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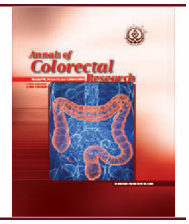

Isolation Set-up and Characterization of Cancer Stem Cells from HT29 Colon Cancer Cell Line

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Abstract

Background: Cancer stem cells (CSCs) have critical roles in tumor initiation, progression, metastasis, resistance to chemoradiotherapy, and recurrence in various cancers such as colon cancer. Therefore, extensive studies are required to understand the CSCs mechanism of action and design novel cancer therapies. Successful isolation and characterization of CSCs from tumor tissues or cancer cell lines provide the proper opportunity for these kinds of studies. This study aimed to isolate and characterize CSCs from HT29 colon cancer cell lines.
Methods: Sphere formation assay was used to isolate CSCs from the HT29 cell line. The expression of four stemness genes, including *LGR5*, *SOX2*, *c-Myc,* and *Oct4,* was assessed by real-time PCR. The CSC markers, CD 44 and CD 24, were also evaluated and compared between the parental HT29 cell line and HT29-derived spheres by flow cytometry. Statistical analysis was performed using GraphPad Prism version 6.0 and the Mann-Whitney U test.
Results: HT29-derived sphere cells were successfully formed in serum-free media. The proportion of CD 24 and CD 44-expressing cells and the expression of *LGR5*, *SOX2*, *c-Myc,* and *Oct4* stemness genes were significantly higher in CSCs isolated from HT29 spheres compared with parental HT29 cells.
Conclusion: Sphere formation assay is a proper method for the isolation of CSCs from the HT29 cell line. The stemness markers *LGR5*, *SOX2*, *c-Myc*, *Oct4*, CD 44, and CD 24 are suitable for the characterization of these cells.

Keywords: Neoplastic stem cell, Colonic neoplasm, HT29 cell

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Introduction

Despite new promising therapeutic approaches, colorectal cancer is still a major cause of morbidity and mortality (1). Recent studies have shown that a small population of cells in tumor tissues has an important role in the initiation and growth of various tumors such as colorectal cancer. Similar to normal stem cells, the main characteristics of this subgroup of tumor cells are self-renewal, asymmetric cell division, and the ability to differentiate into different cell lines. Therefore, they are called cancer

stem cells (CSCs) (2).

Although the origin of CSCs is not exactly clear, evidence suggests that they originate from normal stem cells or progenitor tissue cells due to accumulating genetic mutations and aberrant epigenetic alterations in cell cycle signaling pathways. These uncontrolled stem cells (CSCs) lead to the formation and progression of tumor tissues (3).

CSCs were first identified in acute myeloid leukemia (AML) (4) and then in a variety of tumors such as glioblastoma, melanoma, and lung, breast, prostate, ovary, and colon cancers (5). In addition to tumor initiation, maintenance, and invasion, CSCs play an important role in the resistance of tumors to conventional therapies such as chemotherapy and radiotherapy, resulting in recurrence (6, 7). Therefore, it seems that further characterization of CSCs not only improves the understanding of their key roles in drug resistance, tumor metastasis, and recurrence but also contributes to the design of novel cancer therapies (2, 8, 9).

There are some properties related to the resistance of CSCs to therapeutic agents, including a reduced cell cycle speed, high DNA repair capacity, high expression of multidrug resistance membrane transporters, epithelial-mesenchymal transition (EMT), and high expression of anti-apoptotic proteins (10).

Researchers have taken practical steps to induce apoptosis in various cancers, such as colorectal cancers, by targeting multiple markers in CSCs or even manipulating signaling pathways using RNA interference (RNAi).

Several cell surface markers such as CD 133, CD 44, CD 24, CD 166, leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), epithelial cell adhesion molecule (EpCAM), and aldehyde dehydrogenase (ALDH-1) can be targeted for isolation of CSCs such as colorectal cancer stem cells (CR-CSCs). However, the exact pattern of specific markers in CR-CSCs is still unknown, and the science of targeting CR-CSCs remains controversial (11).

There are also some technical challenges for CSC research, including the constant need for CSCs to conduct sequential studies, the need for access to new tissues for isolation of these cells, the possibility of genetic and epigenetic changes from one tissue sample to another, and the low efficacy of CSC isolation from cell lines. Given these limitations, optimizing the isolation conditions and characterization of CSCs is critical for further studies on these cells (12, 13). Accordingly, the present study aimed to isolate and characterize colon CSCs from HT29 colon cancer cell lines.

