Int J Clin Exp Pathol 2017;10(8):8377-8382
www.ijcep.com /ISSN:1936-2625/IJCEP0055578

Original Article

Isolation and identification of cancer stem cells from PC3 human prostate carcinoma cell line

Yaojun Zhou[1*], Hongqiong Yang[2*], Wei Xia[1*], Li Cui[1], Renfang Xu[1], Hao Lu[1], Zhong Xue[1], Bo Zhang[1], Zinong Tian[1], Yunjie Cao[1], Zhaoyu Xing[1], Shuai Yin[1], Kun Wang[1], Qianqian Shi[1], Xiaozhou He[1]

Departments of [1]Surgical Urology, [2]Geriatric Medicine, The Third Affiliated Hospital of Soochow University, Changzhou, Jiangsu, China. [*]Equal contributors.

Received April 17, 2017; Accepted June 30, 2017; Epub August 1, 2017; Published August 15, 2017

Abstract: Objective: Morphological differences of PC3 clones were dynamically observed, and the expression of CD44 in different clones was detected to compare the tumorigenic ability of different clone cells in nude mice and identify the clones containing prostate cancer stem cells. Materials and methods: Clone formation assay was used for observing and classifying PC3 clones and calculating the cloning efficiency and the proportion of each clone. CD44 expression in different clones was detected by immunofluorescence technique. In addition, different morphologies of clones were isolated to measure the ability of self-renewing, and inoculated into nude mice to observe the tumorigenic ability. Results: PC3 cells could form three morphologies of clones, namely holoclone, meroclone, and paraclone. The cloning efficiency was 10.23%±0.91%, and the proportion of the three clones was 11.7%, 50.0% and 38.3%, respectively. Immunofluorescence showed that the expression of CD44 in holoclone was significantly stronger than meroclone and paraclone. Holoclone had self-renewing ability and strong tumorigenic ability in nude mice. Conclusion: There are differences in morphologies and differentiation of PC3 clones. Moreover, prostate cancer stem cells are abundant in holoclone.

Keywords: PC3, CD44, morphologies, self-renewing, tumorigenic ability

Introduction

Prostate cancer is one of the most common malignancies in the male genitourinary system and is reported to be the second most common cause of male malignancy [1]. In America, the incidence of prostate cancer has exceeded lung cancer and now prostate cancer becomes the first in cancers that harms to male health [2]. In early interventions, the prostate-specific antigen blood test is commonly used, and the incidences of prostate cancer increase, while the mortality rates decrease [1]. To prevent death and disability from prostate cancer and minimize complications related with interventions, a number of treatments were used for prostate cancer, including watchful waiting [2], removing the prostate gland surgery [3] and stereotactic body radiation therapy (SBRT) [4]. However, the mechanism of pathogenesis and progression of prostate cancer are not clearly clarified, which limit the level of diagnosis and treatment of prostate cancer.

Cancer stem cells (CSCs) are a small number of tumor cells that have self-renewing ability, differentiation potential and drug resistance ability [5], which are the roots of progression, recurrence and metastasis [3], suggesting that rare CSCs should be targeted for effective therapeutics to sustain tumor malignancy. Researchers found that there were different morphologies of clones in a variety of tumor cell lines originated from human epithelial in vitro, moreover, tumor stem cells was confirmed to be abundant in one of the clones, which was known as holoclone [4-6]. As reported, holoclone has a high proliferative capacity and was described as large and round colonies [6].

In this study, morphologies of PC3 human prostate cancer cell clones were observed, and the differentiation characteristics were detected. We preliminary identified clones containing CSCs to further explore the mechanism of pathogenesis and progression of prostate can-

cer and provide a theoretical basis for the targeted treatment.

Materials and methods

Cell culture

PC3 human prostate cancer cell lines were purchased from Shanghai Cell Bank of Chinese Academy of Science, and cultured in complete culture medium supplemented with 10% FBS (Gibco, Australia) in 0.5% CO_2 incubator (Sanyo, Japan) under 37°C. PC3 cells were digested with digestion solution containing 0.25% trypsin (Sigma, USA) and 0.02% EDTA (Gibco), and cells in the logarithmic growth phase were collected for the following experiments.

