A possible clinical benefit of the identification and characterization of colon cancer stem cells

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1. Introduction

Colorectal cancer is the fourth most common cancer in men and the third most common cancer in women worldwide, with rapidly increasing incidence[1]. Disease incidence may correlate with diet, lifestyle, hereditary factors, and physical inactivity. The disease risk varies from country to country and even within countries. The risk also varies among individuals.

Surgical resection, radiotherapy, and treatment with cytostatic compounds are likely the most effective therapies for patients suffering from colon and rectum carcinoma[2]. In many age groups, both male and female, tumors eventually spread into the abdomen, pelvis, lung, and, in particular, the liver parenchyma; metastasis to the liver parenchyma is slightly more common in females than in males.

The scientific community has investigated methods for categorizing all tumors and determining the extent of cancer progression. Cancer staging is a process employed by almost all researchers and oncologists to identify a proper treatment for all cases.

Six colon cancer patients and a commercial colon cancer cell line were included in the present study to assess the existence of colon cancer stem cells (CSCs). Furthermore,
the function of the nanog gene and its association with the markers mentioned above was evaluated by repressing its expression permanently within 48 h. The selected group of patients included males and females with different cancer stages.

2. Materials and methods

To investigate the correlation between nanog, oct3/4, sox2, and cd26 expression levels and colon carcinoma, a series of colon cancers were successfully analyzed, and various protocols were performed on a group of patients.

Peripheral blood was collected from six patients diagnosed with various stages of colon carcinoma (one patient suffered from colon cancer stage II, two patients suffered from colon cancer stage III and the last three were diagnosed with stage IV cancer). Consent was provided by all patients. To prevent coagulation, the samples were collected in tubes (BD Falcon 12 mm x 75 mm style 5 mL Polystyrene Round Bottom Tube, 352054, Becton Dickinson Greece) containing ethylene diamine tetraacetic acid and rotated for approximately 30 min before testing. All samples were collected for analysis with patient consent.

A commercial colon CSC cell line was provided by Celprogen Inc, San Pedro, USA, and used as a control. The cells were cultivated according to the supplier’s instructions.

To isolate circulating tumor cells (CTCs) from whole blood samples, Biocoll, an isotonic separating solution with a density of 1.077 g/mL (L6115, Biochrom, Berlin, Germany) was used. This solution (D=1.077 at +20 °C) contains Ficoll 400 (Polysucrose 400), a polymer with a molecular weight of approximately 400000 Daltons. The Biocoll separating solution was aliquoted into 15 mL centrifuge tubes (Cat No. 188271, Greiner Bio-One GmbH, Frickenhausen, Germany), and equal parts of whole blood were carefully applied on top of the Biocoll layer. After centrifuging the samples at 2500 r/min for 20 min, the layer of enriched (70%-100%) lymphocytes between the plasma and Biocoll layer was isolated using a Pasteur pipette. The collected cells were washed twice with phosphate buffer saline (P3621, Sigma Aldrich, Greece) and then divided and cultured in 25 cm² flasks (5520100, Orange Scientific, Greece) with Dulbecco’s modified Eagle’s medium (D5546, Sigma Aldrich, Greece) and a STEMPRO hESC SFM kit (A10007-01, Life Technologies, Greece). Because the CSC population is generally characterized by molecular markers such as nanog, oct3/4, and sox2, a molecular analysis was performed. Furthermore, the cd26 gene, which is, according to many researchers, associated with colon cancer, was also included in this study. RNA was isolated using TRIzol reagent (15596–026, Life Technologies, Greece) and used as template to synthesize cDNA with First Strand cDNA Synthesis (K1612, Fermentas, Greece). To assess gene expression, a quantitative real-time PCR protocol was performed (K0221, Maxima Sybr Green, Fermentas, Greece).

The patient colon cell lines were cultivated in STEMPRO medium (complete medium) in 25 cm² flasks (5520100, Orange Scientific, Greece) at 37 °C in a 5% CO₂ environment. The commercial colon CSCs were cultivated in Celprogen’s ready-to-use growth medium under the same conditions described above.

All experiments were performed during the exponential phase after the culture reached 80%-90% confluence.

