International Journal of Scientific & Engineering Research, Volume 9, Issue 9, september-2018
ISSN 2229-5518

Characterization of colorectal cancer stem cell (CSC) for developing CSC-targeted anti-tumor drugs

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Abstract Background: Cancer stem cell (CSC) is the major cause for tumor development and anti-tumor drugs resistance which we should give it all our attention when starting the plan of cancer treatment. In addition to that CSC has distinct features, it is quiescent, self-renewal, highly tumorigenic and highly resistant to chemo- or radio-therapy. This study focused on characterization of a highly carcinogenic established CSCs and its importance to be the main target for anti-cancer medications. Methods: Cells were assayed for their morphology, holoclone and spheroids forming capacity, Isolation, Purification and Characterization of the Tumor-Initiating Cells using Flow cytometry (FACS), tumor initiation capacity after tumor xenograft and anticancer drug resistance screening using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results: Cells formed holoclones and spheroids, expressed stemness markers 44+(65%),133+(82%)and 166+(98%).CR4 showed cell growth and proliferation with error in DNA and no or low percentage of apoptosis. CR4 cells are highly resistant to anti-cancer drugs like paclitaxel and SB-T-1214. Conclusion: CSCs are the tumor recurrence initiating cells. CR4 CSCs are highly tumorigenic and drug resistant and should be the main target during synthesis of anti-tumor drugs not tumor shrinkage.

Index Terms Cancer stem cell; Colorectal; Holoclone; resistance; Spheroids; Characterization

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Abbreviation

ULA: Ultra low attachment

CSC: Cancer stem cell

CICs: Cancer initiating cells

MTT:3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

FACS: Fluorescence-activated cell sorting

CAFS: Cancer-associated fibroblasts

1. Introduction

Cancer is a disease of anomalous cellular growth. Cancer cells have many features which make it distinct from normal cells. Cancer cells grow without control. Their rate of growth is faster than normal cells, but they do not terminate growth. Cancer cell can grow in petri-dish in multilayer way while normal cell's growth is

International Journal of Scientific & Engineering Research, Volume 9, Issue 9, september-2018
ISSN 2229-5518

stopped after monolayer formation when cellular signals instruct them to halt. Cancer cells frequently grow without any control of hormones and growth factors. Cancer cells are immortal. Cancer cells have no limit of number of replications while normal cells have a limit of replication repeats. Cancer cells differ in last stages in their morphology and size **[1].**

Unlike embryonic stem cells or induced pluripotent cells, they have unlimited replication ability while cancer stem cell does the same but without any regulation during cell proliferation and growth, that is why tumor is formed with high ability of metastasis.

Cancer stem cells are unspecialized primitive cells and have ability to develop into different cell types of the body through differentiation. They are characterized by their ability to self-renew and undergo multilineage differentiation. CSC cancer stem cell theory stated that those cells are small fraction of the cancer cells within a tumor have carcinogenic ability when transplanted into immune-deficient mice, the cancer stem cell sub-population can be isolated by unique surface markers, tumors resulting from the cancer stem cells contain the mixed tumorigenic and non-tumorigenic cells of the original tumor; and the cancer stem cell sub-population can be serially transplanted through multiple generations, indicating that it is a self-renewing population **[2]**.

CSCs hold stemness properties that support cancer progression, self-renewal, cloning, growing, metastasizing and reproliferating. CSCs show unique organizing capacities as they can guide surrounding cells to escape from the defense attacks of immune system, provide a good environment for tumor growth. CSCs propagate heterogeneous cells with a high plasticity potential **[3]**, high resistance to stressful factors in the tumor microenvironment like low oxygen and chemotherapeutic drug **[4]**, and quiescence **[5].**

CSC is the main reason of conventional therapies' resistance and tumor growth is being restored. CSC escape is a real enemy which drugs should target that is why the drug resistance is the major problem in treatment. CSC was assayed before from breast, pancreas and colon that are all-resistant to chemotherapeutic drugs **[6]**.

In this work, we focused on the characterization of colorectal CSC **CR4** cell line established in our laboratory and it is role in cancer propagation that is why it should be targeted**.**

2.1. Materials

Mesenchymal stem cell growth media (MSCGM) from Lonza (Portsmouth, NH), Penicillin, streptomycin and Trypsin-EDTA were obtained from Invitrogen (Grand Island, NY, USA). Collagenases type II and type IV from Sigma-Aldrich, Anti human CD133/2-APC antibody (clone 293C3) from MiltenyiBiotec, CA, USA; CD44-FITC antibody (clone F10-44-2), Ep-CAM-FITC antibody from Biosource, CA, CXCR4-PE antibody from Biosource, USA. Cell Cycle Phase Determination Kit from Cayman

International Journal of Scientific & Engineering Research, Volume 9, Issue 9, september-2018
ISSN 2229-5518

Chemical, USA. Collagen I, Rat Tail, Corning®, USA. NOD/SCID mice (Charles River Laboratories International, Inc., MA) were maintained under defined conditions at SBU animal facility. Ultra-low-adherent (ULA) plates or flasks (Corning), 1X PBS (Phosphate Buffered Saline) Lonza.

