Phenotypic characteristics of CD133$^+$EpCAM$^+$ cancer stem-like cells derived from the human hepatoma HepG2 cell line

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Background/aim

Cancer stem-like cells (CSCs) have been found to be a serious hurdle in the effective treatment of cancer. The rationale of this study was to isolate and characterize CD133$^+$EpCAM$^+$-enriched cells from the human hepatoma HepG2 cell line to prove their stemness phenotype.

Materials and methods

The CD133$^+$EpCAM$^+$ cells were sorted from the HepG2 cell line using magnetic cell sorting and specified by flow cytometry analysis of surface markers [CD13, CD24, CD34, CD44, CD90, CD133, and CD326 (EpCAM)] and transmission electron microscopy to confirm their identity as CSCs. Quantitative real-time PCR analysis was applied for determining the expression level of stemness marker genes: Oct4, Nanog, ALDH1A1, Notch receptors (NOTCH1, NOTCH2, and NOTCH3), and cytokeratins (CK8/18/19). The proliferative ability of the isolated cells was identified through MTT assay, and their sensitivity to chemotherapeutic drugs was measured by cell counting kit-8 assay.

Results

The isolated CD133$^+$EpCAM$^+$ cells from the HepG2 cell line characterized by flow cytometry were positive for CD13 (81.8%), CD24 (24.4%), CD34 (3.36%), CD44 (92.0%), CD90 (39.7%), CD133 (82.3%), and CD326 (2.79%). Moreover, our data clarified from transmission electron microscopy examination that the isolated CD133$^+$EpCAM$^+$ cells exhibited irregular cell morphology and integral cell membrane structure. The sorted CD133$^+$EpCAM$^+$ cells possessed considerable increase in the mRNA level of Oct4, Nanog, ALDH1A1, NOTCH1, NOTCH2, NOTCH3, and CK19 genes, whereas they showed significant decrease in the mRNA level of CK8 and CK18 genes versus CD133$^-$EpCAM$^-$ cells. Moreover, starting from day 4 to day 10, the CD133$^+$EpCAM$^+$ cells showed a significant increase in their proliferation rate and displayed high resistance to chemotherapy (doxorubicin) contrary to CD133$^-$EpCAM$^-$ cells.

Conclusion

On the basis of the aforementioned results, CD133$^+$EpCAM$^+$-enriched cells strictly represented CSC phenotype in the HepG2 cell line. These cells might be valuable for studying the mechanism of CSCs in hepatoma and screening novel targets for cancer therapy.

Keywords:
cancer stem-like cells, CD133, EpCAM, HepG2 cell line, stemness markers

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Introduction

Liver cancer is a prime contributor to the world's cancer burden, and occurrence rates have increased in a great number of countries in recent decades. Hepatoma [hepatocellular carcinoma, (HCC)] is accountable for the great majority of liver cancer deaths [1]. It is the sixth and fourth most popular cancer globally and in Egypt, respectively. Egypt is the third and 15th most populous country for hepatoma in Africa and worldwide, respectively [2].

Various molecularly targeted drugs have been accepted by the FDA for the medication of patients with advanced hepatoma, including angiogenesis inhibitors, kinase inhibitors, and immune checkpoint inhibitors. Despite the fact that these therapies have been established to be beneficial as adjuvant therapy, their effectiveness is moderate, either because they only prolong the patient's survival for a few months or are only successful against a small number of patients [3]. This disease is assumed as a crucial public health concern owing to its high occurrence, persistent recurrence, limitations of the present treatment systems, and high death rate [1].

Hepatoma is a strongly heterogeneous and biologically complex disease. The tumor includes a varied set of cells harboring several molecular signatures with different levels of sensitivity to therapy. Tumor heterogeneity has been classically explicated by the clonal emergence of tumor cells triggering from the cumulative accumulation of genetic and/or epigenetic alterations [4]. Additionally, persuasive clue at present demonstrates that the heterogeneity within a tumor is developed from a subpopulation of cells with progenitor or stem cell properties known as cancer stem-like cells (CSCs) [5].

The CSCs are identified as a rare tumor cell population, competent for generating and maintaining tumors. The three peculiar characteristics of these cells are (a) the capability to proliferate and generate tumors, (b) self-renewal, and (c) the potency to differentiate into different cells [6]. It has been elucidated that hepatic cancer progenitor cells (HPC) exist in precancerous regions comprising foci of altered hepatocytes that exhibit CSC-like features. These foci include various types of cells, including $SOX9^+$, epithelial cell adhesion molecule ($EpCAM^+$), and $CD44^+$ cells, suggesting that these niches might serve as sites for CSC expansion [7].

The expression of surface markers like CD24, CD29, CD44, CD133, aldehyde dehydrogenase 1 (ALDH1), and epithelial-specific antigens was applied to derive and enrich populations of CSC from several tumors [8]. Recent investigations have clarified that the CSC surface marker expression is delicate for every type of tissue and particular for every tumor [9]. Increased protein expression levels of hepatic stem cell or HPC biomarkers, such as cytokeratin 19 (CK19), CD133, ATP-binding cassette super-family G member 2 (ABCG2), CD44, and nestin are closely correlated with tumor angiogenesis and with poor prognosis of hepatoma [10]. Moreover, gene signatures correlated with S2 and CK19, known by upregulation of MYC–AKT signaling and expression of EpCAM and alpha-fetoprotein, have been established to predict the outcomes of patients with hepatoma in relation to liver transplant indications [11].

