Morphological and Molecular Characteristics of Spheroid Formation in HT-29 and Caco-2 Colorectal Cancer Cell Lines

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Abstract

Background: Relapse and metastasis in colorectal cancer (CRC) are often attributed to cancer stem-like cells (CSCs), as small sub-population of tumor cells with ability of drug resistance. Accordingly, development of appropriate models to investigate CSCs biology and establishment of effective therapeutic strategies is warranted. Hence, we aimed to assess the capability of two widely used and important colorectal cancer cell lines, HT-29 and Caco-2, in generating spheroids and their detail morphological and molecular characteristics.

Methods: CRC spheroids were developed using hanging drop and forced floating in serum-free and non-attachment condition and their morphological features were evaluated by scanning electron microscopy (SEM). Then, the potential of CSCs enrichment in spheroids was compared to their adherent counterparts by analysis of serial sphere formation capacity, real-time PCR of key stemness genes (*KLF4*, *OCT4*, *SOX2*, *NANOG*, *C-MYC*) and the expression of potential CRC-CSCs surface markers (CD166, CD44, and CD133) by flow cytometry. Finally, the expression level of some EMT-related (*Vimentin*, *SNAIL1*, *TWIST1*, *N-Cadherin*, *E-Cadherin*, *ZEB1*) and multi-drug resistant (*ABCB1*, *ABCC1*, *ABCG2*) genes was evaluated.

Results: Although with different morphological features, both cell lines were formed CSCs-enriched spheroids, indicated by ability to serial sphere formation, significant up-regulation of stemness genes, *SOX2*, *C-MYC, NANOG* and *OCT4* in HT-29 and *SOX2*, *C-MYC* and *KLF4* in Caco-2 spheroids (*p-value<0.05*) and increased expression of CRC-CSC markers compared to parental cells (*p-value<0.05*). Additionally, HT-29 spheroids exhibited a significant higher expression of both *ABCB1* and *ABCG2* (*p-value=0.02*). The significant up-regulation of promoting EMT genes, *ZEB1*, *Twist1*, *E-cadherin* and *SNAIL1* in HT-29 spheroids (*p-value=0.03*), *SNAIL1* and *Vimentin* in Caco-2 spheroids (*p-value<0.05*) and *N-cadherin* down-regulation in both spheroids were observed.

Conclusion: Based on enrichment of CSC-related features in HT-29 and Caco-2 spheroids, our findings suggest CRC spheroids culture as simple, cost-effective and efficient model to imitate the complexity of *in vivo* CRC tumors including self-renewal, drug resistance and invasion potential for CSC research.

Background

Colorectal cancer (CRC) is the third most common cancer and the cause of cancer related-death in both men and women worldwide [1]. Although the effective therapeutic strategies and interventions including surgery, chemo- and radiotherapy have improved the survival of patient with CRC, recurrence and metastasis are the main causes of CRC related death [2]. There has been accumulating evidence that cancers including CRC originate from tumor initiating or cancer stem cells (CSCs) [3-5]. CSCs are referred to as a sub-population of tumor cells with stem cell-like properties including self-renewal and multi-lineage differentiation capacity which are resistance to conventional therapies [6-9]. In fact, the emerging evidence has shown that deregulation of signaling pathways involved in self-renewal of normal stem

cells leads to uncontrolled development of tumor cells along with functional and proliferative tumor heterogeneity [10].

Most of challenges in cancer treatment such as treatment failure, tumor aggressiveness, relapse, metastasis and poor prognosis are related to CSCs characteristic, most of which are attributed to epithelial to mesenchymal transition (EMT) potential, stemness signaling pathways regulating pluripotency as well as high expression of ABC transporter genes in this subpopulation [11-18]. Hence, the complete regression of tumors requires identifying biological features of CSCs in *in vitro* and *in vivo* CSC models that may help to explore new approaches to target this subpopulation. There are several strategies to target CSCs including self-renewal prevention, altering drug permeability and stimulating them to differentiate [19-21]. Moreover, Signal transduction pathways and microenvironment signals in CSCs are considered as the main therapeutic aspects linked to stem-cell biology that can be targeted [13, 22, 23].

Since it is difficult to fully identify the site of CSCs accumulation and monitor their distinct biological features, the discovery and development of novel techniques for isolation and evaluation of CSCs properties can play a significant role in the clinical diagnosis and treatment of cancer. Different approaches have been applied to isolate, characterize, purify, and CSCs enrichment from various cancers [7, 24]. Immunogenic characteristics of CSCs specially as related to their specific cell surface markers such as CD133, CD44, ALDH1 and CD44 are the well-known methods which apply for isolation and characterization in early diagnosis and targeted therapy of cancer [25], while methods such as sphere formation, clonogenic growth and drug resistance assays lead to functional isolation of CSCs [25]. Therefore, development of feasible and applicable models for enrichment and assessment of CSCs and their characteristics are warranted. Cell-based assays are suitable and valuable tools to obtain information regarding cellular and molecular mechanisms involved in CSCs biology and explore new therapeutic strategies for targeting of this sub-population [24, 26].

It is well accepted that micro tumor structures formed as spheroids are able to present the main characteristics of *in vivo* tumors such as cellular heterogeneity, hypoxia and pH rate, exposure to nutrients and metabolites, cell-cell cohesion and interaction, physical and chemical stresses, and gene expression profiles [27]. Therefore, spheroids with their particular architecture and biology can serve as reliable cancer models in different areas of cancer research including tumor microenvironment modeling and matrix remodeling, migration and invasion, drug discovery and screening, immune interactions and angiogenesis, etc. [26, 28, 29]. There are different systems of spheroid formation including suspension culture, non-adherent surface, hanging drop and microfluidic methods. Spheroids culture as serum free and non-adherent condition is the most frequent and powerful technique to obtain CSC-like properties and expand CSCs that due to the presence of mathematical/testable approaches established based on their structure destruction decrease underestimated results in clinical application and guarantees reproducibility [30, 31]. CRC cell lines have derived from different CRC subtypes and show different phonotypic and molecular properties in *in vivo* and *in vitro* studies [32, 33]. For example, despite similar epithelioid phenotype of HT-29 and Caco-2 cells, HT-29 cells are invasive and metastatic *in vivo*, but not *in*

vitro, whereas, Caco-2 cells are noninvasive [34]. Moreover, the different cell lines can generate spheroids with differences in morphological feature, capacity of CSCs enrichment and CSC characteristics-related gene expression profiles. To overcome these obvious limitations, it is necessary to determine the best-matching cell line for CSC studies through evaluation and comparison their-derived spheroids in terms of stem cell-like characteristic.

Considering these points together, this study aim to gain insight into the biological features of two different types of colonospheres derived from two colorectal cancer cell lines including HT-29 and Caco-2 and assess whether these spheres are suitable models for CSC enrichment as well as evaluation of CSC-related characteristics in terms of gene expression profile and morphological features in compared to their 2D parental cells. Hence, we compared the expression level of genes related to the CSCs properties including genes implicated in stemness, EMT and drug resistance in HT-29 and Caco-2 human colorectal carcinoma (CRC) cell lines grown in spheroids versus 2D cell culture conditions. We applied simple, inexpensive and time-consuming methods for spheroid formation without using special scaffold or biochemical materials.

Materials And Methods

Cell lines and culture conditions

HT-29 and Caco-2 human colon adenocarcinoma cell lines were obtained from the Iranian Biological Research Center (IBRC, Tehran, Iran) and were tested for spheroid formation. Cells were cultured in DMEM/High glucose medium (Gibco, Germany) supplemented with 10% fetal bovine serum (FBS, Gibco, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin antibiotics (Biowest, France), 1% non-essential amino acid (Gibco, Germany) and 2 mM L-glutamine (Gibco, Germany). Cultures were maintained under standard cell culture conditions in 37°C, 5% CO2 and 95% humidified incubator and passaged in 70-90% confluence.