Materials and Methods

Monolayer Cell Culture

Human colon cancer cell line HT29 was purchased from the Bonyakhteh Institute and maintained under standard culture conditions. The traditional culture media consisted of Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco-BRL). Cells were maintained in six-well tissue culture plates in a 37 °C humidified incubator with 5% CO_2. After the formation of a confluent monolayer, cells were rinsed with phosphate-buffered saline (PBS) and detached using trypsin. An equal volume of standard media was used to deactivate the trypsin, and cells were centrifuged at 400 xg for 5 minutes. The cell suspension (10 µL) was combined with 10 µL Trypan blue (Life Technology), before cellular viability was determined by counting the cells.

Sphere Formation Assay

An in vitro sphere formation assay was performed to recognize CSCs and study their properties. According to this method, CSCs are allowed to grow as floating spheres on hydrogel matrices in serum-free media (14).

Preparation of Cell Culture Flasks Coated with Poly-HEMA

The 1.2% poly-2-hydroxyethyl methacrylate (poly-HEMA) solution was prepared by dissolving 1.2 g poly-HEMA (Sigma, USA) in 100 mL 96% ethanol and rotating overnight for the complete dissolving of the polymers. After centrifugation of the solution for 30 min at 400 × g and removing the undissolved particles, the solution was filtered by 0.22 µm filters. Each T 25 culture flask was coated with 2.5 mL poly-HEMA solution under a biosafety hood at room temperature. They were kept under UV light with no lid for at least 24 hours or until the ethanol evaporated. Eventually, the flasks were washed with PBS and stored in a 37 °C incubator for use in the future.

HT29-derived Spheres

For sphere formation, HT29 cells were suspended at 1000 cells/mL concentration and seeded into the poly-HEMA coated flask in serum-free DMEM/F12 supplemented with 1% non-essential amino acid, 2 mM L-glutamine, and 1% penicillin-streptomycin (all purchased from Sigma, USA), 10 ng/mL basic fibroblast growth factor (b-FGF), and 20 ng/mL epidermal growth factor (EGF) (last two purchased from Royan Institute, Iran). Growth factors were added every 24 hours to the medium. Cells were grown as non-adherent spheres in this condition. Primary spheres were collected by centrifugation every week and separated into single cells by 0.05% (v/w) trypsin/ethylene diamine tetraacetic acid (EDTA).

Gene Expression Study

The expression of stemness genes including *Oct4, LGR5*, *SOX2,* and *c-Myc* was evaluated by real-time PCR. For this purpose, RNA was extracted from confluent monolayers of the parental HT29

cell line and seven-day-old CSC spheres using the RiboZol™/chloroform extraction method. The RNA concentration and purity were determined using the Nanodrop ND-1000 (ThermoScientific) by measuring the absorbance at 260 and 280 nm. Then, 1 µg of RNA was converted to cDNA using the cDNA Synthesis kit (ThermoFisher Scientific) according to the manufacturer's protocol. Samples were diluted in RNAse-free deionized water and stored at -20°C. RT-qPCR was conducted using SYBR green DNA PCR master mix (Amplicon, USA) for amplifying primers (Methabion, Germany, summarized in Table 1) with the ABI real-time PCR 7500 system. *GAPDH* was used as the internal reference gene, and the relative expression of target genes were quantified using the $2^{-\Delta CT}$ method.

Flow Cytometric Analysis of CSC Markers

Parental HT29 cell monolayers were trypsinized and pelleted by centrifugation. CSC spheres were also dissociated into single-cell suspensions. Cell pellets were re-suspended in 4% paraformaldehyde and incubated at 37 ℃ for 10 minutes, then placed on ice for one minute prior to re-centrifugation. Paraformaldehyde was removed, and cell pellets were re-suspended in PBS and 0.1% sodium azide and stored at 4 ℃ prior to staining. Samples were diluted in PBS to achieve concentrations of 10^6 cells/mL. Samples were blocked in 100 µL incubation buffer for 10 minutes at room temperature prior to centrifugation and re-suspended in antibody diluted incubation buffer at the following concentration: anti-CD 44-FITC (1:30) and CD 24-PE (1:20) (BD Biosciences, USA) per 10^6 cells. All samples were protected from light and incubated at room temperature for 30 minutes. Following incubation, samples were centrifuged, washed, and re-suspended in PBS. Analysis was performed using a BD Accuuri C6 flow cytometer (BD Biosciences) and FlowJo software.