Among CSC biomarkers, CD133 (prominin-1) is implicated in the promotion of epithelial-to-mesenchymal transition (EMT)-activating signals, like nuclear factor kappa B and autocrine interleukin1 signaling [12,13]. It works as a marker for CSCs of particular cancers in humans [14], and presently, it has been applied to identify CSCs from hepatoma [15]. Many studies on sorting and enriching CSC populations depending on CD133 marker expression have shown favorable results with high purity [9]. The study of Fang *et al*. [16] proved the prosperous derivation of $CD133^+$ cell population in primary colorectal tumors and demonstrated that these cells were competent to generate spheroids and preserve CD133 expression. Sheng *et al.* [17] registered that the sorted and enriched prostate CSCs by magnetic-activated cell sorting (MACS) and propagation in serum-free medium experienced high expression of CD133 and CD44. Additionally, Wang *et al*. [18] derived the subpopulations of cells that are $CD133^-CD44^-$, $CD133^-CD44^+$, $CD133^+CD44^-$, and $CD133^+CD44^+$ from Hep2 and TU-177 cell lines through MACS technique and documented that the cells expressing $CD133^+CD44^+$ had a greater ability for survival, metastasis, invasion, and tumor formation versus the other populations. The differential expression of CD133 in various hepatoma cells is confused and consequently CD133 alone is not appropriate to disclose CSCs in hepatoma [19].

Lately, EpCAM (CD326) has been specified as a potential marker of CSCs in hepatoma [20]. It promptly controls the cell cycle and proliferation, operating as a transcription factor by motivating cyclin A, cyclin E, and c-myc [21]. $EpCAM^+$ hepatoma cells isolated from tumor specimens and cell lines are strongly aggressive and tumorigenic [20].

The main goal of this attempt was to isolate CD133 and EpCAM-enriched cell population from the HepG2 cell line using MACS technique to establish their stemness properties.

Materials and methods

Cell culture

The HepG2 cell line was obtained from VACSERA (The Holding Company for Biological Products & Vaccines, Giza, Egypt), and was maintained in Dulbecco's modified Eagle's medium-high glucose (Biowest, Nuaillé France) containing 10% heat-inactivated fetal bovine serum (Biowest) and 1% penicillin/streptomycin (Biowest). HepG2 cells were cultured in 5% CO_2 humidified atmosphere at 37°C. Cells were passaged at 80–90% confluence by 0.025% trypsin/EDTA (Biowest) [22].

Magnetic-activated cell sorting

The $CD133^+$ and CD326 ($EpCAM^+$) hepatic CSCs were fractionated from the HepG2 cell line using CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany) and CD326 (EpCAM) MicroBeads kit (Miltenyi Biotec) according to the manufacturer's instructions. In brief, the $CD133^+$ and CD326 $(EpCAM)^+$ cells were magnetically labeled with CD133 MicroBeads – Tumor Tissue and CD326 (EpCAM) microbeads. Then, the cell suspension was loaded onto a MACS column (LS Columns; Miltenyi Biotec), which was placed in the magnetic field of a Midi MACS separator (Miltenyi Biotec). The magnetically labeled cells ($CD133^+EpCAM^+$) were retained within the column. The unlabeled cells ($CD133^-EpCAM^-$) were run through; this cell fraction was thus depleted of $CD133^+EpCAM^+$ cells. After removing the column from the magnetic field, the magnetically retained cells were eluted as the positively selected cell fraction. The cells were expanded in the same condition for the HepG2 cell line culture to obtain enough cells for further evaluation.

Flow cytometry analysis

To ensure that the isolated cells were CSCs, they were characterized by flow cytometry analysis for specific surface markers (CD13, CD24, CD34, CD44, CD90, CD133, and CD326). The FITC conjugated-CD13 antibody was procured from Beckman Coulter Co., California (USA). However, the PE-conjugated CD24 and CD133 antibodies as well as the FITC conjugated-CD34 and 326 antibodies were supplied by Miltenyi Biotec. The PE-conjugated CD44 and CD90 antibodies were purchased from R&D Systems, Abingdon (UK). The cells were incubated with the antibody against each of the surface markers for 20 min in dark at room temperature. Concentrations of antibodies were used according to the manufacturer's recommendations. The stained cells were analyzed by flow cytometry analysis using Beckman Coulter Elite XL, California (USA) instrument [23].

Transmission electron microscopy

The freshly isolated cells were fixed with 2.5% glutaraldehyde at 4°C for 4 h, then rinsed with cold phosphate-buffered saline and further fixed in 1% osmium tetroxide at room temperature for 2 h. The samples were first dehydrated using a gradient of ethanol and acetone, then embedded in Epon812 resin and acetone (v/v, 1 : 1) for 30 min, followed by 100% Epon812 resin for 1 h. The Epon812 resin was solidified at 37°C for 24 h and at 60°C for 48 h. Preparation of ultrathin sections was done using an ultramicrotome, which were thereafter stained with uranyl acetate and lead citrate [24] for examination by transmission electron microscopy (TEM) (JEOL JEM – 1400, Tokyo, Japan).

Quantitative reverse transcription PCR

The RNA was extracted from the isolated cells using RNeasy mini kit for total RNA purification from cells (Qiagen, Hilden, Germany) according to the manufacturer's instruction. RNA concentration and purity were evaluated using Nano Drop 2000 (Thermo Fisher Scientific, Rockford, Illinois, USA) using 260/280 nm ratio. Then, cDNA synthesis was performed using Revert Aid first strand cDNA synthesis kit (Thermo Fisher Scientific Inc., Vilnius, Lithuania) according to the manufacturer's instruction. Gene expression analysis of octamer-binding transcription factor 4 (Oct4), Nanog, ALDH1A1, Notch homolog 1, translocation-associated (NOTCH1), NOTCH2, NOTCH3, CK8, CK18, and CK19 was carried out using DNA-Technology Real-Time PCR device (DT lite 4, Moscow, Russia). The reaction mixture (25 μl volume) included 12.5 μl of QuantiTect SYBR Green master mix (Qiagen), 0.75 μl of forward and reverse primer of target gene (Invitrogen, Carlsbad CA, USA), 1.5 μl cDNA template, and 9.5 μl RNase-free water. β-actin was used as a housekeeping gene. Relative mRNA expression versus control value ($CD133^-EpCAM^-$ cells) was assessed using the $2^{-\Delta\Delta Ct}$ comparative method after normalization with β-actin gene.