Preparation of poly-HEMA coated cell culture dishes

To prepare the 1.2% poly-HEMA (Poly-2-hydroxyethyl methacrylate) solution, 1.2 g poly-HEMA (Sigma, USA) was dissolved in 100 mL 96% ethanol by rotating overnight to dissolve the polymers completely. The solution was centrifuged for 30 min at 400 × g to remove unsolved particles and then filtered by 0.22 µm filters. The tissue culture dishes were coated with poly-HEMA solution (1.2 mL per six well plate or 2.5 mL per T25 tissue culture flask) under the biosafety laminar flow hood at room temperature for an overnight to evaporate ethanol completely. Finally, the plates were washed with PBS and stored at 37°C incubator for future use.

Spheroids culture

HT-29 and Caco-2 spheroids were generated by two types of spheroid culture systems; the hanging droplet technique or as free-floating spheroids cultured on poly-HEMA coated dishes. For hanging droplet

spheroids, the cells were detached with 0.05% trypsin/EDTA (Gibco, Germany) and after trypsin inactivation, the resulted single cells were washed twice with PBS and pre-warmed serum free media. Dissociated single cells re-suspended as five or ten thousand cells per 25µL of serum free medium (DMEM/F12, Gibco, Germany), which was supplemented with 20 ng/mL epidermal growth factor (EGF, PeproTech, USA), 10 ng/mL of basic fibroblast growth factor (bFGF, PeproTech, USA), 2% B27 supplement (Gibco, Germany), 1% non-essential amino acid, 2 mM L-glutamine and 1% penicillin-streptomycin. About 60×25µL cellular drops were dispensed on the inverted lids of the 9 cm petri dishes and the lids carefully set on the dishes which had been pre-filled with five mL of PBS to assure high humidity and incubated for 96 hours. In the next step, the droplets were washed with 2 mL of media by gentle shaking and formed spheroids were transfer onto poly-HEMA coated dishes for an additional six days. For free-floating spheroids, single-cell suspensions were seeded into poly-HEMA coated dishes at different cell densities (1-5×10^5 cells/mL) in the serum free medium as described above and were cultivated for up to 10 days. The culture media was supplemented with additional 2% B27, bFGF and EGF every other day.

Secondary sphere formation assay

To examine the ability of colonospheres to generate the next generations, spheroids were harvested and dissociated enzymatically with trypsin/EDTA and mechanically by gently pipetting. The resulting single cells, after counting, were re-plated in serum free spheroid medium at the same densities and culture conditions as mentioned above for three sequential passages (P1-P4).

Scanning electron microscopy

Spheroids were collected by centrifugation for 1 min at 100 g and supernatant were carefully aspirated. Then, the collected spheroids were washed with PBS and fixed with 2.5% (v/v) glutaraldehyde in PBS for 30 min at room temperature. After fixation, spheroids were washed once with PBS and dehydrated using ethanol series (50, 65, 75, 85, and 100%). The samples were sputter-coated with gold–palladium and examined in scanning electron microscope (SEM, Seron Technology, AIS-2100, Korea).

Quantitative real-time PCR analysis

The following genes were selected and examined by real-time PCR: stemness genes; *KLF4*, *OCT4*, *SOX2*, *NANOG* and *C-MYC*, EMT genes; *Vimentin*, *SNAIL1*,*TWIST1*, *N-Cadherin*, *E-Cadherin* and *ZEB1*, ABC transporter genes; *ABCB1*, *ABCC1* and *ABCG2*. Spheroids were harvested a day before structural disintegration (day 10 for HT-29 and day 4 for Caco-2 spheroids). The total RNAs were then extracted from parental and spheroid cells using RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. After measurement of RNA quantity and quality by Nanodrop (ThermoFisher Scientific, USA), cDNA were synthesized with 1 µg of total RNA using cDNA synthesis kit (GeneAll, Korea). Real-time polymerase chain reaction (RT-qPCR) was performed using the SYBR Premix Ex Taq II real-time PCR kit (TaKaRa, Japan) on the Rotor-Gene Q LightCycler (Qiagene, Germany) with the following conditions: 40 two-step amplification cycles of 95°C for 5 seconds and 60°C for 30 seconds. The relative

expression values of target genes were quantified relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), as the internal reference gene, by using the $2^{-\Delta CT}$ method. Real-time PCR primers are listed in Table 1.

Flow Cytometry

Flow cytometry were used to quantify the percentage of CSC markers expression in HT-29 and Caco-2 spheroid cells compared to parental cells. The parental and spheroid cells from each cell line were dissociated with trypsin/EDTA and were washed with PBS twice. The dissociated cells were counted using Trypan blue exclusion assay, and if cell viability was more than 95%, they were evaluated for CSC markers expression. The following antibodies were used: anti-CD44 (1:30), anti-CD133 (1:300), anti-CD166 (1:90) (all from abcam, USA). All antibodies were incubated with 3×10^5 cells for 30 minutes at 4°C. Goat anti-rabbit IgG-FITC (1:100) (Santa Cruz biotechnology, USA) was used as secondary antibody. The percentage of CSC marker positive cells were analyzed using an Atuune NxT flow cytometer (Thermo Fisher Scientific, USA) and data were analyzed using FlowJo VX software.

Statistical analysis

Data were reported as the mean ± standard deviation for each group from three or four independent experiments. The diameter of the spheroids was measured using Image J software (n=25) (IJ 1.46r version, NIH, USA). Student's t-test was used to compare the difference between the control (parental) and spheroids groups using GraphPad Prism version 8.0 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com). A *p-value < 0.05* was accepted as statistically significant difference between groups.

Results

HT-29 and Caco-2-derived spheroids exhibit different spheroidization time and morphological characteristics

First, we tested whether HT-29 and Caco-2 cell lines are able to generate 3D spheroids. Spheroid formation in non-adherent condition on poly-HEMA coated dishes at different cell densities was applied for generation of spheroids from HT-29 and Caco-2 adherent cells. Both cell lines could form spheroids, while HT-29 spheroids were unstable and their structure started to disintegrate after three days (data not shown). Hence, we used hanging drop method as well-established technique to spheroid formation from HT-29 cells. As observed from the Fig.1, both cell lines formed 3D spheroids in serum free media using hanging drop method for HT-29 and free floating culture in non-adherent condition for Caco-2. Phase contrast microscope images from spheroids revealed different growth patterns; HT-29 cells formed spheroids with round-type, smooth surface and compact morphology after 96 h incubation as hanging drops and became more compact and dense during ten days of culture (Fig. 1b and c). By contrast, Caco-2 cells started spontaneous formation of round-shape structure and after cultivation for three days; they formed hollow spheroids with bubble like structures (Fig. 1e and f). Furthermore, Caco-2 spheroids were

significantly smaller than HT-29 spheroids after four days of incubation (*p-value <0.01*). The average diameter of Caco-2 spheroids was 66.9±14.88 µm compared to 82.52±22.56 µm in HT-29 spheroids (Fig. 2).

However, Caco-2 cells exhibited shorter spheroidization time, two days following plating in compared to four days in HT-29, whereas HT-29 spheroids were more stable and maintained compact spheroid architecture even up to 12 days of cultivation. In contrast, Caco-2 spheroids remained stable for five days. HT-29 spheroids showed greater increase in diameters over time than Caco-2 spheroids. Whereas, the spheroids derived from Caco-2 cells were slowly growing in size and displayed more stable diameter and less variability in size.

In order to assess spheroid morphological parameters in more details and increase the spatial resolution, micrographs of spheroids were acquired by SEM. Both HT-29 and Caco-2 colonospheres showed continuous and smooth surface without any plasma membrane projections or microvilli so that it was hard to distinguish individual cells. Interestingly the hollow structures were observed in many of Caco-2 spheroids that were its obvious difference compared to HT-29 spheroids with conglomerated appearance (Fig. 3a-c).

