Self-Renewal Assessment and Isolation of CD133 Positive Cancer Stem Cells from the Ovarian A2780 Cell Line

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Research

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Abstract

**Background:** Ovarian cancer (OC) is the 7th most common type of cancer and the 5th cause of cancer-related death among women worldwide. It is a heterogeneous disease which is quite variable from the genomic and histopathological aspect. In addition to the usual treatments for ovarian cancer, its recurrence is quite common, mainly due to lack of complete eradication of cancer stem cells. These cells have different properties such as self-renewal ability and stemness property, including proliferation.

**Method:** In the present study, we isolated cancer stem cells with the CD133 surface marker from the ovarian A2780 cell line and examined the stemness property and self-renewal ability of these cells. Initially, CD133 surface marker expression in this cell line was assessed by the flow cytometry technique. Then, the isolation of these cells was performed by the Magnetic-activated cell sorting (MACS) method. Flow cytometry (FCM) was also used to confirm the isolation efficiency. The levels of mRNA expression were evaluated in several stem cell markers in CD133+ cells compared with CD133- cells. Moreover, the self-renewal ability of the isolated cells was investigated in serum-free culture medium.

**Results:** Ovarian cancer stem cells (OCSCs) with CD133 surface marker showed high expression of some stemness markers such as Sox2, Nanog, Oct4, ABCG2, ALDH1, LGR5 and Msi. These cells also had the ability to regenerate themselves in a serum-free environment.

**Conclusion:** Cancer stem cells can be isolated through surface markers by the MACS technique; they have stemness and self-renewal properties. So, the CD133 surface marker can be introduced as a key CSC marker in the isolation, characterization and targeted therapy of ovarian cancer patients.

Introduction

Ovarian cancer is one of the most common heterogeneous cancers with very diverse genomic and histopathological properties (1). One of the most common types of ovarian cancer is epithelial ovarian cancer which accounts for about 90% of all ovarian cancers and consists of different subtypes including serous, endometroid, and mucinous (2). Ovarian cancer has about 239,000 new cases and 152,000 annual deaths worldwide. Worldwide there are nearly 600,000 women living within five years of ovarian cancer identification (5-year incidence). The highest incidence of this disease has been observed in Central and Eastern Europe (3). This disease is usually diagnosed in the late stages which at this time its 5-year survival rate is only 29%, whereas in patients diagnosed in the early stages the 5-year survival rate is about 92% (4). In addition to the conventional platinum-based chemotherapy for ovarian cancer, a number of new therapeutic modalities are being used (5–8). Conventional and traditional treatments for ovarian cancer have always been associated with tumor recurrence in patients due to the inability to completely eradicate a certain type of cancer cells. These cells, known as cancer stem cells (CSCs), have fundamental properties and are able to regenerate cancerous masses. These cells also have abilities such as regeneration, tumorigenesis and chemo-resistance (9). Most of these features are due to mutations that have allowed cells to obtain cancerous stem-like properties. CSCs and natural stem cells
(NSCs) have a number of common features such as increased detoxification ability (10) by altering drug transporters (11) and the DNA repair ability (12); both cells have common signaling pathways including Wnt, Notch, and Hedgehog (13–15). However, they differ in some other features; NSCs, for example, have a highly regulated hemostatic balance, ability to differentiate, and regenerate, and have a normal karyotype, while CSCs have lost these characteristics (16). Specific cell surface markers are used to identify and isolate CSCs, including CD133, CD44, and CD54 (17). CD133 (prominin-1) is an indicator marker for hematopoietic stem and progenitor cells (18) and is one of the most common cell surface markers used to isolate CSCs from a variety of cancer cells including breast cancer, glioblastoma and prostate, colon and ovarian cancers (19). Recent studies have shown that CD133+ cells have a higher cloning and proliferation potential in NOD/SCID mice compared to CD133- cells (20, 21). This marker is referred to as CSC biomarker and its high expression is regarded as a diagnostic indicator for disease progression (22). The increased expression of this marker in ovarian cancer was first performed by Fradina et al. (23). This marker regulates a number of intracellular and extracellular factors, including epigenetic factors, signaling pathways, and miRNAs (24). It is used alone or in combination with other markers to isolate CSCs. This isolation is done by using the MACS or Fluorescence-activated cell storing method (FACS) techniques. In this study, we hypothesized that CSCs could be a major cause of recurrence, metastasis, and malignancy in ovarian cancer. Therefore, in order to target these cells directly in subsequent studies, we isolated these cells from the A2780 ovarian cell line using the CD133 surface marker and examined their fundamental and self-regeneration properties.