Statistical Analysis

Data were explained as the mean±standard deviation (SD) of three independent experiments for each group. The difference between groups was compared with the Mann-Whitney U test using GraphPad Prism software version 6.0 for Windows (GraphPad Software, La Jolla, CA, USA). P-values less than 0.05 signified statistically significant differences between groups.

Results

Sphere Formation from HT29 Cell Lines

Cancer stem cell isolation from HT29 parental cell lines was evaluated via a sphere formation assay in non-adherent conditions on poly-HEMA coated dishes of stem cell medium (serum-free medium). As shown in Figure 1, HT29 cells formed spheres with a round shape, smooth surface, and compact morphology without any plasma membrane projections or microvilli after three days of incubation. They became more compact and dense during five days of culture, with stable sphere architecture until seven days of cultivation. After that, spheres were degenerated and necrosed.

Increased Gene Expression of CSC Markers in HT29-derived Spheres

RT-qPCR was used to analyze the gene expression of four colorectal CSC markers, *Oct4*, *LGR5*, *SOX2,* and *c-Myc* in parental HT29 cell lines and CSCs isolated from HT29 spheres. As shown in Figure 2, the expression of all four CSC markers was significantly higher in CSCs isolated from HT29 spheres compared with parental HT29 cells ($P<0.01$ for LGR5 and *c-Myc*, $P<0.001$ for *Oct4*, and $P<0.0001$ for SOX2).

Evaluation of CSC Markers by Flow Cytometry

Some markers, such as CD 44 and CD 24, have been reported as the characteristic features of the colon CSCs. Therefore, we evaluated their expression by flow cytometry in both HT29 parental cells and HT29 spheres**.** We found that non-adherent forms of HT29 spheres contained more CSC-like cells than their parental counterparts (Figure 3).

Table 1: Quantitative reverse transcription PCR primer sequences

Gene	Primer sequence
GAPDH	
Forward	5'- CGACCACTTTGTCAAGCTCA -3'
Reverse	5'- AGGGGTCTACATGGCAACTG -3'
OCT4	
Forward	5 '- GAGAACCGAGTGAGAGGCAACC -3'
Reverse	5' - GCTGGGCGATGTGGCTGAT -3'
C-MYC	
Forward	5 '- GGAGTTGGGAGGAAGGTGAGGAA -3'
Reverse	5' - TGGTTGTGAAGGCAGCAGAAGC -3'
LGR5	
Forward	5 '- GGATGTTGCTCAGGGTGGA -3'
Reverse	5' - TAGGTAGGAGGTGAAGACGCT -3'
SOX2	
Forward	5 '- ATGTCCCAGCACTACCAGA-3'
Reverse	5 '- CCTCCCATTTCCCTCGTTT-3'

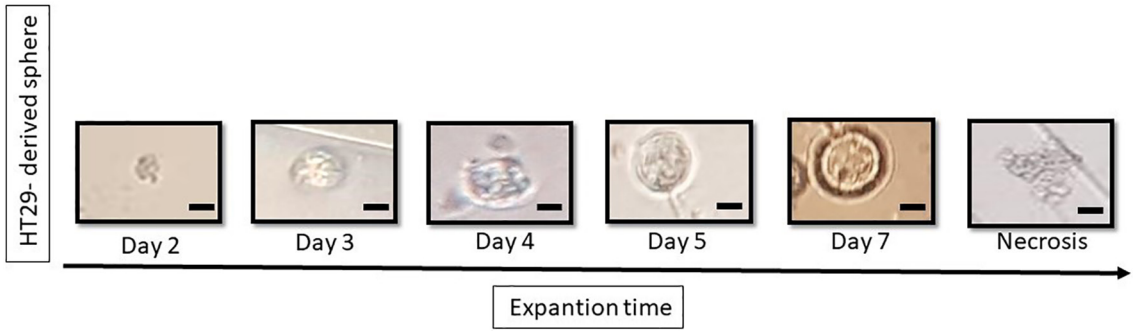

Figure 1: HT29 sphere formation over seven days. Images were taken of parental HT29 cells seeded in six-well ultra-low adhesion plates for seven days.