Enrichment of cancer stem cell-like sub-populations in generated spheroids

To investigate CSC enrichment in formed spheroids as compared to their adherent counterparts, we applied three separate techniques: (1) secondary sphere formation capacity whereby self-renewal properties of CSCs are exploited for maintaining stem-like features of cancer cells; (2) assessment of key stemness genes expression including *SOX2*, *C-MYC*, *OCT4*, *KLF4* and *NANOG*, as master regulators of pluripotency and self-renewal capacity in CSCs; and (3) quantifying the expression of potential CSCs surface markers by flow cytometry.

A secondary sphere formation ability of generated spheroids was investigated by serial passaging. Seeding of single cells from early passage of spheroids showed that spheroids derived from both HT-29 and Caco-2 cells had still the capacity to generate spheroids, even after four sub-cultures (Fig. 4a-h). Thus, these data suggest that spheroids maintain their self-renewal capacity over several passages. Furthermore, RT-qPCR analysis confirmed a definitive upregulation in most of examined stem cell-related genes in both HT-29 and Caco-2 spheroids as compared to the 2D monolayers (Fig. 5a and b). HT-29 spheroids displayed the significant upregulation of *SOX2*, *C-MYC, NANOG* and *OCT4* (*p-value <0.03*) stemness genes compared to HT-29 parental cells with highest expression level of *NANOG* (*p-value <0.03*) (Fig. 5a). Whereas, Caco-2 spheroids showed significant high expression of *SOX2*, *C-MYC* and *KLF4* in compared to Caco-2 parental cells with highest expression of *SOX2* (*p-value <0.029*) (Fig. 5b).

To further corroborate these findings, the expression of putative CSC surface markers CD166, CD44 and CD133 were investigated. Similar to our observations from stemness genes expression analysis, flow cytometry analysis also revealed that the expression of CD166, CD44, CD133 CSC markers were elevated in both spheroid models compared to differentiated 2D cultures which was statistically significant

(CD166, CD44, CD133 (*p-value <0.03*) in HT-29 spheroids and CD166 (*p-value <0.0008*), CD44 (*p-value <0.0004*) and CD133 (*p-value <0.03*) in Caco-2 spheroids) (Fig. 6a and b) (Table 2).

CSC-like enriched spheroids exhibited high expression of multi-drug resistant (MDR) genes

Resistance against chemotherapeutics is another key feature of CSCs that is mediated by a family of ATP-binding cassette (ABC) proteins. Accordingly, we assessed the expression pattern of a panel of major multi-drug resistant (MDR) genes; *ABCB1*, *ABCC1* and *ABCG2* in generated spheroids compared to parental cells. Specifically, HT-29 derived spheroids exhibited a significant higher expression of both *ABCB1* and *ABCG2* compared to HT-29 parental cells (*p-value=0.02*). The expression of *ABCC1* was also increased in HT-29 derived spheroids than their parental cells, although this increase was not significant (*p-value=0.14*) (Fig. 7a). In spite of the ABC expression pattern of HT-29 spheroids, Caco-2 spheroids did not show any significant differences in expression patterns of *ABCB1*, *ABCC1* and *ABCG2* compared to Caco-2 parental cells (*p-value>0.99*) (Fig. 7b).

CRC spheroids were driven toward the EMT compared to 2D adherent cells

To characterize the EMT properties in generated spheroids, we next compared the expression profile of EMT inducer genes including *Twist1*, *SNAIL1*, ZEB1, Vimentin, *E-cadherin* and *N-cadherin* in both spheroids and parental cells. The expression of EMT-promoting genes; *ZEB1*, *Twist1* and *SNAIL1* were significantly up-regulated in HT-29 spheroids compared to 2D monolayer cultures (*p-value=0.03*). Unexpectedly, the expression of *E-cadherin* was up-regulated in HT-29 spheroids (Fig. 8a). A significant up-regulation of *SNAIL1* and *Vimentin* were also observed in Caco-2 spheroids than their parental cells (*p-value<0.05*). Although *ZEB1* showed a higher expression in Caco-2 spheres than 2D counterparts, this upregulation was not statistically significant (*p-value>0.05*) (Fig. 8b). On the contrary, the expression of *N-cadherin* was down-regulated in both HT-29 and Caco-2 spheroids compared to parental cells (Fig. 8).

Discussion

To overcome the treatment failure due to the cancer recurrence and metastasis related to CSCs biological features and their interference with intrinsic drug resistance mechanisms, design of appropriate preclinical models is one of the most pressing issue to test targeted therapies [35, 36]. Identification of the ideal tools to clarify CSC biology is still proceeding, and great efforts have been paid to improve isolation and treatment modalities in CSCs research [37]. Spheres culture in which CSCs are trapped and enriched, has been recommended as an extremely effectual CSC isolation method for cancer cell lines and solid tumors [37-39]. Such an anchorage independent sphere forming system under non-adherent, nutritional deficient and serum free conditions prevents the differentiation of stem-like cells and leads to eliminate non-CSCs cells through apoptosis [40-42]. The superiority of this method is based entirely on the intrinsic properties of CSCs and leads to enriching the CSC subpopulations regardless of the expression patterns of cell surface markers [43, 44]. Moreover, spheroid cultures offer an ideal platform for routine toxicity and drug efficacy testing to determine safe exposure doses in designed cellular models [45-48]. In addition, previous studies have shown that different cancer cell lines form spheres with

distinctive morphological and functional features based on sphere-formation method [49-51]. Hence, the status of morphological characteristics, CSC properties and genes expression profile of EMT and drug resistance is important in selecting appropriate model.

In this regard, in the current study, the forced floating using low attachment plates and hanging drop methods, as more applicable and feasible methods were used to generate CSC-enriched spheroids from Caco-2 and HT-29 cell lines, respectively. We chose these two cell lines because of their widespread use in research and drug discovery. Caco-2 cell line is widely used in pre-clinical investigations, drug permeability and solubility studies and nanoparticle translocation. The cell line is accepted by the U.S. Food and Drug Administration (FDA) to support new drug applications. In addition both Caco-2 and HT-29 cells are widely applied in the study of intestinal transport and predict bioavailability [52-54].

To generate spheroids serum-free condition was applied to prevent the differentiation of stem-like cells and elimination of non-CSCs population using suspension culturing in low attachment plates [55-58]. Although, both cell lines showed capacity to form spheroids, the HT-29 cells generated loose and disintegrating spheres in poly-HEMA plates, whereas, they formed compact spheroids when were cultured as hanging drops. The comparison of morphological and molecular characteristics of Caco-2 and HT-29 derived spheroids has been summarized in Table 3.

Generation of dense and practical spheroids strongly depend on cellular interactions through the gap junctions between single cells [59, 60]. Hence, hanging drop method was selected to generate HT-29 spheroids with compact structure due to its advantages in improvement of cells accumulation and adhesion potential with least damage to the spheres through gravitational force. Our observation was in line with the previous study in rat pancreatic beta cells which hanging drop method enhanced connexin protein accumulation and generation of tight spheres [60]. Moreover, murine and human brain tumor cell lines showed more consistent structures in brain tumor spheroids with applying of hanging drop method compared to spinner culture [61]. Therefore, it seems that different cell lines can be compatible with different spheroid formation methods, as in our study as well; hanging drop and low attachment poly-HEMA coated plates were suitable, rapid and time efficient for spheroid formation from HT-29 and Caco-2 cells, respectively. Some limitations of hanging drop including possible shattering of spheroids due to mechanical shock in transferring of spheroids to the conventional culture plates and high spheroid-diameter variation compared to low attachment culture can lead to changes in initiate characteristics in HT-29 spheroids. Whereas, forced floating method using low attachment culture with more reproducibility than hanging drop could be the most suitable method for high-through put screening. Therefore, appropriate cells and spheroid formation methods must be accurately chosen based on aim of study and tumor type.