Observation of clonal morphology

PC3 cells in the logarithmic growth phase were collected and digested. Then complete culture medium was added to prepare single cell suspension. The density of cells were adjusted to 10 cells/ml and seeded in 96-well plates with 0.1 ml cell suspension/well. Wells with only one cell were selected and marked, and dynamically observed the process of cloning under inverted microscope. The clone criterion was that the isolated cell population with more than 50 cells two weeks later, and the cloning efficiency was calculated. Morphological classification of clones was carried out, and the proportions of different types of clones were counted.

$$\text{cloning} = \frac{\text{The number of clones}}{\text{The number of wells containing single cell}} \times 100\%$$

MTT assay

Randomly selected 5 holoclones, 5 meroclones and 20 paraclones, and digested into cell suspensions. The clones were seeded into culture dish with a diameter of 100 mm. Cells of holoclones, meroclones, paraclones and PC3 were seeded into 96-well plates at a concentration of 50 cells/well. The solution was changed every two days. A 96-well plate at the same time every day was taken out from the third day to the tenth day. MTT solution (20 μl) was added into each well and incubated at 37°C for 4 h. Then, supernatant was removed, and 150 μl DMSO was added into each well and shocked for 15 min. The absorbance (A) was read at 490 nm for MTT by using an iMark microplate reader (Bio-Rad, Hercules, CA, USA). Each group had eight parallel wells, and the average value was calculated to draw the proliferation curve.

Continuous colony forming experiments

Cells from holoclones, meroclones and paraclones were selected and seeded into 96-well plates at a concentration of 1 cell/well. After 2 weeks of static culture, colony forming experiment was performed again to observe the self-renewing ability of cells originated from different clones.

Immunofluorescence staining

PC3 cells were digested and counted, and seeded into culture dish with a diameter of 30 mm at a concentration of 300-400 cells/well. After 2 weeks of static culture, the procedures were as follows: medium removal, cells washed with PBS for twice, fixed with 4% paraformaldehyde, washed with PBS, transparent with 0.2% Triton × 100 for 5 min, washed with PBS for three times, and blocked with BSA for 45 min. Primary antibody CD44 (1:100) was added and incubated at 4°C overnight. Then, goat anti-rabbit secondary antibody labeled with fluorescein isothiocyanate (1:50) was added and incubated at 37°C for 1 h. Nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI). The fluorescent intensity was detected by using laser scanning confocal microscope.

Tumor formation in nude mice

PC3 cells, holoclone, meroclone and paraclone cells were digested, centrifuged and suspended into single-cell suspension at a concentration of 10^3 cells/mL. Twenty-four female nude mice (SPF) were weighted and divided into four groups, namely the PC3 group, holoclone group, meroclone group and paraclone group. Each group has six mice. Each mouse was subcutaneously injected with 0.1 ml of cell suspension in the axilla. Because paraclone cells grew extremely slowly, only two nude mice were successfully inoculated in paraclone group. The tumor growth was measured every five days, and the length diameter and short diameter were measured by vernier calipers. Tumor volume was calculated (tumor volume = π × length diameter × short diameter^2/6). The nude mice were sacrificed 45 days later, and tumor growth curve was drawn.

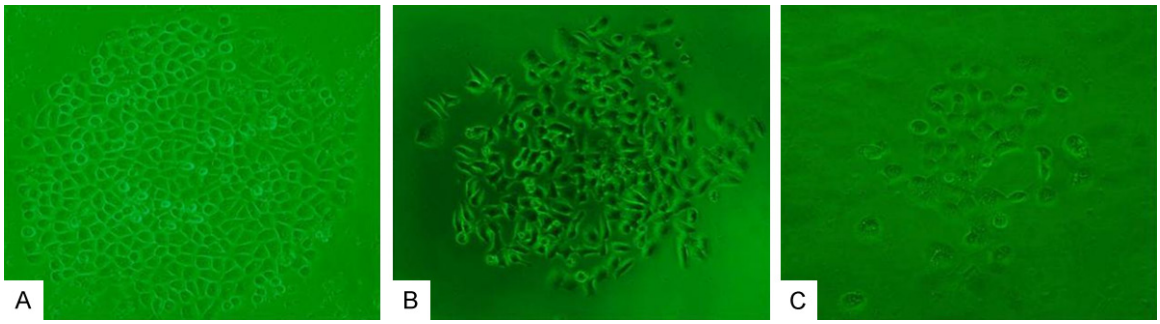

Figure 1. Observation of the morphology of PC3 clones (× 100, on the 14th day). A. The morphology of holoclones; B. The morphology of meroclone; C. The morphology of paraclone.