**Materials And Methods**

**Cell culture**

The A2780 Ovarian cell line was purchased from the Biotechnology Research Center of Bouali Research Institute of Mashhad (Mashhad, Iran). STR Profiling analysis was performed to confirm the validity of this cell line. The A2780 cell line was cultured in RPMI-1640 culture medium containing 10% fetal bovine serum (FBS) (Gibco, UK), 100 units/ml penicillin and streptomycin (Gibco, UK) at 37°C, 95% humidity and 5% CO2.

**CD133 expression analysis by FCM**

Flow cytometry was performed to evaluate the expression level of CD133 surface marker in the A2780 cell line. For this purpose, after the cells reached 80% confluency, they were washed with PBS buffer and trypsinized to make single-cells. Single cells with a number of at least $10^5/100\mu l$ were dissolved in PBS buffer containing 5% serum mixed with 10ml of FITC dye (Miltenyi Biotec, Germany)-conjugated CD133 antibody according to the instructions and was incubated for 10-15 minutes at 4°C in the dark. The labeled cells were analyzed by FACS Calibur flow cytometer and the results were studied by the FlowJo 7.6.2 software (Tree Star, Ashland, OR).
Isolation of CD133+ cells by the MACS technique

After accurately calculating the expression level of CD133 marker in this cell line, cell culture was performed in high volume and isolation was done by the MACS technique using specific CD133 antibody-conjugated microbeads. For this purpose, $10^8$ A2780 cells were cultured in several T75 flasks at 37°C and after reaching a suitable confluency, they were trypsinized and single-celled. The single cells were passed through a 30 µm filter to remove clumps and multicellular masses. The cells were then counted accurately by trypan blue staining. The cell pellet was dissolved in 600µl of a combined buffer kit (MACS BSA Stock Solution 1:20 autoMACS Rinsing Solution). 200µl of the FcR Blocking Reagent was added to the mixture and finally 200µl of CD133 microbeads (Miltenyi Biotec, Germany) was added and mixed well. The mixture was placed at 4°C for 15 minutes and shaken gently every 2 minutes for better bonding of the microbeads. The cells were then washed with 10-20 ml of buffer and after centrifugation at 300xg for 10 minutes, were dissolved in 5 ml of buffer and then the isolation process was performed by magnet and column according to the kit instructions. Then, in order to confirm the isolation accuracy of CD133+ cells, $10^5$ of these cells were counted and dissolved in 100 µl of buffer. CD133 antibody (10 µl) was added to the buffer and placed at 4°C for 10 minutes. The cells were then washed with 1-2 ml of buffer, centrifuged at 300xg for 10 minutes, and the cell pellet was dissolved in 400 µl of buffer for flow cytometry analysis.

Evaluation test for sphere formation of CD133 positive cells

To evaluate the self-regenerative nature of CD133+ cells isolated from the A2780 ovarian cell line, these cells were cultured as $2\times10^5$ in number in Ultra low attachment 6-cell plates (Sigma-Aldrich, Corning) containing 2 ml of serum-free DMEM/F12 medium including 20 ng/mL h-EGF, 20 ng/mL h-bFGF (Sigma-Aldrich), 2% B27 supplement, 5 µg/ml Insulin and 1% penicillin/streptomycin. Cell growth was examined daily with an inverted microscope (OPTIKA, Italy) and the size and number of spheres formed in CD133+ and CD133- cells were compared and analyzed.