We employed the siRNA–based gene knockdown method to investigate the correlation between nanog expression and colon cancer development. The nanog gene was repressed permanently using 1 μL (25 pmol/μL) of siRNA and Lipofectamine reagent (11668–019, Lipofectamine® 2000 Transfection Reagent, Life Technologies, Greece). The results of the knockdown experiment were analyzed in a time frame of 48 h. All experiments were performed in duplicate. The siRNA assay was designed to target nanog gene mRNA based on the rational siRNA design literature. A siRNA that targets the mRNA sequence of a gene should not share significant homology with other genes or sequences in the genome; therefore, an homology search is essential for preventing off-target effects. For that reason, the NCBI BLAST tool was used to perform this homology search[6].

As CSCs lose their ability to detach, form spheres, and develop a mesenchymal phenotype under special conditions via the epithelial–mesenchymal transition, a sphere formation evaluation method was used to analyze the transfected samples. Colon spheres are spheroid structures that can be passaged serially to generate daughter spheres with a similar composition, thereby demonstrating that sphere-forming cells are capable of self-renewal[7,8]. Using light microscopy, the spheres can be observed in semi-suspension in culture.

The real–time polymerase chain reaction (PCR) method, which is the most sensitive, simple, and quick method for studying gene expression, was also utilized. This method requires low levels of template and is thus a widely used research tool.

Because the CSC population is generally characterized by molecular markers such as nanog, oct3/4, and sox2, a molecular analysis was performed. Furthermore, the cd26 gene, which is, according to many researchers, associated with colon cancer, was also included in this study. RNA was isolated using TRIzol reagent (15596–026, Life Technologies, Greece) and used as template to synthesize cDNA with First Strand cDNA Synthesis (K1612, Fermentas, Greece). To assess gene expression, a quantitative real–time PCR protocol was performed (K0221, Maxima Sybr Green, Fermentas, Greece).
undesired genes. The real-time PCR protocol included a denaturation program (94 °C for 10 min), 50 cycles of amplification and quantification (94 °C for 15 seconds, 59 °C for 15 seconds, and 72 °C for 30 seconds) with a single fluorescent measurement, and a melting curve program (55–95 °C with a heating rate of 0.5 °C per second and a continuous fluorescent measurement). Each sample was amplified in triplicate. At the end of the reaction, the cycle–threshold (which is the level of detection at which a reaction reaches a fluorescence intensity above background) was determined. Finally, relative gene expression was calculated and compared to untreated cells using the 2−ΔΔCt (Livak) method. All samples were tested in triplicate⁹.

All analyses were performed in transfected and non-transfected samples.

3. Results

The ability of CSCs to form spherical colonies under enabling conditions was confirmed in the sphere–formation evaluation assay. The commercial colon CSC phenotype was recommended by Celprogen Inc. (Figure 1).

When all cell populations (patient cell lines) were cultivated using the STEMPRO hESC SFM kit, spherical colonies were observed in the supernatant (Figures 2–4).

According to the qPCR analysis performed using relative quantification with normalization to an endogenous gene (18S rRNA), a reduction in sox2 gene expression was observed in all patients including the commercial colon CSCs following nanog gene repression. When the expression of nanog was compared with that of the oct3/4 gene, it was observed that when the nanog gene was repressed, the expression of oct3/4 was reduced in five of the six patient samples. Finally, nanog expression levels correlated with cd26 gene levels, and repression of nanog expression increased the expression of cd26 in five of the six patients. All analyses were performed using the Livak method (Tables 1 and 2).

### Table 1

| Description of the gene expression level changes post–transfection with the nanog gene. |
|---------------------------------|---------------------------------|-------------------|-------------------|
| Colon cancer patients and colon CSC line gene expression analysis profile |
| nanog in correlation with oct3/4 |
| nanog in correlation with sox2 |
| nanog in correlation with sox2 & oct3/4 |

As the nanog gene was repressed, the other panels show the total change for each gene separately. ↓ indicates a decrease; ↑ symbol indicates an increase; and – indicates no change in the expression level.

### Table 2

| Total results | Stage | nanog | oct3/4 | sox2 | cd26 | nanog knockdown percentage (%) |
|---------------|-------|-------|--------|------|------|-------------------------------|
| Colon CSCs    | –     | ↓     | ↓      | ↓    | –    | 45                            |
| 11CO          | II    | ↓     | ↓      | ↓    | ‑    | 82                            |
| 14CO          | III   | ↓     | –      | ↓    | ‑    | 84                            |
| 7CO           | III   | ↓     | ↓      | ↓    | ‑    | 72                            |
| 13CO          | IV    | ↓     | ↓      | ↓    | ‑    | 38                            |
| 15CO          | IV    | ↓     | ↓      | ↓    | ‑    | 86                            |
| 2CO           | IV    | ↓     | ↓      | ↓    | ‑    | 43                            |

As the nanog gene was repressed, the other panels show the total change for each gene separately. ↓ indicates a decrease; ↑ symbol indicates an increase; and – indicates no change in the expression level.