The PCR cycling was set as follows: initial denaturation step at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 60°C for Oct4, ALDH1A1, NOTCH3, CK8, CK18, and CK19 genes, 58°C for Nanog gene, 53°C for NOTCH1 gene, and 55°C for NOTCH2 gene for 30 s, and extension at 72°C for 30 s. The primer sequences of each target gene are delineated in Table 1.

Cell proliferation assay

The proliferative abilities of the isolated cells were determined using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT; Sigma, Missouri, USA) assay, as described in detail previously [31]. In brief, the isolated cells were cultured in 96-well plates at a density of 1×10^3 cells/well. At 2, 4, 6, 8, and 10 days of culture, MTT dissolved in phosphate-buffered saline was added to each well at a final concentration of 5 mg/ml, and the samples were incubated at 37°C for 4 h. Water-insoluble dark blue formazan crystals that formed during MTT cleavage in actively metabolizing cells

Table 1. List of gene-specific primers in reverse transcription PCR

Gene	Forward	Reverse	References
Oct4	TCGCAAGCCCTCATTTCACC	GCCAGGTCCGAGGATCAAC	Asadi *et al.* [25]
Nanog	GCTGGTTGCCTCATGTTATTATGC	CCATGGAGGAAGGAAGAGGAGAGA	Ambady *et al.* [26]
ALDH1A1	TTACCTGTCCTACTCACGATT	GCCTTGTCAACATCCTCCTTAT	Gangavarapu *et al.* [27]
NOTCH1	CCCGCCAGAGTGGACAGGTCAGTA	TGTCGCAGTTGGAGCCCTCGTTA	Xiao *et al.* [28]
NOTCH2	CCCACAATGGACAGGACA	GAGGCGAAGGCACAATCA	
NOTCH3	TCTCAGACTGGTCCGAATCCAC	CCAAGATCTAAGAACTGACGAGCG	
CK8	TCATAGACAAGGTACGGTTCC	GCCTAAGGTTGTTGATGTAGC	Li *et al.* [29]
CK18	GAGCTGCTCCATCTGTAGGG	CACAGTCTGCTGAGGTTGGA	
CK19	CATGAAAGCTGCCTTGGAAGA	TGATTCTGCCGCTCACTATCAG	
β-actin	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG	Song *et al.* [30]

were then dissolved in dimethyl sulfoxide. Absorbance was measured at 492 nm, using a microplate reader (Model 500; BIORed Instrument Inc., California, USA). The cell proliferation (%) was calculated and compared with the control (CD133$^-$EpCAM$^-$ cells).

Chemotherapy sensitivity assay

The sensitivities of the isolated stem cells to chemotherapeutic drugs were measured by cell counting kit-8 assay using WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium (Sigma-Aldrich). In brief, 4000 cells/well were seeded in 96-well plates, and various concentrations (0.01, 0.1, 1, 10, and 100 μg/ml) of doxorubicin (Adricin, Doxorubicin Hcl; Hikma Specialized Pharmaceuticals, Cairo, Egypt) were added at the beginning [32]. After 72 h of incubation, viable cells were measured by cell counting kit-8 assay following the manufacturer's instruction at 450 nm, using a microplate reader (Model 500; BIORed Instrument Inc.).

Statistical analysis

The attained results were delineated as means ±standard deviations. Data were analyzed by one-way analysis of variance using the Statistical Package for the Social Sciences (SPSS, IBM, Hong Kong SAR, China) program, version 14, followed by least significant difference to compare the significance between groups. The level of significance was set at *P* value less than 0.05.

Results

Flow cytometry analysis of CD133$^+$EpCAM$^+$ cells

The isolated CD133$^+$EpCAM$^+$ cells from the HepG2 cell line characterized using flow cytometry were positive for CD13 (81.8%), CD24 (24.4%), CD34 (3.36%), CD44 (92.0%), CD90 (39.7%), CD133 (82.3%), and CD326 (2.79%) (Fig. 1).

Transmission electron microscopy analysis of CD133$^+$EpCAM$^+$ cells

The structural details of isolated CD133$^+$EpCAM$^+$ cells from the HepG2 cell line were examined by TEM. Irregular cell morphology and integral cell membrane structure were visible. Furthermore, a small number of vacuoles were observed (Fig. 2).

Expression of cancer stem-like cells markers in isolated CD133$^+$EpCAM$^+$ cells

To further characterize the isolated CD133$^+$ and EpCAM$^+$-enriched cell population, real-time PCR analysis was carried out to detect the expression level of different CSCs-related genes. The attained results revealed that the mRNA expression level of Oct4, Nanog, ALDH1A1, NOTCH1, NOTCH2, and NOTCH3 genes was significantly ($P<0.05$) up-regulated in the isolated CD133$^+$EpCAM$^+$ cells when compared with CD133$^-$EpCAM$^-$ cells (Fig. 3). However, they exhibited significant ($P<0.05$) down-regulation in the mRNA levels of CK8 and CK18 genes versus CD133$^-$EpCAM$^-$ cells. Moreover, CD133$^+$EpCAM$^+$ cells showed insignificant ($P>0.05$) up-regulation in the mRNA expression level of CK19 genes relative to CD133$^-$EpCAM$^-$ cells (Fig. 3).