Although both spheroids derived from HT-29 and Caco-2 cells exhibited similar roundness and smooth surfaces, the light and electron microscopic examination revealed some differences. The Caco-2 spheroids presented hollow core structure as distinct morphological feature which has been reported before by others [62, 63]. Samy et al, also found that after 5 days culturing in Matrigel, the Caco-2 cells

self-organized into intestinal epithelial like cells as spheroids with a confluent monolayer surrounding a hollow lumen [64]. This hollow structure in Caco-2 cells is account to the functional polarization through E-cadherin-dependent cell-cell adhesion [65, 66]. Since the hollow core is the common feature of the Caco-2 spheres formed by different methods [62, 63], it can inference that this feature is cell line dependent and may not related to the sphere formation methods.

The Caco-2 spheroids features in expression of drug transporters similar to human intestinal and also their ability to recreate the spatial organization similar to intestinal epithelial cells have been made them as an improved platforms in drug screening [64, 67, 68]. However, Caco-2 spheroids with round shape and hollow lumenized morphology have widely employed as a reproducible *in vitro* model for studying intestinal features and functions as well as intestinal drug metabolism and uptake [64, 69], they are not compatible with anti-cancer drug researches which are based on evaluation of the spheroid size reduction and drug penetration into inner layers. The roundness and smooth surface of spheroids which hide individual cells, is ascribed to high ECM secretion and strong cell-cell adhesion and could help analysis of drugs efficacy more accurately [49, 70]. Accordingly HT-29 spheroids with compact round shape may be more suitable platforms for anti-cancer drug testing in CRC than Caco-2 spheres. In general, in order to create more practical and functional CRC spheroid models, it is essential to characterize the gene expression alterations, invasion, and drug transporters in spheroids to standardize them based on research requirements [61, 64, 71-73]. Hence, CRC spheroid models were further assessed to determine their potential in enrichment of CSCs-related characteristics, including the cell surface marker patterns, serial sphere formation capability, and gene expression profiles of multipotency, EMT and drug resistance transporters compared to the parental cells. Our findings showed that the expression levels of stemness genes could be affected by CRC spheroid culture, and both spheroids displayed similar high expression levels of pluripotent stem cell genes (*KLF4*, *OCT4* and *C-MYC*) when compared to their parental counterparts, while drastic higher expression level of *SOX-2* and *NANOG* was found in Caco-2 and HT-29 spheres, respectively. It has been also documented that the overexpression of *NANOG* as an oncogene along with *OCT4* is a prominent characteristic of CSCs and is associated with EMT transition of CSCs and drives tumor progression and poor prognosis in patients with breast and colorectal cancer [74-79]. Furthermore, many studies have proved that *SOX-2* overexpression is correlated with self-renewal capacity, a poorly differentiated-aggressive phenotype and clinicopathological characteristics of CRC patients [80-82]. Therefore, high expression levels of stemness genes, specially, *SOX2* and *NANOG* that were observed in our CRC spheroids could be served as an indicator of self-renewal potential.

We extended our study by assessing the expression of CSC surface markers in CRC spheroids compared to the parental cells. Our findings independently corroborated the expression profiles of stem cell-related genes which evidenced by enriching the CD166, CD44, CD133 positive populations of CSCs in CRC spheroids. This was in line with other reports in various cancers, where non-adherent spheroid cultures possessed more characteristics of CSCs [44, 83-86]. Moreover, serial sphere formation which has been applied as an appropriate platform for long-term expansion of cells with self-renewal capacity and imitates the tumor heterogeneity [24], was maintained in CRC spheroids cells during the long-term

cultures. Thus, these results further advocated the efficiency of the serum free and non-adherent condition in enrichment of CSCs.

The complexity of cross-talks between stem cell–related genes and EMT are still unclear and need to be more clarified. To determine the association of the EMT genes expression level with the enriched CSC nature in spheroids, we compared the expression of *Twist1*, *Snail1*, *ZEB1*, *Vimentin*, *E-cadherin* and *N-cadherin* in spheroids than parental cells. Our results showed that in spite of up-regulation of *Twist1*, *Snail1*, *ZEB1* and *Vimentin*, unexpectedly *E-cadherin* was up-regulated and *N-cadherin* was down-regulated in spheroids which are in contrast to the results of EMT process from other similar studies [87-90]. Several reports suggested the strong correlation of E-cadherin down-regulation and *N-cadherin* up-regulation as the main hallmark of EMT [77, 87-91]. It can postulate that the expression level of EMT genes, might be varied widely due to the absent of definite correlation between the gene transcripts levels and their corresponding proteins and do not necessarily reflect their protein levels in CSCs [92-96]. In addition, Jolly, Jia et al. displayed that the partial EMT is associated with stemness [97], deciphering the coupling of EMT and stemness needs further investigations.

The increased expression of ATP-binding cassette (ABC) transporter genes such as *ABCB1*, *ABCC1*, and *ABCG2*, as other CSC-related characteristic is involved in regulation of self-renewal and multidrug resistance (MDR) in ovarian and colon cancer cell lines [98-103]. In agreement with Collura et al. study [104], our results also demonstrated significant increased expression of *ABC B1* and *G2* genes in HT-29 spheres compared to monolayers and Caco-2 spheres which further verified the CSCs enrichment.

Conclusions

In summary, we present here the first study demonstrating CSC-enrichment in Caco-2 cells without using special scaffold or biochemical materials. Despite the hollow core structure and no increase expression of ABC transporter genes in Caco-2 spheroids, our findings indicated that spheroids culture from both HT-29 and Caco-2 cell lines is capable to enhance the expression of genes involved in CSCs regulation. It enables us to recreate the complexity of *in vivo* tumors including presence of CSCs subpopulation, resistance to chemotherapeutics and invasion potential and should be considered as more realistic CRC *in vitro* models for further investigation in CSC research.

Abbreviations

CRC: Colorectal Cancer; CSCs: Cancer Stem-Like Cells; SEM: Scanning Electron Microscopy; EMT: Epithelial-to-Mesenchymal Transition; poly-HEMA: Poly-2-Hydroxyethyl Methacrylate; EGF; Epidermal Growth Factor; bFGF: Basic Fibroblast Growth Factor; RT-qPCR; Real-Time Polymerase Chain Reaction; *GAPDH*: Glyceraldehyde-3-Phosphate Dehydrogenase; MDR: Multi-Drug Resistant; ABC: ATP-Binding Cassette; FDA: Food and Drug Administration.

Declarations

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Authors' contributions

Zahra Madjd and Roya Ghods designed and supervised the project, and approved all parts of the manuscript. **Fatemeh Atyabi** designed and supervised the project. **Elmira Gheytanchi and Marzieh Naseri** planned and performed the experiments, analyzed and interpreted the data, and wrote the manuscript. **Feridoun Karimi-Busheri** critically revised the manuscript. **Ensie Sadat Mirsharif** performed the flow cytometry. **Mahmood Bozorgmehr** analyzed the flow cytometry data.

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Availability of data and materials

The analyzed data during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All procedures performed in this study were in line with the ethical standards of the institution at which this study was conducted.

Consent for publication

All listed authors discussed results and commented on the submitted manuscript.

Competing interests

The authors declare no conflict of interests.

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Tables

Table1. Primers used for quantitative RT-PCR.