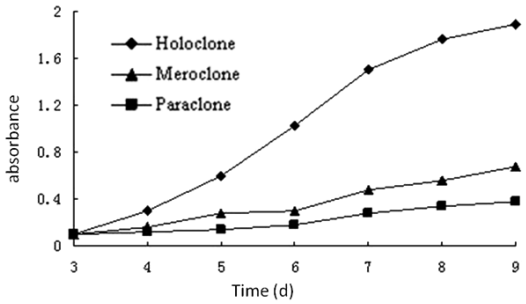

Figure 2. In vitro growth curve of the three clones of PC3.

Statistical analysis

We used SPSS 13.0 for the data analysis. Measurement data were expressed as $\bar{x} \pm s$.

Results of in vitro proliferation experiments and tumor volume were compared by one-way ANOVA test, test level $\alpha = 0.05$.

Results

Clonal morphology and cloning efficiency

The process of clone formation was observed under inverted phase contrast microscope. Clones similar to holoclone, meroclone and paraclone that derived from keratinocytes/epithelium tumor cells as reported were formed after two week's cultivation. Holoclone had regular morphology, small cell, large numbers and dense arrangement. Paraclone had irregular morphology, large cell, low numbers and loose arrangement. The morphology, cell size, and numbers of meroclone were between holoclone and paraclone (**Figure 1**).

There were 645 wells that corresponded to the screening criteria of one living cell per well in ten 96-well plates. Then, after two week's observation, sixty clones that corresponded to the criteria of isolated cell numbers more than 50 were formed, including 7 holoclones (11.7%), 30 meroclones (50%) and 23 paraclones (38.3%). The cloning efficiency was 10.23%±0.91%.

In vitro proliferation of clones

The proliferation level of holoclone cell was significantly higher than meroclone and paraclone cells, and the differences aggravated with the prolongation of time ($F = 34.050$, $P < 0.05$; **Figure 2**), indicating that cells of holoclone had strong in vitro proliferation ability.

Self-renewing ability

After two week's cultivation of three typical clones, holoclone cells formed second holoclones that were similar to original clone morphology, and formed a small number of meroclone and paraclone. Meroclone cells only formed second meroclone and paraclone. Only a small number of paraclone cells were able to grow, and most of them stopped proliferation with no formation of clones.

Expression of CD44 in clones

According to the result of immunofluorescence assay, the expression of CD44 was strongest in holoclone cells, and weakest in paraclone cells. The expression of CD44 in meroclone cells was between holoclone and paraclone cells (**Figure 3**).

Formation of tumor in nude mice

Transplanted tumor was formed on the 10th day of inoculation in holoclone group, and the tumor