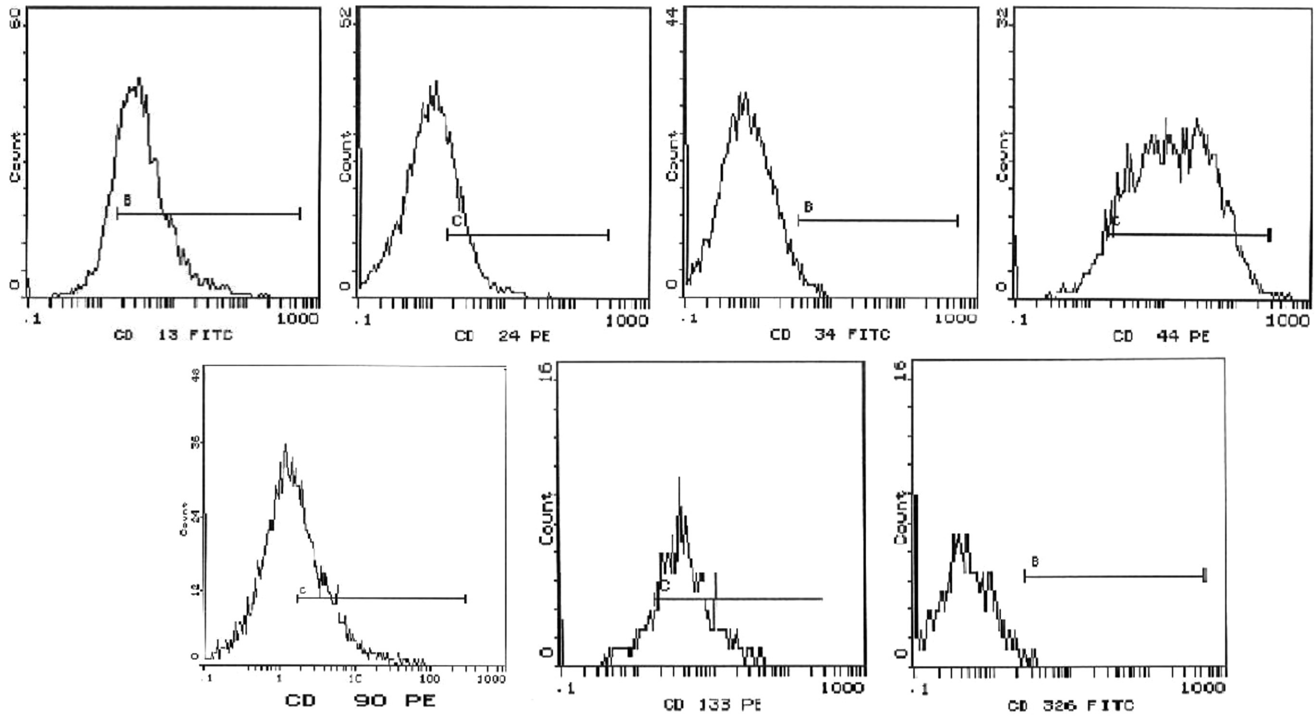
Proliferative abilities of isolated CD133$^+$EpCAM$^+$ cells

One of the key features of CSCs is fast proliferation. During 10 days of culture, the isolated CD133$^+$EpCAM$^+$ cells proliferated rapidly (Fig. 4). Starting from day 4 to day 10, the CD133$^+$EpCAM$^+$ cells showed significant ($P<0.05$) increase in their proliferation rate in comparison with CD133$^-$EpCAM$^-$ cells.

Resistance of isolated CD133$^+$EpCAM$^+$ cells to chemotherapy

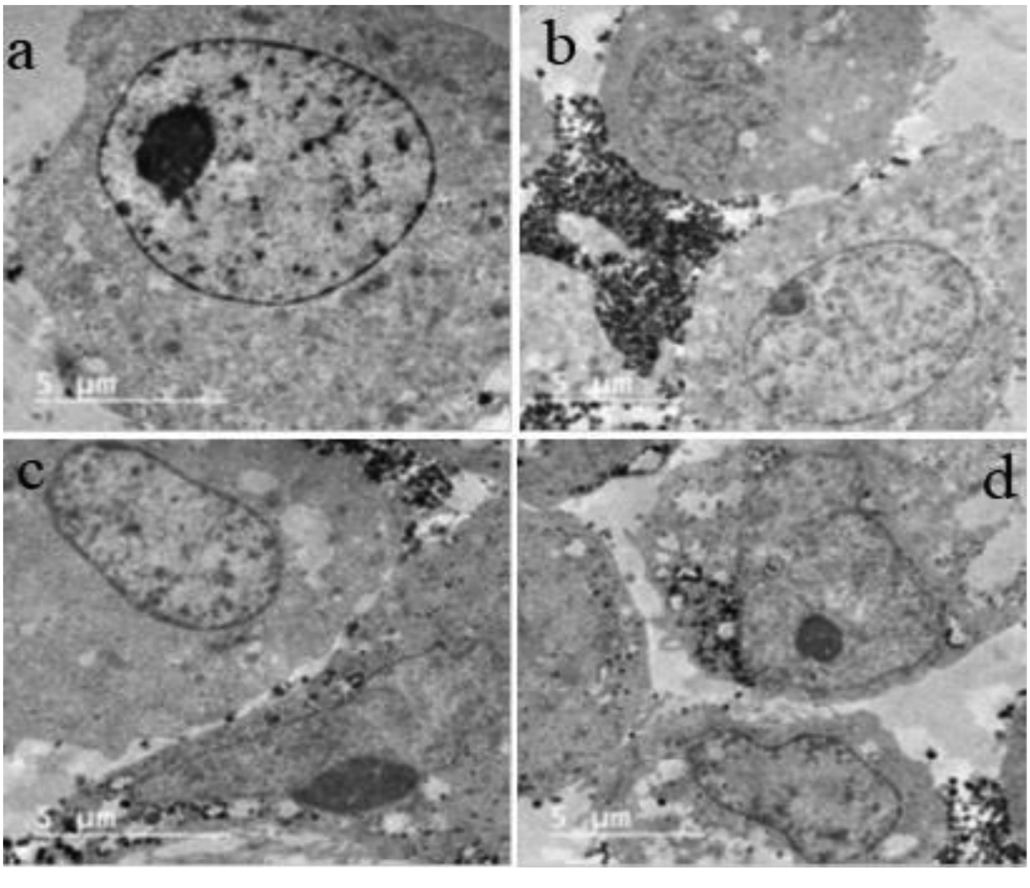
To explore whether the isolated CD133$^+$EpCAM$^+$ cells from the HepG2 cell line have increased chemoresistance, the sensitivity of CD133$^-$EpCAM$^-$

Figure 1.