Genes groups	Gene name	Primer Sequence (5´→3´)
Housekeeping gene	*GAPDH*	F-CATGAGAAGTATGACAACAGCCT
		R-AGTCCTTCCACGATACCAAAGT
Stemness genes	*C-MYC*	F-ACACATCAGCACAACTACG
		R-CGCCTCTTGACATTCTCC
	Klf4	F-CCTCGCCTTACACATGAAGAG
		R-CATCGGGAAGACAGTGTGAAA
	SOX2	F-AATGGGAGGGGTGCAAAAGAGG
		R-GTGAGTGTGGATGGGATTGGTG
	NANOG	F-AGCTACAAACAGGTGAAGAC
		R-GGTGGTAGGAAGAGTAAAGG
	OCT4-A	F-GTGGAGAGCAACTCCGATG
		R-TGCAGAGCTTTGATGTCCTG
EMT genes	*Vimentin*	F-TCTACGAGGAGGAGATGCGG
		R-GGTCAAGACGTGCCAGAGAC
	Snail1	F-CCAGAGTTTACCTTCCAGCA
		R-GATGAGCATTGGCAGCGA
	Twist-1	F-TTCTCGGTCTGGAGGATGGA
		R-CCACGCCCTGTTTCTTTGAAT
	N-Cadherin	F-GCCCAAGACAAAGAGACCC
		R-CTGCTGACTCCTTCACTGAC
	E-Cadherin	F-CAGGAGTCATCAGTGTGGT
		R-GGAGGATTATCGTTGGTGTCAG
	Zeb1	F-CTTCTCACACTCTGGGTCTTATTC
		R-CGTTCTTCCGCTTCTCTCTTAC
ABC Transporte	*ABCG2*	F-TTCCACGATATGGATTTACGG
		R-GTTTCCTGTTGCATTGAGTCC
	ABCB1	F-GTTCAGGTGGCTCTGGATAAG
		R-AGCGATGACGTCAGCATTAC
	ABCC1	F-CGCCTTCGCTGAGTTCCT

	R-TGCGGTGCTGTTGTGGTG

Table 2. The expression percentage of CD44, CD166, and CD133 common CRC-CSCs markers in spheroids compared to their parental cells.

	HT-29		Caco-2	
Cell types / Markers	**Parental**	**Spheroid**	**Parental**	**Spheroid**
CD44%	1.80±0.77	19.3±6.60*	1.59±0.28	41.05±3.53***
CD133%	1.35±0.19	11.75±4.69*	1.25±0.45	28.25±17.45*
CD166%	1.66±0.37	22.40±5.92*	1.78±0.18	37.15±4.02***

Data are shown as mean ± SD of four independent experiments. A significant upregulation of CSC markers in spheroids compared to parental cells are presented as * = P<0.05, *** = P<0.001, ns = not significant.

Table 3. The comparison of morphological and molecular characteristics of Caco-2 and HT-29 derived spheroids.

Characteristic \ Cell type	Caco-2	HT-29
Sphere formation method	Free floating (suspension) culture	Hanging drop method
Complexity of setup	Simple (spontaneous formation)	Moderate
Cost and reproducibility	• Low cost • High reproducibility	• Low cost • High reproducibility • Time consuming and extensive handling necessary
Spheroid formation rate (spheroidization time)	3 days	4 days
Spheroid structure maintenance	Moderate (stable for 5 days)	High (stable up to 12 days)
Morphological characteristics	Round-shape, hollow structures with bubble like structures	Round-shape, smooth surface and compact morphology
Secondary sphere formation ability	Yes	Yes
Spheroid size (average)	Medium (66.9±14.88 µm)	Large (82.52±22.56 µm)
CSC enrichment capacity **Up-regulated stemness genes** **CSCs surface markers up-regulation**	Yes *SOX2, C-MYC, KLF4* ++	Yes *SOX2, C-MYC, OCT4, NANOG* +
MDR genes up-regulation	No	Yes (*ABCB1 and ABCG2*)
Up-regulated EMT genes	*SNAIL1, E-cadherin, Vimentin*	*Twist1, SNAIL1, ZEB1, E-cadherin*

Figures

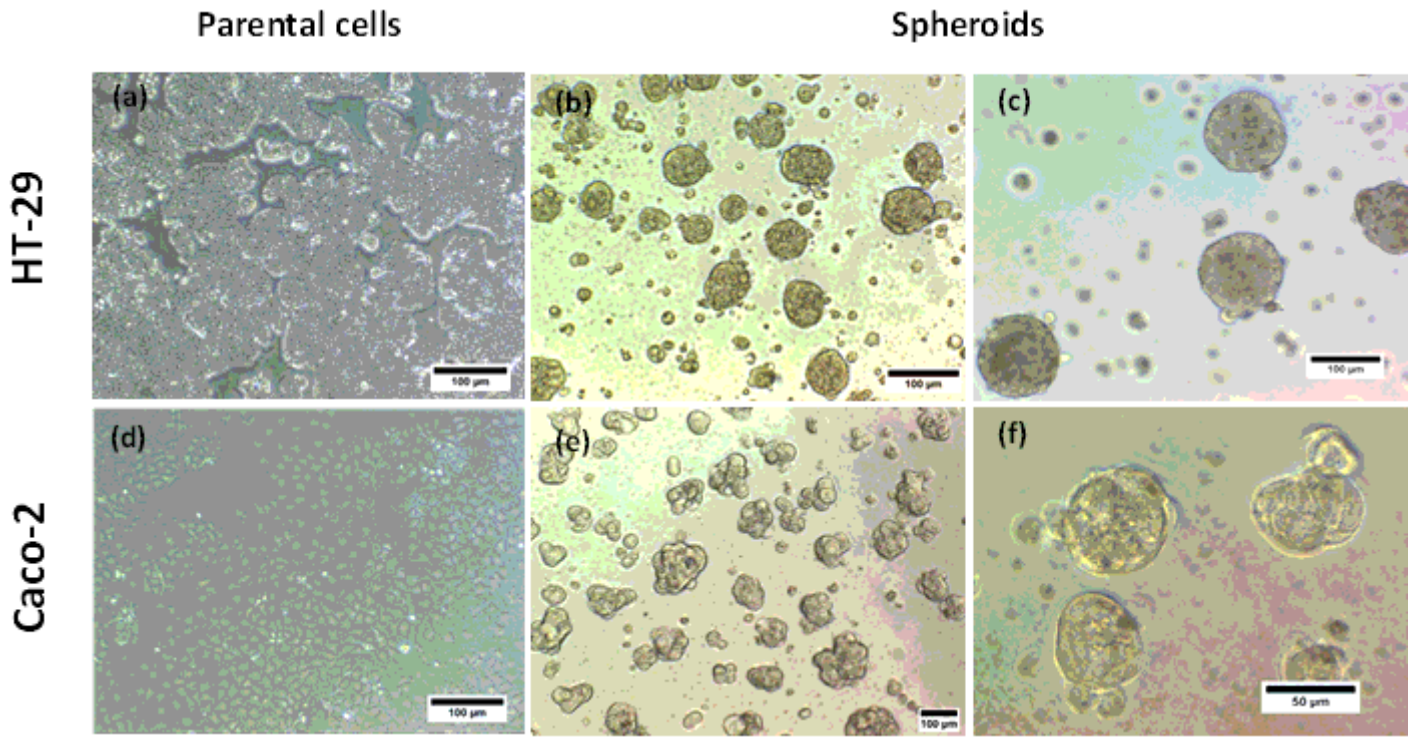

Figure 1

Morphology of HT-29 and Caco-2 parental adherent monolayer cells and their derived spheroids. (a) Parental HT-29 cells grew as an adherent monolayer (b and c) HT-29 derived spheroids cultured at nonadherent and serum free condition showed well-round shape and compact morphology. (d) Caco-2 parental cells as monolayer and, (e,f) Caco-2 derived spheroids displayed small and round shape morphology.

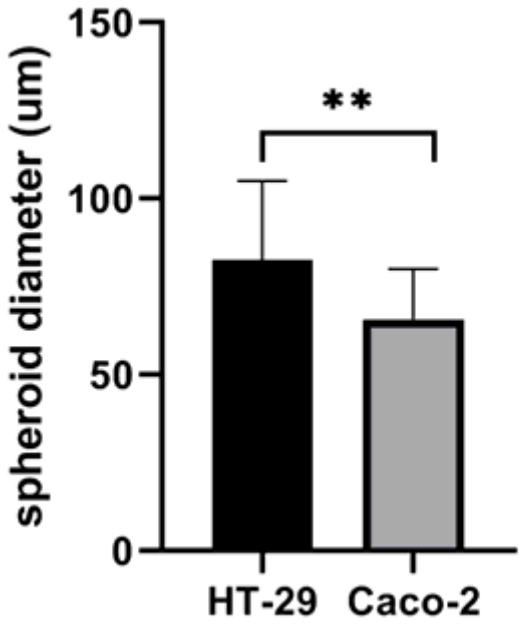

Figure 2

Size comparison of HT-29 and Caco-2 derived spheroids. Caco-2 spheroids showed significantly smaller size in compared to HT-29 spheroids. Data are presented as mean ± SD as ** = P<0.01, (n=25).

