD133+ cells counted in vitro. The amount of CD133+ cells were significantly increased after the procedure ($P<0.05$).

sity. For the purpose of the staining analysis, we examined only cores in which greater than 50% of the tissue remained intact on the glass slide. Based on this criterion we had 12 patients for whom we calculated the PI score using a single core, and 278 in which we took the average PI score from 2 cores, for a total of 290 patients analyzed.

Statistical analysis

All experiments were performed at least three times and data were exhibited as mean ± SD. Statistical analysis was conducted using the chi-square test and Student's t-test. $P< 0.05$ was considered significant.

Patient survival was calculated from date of diagnosis to date of death, or entrance into hospice care when not available and converted from days to months by dividing the number of days by 30. Overall survival was analyzed by Kaplan-Meier plots and statistical significance inferred by log-rank tests. Correlations to clinical parameters were made using chi-square analysis. Statistical analyses were performed using the Prism 6 software (Graphpad, La Jolla CA).

Results

Passage of ovarian epithelial cancer in NOD/SCID mice

The ovarian cancer tissues were collected from 9 patients with primary high-grade serous carcinomas. As shown in **Table 1**, the tumor tissues from 6 patients (#2, 3, and 5-8) were successfully engrafted in NOD/SCID mice with varying tumor growth or development. Three tumor samples (#1, 4, and 9) did not grow. Samples 2, 3 and 7 grew only 2 passages, while sample #6 had 3 passages. There were 2 tumor samples, which were successfully passaged 4 (#8) and 5 (#5) generations, respectively. The tumor cells from these 2 "long lived" samples were collected, cultured, and sub-cultured. In particular, the sample #5 was selected because of its fast in ex vivo tumor formation and better growth in cultures. The successful serial transplantation of the ovarian tumors in NOD/SCID mice sug-