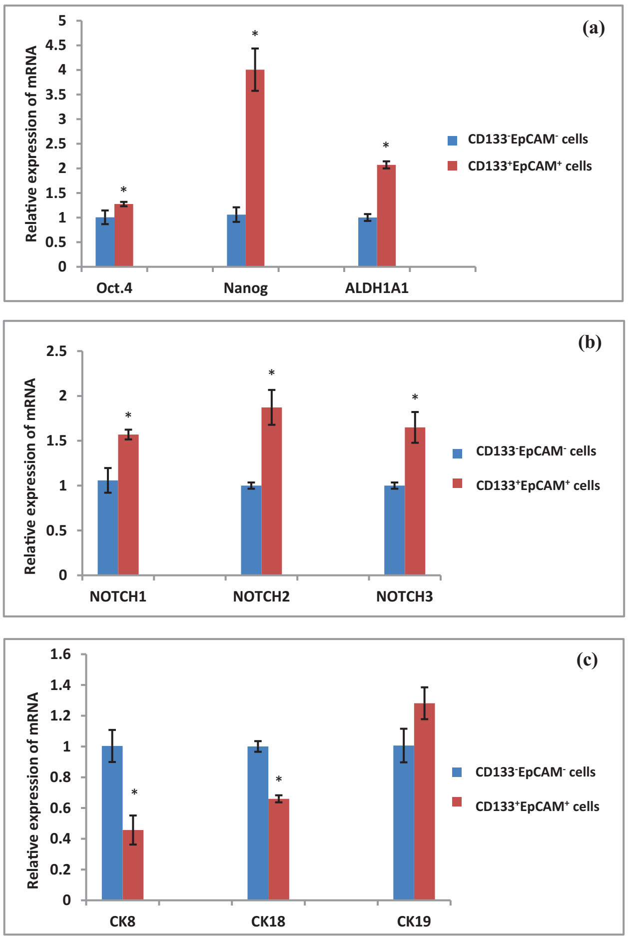
Flow cytometric analysis of isolated CD133$^+$EpCAM$^+$ cells from the HepG2 cell line after staining with CD13, CD24, CD34, CD44, CD90, CD133, and CD326 antibodies.

Figure 2.



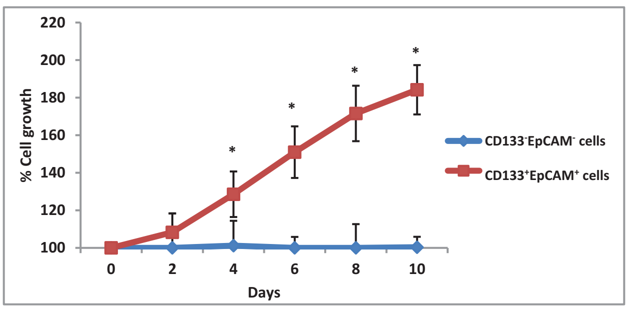
TEM images of isolated CD133$^+$EpCAM$^+$ cells from the HepG2 cell line. (a, b) Irregular cell morphology and integral cell membrane structure. (c, d) A small number of vacuoles. TEM, transmission electron microscopy.

Figure 3.



Expression of CSC-related genes in isolated CD133$^+$EpCAM$^+$ cells from the HepG2 cell line. (a) Oct4, Nanog, and ALDH1A1 genes; (b) NOTCH1, NOTCH2, and NOTCH3 genes; and (c) CK8, CK18, and CK19 genes. All experiments were repeated three times. Data are displayed as the mean±SD. **P* value less than 0.05 compared with CD133$^-$EpCAM$^-$ cells. CSC, cancer stem-like cells.

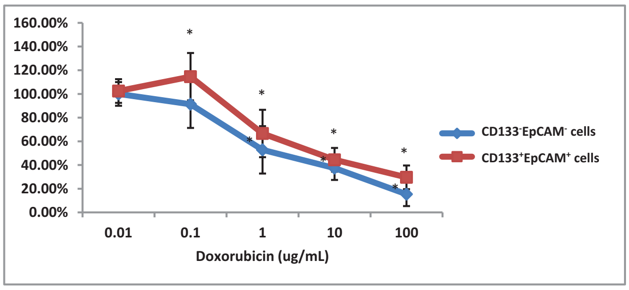
Figure 4.



Proliferative capacities of the isolated CD133$^+$EpCAM$^+$ cells from the HepG2 cell line. Data are displayed as the mean±SD. **P* value less than 0.05 compared with CD133$^-$EpCAM$^-$ cells.

cells and isolated CD133$^+$EpCAM$^+$ cells toward different concentrations of doxorubicin was assessed. Compared with CD133$^-$EpCAM$^-$ cells, the isolated CD133$^+$EpCAM$^+$ cells were much more resistant to doxorubicin (Fig. 5).

Figure 5.



Resistance of the isolated CD133$^+$EpCAM$^+$ cells to doxorubicin. Data are displayed as the mean±SD. **P* value less than 0.05 compared with CD133$^-$EpCAM$^-$ cells.

Discussion

CSCs have been specified in various tumors and theorized to have a role in tumorigenicity, angiogenesis, metastasis, invasion, migration, chemoresistance, reiteration of malignant tumor, and malignant transformation. The current therapeutic approaches for cancers primarily target somatic tumor cells rather than CSCs. For developing effective treatments to curb CSCs, it is imperative to derive and identify CSCs from cell lines or tumor tissues and disclose their characteristics as well as study the mechanisms implicated in their stemness [28]. In the current setting, CD133$^+$EpCAM$^+$ cells were successfully isolated and characterized from the HepG2 cell line using the MACS system.