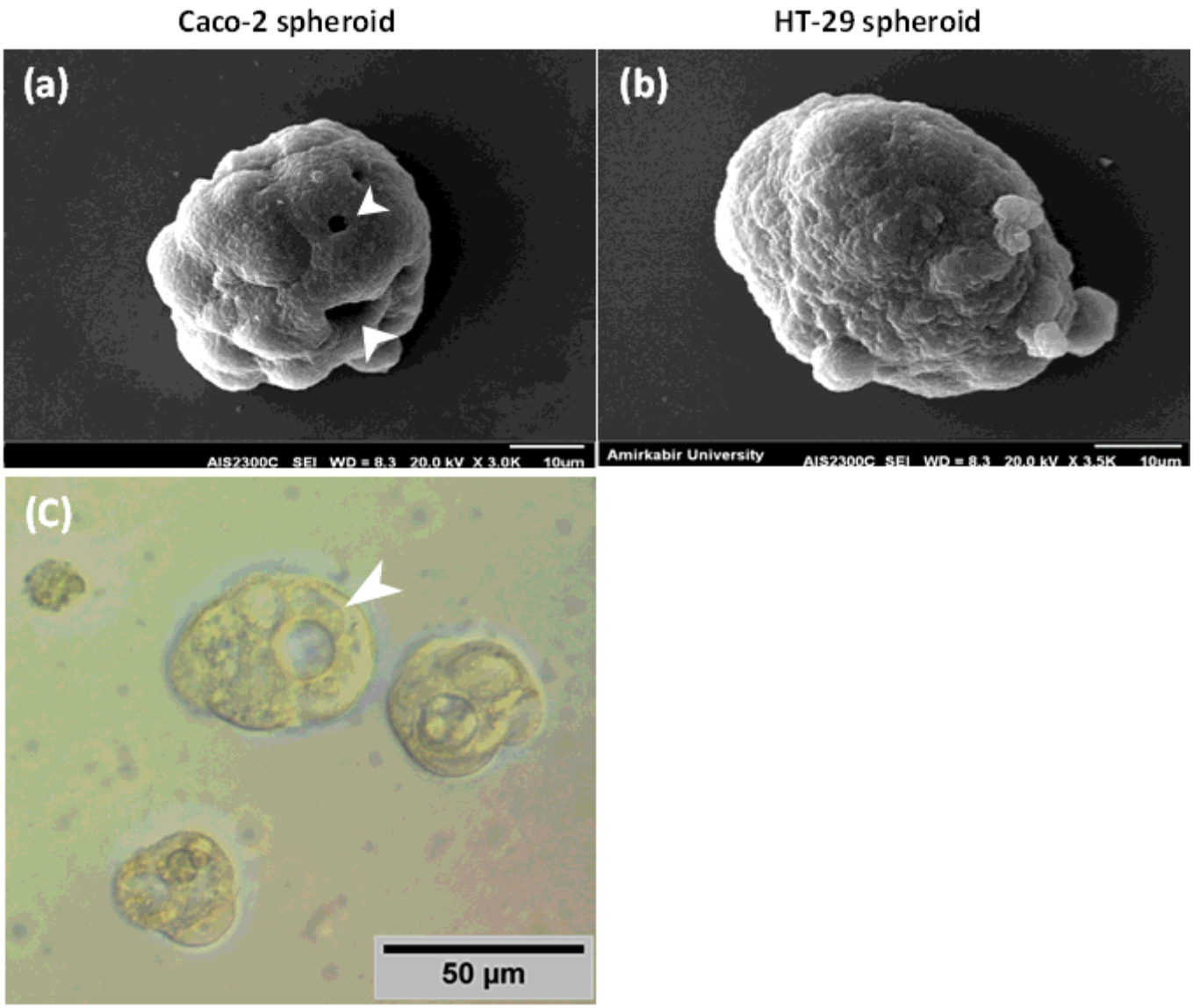

Figure 3

Scanning electron microscope (SEM) imaging of HT-29 and Caco-2 derived spheroids. Representative SEM images of spheroids show roundness structure and smooth surfaces of both (a) Caco-2 and (b) HT-29 derived spheroids so that it is not possible to distinguish of individual cells. (c) Representative phase contrast images of hollow core in Caco-2 derived spheroids. Arrows indicate hollow core of Caco-2 spheroids.

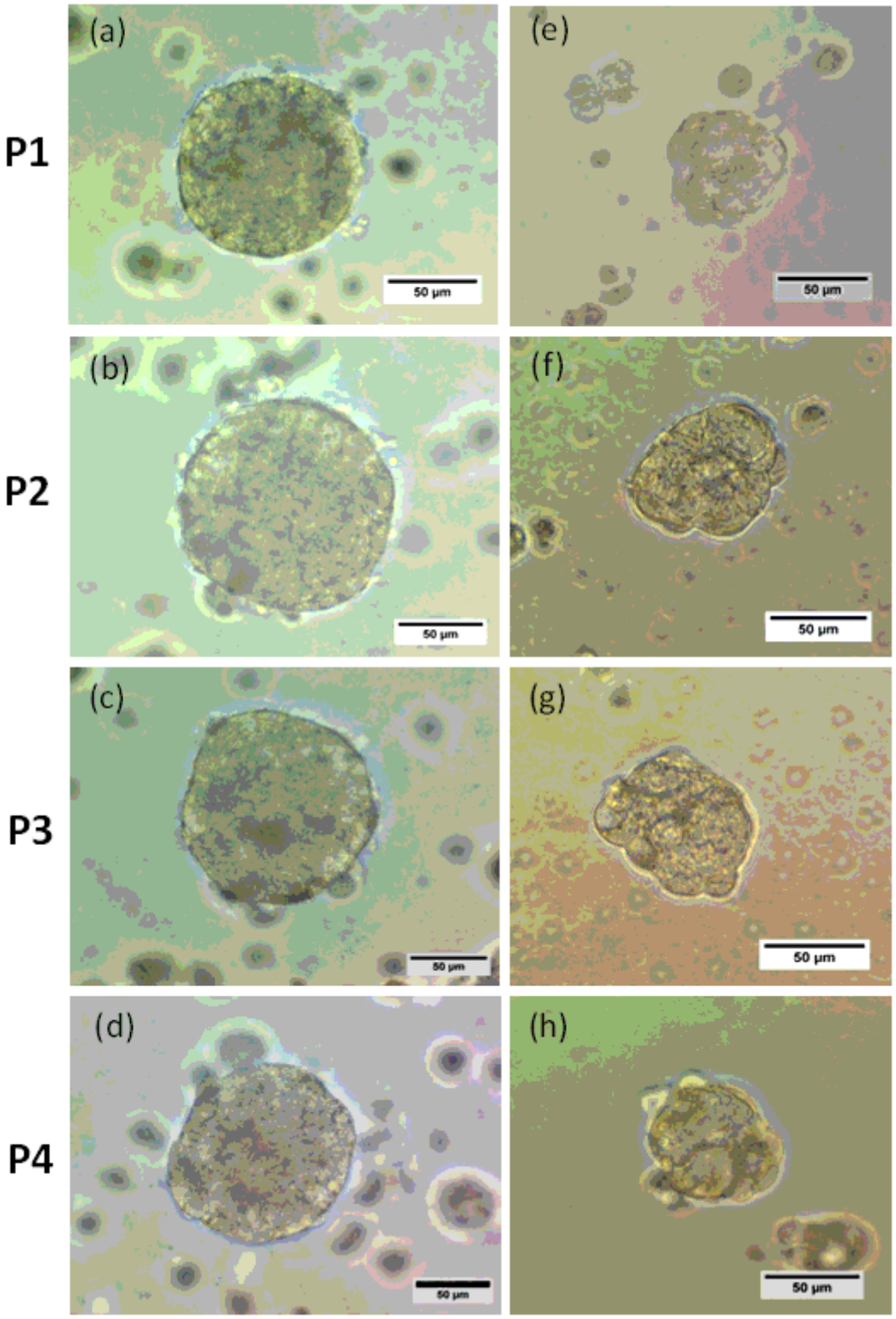

Figure 4

Serial spheroid formation capacity of colonospheres. (a-f) HT-29 and (e-h) Caco-2 spheroids derived cells generated spheroids for four consequence passage (P1-P4) and there were no obvious differences in their size and morphology in different passages.