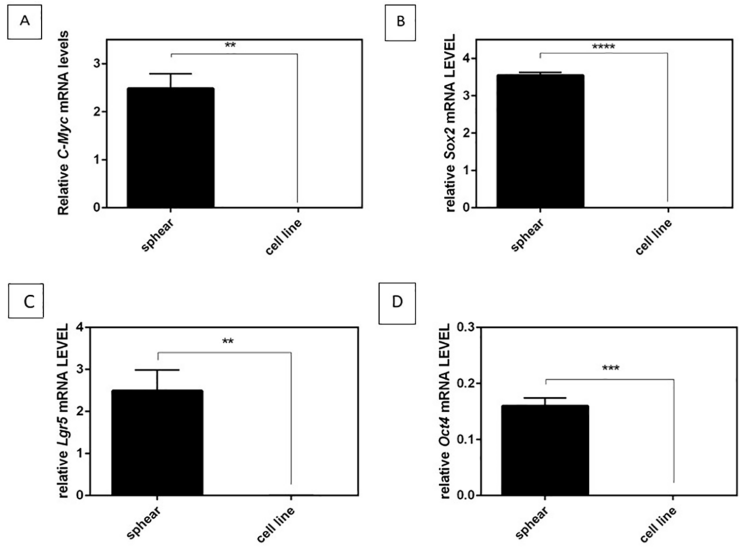

Figure 2: RT-q PCR analysis. Gene expression of four HT29 CSC markers, *C-MYC* (A), *SOX2* (B), *LGR5* (C), and *OCT4* (D), was quantified in HT29 parental cells and CSCs isolated from HT29 spheres. Target gene expression was normalized to the expression of the reference gene, *GAPDH*. *: P< 0.05, **: P< 0.01 ***: P<0.001 and ****: P<0.0001 were considered as significant.

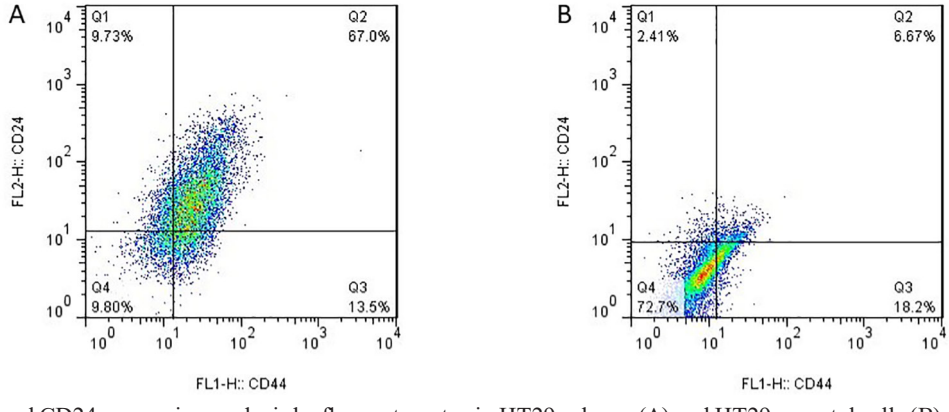

Figure 3: CD44 and CD24 expression analysis by flow cytometry in HT29 spheres (A) and HT29 parental cells (B). Both CD surface markers were more strongly present in HT29 sphere cells compared with parental HT29 cells.

The proportion of CD 24 and CD 44-expressing cells was higher in HT29 sphere cells compared with HT29 parental cells (P<0.05). There were no significant differences between the expression of CD 44 and CD 24 in HT29 parental cells (P>0.05); however, HT29 sphere cells were found to express higher amounts of CD 44 (P<0.05) (Figure 4, Table 2).

Discussion

Similar to other cancers, cancer stem cell formation plays an important role in the development of colon cancer. Cancer stem cells are formed during the clonal expansion of a single normal stem cell or progenitor tissue cells during the genetic and epigenetic transformations in the cell cycle signaling pathways (3). The critical roles of CSCs in tumorigenesis, tumor progression, invasion, and radio- and chemo-resistance highlight why they should be extensively studied (2, 6, 7).

As the first step of CSC studies, the cells should be isolated from tumor tissues or cancer cell lines. Although the model of cancer stem cells was an old idea, the first isolation of CSCs dates back to the