![Figure 1. Commercial colon CSCs, passage 29.](image1.png)

A represents the matrix of cells, pre–transfection. B represents the cell phenotype 48 h post–transfection with nanog siRNA (25 pmol/µL). A difference in phenotype can be observed in A and B.

![Figure 2. Colon CSCs isolated from a patient with stage IV colon carcinoma.](image2.png)

A represents the matrix of cells pre–transfection. B represents the cell phenotype 48 h post–transfection with nanog siRNA (25 pmol/µL). A difference in phenotype can be observed in A and B.

![Figure 3. Colon CSCs obtained from a second patient with stage IV colon carcinoma.](image3.png)

A represents the matrix of cells pre–transfection. B represents the cell phenotype 48 h post–transfection with nanog siRNA (25 pmol/µL). A difference in phenotype can be observed in A and B.

![Figure 4. Colon CSCs derived from a patient with stage III colon carcinoma.](image4.png)

A represents the matrix of cells pre–transfection. B represents the cell phenotype 48 h post–transfection with nanog siRNA (25 pmol/µL). A difference in phenotype can be observed in A and B.
4. Discussion

The crucial aspect of this study was the discovery of a small population of cells inside the tumor mass termed CSCs. CSCs have been the object of study because of their unique characteristics. They have the ability to self-renew and sustain cancer, much like normal stem cells, renew and sustain organs and tissues. The field of cancer research has reached a new phase. It is believed that there is a stage between the primary tumor and the metastatic loci during which CSCs cease dividing but survive in a quiescent state while waiting for appropriate environmental signals to begin proliferation[10,11]. Molecular mechanisms and models have been established to characterize the cancer cell dormancy process[12-14].

CSCs are identified within a population of cells using molecular signatures, which may be unique gene expression patterns or posttranslational modifications that determine gene function. The expression of these genes controls the establishment, survival, and maintenance of CSCs in a tumor. Many growth factors and signaling molecules in the body regulate specific gene expression patterns. The nanog gene is a critical transcription factor that is expressed in many tumors and regulates their progression. Nanog expression may be correlated with colorectal cancer and metastasis into the liver[15-19]. The association between the nanog gene and the oct3/4 and sox2 genes is well characterized and extensively used to analyze the stemness ability of a distinct population of cancer cells[20]. Furthermore, cd26/dipeptidyl peptidase IV (DPPIV) is correlated with tumor progression and specifically colon cancer.

The present scientific study attempted to investigate the presence of CSCs within the population of CTCs. CTCs were isolated from blood, which was collected from six colon patients with stages II, III, and IV cancer. Consent was provided by all patients. After cultivation of the cells for one week, the remaining population included the desired cells with a potential stemness.

To analyze the function of nanog in the sub-population of cells, which may be CSCs, nanog was permanently repressed using a siRNA-based method and Lipofectamine. The results of the gene knockdown were analyzed 48 h post-transfection. As a control, cells in which the expression of nanog was not modified (non-transfected cells) were also included.

According to the literature, CSCs display identical formation. They are typically cultivated in vitro under non-adherent conditions as spheres or under adherent conditions in either two-dimensional culture or a three-dimensional matrix. Sphere-forming assays are widely used in stem cell biology because both self-renewal and differentiation can theoretically be evaluated at the single-cell level. In semi-suspension, CSCs form spheres[21-23]. Analyzing the phenotypes of the two groups of cells (colon CSCs) revealed that transfected cells were in suspension and round in shape, while non-transfected cells formed spheres. This phenomenon was observed in all samples tested as well as in the commercial cancer cell line (colon CSCs), even though the knockdown percentage varied in each case (ranging from 43% to 86%; nanog gene knockdown)[24]. Concerning the molecular analysis, nanog gene expression knockdown reduced self-renewal and decreased the expression levels of sox2, the other stem cell–related gene, in all patient samples selected for the project and the colon CSCs obtained from Celprogen Inc. The sox2 gene encodes a member of the SRY–related HMGBox (SOX) family of transcription factors involved in the regulation of embryonic development and determination of cell fate. Sox2 contributes to colorectal carcinogenesis and was associated with poor prognosis in colon cancer when its expression was analyzed separately from the nanog gene[25]. Other research studies have investigated the correlation, in relative expression, between sox2 and nanog gene expression as well as with oct3/4[26]. The expression of oct3/4 was reduced in five of the six colon patients in the present study. Oct3/4 (octamer–binding transcription factor 4), also known as POU5F1 (POU domain, class 5, transcription factor 1), represents the third well–characterized stemness marker[27,28]. The protein is encoded by the POU5F1 gene in humans and is a homeodomain transcription factor of the POU family. This protein may be critically involved in the self–renewal of undifferentiated embryonic stem cells.