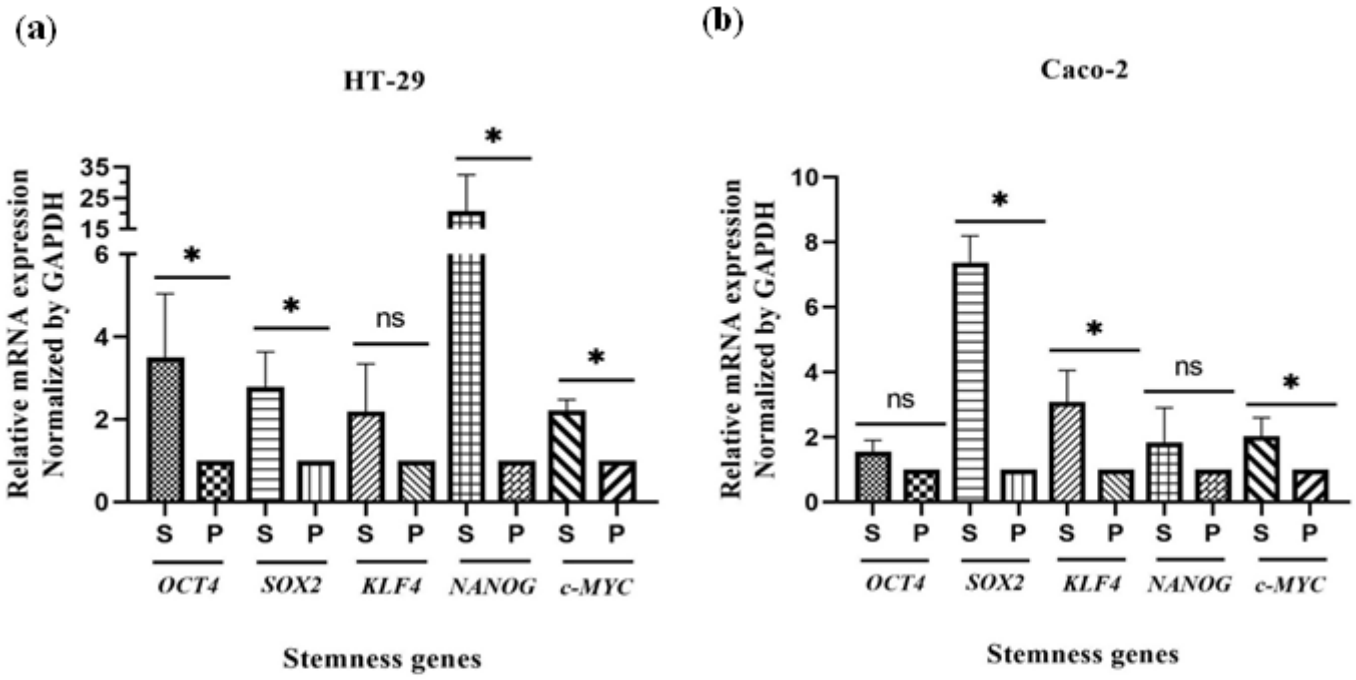

Figure 5

Increased expression of stemness regulator genes in HT-29 and Caco-2-derived spheroids compared to adherent counterparts. (a) Quantitative real-time PCR analysis of HT-29 spheroids revealed the high expression of key stemness genes OCT4, SOX2, NANOG and C-MYC in compared to HT-29 parental cells. (b) Caco-2 spheroids showed an increased expression of SOX2, C-MYC and KLF4 in compared to Caco-2

parental cells. Data are presented as mean ± SD from four independent experiments as * = P<0.05, ns = not significant.

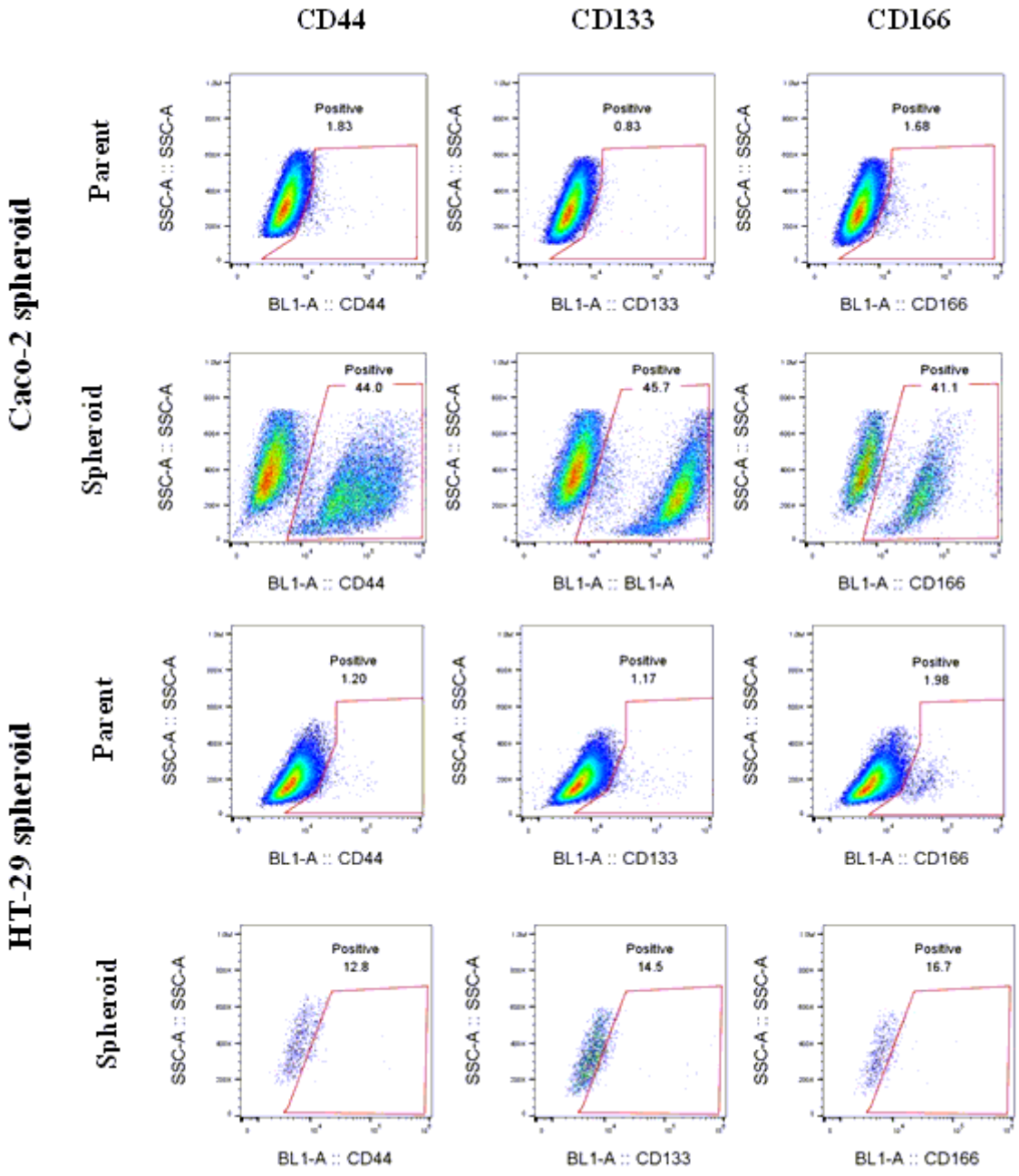

Figure 6

Flow cytometry analysis of CRC-CSC markers expression in HT-29 and Caco-2 spheroids compared to their parental cells. Both HT-29 and Caco-2 spheroids showed higher expression of CD166, CD44 and CD133 CRC-CSC markers as compared to their parental cells.