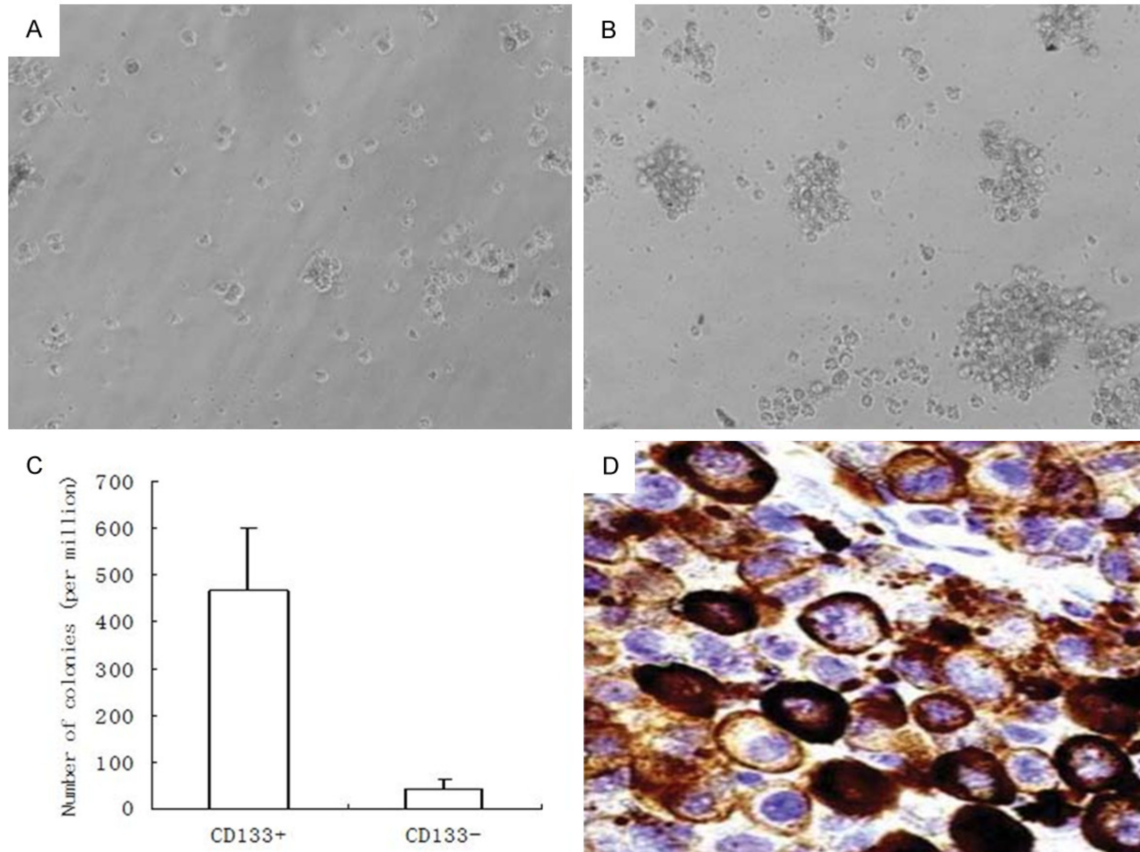

Figure 2. Clonogenic ability assayed with semi-solid agar culture. The CD133+ and CD133- cells were cultured in semi-solid agar. The images were taken and the colonies were counted under a light microscope at day 21. CD133- cells show a sparse and small colony formation (A), while CD133+ cells form lot more large colonies in an identical condition (B). (C) Represents the results the number of colonies from 3 experiments expressed by mean numbers ± SD. (D) Shows cells with positive expression of CD133 in cytoplasm and cell membrane, but not in nuclei.

gests the presence of a self-renewing cell population in the implanted tumor tissue.

Growing ovarian cancer cells in vitro

A total of 16 regenerated tumor tissue samples collected from the 4th and 5th passages of the tumor samples was processed and cultured. Fifteen (94%) of the samples were successfully passaged and propagated with a similar cell proliferation index. The majority of the cancer cells displayed irregular shape adhering to the flask surface. The number of cultured tumor cells increased exponentially with a doubling time of 18 hours (data not shown).

Expression of CD133 and isolation of the CD133+ cells

Flow cytometry was used to separate CD133+ from negative cells and the CD133+ cells were significantly enriched (**Figure 1**). After separation with flow cytometry, the ratio of CD133+ to CD133- cells for each batch of the cell culture and sub-culture was analyzed. The percentage of CD133+ cells increased from 15% (5.4±0.43 cells per high power field) in average at the time of initial seeding to 88% (88.7±13.3 cells per high power field) on day 6. It then dropped to 36% (12.4±5.7 cells per high power field) on day 7 right before subculture. The detailed data is summarized in **Figure 1C**.

The clonogenic ability of the CD133+ cells

To test the clonogenic ability, the CD133+ or CD133- cells were plated on semi-solid agar and cultured up to 3 weeks. As expected, 105 CD133+ cells generated 468±132 colonies, with the colony-forming efficiency of 0.47%. In contrast, CD133- cells with 105 density gener-

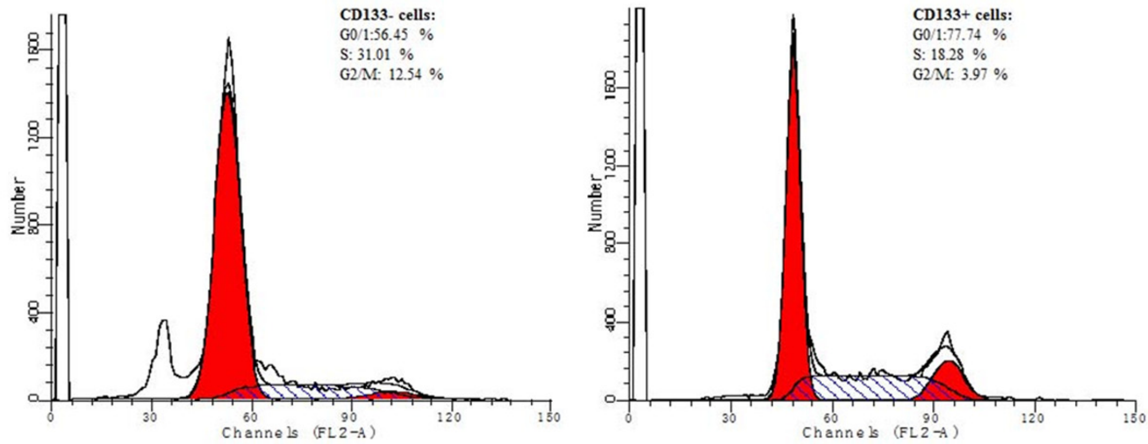

Figure 3. Cell cycle distributions analysis of CD133 cells with flow cytometry assay. The cell cycle stage distribution of the enriched CD133+ were analyzed with flow cytometry and compared with that of the CD133- cells. The proportion of cells in G1/G0 stage cells with CD133+ cells was 77.74%, which was significantly higher than those CD133- cells (56.45%) (P<0.05).