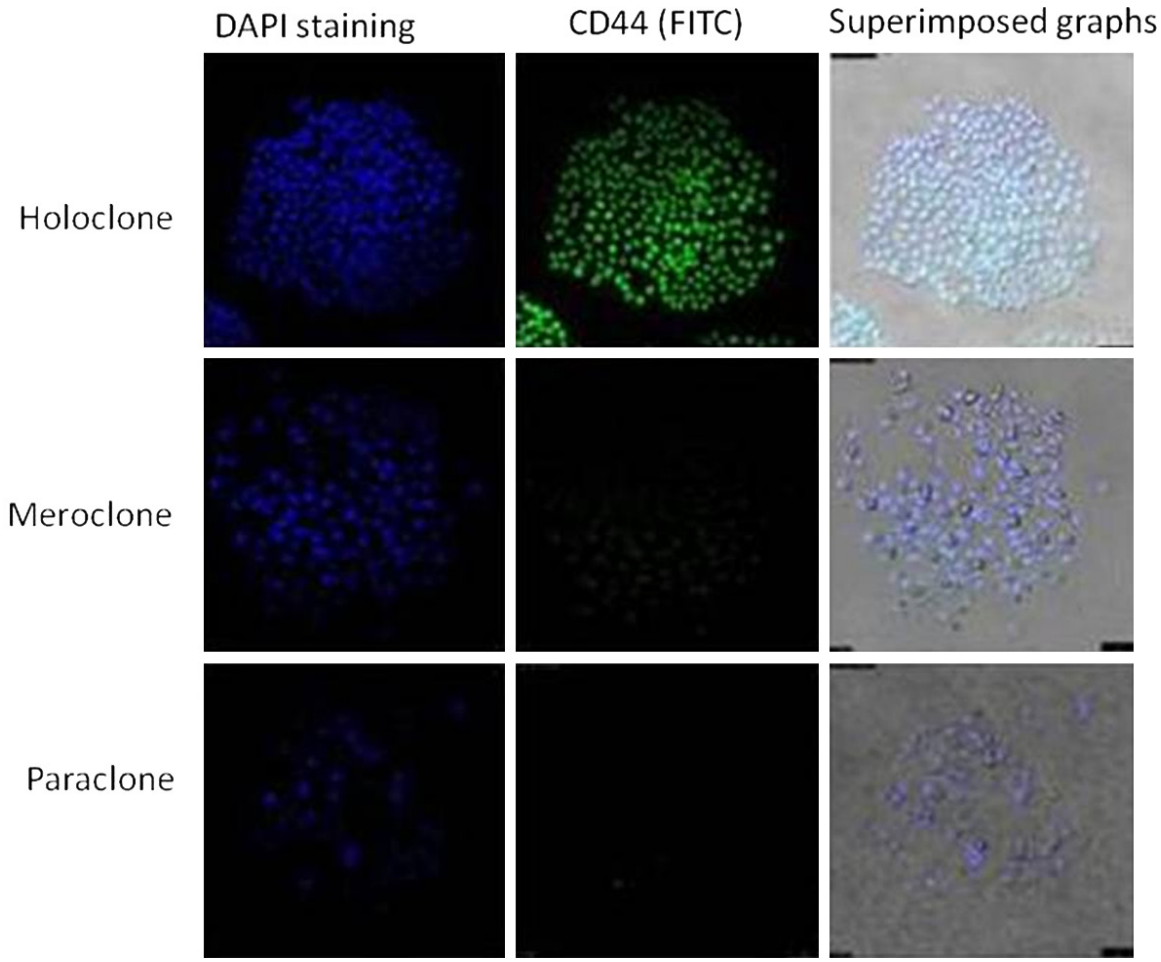

Figure 3. CD44 expression of different morphologies of PC3 clones by immunofluorescence assay.

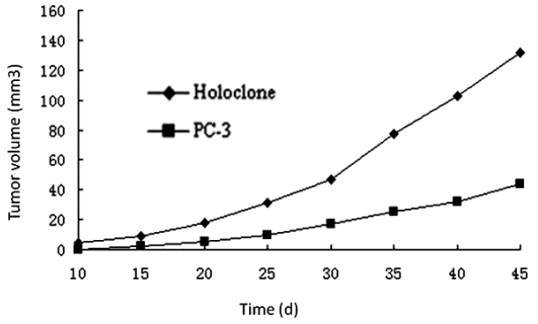

Figure 4. In vitro growth curve of transplanted tumor from nude mice inoculated with PC-3 cell lines and holoclone cells.

formation rate was 100% (6/6). Transplanted tumor was formed on the 15th day of inoculation in PC3 group, and the tumor formation rate was 66.7% (4/6) (**Figures 4** and **5**). There was significant difference in volume of transplanted tumor between holoclone group (33.45±10.21 mm^3) and PC3 group (9.02±1.56 mm^3) since the 25th day, indicating that holoclone had strong tumorigenic ability, and meroclone and paraclone didn't have tumorigenic ability.

Discussion

Researchers have isolated CSCs and proved the existence of CSCs in many tumors, such as gastric cancer, colorectal cancer, cervical cancer, glioma, etc [7-10]. CSCs are considered to be the basis of tumorigenesis and development, and play a decisive role in maintaining malignant proliferation, invasion, metastasis, recurrence and drug resistance of tumors [11, 12]. There are differences in morphology, proliferation and differentiation between CSCs and differentiated tumor cells [13, 14]. In vitro culture, there are differences in clone formation ability and cloning phenotype. Previously, researchers have found that clones formed by tumor cells, such as pancreatic cancer, malignant glioma, gastric cancer and colorectal can-

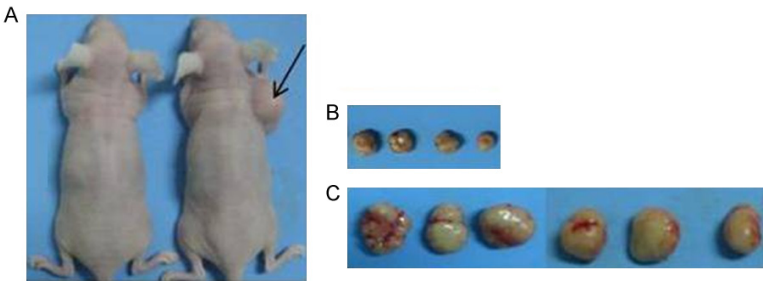

Figure 5. Tumor formation of PC3 cell lines and holoclone cells in nude mice. A. Tumor formation of holoclones in nude mice (arrow). B. Tumor formation of PC3 cell lines in nude mice. C. Tumor formation of holoclone cells in nude mice.

cer, have morphological heterogeneity and can form three different morphologies of clones, namely holoclone, meroclone and paraclone [15-17].