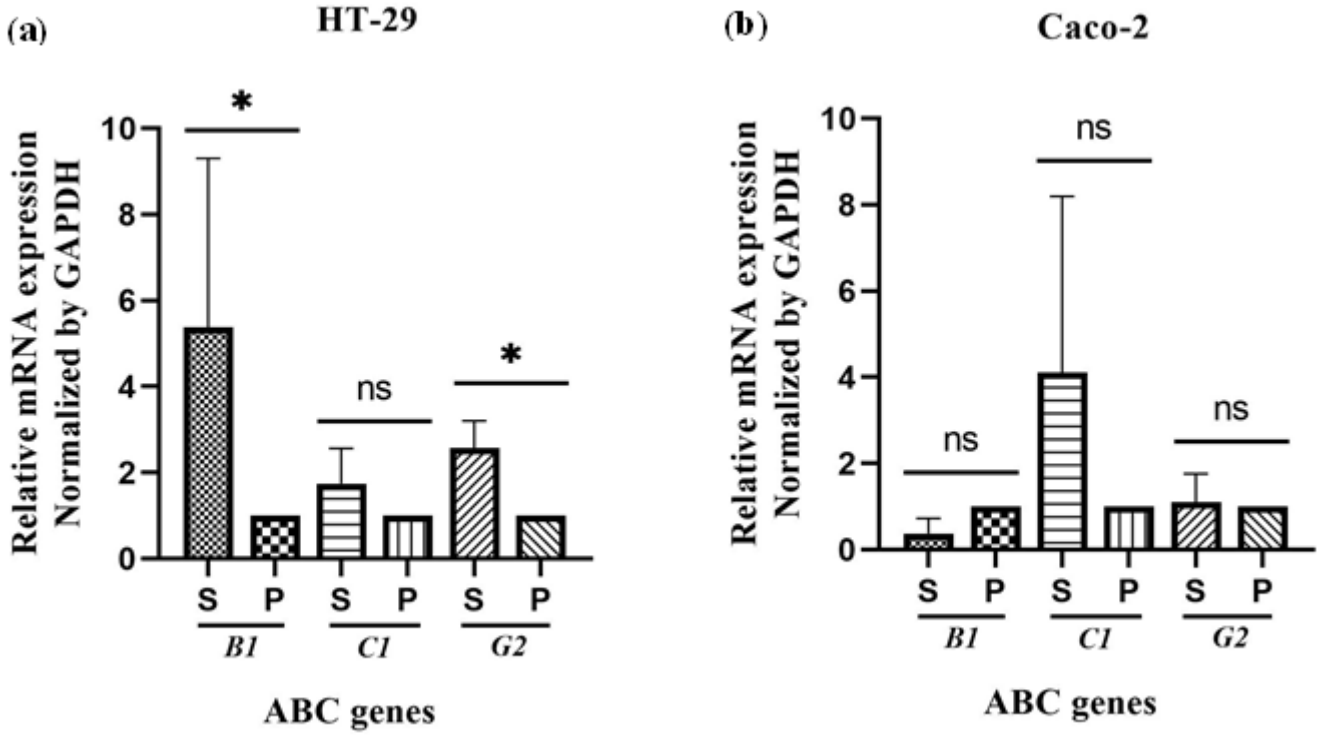

Figure 7

The expression profile of drug resistance genes in HT-29 and Caco-2 derived spheroids compared to their adherent counterparts. The comparative real-time PCR analysis of ABC transporter genes; ABCB1, ABCC1 and ABCG2 in generated spheroids and their parental cells showed (a) the higher expression of ABCB1 and ABCG2 in HT-29 derived spheroids than HT-29 parental cells, (b) whereas there was not observed a significant differences between Caco-2 spheroids and their parental cells. Data are presented as mean ± SD from four independent experiments as * = P<0.05, ns = not significant.

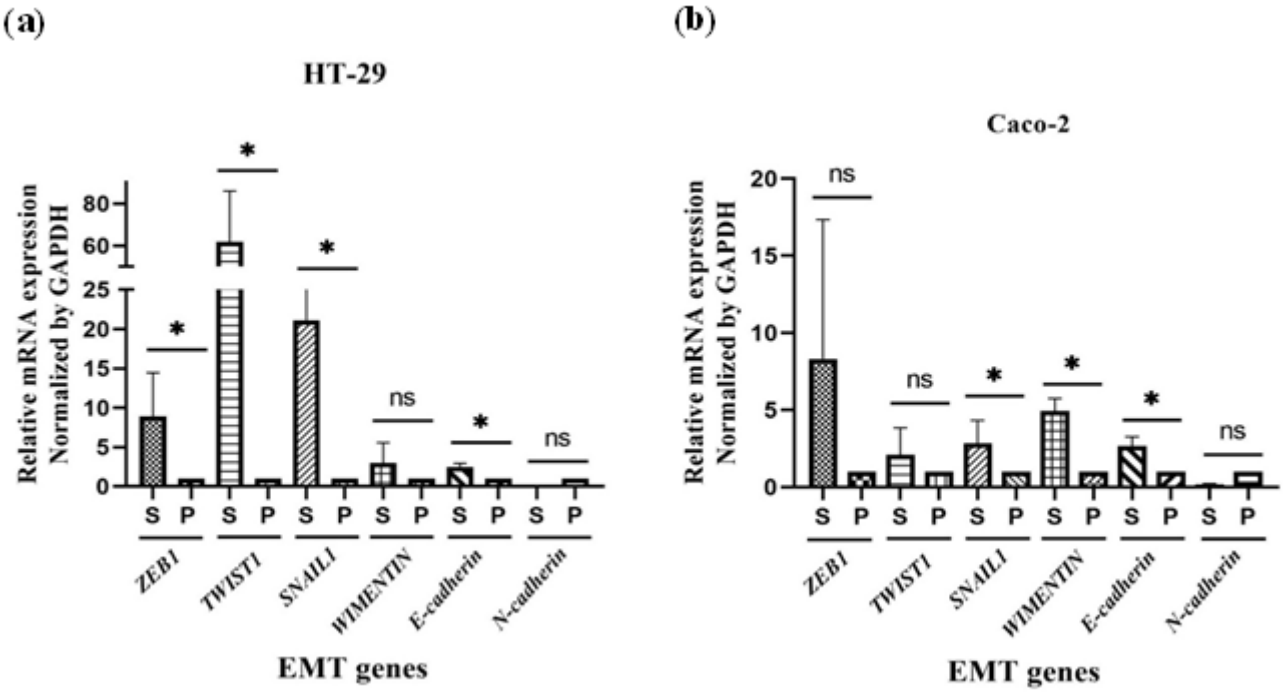

Figure 8

The expression profile of EMT regulator genes in HT-29 and Caco-2 derived spheroids compared to adherent counterparts. Graphs showing relative expression of genes involved in EMT; Vimentin, SNAIL1,TWIST1, N-Cadherin, E-Cadherin and ZEB1 in generated spheroids. (a) The expression of ZEB1, TWIST, Snail1 and E-cadherin was significantly higher in HT-29 spheroids than parental cells. (b). Caco-2 spheroids showed higher expression of Snail1, vimentin and E-cadherin compared to parental cells. Data are presented as *P<0.05, ns = not significant.