Table 2. Comparison of gene expressions assayed with qRT-PCR

	Oct4/GAPDH	Sox2/GAPDH	Nanog/GAPDH	Mcl-1/GAPDH
CD133+ cells	4.46E-02	2.03E-03	2.94E-02	8.25E-02
CD133- cells	6.37E-04	0.56E-03	1.55E-03	1.38E-04
Fold of increase	170.01	3.63	68.97	597.83
P values	0.017	0.696	0.043	<0.0001

Note: all CSC markers, after normalization with GAPDH, were significantly more expressed in CD133+ cells compared to those CD133- cells except Sox2.

ated only 43±19 colonies with the colony-forming efficiency of 0.01%. Compared to the CD133- cells (**Figure 2A**), the CD133+ cells had at least 10-fold higher clonogenic capacity (**Figure 2B**). That meant the CD133+ cells had a significantly more potent clonogenic ability than that of CD133- cells (**Figure 2C**, P<0.001). When the colonies were harvested and plated on semi-solid agar, the CD133+ cells were able to repeatedly generate colonies, whereas the CD133- cells did not. A representative immunocytochemistry picture of positive CD133 cells is presented in **Figure 2D**. These results demonstrated that CD133+ cells possess self-renewal and growth potential.

Cell cycle analysis of the CD133 labeled cells

After the cells with positive or negative CD133 marker expression were largely separated by flow cytometry, we further tested the cell cycle distribution of these two groups of the cells to see if they were different in cell growth status. Compared with that of the CD133- cells, the ratio of G1/G0 phase of those cells with CD133+ immune-phenotype was 77.74%, which was much higher than that of CD133- cells (56.45%) (**Figure 3**).

The expression of Oct-4, Sox2, Nanog, and Mcl-1 in the CD133+ cells and CD133- cells

It is commonly believed that the cells possessing stem cell properties express specific CSC biomarkers, such as OCT4, Sox2, Nanog and Mcl-1. To further confirm that CD133+ cells we obtained have the properties of CSC, the above biomarkers were investigated in those samples derived from the ovarian epithelial cancers by using qRT-PCR analysis. Compared to the cells without CD133 expression, the cells with positive CD133 expression showed a significantly increased copy number of these CSC related genes except Sox2. The increment ranged from 3.6 to 597.8 folds after GAPDH normalization. All the increment of the gene expression was statistically significant except Sox2. One particular gene Mcl showed about 600-fold increase of expression in the CD133+ cells (P<0.0001). The detailed data is summarized in **Table 2**.

Ability of in vivo tumor formation

To regenerate a tumor is a hallmark of tumor stem cell. To further determine the role of CD133 in tumor formation, the CD133+ and

Table 3. Patient demographic in a ovarian epithelial cancer tissue microarray of 290 patients

Characteristics	Total number of cases (N = 290)
Age of diagnosis	
Mean	63
Medium	62.2
Range	31.5 to 92.3
FIGO Stage, n (%)	
I	6 (2.0)
II	10 (3.4)
III	249 (85.9)
IV	25 (8.6)
FIGO grade, n (%)	
1	10 (3.4)
2	35 (12)
3	245 (84.5)
Debulking status, n (%)	
No residual	115 (39.7)
Regional recurrence	78 (26.9)
Never disease free	65 (22.4)
Unknown	32 (11.0)

CD133- cells were injected into SCID mice, respectively. The results showed that inoculation of 106 CD133+ cells regenerated a tumor within 6 weeks with 100% efficiency (8/8). The efficiency rate was reduced to 25% (2/8) when 104 cells were inoculated. The tumors regenerated from CD133+ cells were able to be re-implanted for the next generations with a noticeable faster tumor growth. In contrast, injection of 104 CD133- cells failed to regenerate a tumor during a 6-week observation, and only one out of 8 inoculations was successful following an injection of 106 CD133- cells, which was significantly less efficiency of tumor generation compared with those CD133+ cells (P<0.0001).