According to the data of flow cytometry analysis, the proportion of CD133 and EpCAM marker expression in CD133$^+$EpCAM$^+$ HepG2 cell subpopulation was 82.3 and 2.79%, respectively. Furthermore, these cells exhibited positive expression for CD13 (81.8%), CD24 (24.4%), CD34 (3.36%), CD44 (92%), and CD90 (39.7%). These findings are in harmony with those of Chen *et al.* [33]. Feng *et al.* [34] registered that the enriched stem cell-like HepG2 spheres expressed elevated level of CD133 in comparison with parental HepG2 cells. Meanwhile, Suetsugu *et al.* [35] cited that CD133$^+$ hepatic CSCs, derived from the Huh7 cell line, manifested greater proliferative and metastatic capacity relative to the CD133$^-$ counterparts. Moreover, the research by Ma *et al.* [23] denoted that CD133$^+$ cells exhibited a substantial colony-forming property, considerable tumorigenicity, and prospects to differentiate into angiomyogenic-like lineages. Moreover, these investigators demonstrated that CD133$^+$ population displayed greater tumorigenicity *in vivo*. Liu *et al.* [36] proved that CD133 was important to detect the migratory competence of liver CSCs, tumor-originating features, and the EMT process. Additionally, Tang *et al.* [37] uncovered that CD133$^+$ liver tumor-initiating cells showed angiogenic capacity and exhibited a fundamental role in controlling tumorigenesis of hepatic tumor-initiating cells through controlling network identified neurotensin, interleukin-8, CXCL1, and MAPK signaling. Moreover, Li *et al.* [38] established that the hepatoma cells with positive expression for CD133 could avail autophagy to ensure their existence. Of note, hepatic CD133$^+$ CSCs are known to exhibit great resistance to radiotherapy [39] and chemotherapy [40].

EpCAM has been identified as a cell surface antigen expressed in epithelial tumors [41]. Mu *et al.* [42] disputed that EpCAM expression in hepatoma tissues was as low as 30% and could serve as a biomarker for CSCs in hepatoma. It has been cited that the EpCAM$^+$ hepatoma cells possessed CSC-like features comprising an increased self-renewal potential and differentiation ability and were competent to generate extremely tumorigenic cancers in NOD/SCID mice. Moreover, EpCAM has been recognized as one of the target genes in Wnt-β-catenin signaling pathway [20]. A set of microRNAs such as miR-155, miR-18,1 and miR-216a/217 have been evinced to be implicated in controlling stemness of EpCAM$^+$ hepatoma cells [43,44] and be responsible for chemoresistance and tumor metastasis. Ji and Wang [45] mentioned that a majority of EpCAM$^+$ HCC cells expressed CD133.

CD13 (aminopeptidase N) is a membranous glycoprotein that has a primary role in cancer progression [46]. It serves as an indicator of dormant or semi-quiescent CSCs in hepatoma cell lines. Haraguchi *et al.* [47] documented that CD13 is mainly distributed during the G1/G0 phase in hepatoma cells and generally enriched in a side population derived from hepatoma cell lines. Moreover, CD13$^+$ hepatoma cells exhibit a great tumorigenic capacity in NOD/SCID mice. The data proved that CD13$^+$CD90$^-$ fractions in PLC/PRF/5 and CD13$^+$CD133$^+$ cells in Huh7 could generate tumor successfully. Additionally, it has been demonstrated that CD13$^+$ cells are highly chemoresistant, proposing that CD13$^+$ cells exemplify the dormant hepatic CSCs in hepatoma as they decreased ROS-induced DNA damage after genotoxic chemo/radiation stress and protected cells from apoptosis [47].

CD24 is mainly expressed in stem/progenitor cells and CSCs originated from pancreatic, colon, breast, and ovarian cancers as well as squamous cell head-neck cancer. Lee *et al.* [48] clarified that CD24$^+$ hepatoma cells are competent for self-renewal and tumor emergence as well as chemoresistance to cisplatin. It has been cited that CD24$^+$ hepatoma cells could form larger and more hepatospheres versus CD24$^-$ hepatoma cells, which means that these cells are capable of generating tumors. As few as 500 CD24$^+$ cells from hepatoma cell lines are adequate for tumor formation in NOD/SCID mice. Moreover, CD24 expression has been documented to be needed for maintaining self-renewal, differentiation, and metastasis of tumors and to considerably affect patients' clinical outcome. Furthermore, most of CD24$^+$ hepatoma cells expressed EpCAM and CD133, as it has been registered that CD24 expression was found to overlap with that of CD133 and EpCAM, proposing that these three indicators share comparable self-renewal properties [45].

Our data also revealed that the isolated CD133$^+$EpCAM$^+$ cells exhibited positive expression for CD34. It has been reported that CD34 plays a key role during liver development and regeneration [49]. Zeng *et al.* [50] recorded that a population of CD34$^+$ stem cells served as liver CSCs by forming three kinds of human liver carcinoma (combined cholangiohepatocellular carcinoma, HCC, and cholangiocarcinoma), demonstrating that this liver CSCs evince bipotency, to differentiate into hepatic and cholangiocytic lineages. Moreover, CD34$^+$OV6$^+$ cells and their derivatives, OV6$^+$ cells, generated human liver carcinoma xenografts; accordingly, it has been proposed that these liver CSCs might be acquired from liver progenitor cells [51].

CD44 is implicated in cell–cell adhesion and migration and has been demonstrated to be accompanied with migration of tumor cell in hepatic cancer [52]. CD44 has been specified as a CSC indicator in pancreatic, gastric, colorectal, and breast cancers. In hepatoma, CD44 is a substantial indicator that is applied together with other indicators for CSC to identify hepatic CSCs. Cells co-expressing CD90 and CD44, or CD133 and CD44, display a great invasive phenotype than the cells expressing CD133 or CD90 alone. In agreement with these findings, our data showed that the isolated cells exhibited positive expression for CD44. Yang *et al.* [53] presented that CD44$^+$ cells generate tumor nodules in immunodeficient mice faster than CD44$^-$ cells, whereas lung metastases are only detected in immunodeficient mice implanted with CD90$^+$CD44$^+$ cell population. Moreover, CD44 was evinced to be favorably expressed in a CD133$^+$ subpopulation sorted from four hepatoma cell lines: SMMC7721, MHCC-LM3, MHCC97L, and Huh7. In comparison with CD133$^+$CD44$^-$ cells, CD133$^+$CD44$^+$ hepatoma cells are highly tumorigenic, exhibiting chemoresistance and expressing an elevated level of stemness-associated genes [52].