In the present study, three different morphologies of clones were found in PC3 cell line by morphological separation, which showed the characteristic holoclone. We also compared the differentiation characteristics of different clones by examining the expression of CD44, which showed that CD44 expressed strongest in holoclone. CD44 is a member of the adhesion molecules family. As the cell surface components, it interacts with extracellular matrix and involves in cell migration, moreover, CD44 overexpression is closely related with tumor metastasis and drug resistance. According to previous reports, CD44 is a surface molecule marker of various CSCs, such as colorectal cancer, head and neck squamous cell carcinoma, gastric cancer, nasopharyngeal carcinoma [18, 19]. In this study, we speculated that prostate cancer stem cells might be abundant in holoclones through the heterogeneity of the morphology and differentiation of PC3 clones.

We performed the tumor formation assay in nude mice and proved that holoclone had tumorigenesis ability in vivo, which might be the source of tumor heterogeneity in PC3 cells. There are three main separation technologies, namely the flow cytometry, immunomagnetic beads and cell line establishment and culture [20-22]. Flow cytometry and immunomagnetic beads have high requirements to equipment and technologies, and are very expensive. Cell line establishment and culture uses serum-free medium supplemented with growth factors, which might affect the biological characteristics of tumor stem cells. The morphology separation method of CSCs is simple and has low requirement to equipment, thus having practical values. Hence, combined with the ease of use, separation efficiency and economic factors, we chose cell line establishment and culture method.

In conclusion, prostate cancer stem cells might be abundant in holoclone according to the measurement of related molecule expression CSCs. CD44 might be a stem cell marker for PC3 cell line. Target therapy strategies for prostate cancer stem cells could be designed using targeted surface markers of prostate cancer stem cells, which have a meaningful impact on the treatment of prostate cancer.

Disclosure of conflict of interest

None.

Address correspondence to: Xiaozhou He, Department of Surgical Urology, The Third Affiliated Hospital of Soochow University, Changzhou 213003, Jiangsu, China. Tel: 86-519-68871251; Fax: 86-519-86621235; E-mail: Xiaozhou_He@163.com

References

[1] Wilt TJ, Macdonald R, Rutks I, Shamliyan TA, Taylor BC and Kane RL. Systematic review: comparative effectiveness and harms of treatments for clinically localized prostate cancer. Ann Intern Med 2008; 148: 435-448.

[2] Bill-Axelson A, Holmberg L, Ruutu M, Garmo H, Stark JR, Busch C, Nordling S, Häggman M, Andersson SO, Bratell S, Spångberg A, Palmgren J, Steineck G, Adami HO, Johansson JE; SPCG-4 Investigators. Radical prostatectomy versus watchful waiting in early prostate cancer. N Engl J Med 2011; 364: 1708-17.

[3] Katz A, Ferrer M, Suárez JF; Multicentric Spanish Group of Clinically Localized Prostate Cancer. Comparison of quality of life after stereotactic body radiotherapy and surgery for early-stage prostate cancer. Radiat Oncol 2012; 7: 194.

[4] Chen LN, Suy S, Uhm S, Oermann EK, Ju AW, Chen V, Hanscom HN, Laing S, Kim JS and Lei S. Stereotactic body radiation therapy (SBRT) for clinically localized prostate cancer: the Georgetown university experience. Radiat Oncol 2013; 8: 58.