CD133 staining in primary ovarian epithelial cancers associated with shorter survival analyzed by tumor microarray immunohistochemistry

To confirm the trends observed in the in vitro experimental analysis suggesting an aggressive tumor growth was associated with CD133 expression, we decided to examine the protein levels of CD133 expression in human ovarian cancer samples. Cytoplasmic expression of CD133 protein was analyzed in a cohort of 290 ovarian epithelial cancers by immunohistochemistry using a specific CD133 antibody in an ovarian cancer tissue microarray. All cancer samples were of epithelial origin with clinical pathologic characteristics summarized in **Table 3**. All primary tumor microarray samples examined were taken at the time of initial debulking surgery. Diffuse and focal staining was observed in the cytoplasm of glandular or columnar epithelial cells, but not in the tumor stroma in any of the cores. There is no nuclear staining. Representative pictures of positive and negative immunohistochemical staining of CD133 in ovarian epithelial cancers are presented in **Figure 4**. Total intensity of positive pixels and total number of positive pixels, as determined by Imagescope software, were combined into a positive intensity score (PI), which was averaged across the two representative cores per patient, and used for patient stratification. We chose the top and bottom 10 and 20 percent of patients to compare survival differences between low and high expressers of CD133 protein, and found a significant decrease in survival for both top 10% and 20% of patients expressing high levels of CD133, with a median survival of 28 and 27 months when compared to patients expressing the lowest 10% (P = 0.026) and 20% (P = 0.035), with a median survival of 47 and 50 months respectively. The composition of the top and bottom 10% and 20% of patients was similar when examining clinical parameters of FIGO grade, stage, and age. Part of the data is presented in **Table 4**.

Discussions

Previous studies have stated that CSC influences the progress of OECs. Residual cancer cells, especially the CSC, have been hypothesized to contribute to the relapse of OEC. Indeed, there are considerable lines of evidence showing that CSC promotes ovarian cancer cell proliferation. However, identifying ovarian CSC with a specific biomarker is the main obstacle to isolate CSC. So far, some stem cell associated biomarkers have been used to isolate CSC, for example, CD34+ CD38- Thy- phenotype for acute myelogenous leukemia stem cells [4] and CD44+ CD24- and ESA+ for breast cancer stem cells [13]. Although the biomarkers for ovarian cancer stem cells remain elusive, it is believed that ovarian epithelial can-