CD90 (Thy-1) has been theorized as an indicator for several types of stem cells, including murine breast CSCs and hematopoietic stem cells [54]. Some investigations have proposed CD90 as an additional indicator for CSCs and hepatic stem cells [53]. Yang *et al.* [53,55] proved a considerable positive correlation of CD90 expression with tumorigenicity and metastatic capabilities in hepatoma cell lines. Moreover, some researchers used CD90 with CD45, to sort CD90$^+$ cells from tumor samples and blood of patients with hepatoma. It has been recorded that CD90$^+$ cells derived from hepatoma cell lines and CD90$^+$CD45$^-$ cells sorted from primary tumor and blood samples of patients with hepatoma are able to generate hepatoma nodules upon intrahepatic injection into SCID/Beige mice [53,55], and this explains the relation between CD90 expression and tumorigenicity in hepatoma cell lines.

The morphological details of the isolated CD133$^+$EpCAM$^+$ HepG2 population by TEM examination revealed that these cells exhibited irregular cell morphology with integral cell membrane structure, and also, a small number of vacuoles were observed. Our findings mirror prior a study by Nguyen *et al.* [9], who demonstrated that the isolated CD133$^+$ HepG2 cells by magnetic cell sorting method were larger in size and had a nonspecific shape compared with the CD133$^-$ populations.

A growing body of evidence has denoted that CSCs could express stem cell-associated genes [56]. To ensure the stem cell-like identities of the isolated CD133$^+$EpCAM$^+$ cells from the HepG2 cell line, the mRNA expression levels of Oct4, Nanog, ALDH1A1, NOTCH1, NOTCH2, NOTCH3, CK8, CK18, and CK19 genes were analyzed. Our data clarified that CD133$^+$EpCAM$^+$ cells expressed higher mRNA levels of Oct4, Nanog, ALDH1A1, NOTCH1, NOTCH2, NOTCH3, and CK19 genes, whereas they expressed lower mRNA levels of CK8 and CK18 genes versus CD133$^-$EpCAM$^-$ cells.

Oct4 is considered as a substantial stem cell factor and master regulator in the maintenance of stem cell potency. Robust Oct4 can promptly control two downstream stem cell regulator genes, Nanog and SOX2, motivating liver CSC phenotypes. Diverse studies have determined the correlation between Oct4 and liver CSCs. Nanog has been suggested as a paramount regulator influencing the phenotype of CSCs in several cancer types [57]. It has been recorded that these stem cell-related genes are dominant for proliferation, self-renewal, and differentiation of stem cells [58]. In the present investigation, CD133$^+$EpCAM$^+$ cells expressed greater mRNA level of Oct4 and Nanog versus CD133$^-$EpCAM$^-$ cells. These results are supported by those of Lee-Theilen *et al.* [59] who cited that the HepG2 CSCs had elevated mRNA levels of Oct4 and Nanog. Moreover, the study by Li *et al.* [32] clarified that hepatoma stem cells exhibited significant upregulation of Oct4 and Nanog gene expression levels as compared with their parental cells. Moreover, Jia *et al.* [60] recorded a considerable overexpression of Oct4 and ABCG2 in CD90$^+$CD133$^+$-enriched liver CSCs sorted from the HepG2 cell line versus parental cells. Furthermore, Yin *et al.* [61] concluded that the coexpression of Oct4 and Nanog initiated stem cell features in hepatoma and motivated EMT, contributing to tumor migration, invasion, and metastasis *in vitro* and *in vivo*. This was accomplished via the promotion of Stat3/Snail signaling. Specifically, coexpression of Oct4 and Nanog stimulated the expression of stem cell markers CD133, ALDH1, and BMi-1. Moreover, Oct4 and Nanog expression ultimately induced the expression of ABCG2 and MDR1, two major ABC transporters that contribute to drug resistance [61].

ALDH1 is a crucial regulator for the retinoic acid signaling pathway, which modulates a wide variety of biological processes like cell proliferation, differentiation, cell cycle arrest, and apoptosis [62,63]. Based on these stemness properties, the ALDH1 family is theorized to be a stem cell indicator [64]. Our data clarified that CD133$^+$EpCAM$^+$ cells showed elevated mRNA level of ALDH1A1 versus CD133$^-$EpCAM$^-$ cells. This observation comes in line with that of Ma *et al.* [65], which indicated the preferential expression of ALDH1A1 in the CD133$^+$ subpopulation isolated from hepatic cancer cell lines. It has been demonstrated that ALDH$^+$ cells possessed enhanced metastatic capacity and chemotherapeutic drug resistance relative to ALDH$^-$ counterparts [66]. Chen *et al.* [67] denoted that FOXM1 stimulates ALDH2 expression via binding to its promoter region and hence affect the expression of

Nanog, Oct4, and Sox2 in liver CSCs and lead to the conclusion that silencing FOXM1 suppresses the stemness of hepatic CSCs and stimulate their apoptosis through reducing the expression of ALDH2.