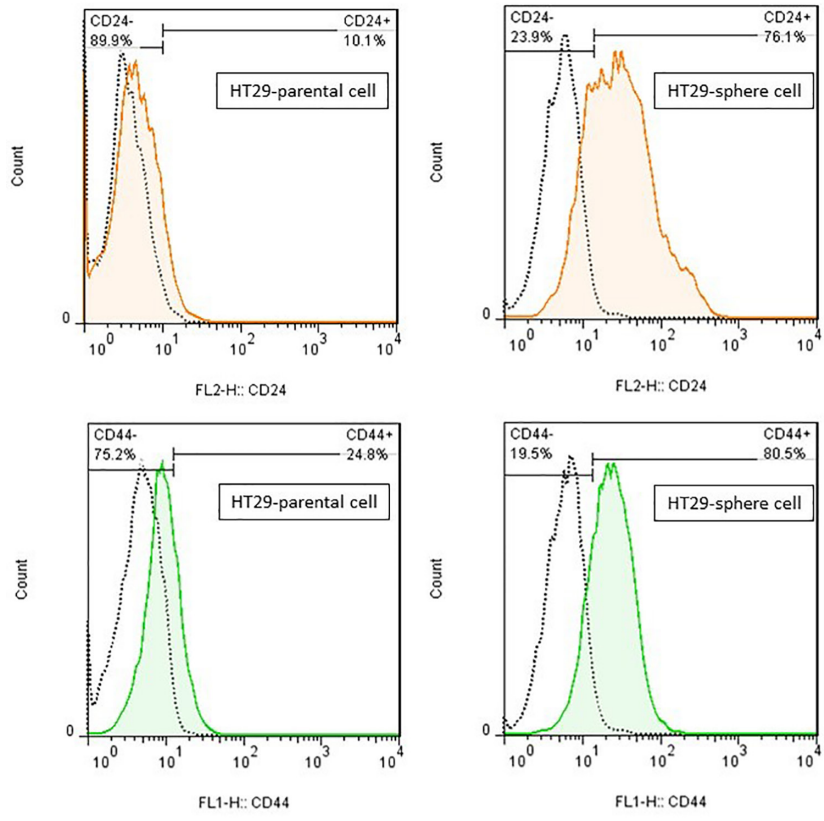

Figure 4: The sub-populations of markers associated with stemness analyzed by flow cytometry. The histograms display sub-populations of CD24 in HT29 parental cells (A), CD24 in HT29 sphere cells (B), CD44 in HT29 parental cells (C), and CD44 in HT29 sphere cells (D).

Table 2: The expression of CD24 and CD44 markers in HT29 spheres and parental HT29 cells

Cell type / Markers	HT29-Parental cells	HT29-sphere cells
CD24 (%)	10.4±1.2	76.73±0.31*
CD44 (%)	24.6±0.86	80.05±0.9*

Data are shown as mean±SD of three independent experiments. The significant upregulation of CSC markers in HT29 sphere cells compared with parental cells are presented as *P<0.05

year 1997 in AML patients and subsequently in other cancers such as colorectal cancer (5, 15).

Identification of colorectal cancer stem cells is possible through the expression of some specific cell surface biomarkers. Multiple cell surface markers can be targeted for isolation of colorectal cancer stem cells (CR-CSCs), including CD 133 (16), CD 44 (17), CD 24, CD 166 (18), *c-Myc* (19), octamer-binding transcription factor 4 (*Oct4*), homeobox protein NANOG, (20), sex-determining region YBX2 (SOX2) (21), LGR5 (22), EPCAM, ALDH1 (23), signal transducer and activator of transcription 3 (STAT3), Sal-like protein 4 (SALL4), Kruppel-like factor 4 (KLF4), ATP-binding cassette superfamily G member 2 (ABCG2) (24). However, the exact pattern of specific markers for CR-CSCs is still unknown, and the science of targeting CR-CSCs remains controversial.

On the other hand, the low efficacy of CSC isolation from tissue and cell lines is known as an important technical challenge related to CSC research (12). In this regard, this study aimed to set up the isolation and characterization of CSCs from the HT29 colon cancer cell line. Obtained results indicated successful isolation of CSCs from the HT29 cell line using sphere formation conditions described above. We also found that *SOX2*, *c-Myc*, *LGR5,* and *Oct4* are significantly overexpressed in sphere-derived HT29 cells compared with parental HT29 cells. Both CD 44 and CD 24 markers were present at higher concentrations in sphere-derived HT29 cells than parental HT29 cells. This increase was more considerable for CD 44.

Conclusion

Successful isolation of colon CSCs is critical for studies on colon cancer. These studies contribute to understanding the key roles of CSCs in colon tumorigenesis, metastasis, recurrence, and drug resistance, besides facilitating the design of novel cancer therapies. CSCs can be successfully isolated

from HT29 colon cancer cell lines using the sphere formation assay, and can be characterized by stemness markers including *SOX2*, *c-Myc*, *LGR5*, *Oct4*, CD 44, and CD 24.

Conflicts of interest: None declared.

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