[5] Li Y, Kong D, Ahmad A, Bao B and Sarkar FH. Targeting cancer stem cells for overcoming drug resistance and cancer progression. John Wiley & Sons 2014.
[6] Doherty RE, Haywood-Small SL, Sisley K and Cross NA. Aldehyde dehydrogenase activity selects for the holoclone phenotype in prostate cancer cells. Biochem Biophys Res Commun 2011; 414: 801-7.
[7] Wang L, Guo H, Lin C, Yang L and Wang X. Enrichment and characterization of cancer stem-like cells from a cervical cancer cell line. Mol Med Rep 2014; 9: 2117-2123.
[8] Mao J, Fan S, Ma W, Fan P, Wang B, Zhang J, Wang H, Tang B, Zhang Q and Yu X. Roles of Wnt/β-catenin signaling in the gastric cancer stem cells proliferation and salinomycin treatment. Cell Death Dis 2014; 5: e1039.
[9] Todaro M, Gaggianesi M, Catalano V, Benfante A, Iovino F, Biffoni M, Apuzzo T, Sperduti I, Volpe S, Cocorullo G, Gulotta G, Dieli F, De Maria R, Stassi G. CD44v6 is a marker of constitutive and reprogrammed cancer stem cells driving colon cancer metastasis. Cell Stem Cell 2014; 14: 342-56.
[10] Yan K, Wu Q, Yan DH, Lee CH, Rahim N, Tritschler I, Devecchio J, Kalady MF, Hjelmeland AB and Rich JN. Glioma cancer stem cells secrete Gremlin1 to promote their maintenance within the tumor hierarchy. Genes Dev 2014; 28: 1085-1100.
[11] Holah NS, Aiad HA, Asaad NY, Elkhouly EA and Lasheen AG. Evaluation of the role of ALDH1 as cancer stem cell marker in colorectal carcinoma: an immunohistochemical study. J Clin Diagn Res 2017; 11: EC17-EC23.
[12] Yoshida GJ. The heterogeneity of cancer stem-like cells at the invasive front. Cancer Cell Int 2017; 17: 23.
[13] Ma X, Zhang Y, Kang Y, Li L and Zheng W. A recombinant protein TmSm (T34A) can inhibit proliferation and proapoptosis to breast cancer stem cells (BCSCs) by down-regulating the expression of cyclin D1. Biomed Pharmacother 2016; 84: 373-381.
[14] PLOS ONE Editors. Expression of concern: concomitant targeting of multiple key transcription factors effectively disrupts cancer stem cells enriched in side population of human pancreatic cancer cells. PLoS One 2017; 12: e0174194.
[15] Heeschen C and Sancho P. More challenges ahead-metabolic heterogeneity of pancreatic cancer stem cells. Mol Cell Oncol 2016; 3: e1105353.
[16] Jung J, Gilbert MR and Park DM. Isolation and propagation of glioma stem cells from acutely resected tumors. Methods Mol Biol 2016; 1516: 361-9.
[17] Chen XL, Chen XZ, Wang YG, He D, Lu ZH, Liu K, Zhang WH, Wang W, Li CC and Xue L. Clinical significance of putative markers of cancer stem cells in gastric cancer: a retrospective cohort study. Oncotarget 2016; 7: 62049-62069.
[18] Ismaiel NE, Sharaf WM, Helmy DO, Zaki MM, Badawi MA and Soliman AS. Detection of cancer stem cells in colorectal cancer: histopathological and immunohistochemical study. Open Access Maced J Med Sci 2016; 4: 543-547.
[19] Watanabe T, Okumura T, Hirano K, Yamaguchi T, Sekine S, Nagata T and Tsukada K. Circulating tumor cells expressing cancer stem cell marker CD44 as a diagnostic biomarker in patients with gastric cancer. Oncol Lett 2017; 13: 281-288.
[20] Choi HS, Kim DA, Chung H, Park IH, Bo HK, Oh ES and Kang DH. Screening of breast cancer stem cell inhibitors using a protein kinase inhibitor library. Cancer Cell Int 2017; 17: 25.
[21] Malara N, Trunzo V, Foresta U, Amodio N, De Vitis S, Roveda L, Fava M, Coluccio M, Macrì R, Di Vito A, Costa N, Mignogna C, Britti D, Palma E and Mollace V. Ex-vivo characterization of circulating colon cancer cells distinguished in stem and differentiated subset provides useful biomarker for personalized metastatic risk assessment. J Transl Med 2016; 14: 133.
[22] Takaya A, Hirohashi Y, Murai A, Morita R, Saijo H, Yamamoto E, Kubo T, Nakatsugawa M, Kanaseki T, Tsukahara T, Tamura Y, Takemasa I, Kondo T, Sato N and Torigoe T. Establishment and analysis of cancer stem-like and non-cancer stem-like clone cells from the human colon cancer cell line SW480. PLoS One 2016; 11: e0158903.