The final gene studied was cd26. Cd26/dipeptidyl peptidase IV (DPPIV) is a 110–kDa glycoprotein that is expressed on numerous cell types and exerts multiple biological functions. It plays a crucial role in tumor progression and development. According to published data, it has a tumor suppressor function and regulates the activities of mitogenic peptides implicated in cancer development[29-31]. These findings are in agreement with those of this study, which demonstrated that the expression of cd26 increased when nanog gene expression was repressed. Furthermore, the cd26 gene may be associated with colon cancer. Patients with cd26–positive cells (CSCs) developed distant metastasis according to follow–up documents[32,33].

The data analysis described above indicates that the
population of colon CSCs plays a crucial role in disease progression and treatment. CSCs are present in all clinical cases. The key to improving patient life expectancy will be the identification of a technique to limit the proliferation of CSCs and lock the cells in a dormant state.

**Conflict of interest statement**

We declare that we have no conflict of interest.

**Comments**

**Background**

Colorectal cancer is the third most common cancer and with nearly 1.4 million new cases diagnosed in 2012. Incidence and death rates are highest in blacks and lowest in Asians/Pacific Islanders. Progress in reducing colorectal cancer death rates can be accelerated by improving access to and use of screening and standard treatment in all populations. The aim of this study is to evaluate the prognostic value of colon CSCs in a population of stage II, III and IV colon cancer patients. The scientific community has proven the importance of CSCs in metastasis. By correlating the existence of this population in cancer patients with their cancer stage, this may be a key to target them and to prevent and/or to minimize its side effects.

**Research frontiers**

Studies have been performed in order to determine the correlation between the population of colon CSCs and the stage in colon cancer patients. The disease progression in correlation with stemness was also under evaluation. Circulated tumor cells were isolated from six patients who suffered from colon carcinoma and cultivated in appropriate culture conditions. Cancer stem cells were observed and characterized under light microscope and by using molecular methods. A commercial colon cancer stem cell line was also used.

**Related reports**

According to the literatures (Liu et al. 2012, Ohata et al. 2012, Pastrana et al. 2011), colon CSCs are characteristically grown up in semi suspension, forming sphereres. This finding agreed with the results in which authors investigate tumorospheres in all tested samples. Furthermore, the correlation between nanog and the other stemness markers (oct3/4, sox2 and cd26) have already been observed by other researchers (Saigusa et al. 2009, Okumura et al. 2005, Rizzino et al. 2009, Wesley et al. 2005, Aytac et al. 2004, Pro and Dang, 2004). In this study, it has also been pointed out the correlation, in relative expression, between the above mentioned markers.

**Innovations & breakthroughs**

Correlation between the relative expression of nanog and oct3/4, sox2 and cd26 gene after repressing permanently the first, in colon CSCs populations obtained from patients with stages II, III and IV.

**Applications**

It may be significant to investigate the presence of CSCs within the population of circulating tumor cells. This small population may be correlated with a patient’s cancer stage.

**Peer review**

The existence of CSCs has several implications in terms of future cancer treatment and therapies including disease identification, selective drug targets, prevention of metastasis, and development of new intervention strategies. CSCs may generate tumors through the stem cell processes of self-renewal and differentiation into multiple cell types. These cells are can persist in tumors as a distinct population and cause relapse and metastasis. The merit of this paper lies in the attempt to identify a distinct gene expression pattern of nanog, oct3/4, cd26 and sox2 gene in CSCs. By selectively targeting CSCs, it would be possible to treat patients with aggressive, non-resectable tumors, as well as preventing patients from metastasizing and relapsing. The CSC elimination can result to tumor regression due to differentiation and/or cell death.

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