RNA extraction and cDNA synthesis from CD133+ and CD133- cells

RNA was obtained from $8\times10^5$ CD133+ and CD133- cells with the Pars Toos extraction kit according to the company’s instructions. The amount of light absorption at 260, 280, 230 nm wavelengths and A260/A280, A260/A230 ratios were measured with the nanodrop device. RNA samples were treated with DNasel (Thermo Fisher Scientific, US) and cDNA synthesis was performed with the Pars Toos kit.

Fundamental markers expression analysis in CD133 + and CD133- cells
Comparative real-time PCR analysis was performed in duplicate using Master Mix Cybergreen (AMPLICON, Denmark) and real-time thermocycler (Light Cycler 96, Roche, Germany). The GAPDH gene was used as an interior control. Fold changes greater than 1 were considered as increased expression, less than -1 as decreased expression, and between 1 and -1 were considered as no change in expression. Nucleotide primers were designed for real-time PCR reaction for the internal control gene and stemness markers were designed by the Oligo 7 software and by the Primer Blast software, the specificity of the primers was confirmed (Table 1). Real-time PCR was performed by using 5μl of SYBR Green PCR Master Mix, 10 pM of Forward and Reverse primers of each gene and 100 ng/μl of the cDNA synthesized in the previous step for each reaction in the final volume of 10 μl under the conditions provided by the manufacturer.

Table 1. Specifications of the primers used in real-time PCR

| Sequence | Length (base pairs) | Melting point (°C) | GC%  | Product length (base pairs) | Primer  |
|----------|---------------------|-------------------|------|-----------------------------|---------|
| F: GGAAGGTGAAGTCCGGAGTCGA R: GTCATTGATGGCAACAATATCCACT | 21 | 60 | 57.14 | 101 | GAPDH |
| F: AACAGCCCGGACCACGTCGA R: TCGCAGCCGCTTAGCCTCGT | 20 | 65 | 65 | 189 | SOX2 |
| F: GCAATGGGTGTGACGCAGAAGGC R: GCTCCAGGTTGAATTTGCCAGGTC | 22 | 65 | 59.09 | 137 | NANO2 |
| F: CCTGAAGCAGAAAGACGATCA R: CCGCAGCTACACATGTTCCT | 20 | 63 | 50 | 148 | OCT4 |
| F: TGAGGTTTGGAACTGTGG R: GATTCTGACGCACACCTGG | 19 | 65 | 52.63 | 155 | ABCG2 |
| F: CCTGACAGCTACACATGTTCCT | 20 | 63 | 50 | 50 | OCT4 |
| F: GATCCCGTGGCTACTATG R: TGGATCTTGTCAGGCACAACC | 20 | 62 | 55 | 202 | ALDH1 |
| F: CTTCCACCTCAGCGTCTT R: AGGGATTGAAGGCTTCGCAA | 20 | 60 | 55 | 248 | LGR5 |
| F: GAGACCTGACGCGCCAGGCC R: CGCCTGGTCCATGAAAGTGACG | 20 | 60 | 75 | 213 | Msi1 |
Real-time PCR data analysis was performed by the ΔΔCT method. Statistical differences were calculated using the GraphPad Prism 8.0 software and One-way ANOVA (parametric) test; a P <0.05 was considered as statistically significant.

Results

Expression of CD133 surface marker in A2780 cell line

Flow cytometry was used to assess the expression level of CD133 surface marker in the A2780 ovarian cell line. The expression percentage of CD133+ cells in this cell line was 0.1% (Fig. 1). Regarding this level of expression, this cell line was used to isolate CSCs by the CD133 surface marker via applying the MACS technique.