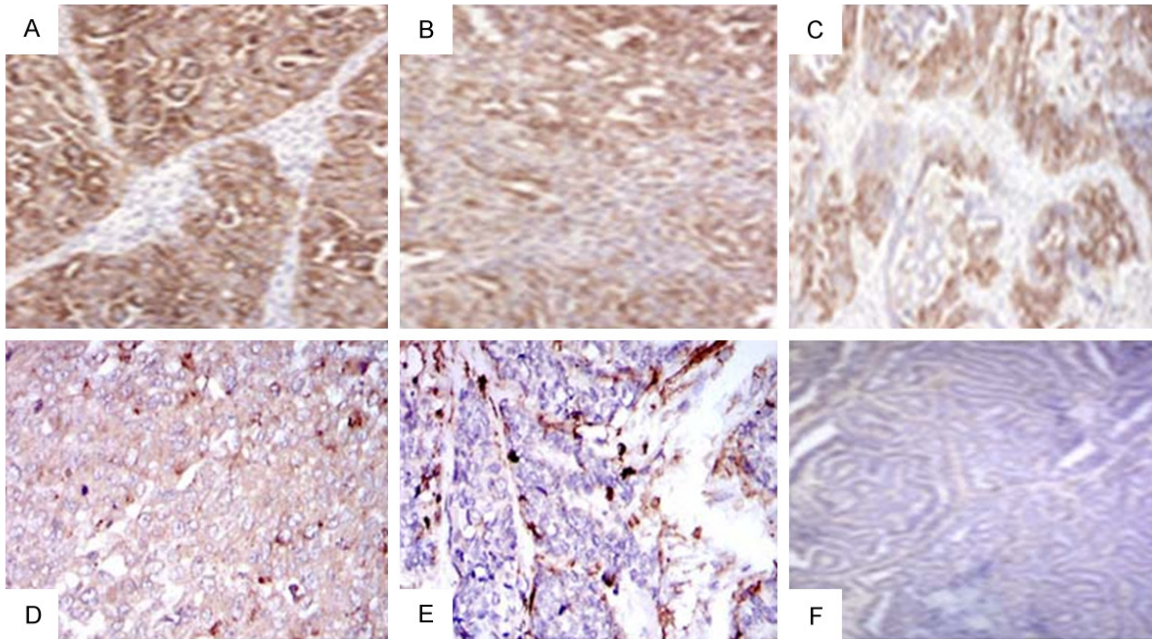

Figure 4. CD133 immunohistochemical staining of ovarian epithelial cancer. Based on the a positive intensity score (PI) of the cancer cells, (A) (poorly differentiated serous carcinoma) and (B) (poorly differentiated endometrioid carcinoma) represent the high PI score, (C) (moderately differentiated endometrioid carcinoma) as an intermediate PI score, while (D) (poorly differentiated clear cell carcinoma) and (E) (undifferentiated carcinoma) represent low PI score. (F) (well differentiated endometrioid carcinoma) is negative. Original magnifications 100 ×.

Table 4. Multivariate analysis using Cox proportional hazard ratio of CD133 expression in 290 patients with ovarian epithelial cancer

Age	Hazard Ratio	Lower 95% CI	Upper 95% CI	*P* value	Significance
CD133	1.0382	0.7113	1.0534	0.0032	**
Age	1.0382	1.0143	1.0485	0.0187	*
FIGO Stage 3	0.9356	0.7321	1.1033	0.4576	
FIGO Grade 3	1.2631	0.7837	2.5378	0.3521	

Note: CD133 = standard deviations from the mean of log2 transformed expression level of CD133 by immunohistochemistry; CI = confidence interval; Significance: *<0.05; **<0.01. The number of cases for stage I, II, and IV were too low to be calculated for the analysis. The same situation applies to the FIGO grade 1 and 2 tumors.

cers should have a similar CSC like properties of other epithelial cancers in the process of cancer development and metastasis [14-17]. To find a biomarker to separate the ovarian CSC cells, we went through all references in this field and scanned most of the candidate markers, and found that CD133 could be a promising candidate marker to identify CSCs within the ovarian epithelial cancer samples. CD133 has been reported to be associated with CSCs in brain, liver, and colon cancers. There are preliminary data suggest that CD133 could also be used to identify ovarian cancer stem cells [18]. However, there is no method to enrich those CD133+ cells from the ovarian epithelial cancers. In this study, we have identified a small subpopulation of CD133+ cells from high-grade ovarian serous cancer samples through flow cytometry analysis and successfully enriched these cells by series tumor cell implantation into SCID mice. The isolated CD133+ cells showed much more potent in vitro clonogenic ability and in vivo tumor formation ability than that of CD133- cells. In addition, the CD133+ cancer cells enriched from the clinical cancer samples can be passaged for several generations in semi-solid agar culture and can engraft tumors repeatedly in SCID mice. These features strongly suggest that the CD133+ cells enriched from ovarian cancers have all the biologic features of the cancer stem cells.

It is commonly believed that CSCs were not actively proliferating cells, usually with overexpression of multidrug resistance molecules and anti-apoptotic mediators. Consistent with this result, our study showed that CD133+ cells

contained a higher proportion of non-cycling cells than CD133- cells. On the other hand, the cells possessing stem cell properties also express classical CSC markers, including Oct4, Sox2, Nanog and Mcl-1. Among the markers, Mcl-1, Oct4 and Nanog significantly overexpressed in CD133+ cells compared with CD133- cells, especially the Mcl-1 showed almost a 600-fold of increment in CD133+ cells. Mcl-1 is an anti-apoptotic member of the Bcl-2 family. Similar to Bcl-2, Mcl-1 can interact with BAX and/or BAK1 to inhibit mitochondria-mediated apoptosis. Recent studies demonstrate that Mcl-1 is essential to the survival of hematopoietic stem cells. The human hematopoietic stem cells seems to be dependent on Mcl-1 to maintain the self-renewal ability, and it is found that Mcl-1 seems to be the only member selectively overexpressed in human hematopoietic stem cells among the Bcl-2 family [19]. In the present study, Mcl-1 was found to be overexpressed in CD133+ cells compared with CD133- cells. The significance of Mcl-1 overexpression warrants further study and could be explored for therapeutic targeting of ovarian CSC.