Many studies indicated that Notch pathway may stimulate the survival, proliferation, self-renewal, differentiation, angiogenesis, and migration of CSCs in various malignant tumors [68]. The abnormal motivation of Notch signaling may lead to the generation of many tumors such as glioma and melanoma as well as breast, colon, and cervical cancers [69]. On the contrary, it has been registered that Notch signaling may act as an inhibitor in some tumors, such as forebrain tumor subtypes [70]. The results of the present study demonstrated that $CD133^+EpCAM^+$ cells exhibited greater mRNA levels of NOTCH1, NOTCH2, and NOTCH3 *versus* $CD133^-EpCAM^-$ cells. Lee and Hong [71] cited that aberrantly elevated Notch signaling was observed in $CD133^+$ cells when compared with $CD133^-$ compartments in the HepG2 cell line. Furthermore, Wu and Pan [72] stated that the Notch signaling cascade is implicated in CD133 signaling.

CK is an essential cytoskeletal component responsible for nucleus fixation and maintenance of cell morphology. CK8 and CK18 are substantial protein components of the cytoskeleton [73] that take part in cell movement and division as well as cytoplasmic transport [74]. CK18 is known as an epithelial-specific indicator of the EMT process [75]. A growing body of evidence has suggested their implication in the invasion or expansion of tumors [76] and drug resistance through stimulation of EMT [77]. It has been registered that CK8 and CK18 expression levels may not directly associate with malignant transformation, but they may be altered accompanying with other malignant signals, for example, EMT [78]. Decrease of CK18 has been detected to stimulate EMT and motivate metastasis [79]. Our findings revealed that the isolated $CD133^+EpCAM^+$ cells expressed lower mRNA levels of CK8 and CK18 genes versus $CD133^-EpCAM^-$ cells. Lai *et al.* [80] registered that of 83 patients with hepatoma, 72.3 and 59.0% had down-regulated expression of CK8 and CK18, respectively.

CYFRA 21-1 is a fragment of CK19 that is typically associated with epithelial cell cancers and is a novel CSC surface marker correlated with EMT and TGFβ/Smad signaling cascade [81]. Furthermore, the PDGFRα-laminin B1-CK19 pathway leads to tumor generation at the invasive front of hepatoma [82]. Rhee *et al.* [83] denoted that CK19 expression in hepatoma is regulated by fibroblast-derived hepatocyte growth factor via a MET-ERK1/2-AP1 and SP1 axis. $CK19^+$ cells exhibited great proliferative capacity and chemotherapy resistance [81,84]. It has been reported that CK19 expression exhibited a great relation with high tumorigenicity, low tumor differentiation capacity, metastasis, and invasion in hepatoma [84,85]. The profiling study of Govaere *et al.* [84] showed that $CK19^+$ hepatoma highly expresses invasion or metastasis-related genes, biliary/HPC markers, and members of the miRNA-200 family. Our data indicated that $CD133^+EpCAM^+$ cells expressed higher mRNA level of CK19 than $CD133^-EpCAM^-$ cells. This result is in contrary to that of Yoshikawa *et al.* [86] who mentioned that most $CD133^+$ cells were $CK19^+$. In human hepatoma and cholangiocarcinoma cell lines, $CD133^+$ cells coexpressed CK19 and alpha-fetoprotein. Yamashita *et al.* [87] denoted that EpCAM-positive hepatoma exhibited an exclusive molecular signature with properties of hepatic progenitor cells and expressed stem/progenitor markers, such as CK19 and c-Kit, whereas EpCAM-negative hepatoma expressed genes associated with features of mature hepatocytes.

It has been reported in a former work that CSC self-renewal and proliferation are involved in HCC tumorigenesis [88]. Our data clarified that during 10 days of culture the isolated $CD133^+EpCAM^+$ cells proliferated rapidly than $CD133^-EpCAM^-$ cells. This finding is on par with that of Nguyen *et al.* [9]. The enhanced proliferation of $CD133^+EpCAM^+$ cells than $CD133^-EpCAM^-$ cells could be attributed to the activation of the Notch pathway, which is known to have a substantial role in stem cell proliferation [89].

One of the prime properties of CSCs is a great resistance to chemotherapeutic drugs [90]. In the current study, the sensitivity of $CD133^-EpCAM^-$ cells and isolated $CD133^+EpCAM^+$ cells toward different concentrations of doxorubicin was assessed. In accordance with the previous study by Chen *et al.* [33], our data demonstrated that the isolated $CD133^+EpCAM^+$ cells were much more resistant to doxorubicin than $CD133^-EpCAM^-$ cells, and this could be related to that CSCs are greatly competent to express drug-resistant proteins, including the ABC transporter. Furthermore, CSCs exhibited greater efficiency for repairing the damage of DNA than normal cancer cells [91]. Chartrain *et al*. [92] demonstrated that melanoma cells enriched with CSCs after temozolomide treatment displayed elevated expression of ABCB5 channels, which enhanced their anticytotoxic characteristics and stem-like features. Furthermore, in the investigation of Cox and Weinman [93], it has been noted that there are subpopulation of cancer cells able to pump drugs out through the ABC transporter family. Consequently, elevated expression of these transport channels in hepatoma cells is a reason of drug resistance. Moreover, Peetla *et al*. [94] have proved that membranes of drug-resistant cancer cells have various lipid components versus other cancer cells in the population. This variation makes the resistant cell membrane very flexible by modulating their fluidity, structure, and lipid density. In turn, the combination with different protein components can decrease the drug permeability via the membrane. Moreover, Wang *et al.* [95] explored the multidrug resistance of side population cells in prostate cancer and registered that the survival rate of side population cells considerably elevated relative to non-side population cells.

Conclusion

Based on the aforementioned data, the current study provides a meaningful evidence for the stemness characteristics of $CD133^{+}EpCAM^{+}$-enriched cells as they exemplified the phenotypic features of CSCs in the HepG2 cell line, including self-renewal, proliferation, and tumorigenicity (they exhibited elevated expression of Oct4, Nanog, NOTCH1, NOTCH2, NOTCH3, and CK19 genes) as well as chemoresistance (they experienced higher expression of ALDH1A1 and lower expression of CK8 and CK18 genes). This attempt represents the basic research in the field of CSCs and drug screening.

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Conflicts of interest

None declared.

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