Isolation Of Cd133+ cells From The A270 Cell Line

The MACS technique was used to isolate CD133+ cells from the A2780 ovarian cell line and flow cytometry was used to confirm the isolation accuracy. The percentage of CD133+ cells among the cells isolated by the MACS technique was 90.5% (Fig. 2).

Evaluation Of Cd133+ cells Sphere Formation

To evaluate the self-regenerating properties of CD133+ cells isolated from the A2780 cell line, the spherogenesis test was performed. For this purpose, the isolated CD133+ and CD133- cells were cultured in serum-free medium containing specific growth factors. Five days after the initial culture, the CD133+ cells formed spheroidal colonies of different sizes and asymmetric shapes, while CD133- cells did not form such colonies (Fig. 3). This could indicate the self-regenerating nature of CD133+ cells.

Stemness Markers Expression Analysis In Cd133+ and Cd133- Cells

To ensure that CD133+ cells have stemness properties in comparison to CD133- cells, the expression analysis of several important stemness markers was performed by the real-time PCR technique. In this analysis, the expression level of Sox2, LGR5, ALDH1, ABCG2, Oct4, Nanog and Msil markers increased in CD133+ cells (Fig. 4); the data analysis is presented in Table 2.
Table 2
Analysis of stemness markers data in CD133+ cells compared to CD133- cells

| Stemness markers | ΔΔCt  | -ΔΔCt | 2^-ΔΔCt | Change of expression |
|------------------|-------|-------|---------|---------------------|
| SOX2             | -1.66 | 1.66  | 3.16    | Increase            |
| NANOG            | -1.25 | 1.25  | 2.37    | Increase            |
| OCT4             | -2.81 | 2.81  | 7.01    | Increase            |
| ABCG2            | -1.25 | 1.25  | 2.37    | Increase            |
| ALDH1            | -4.6  | 4.6   | 24.25   | Increase            |
| LGR5             | -3.86 | 3.86  | 14.52   | Increase            |
| Msi1             | -1.18 | 1.18  | 2.26    | Increase            |