Although the relationship between positive expression of CD133 and prognosis was controversial in the past, in this study, we have shown that patients with ovarian epithelial cancer showing positive CD133 expression have a significantly worse 5-year survival than those cancers with negative or lower CD133 expression. Ferrandina et al. were the first to identify CD133 expression in human ovarian cancer tissue and they found that CD133 expression did not provide additional prognostic information for ovarian cancer patients [20]. However, more recent studies [21] including one study from MD Anderson found an association between CD133 status and prognosis, which used a 400-patient cohort with a good long-term patient follow-up data set [22]. That study showed that positive CD133 expression is associated with various clinicopathologic characteristics of primary ovarian cancer patients, with shorter disease-free survival time, and with lack of response to chemotherapy [22]. In this study, we investigated that expression of CD133 in an independent cohort of 290 patients with ovarian epithelial cancers in a tumor tissue microarray. When patients were stratified based on expression levels to compare the top 10% and 20% to the bottom 10% and 20% respectively, we found a statistically significant reduced survival in the highest expressing patients compared to the lowest expressing patients, with a 65% and 78% reduction in median survival time respectively, which correspond to a difference of over 24 months. The predictive power of CD133 staining in cancer tissue is stronger in older patients, suggesting it is best used for prognosis in patients above the age of 60. This is also in agreement with findings that CD133 expression has prognostic value in hepatocellular carcinoma [23] colon and rectal adenocarcinoma [24], invasive ductal breast carcinoma [25], and non-small cell lung carcinoma [26].

In conclusion, CD133 is a useful and efficient biomarker to identify ovarian CSCs. Enrichment of CD133+ CSCs can be effectively generated through repeated engrafts of cancer tissue in SCID mice. The method of generating ovarian CSCs provides a feasible and practical way for future ovarian cancer research, particularly in the area of mechanism studies for chemoresistance and cancer recurrence. Together with CD133, a combination with other stem cell markers such as Mcl-1 needs to be further explored for potential clinical usage. A targeted therapy by eliminating CD133+ cells may have a clinical value for ovarian cancer treatment. The immunohistochemical assessment of CD133 expression, which serves as an independent poor survival marker, may have a potential clinical value in predicting disease progression and prognosis in ovarian cancer.

Acknowledgements

This work was partially supported by the National High Technology Research and Development Program ("863" Program) of China (2014AA020605, 2012AA02A507), National Natural Science Foundation of China (81272857, 81370074), National Science and Technology Project (2015BAI13B05), and the Shanghai Municipal Public Health Bureau (grant number XYQ2013119), and the "Chenxing Project" from Shanghai Jiao Tong University to Z.Z. The work was also partially supported by Mark and Jane Gibson endowment fund and UTSW Medical Center start up fund to WZ.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Wenxin Zheng, Obstetric and Gynecology, UT Southwestern Medical Center, 6000 Harry Hines Blvd, NB6.408, USA. Tel: 214-648-1190; E-mail: wenxin.zheng@utsouthwestern.edu

References

[1] Werbowetski-Ogilvie TE and Bhatia M. Pluripotent human stem cell lines: what we can learn about cancer initiation. Trends Mol Med 2008; 14: 323-332.

[2] de Figueiredo-Pontes LL, Pintao MC, Oliveira LC, Dalmazzo LF, Jacomo RH, Garcia AB, Falcao RP and Rego EM. Determination of P-glycoprotein, MDR-related protein 1, breast cancer resistance protein, and lung-resistance protein expression in leukemic stem cells of acute myeloid leukemia. Cytometry B Clin Cytom 2008; 74: 163-168.

[3] Ho MM, Hogge DE and Ling V. MDR1 and BCRP1 expression in leukemic progenitors correlates with chemotherapy response in acute myeloid leukemia. Exp Hematol 2008; 36: 433-442.

[4] Jamieson CH. Chronic myeloid leukemia stem cells. Hematology Am Soc Hematol Educ Program 2008; 436-442.

[5] Blank U, Karlsson G and Karlsson S. Signaling pathways governing stem-cell fate. Blood 2008; 111: 492-503.

[6] Morrison R, Schleicher SM, Sun Y, Niermann KJ, Kim S, Spratt DE, Chung CH and Lu B. Targeting the mechanisms of resistance to chemotherapy and radiotherapy with the cancer stem cell hypothesis. J Oncol 2011; 2011: 941876.

[7] Collins AT, Berry PA, Hyde C, Stower MJ and Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 2005; 65: 10946-10951.

[8] Dalerba P, Dylla SJ, Park IK, Liu R, Wang X, Cho RW, Hoey T, Gurney A, Huang EH, Simeone DM, Shelton AA, Parmiani G, Castelli C and Clarke MF. Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci U S A 2007; 104: 10158-10163.

[9] O'Brien CA, Pollett A, Gallinger S and Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 2007; 445: 106-110.

[10] Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C and De Maria R. Identification and expansion of human colon-cancer-initiating cells. Nature 2007; 445: 111-115.

[11] Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD and Dirks PB. Identification of human brain tumour initiating cells. Nature 2004; 432: 396-401.

[12] Wei W, Mok SC, Oliva E, Kim SH, Mohapatra G and Birrer MJ. FGF18 as a prognostic and therapeutic biomarker in ovarian cancer. J Clin Invest 2013; 123: 4435-4448.

[13] Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D, Pilotti S, Pierotti MA and Daidone MG. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. Cancer Res 2005; 65: 5506-5511.

[14] Alvero AB, Chen R, Fu HH, Montagna M, Schwartz PE, Rutherford T, Silasi DA, Steffensen KD, Waldstrom M, Visintin I and Mor G. Molecular phenotyping of human ovarian cancer stem cells unravels the mechanisms for repair and chemoresistance. Cell Cycle 2009; 8: 158-166.

[15] Hosonuma S, Kobayashi Y, Kojo S, Wada H, Seino K, Kiguchi K and Ishizuka B. Clinical significance of side population in ovarian cancer cells. Hum Cell 2011; 24: 9-12.

[16] Steffensen KD, Alvero AB, Yang Y, Waldstrom M, Hui P, Holmberg JC, Silasi DA, Jakobsen A, Rutherford T and Mor G. Prevalence of epithelial ovarian cancer stem cells correlates with recurrence in early-stage ovarian cancer. J Oncol 2011; 2011: 620523.

[17] Zhang S, Balch C, Chan MW, Lai HC, Matei D, Schilder JM, Yan PS, Huang TH and Nephew KP. Identification and characterization of ovarian cancer-initiating cells from primary human tumors. Cancer Res 2008; 68: 4311-4320.

[18] Curley MD, Therrien VA, Cummings CL, Sergent PA, Koulouris CR, Friel AM, Roberts DJ, Seiden MV, Scadden DT, Rueda BR and Foster R. CD133 expression defines a tumor initiating cell population in primary human ovarian cancer. Stem Cells 2009; 27: 2875-2883.

[19] Campbell CJ, Lee JB, Levadoux-Martin M, Wynder T, Xenocostas A, Leber B and Bhatia M. The human stem cell hierarchy is defined by a functional dependence on Mcl-1 for self-renewal capacity. Blood 2010; 116: 1433-1442.

[20] Ferrandina G, Martinelli E, Petrillo M, Prisco MG, Zannoni G, Sioletic S and Scambia G. CD133 antigen expression in ovarian cancer. BMC Cancer 2009; 9: 221.

[21] Liang J, Yang B, Cao Q and Wu X. Association of Vasculogenic Mimicry Formation and CD133 Expression with Poor Prognosis in Ovarian Cancer. Gynecol Obstet Invest 2016; [Epub ahead of print].

[22] Zhang J, Guo X, Chang DY, Rosen DG, Mercado-Uribe I and Liu J. CD133 expression associated with poor prognosis in ovarian cancer. Mod Pathol 2012; 25: 456-464.

[23] Sasaki A, Kamiyama T, Yokoo H, Nakanishi K, Kubota K, Haga H, Matsushita M, Ozaki M, Matsuno Y and Todo S. Cytoplasmic expression of CD133 is an important risk factor for overall survival in hepatocellular carcinoma. Oncol Rep 2010; 24: 537-546.
[24] Li CY, Li BX, Liang Y, Peng RQ, Ding Y, Xu DZ, Zhang X, Pan ZZ, Wan DS, Zeng YX, Zhu XF and Zhang XS. Higher percentage of CD133+ cells is associated with poor prognosis in colon carcinoma patients with stage IIIB. J Transl Med 2009; 7: 56.
[25] Zhao P, Lu Y, Jiang X and Li X. Clinicopathological significance and prognostic value of CD133 expression in triple-negative breast carcinoma. Cancer Sci 2011; 102: 1107-1111.
[26] Xu YH, Zhang GB, Wang JM and Hu HC. B7-H3 and CD133 expression in non-small cell lung cancer and correlation with clinicopathologic factors and prognosis. Saudi Med J 2010; 31: 980-986.