Discussion

Ovarian cancer is a heterogeneous malignancy and is the seventh most common cancer in the world (25). Although many therapeutic approaches have been adopted to treat this cancer, which has increased life expectancy in these patients over the past decades, the 5-year survival rate of patients with ovarian cancer after initial treatment is still very low and recurrence of the disease has been observed after standard chemotherapy; this indicates the need for new treatment strategies to overcome resistance to chemotherapy (26, 27). One of the main factors in resistance to chemotherapy in cancers, especially ovarian cancer, is the presence of CSCs that have the abilities of regeneration, differentiation, tumorigenesis and chemo-resistance (28). Given the importance of ovarian CSCs in drug resistance and tumor recurrence, their elimination can be considered as an effective treatment for reducing chemical resistance and recurrence in ovarian cancer (29). Therefore, different strategies can be used. Signaling pathways could be a good choice for which by their targeting the survival and growth of CSCs can be prevented (19). Surface markers can also be used as a precise target for the suppression and eradication of CSCs. CSC surface markers such as CD24, CD44, CD117 and CD133 can be targeted by several strategies (30). As an example the CD44 cells isolated from the SKOV3 cell line were targeted by hyaluronic acid-paclitaxel (HA-TXL), which caused in tumor weight loss (31). In another report, CD133 cells isolated from the OVCAR5-luc cells were targeted, resulting in significant reduction in tumor progression (32). Inhibition of CD24 cells by inducing apoptosis in the SKOV3 cell line resulted in reduced cancer cell viability and decreased tumor growth in nude mice (33). There is an association between the CD117 surface marker and drug resistance in ovarian cancer (34). Activation of the Wnt/β-catenin-ABCG2 pathway for resistance towards cisplatin/paclitaxel by CD117 has occurred in ovarian CSCs. Imatinib Mesylate has been used as a CD117 inhibitor to treat a variety of tumors and ovarian tumors resistant to chemotherapy (35, 36). The growth of CS44+ and CD117+ chemically resistant ovarian CSCs was also inhibited by paclitaxel and salinomycin (37). Metformin is another drug associated with increased 5-year survival in patients with ovarian cancer. Metformin has been shown to inhibit CD44+ and CD117+ CSCs.
and the EMT process in SKOV3 and A2780 cell lines (38). Therefore, it is very important to isolate these cells and conduct extensive research on them. CSCs can be identified and isolated in different ways. MACS and FACS methods are efficient techniques for isolating CSCs from solid tumors or cell lines based on cell surface or intracellular cell markers. MACS is a quick and easy method (9). On the other hand, CD133 is one of the most common cell surface markers used to isolate CSC from various types of tumor cells such as breast cancer, glioblastoma, prostate cancer, colon cancer, and hepatic cancer. CD133 + glioma cells have been reported to be capable of tumorigenesis. Human lung cancer CD133 + cells and glioma cell lines in mice were also capable of self-regeneration and tumorigenesis (39, 40). Another study has reported that CD105 + cells have higher CSC characteristics compared to CD105- cells following MACS isolation (41). CXCR4 + cells isolated by the FACS method also had higher ability to form spheres and tumorigenesis compared to CXCR4- cells (42). CD133+/CD24+/CTR2 + cells have been shown to have stem-like properties in renal cell carcinoma (43). Numerous markers such as CD133+, ALDH1/2, LGR5, EpCAM, CD44, CD34, CD24, CD117, MyD88 and CDH1 were used to isolation of CSCs from ovarian cell lines (20, 21, 44–51). Another method of CSC isolation based on aldehyde dehydrogenase (ALDH) is using the Aldefluor method which is less specific than cell surface methods (52). Another method for CSC isolation is based on side population (SP) cells with ABC transmitters’ expression using Hoechst staining. In this method, SP cells remove Hoechst dye through a transmitter. This is a mechanism for expelling chemotherapy drugs and creating resistance to chemotherapy (53). This method is also used to isolate CSCs without cell surface markers, yet compared to other methods, it has lower specificity and accuracy besides having toxic effects on isolated cells (54).

**Conclusions**

Given the importance of targeted CSC treatment in the definitive treatment of ovarian cancer, the accurate isolation of these cells is of great importance and therefore the method of isolating CSCs through common surface markers and examining the properties of these cells can be very useful and effective for future research.

**Abbreviations**

Cancer stem cells (CSCs)

Ovarian cancer stem cells (OCSCs)

Ovarian cancer (OC)

Magnetic-activated cell storing method (MACS)

Fluorescence-activated cell storing method (FACS)

Flow cytometry (FCM)
Natural stem cells (NSCs)
Fetal bovine serum (FBS)
Hyaluronic acid-paclitaxel (HA-TXL)
Aldehyde dehydrogenase (ALDH)
Side population (SP)

Declarations

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
VK was involved in experiments and drafting. MM edited and revised the draft. MRA and SRK supervised the project. All authors read and approved the final manuscript.

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Figures

**Figure 1**

Flow cytometry analysis of CD133 surface marker expression in A2780 cell line. Cells were stained with PE conjugated to anti-CD133 monoclonal antibody. The frequency of CD133+ cells in the A2780 cell line was 0.1%.  

Figure 3

Spheroid colony formation in CD133+ cells compared to CD133- cells isolated from the A2780 cell line. CD133+ cells in serum-free culture medium containing bFGF, EGF, B27 supplements and insulin from left to right on the second, third, fourth and fifth day of culture formed spheroid colonies of different sizes.

Figure 4

Analysis of CD133+ stem cell markers expression compared to CD133- cells. Increased expression of Sox2, Nanog, Oct4, ABCG2, ALDH1, LGR5 and Msi1 markers was observed in CD133+ cells.