1.2. Methods

1.2.1. Ethics Statement

All experiments involving the use of animals were carried out with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, via a research protocol which was approved by Stony Brook University Institutional Animal Care and Use Committee (IACUC).

2.2.2. Culture, Isolation, Purification and Characterization of Tumor-Initiating Cells:

The human colorectal cancer stem cells (CR4) was isolated in our lab from liver metastasis of colon cancer patient and became established cell line **[7].** CR4 cells were cultured on rat collagen type1 coated tissue culture dishes as monolayer and for inducing floating 3D spheroid culture, these cells were seeded on ultra-low-adherent (ULA) plates or flasks (Corning) under 5% CO2 atmosphere at 37°C. Isolated cells were tested functionally for their ability to induce round colonies (holoclones) and 3D spheroids under non-adherent culture conditions. For cell culture from primary mouse tumors, tumor tissues were mechanically and enzymatically disaggregated into single cell suspension at sterile conditions, rinsed with Hank's balanced salt solution and incubated for 1.5 hours at 37°C in a serum-free Mesenchymal stem cell medium (MSCBM; Lonza, Walkersville, MD) containing 200 units/ml Collagenases type II and type IV, 120 μg/ml penicillin and 100 μg/ml streptomycin.

1.2.2. Isolation, Purification and Characterization of the Tumor-Initiating Cells using Flow cytometry (FACS)

Cells were sorted with multiparametric flow cytometry with BD FACSAria cell sorter (Becton Dickinson, CA) at sterile conditions. Cells were labeled with one or several markers conjugated with different fluorescent dyes, including anti-human CD133/2-APC (clone 293C3; MiltenyiBiotec, CA, USA); CD44-PE (clone F10-44-2; Invitrogen/Biosources, USA); EpCAMFITC (Biosource, CA, USA); CXCR4-PE antibody from Biosource, USA. Antibodies were diluted in buffer containing 5% BSA, 1mM EDTA and 15-20% blocking reagent (MiltenyiBiotec) to inhibit unspecific binding to non-target cells. After 15 min incubation at 4°C, stained cells were centrifuged at 950 g for 5 min at 4°C, wash with buffer with containing 5% BSA, 1mM EDTA and fix it with 1% formaline Sort and analyze with multiparametric flow cytometer BD FACSAria (Becton Dickinson, CA).

1.2.3. Cell cycle analysis Assay

Cell cycle analysis according to **[8]**. Seed cells in

International Journal of Scientific & Engineering Research, Volume 9, Issue 9, september-2018
ISSN 2229-5518

a 6-, 12-, or 24-well plate at a density of 105 -106 cells/well in 2, 1, or 0.5 mL of culture medium. Culture the cells ina 5 %CO2 incubator at 37°C for at least 24 hours before treatment. Change media to serum-free or low-serum medium to facilitate cycle synchronization. Trypsinize (adherent cells), Centrifuge to pellet the cells, washing twice with Assay Buffer. Resuspend the cell pellet to a density of 106 cells/mL in assay buffer. Add 1 mL fixative to each sample to fix and permeabilize the cells. Cells must be in fixative for at least two hours prior to PI staining. Centrifuge the fixed cells at 500 x g for five minutes. Suspend the cell pellet in 0.5 mL Staining Solution. Incubate for 30 minutes at room temperature in the dark. Analyze the samples in the FL2 channel of a flow cytometer with a 488-nm excitation laser.

1.2.4. Cell Viability Analysis

The cell viability study was performed with Paclitaxel drug. For this purpose, CR4 cells were seeded into collagen coated 96-well plates at a density of $1X10^4$ cells per well. After 24 h, Paclitaxel or SB-T-1214 was added to the monolayer cells along with respective controls. Cells treated with media and DMSO alone (without any drugs) were used as negative control. Eight replicates were made for each test condition. Following 48h incubation period, cell viability was determined with CellTiter assay according to manufacturer's instructions. The absorbance of the plate was read at a wavelength of 570nm using a BioTek-HT UV–Vis/fluorescence microplate reader. The percent cell viability was calculated based on the absorbance of the drug-treated cells over the absorbance of control (media alone) cells and multiplied by one hundred. 50% inhibition of cell viability (IC50) produced by paclitaxel or SB-T-1214 can be calculated.

1.2.5. Mice Tumor Xenografts

All experiments involving the use of animals were carried out in strict accordance with their commendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, via a research protocol that was approved by Stony Brook University Institutional Animal Care and Use Committee (IACUC). Briefly, after sufficient propagation, clonogenic cells expressing high levels of CD133, CD44, CD44v6, CD166 and EpCAM were resuspended in 1:1 MSCGM/Matrigel and injected to 6weeks old NOD/SCID mice (up to 1million cells per mice; subcutaneously). Tumor development was monitored weekly. The primary tumor sizes were measured, and weights determined using the formula $0.5ab^2$, where b is the smaller of the two perpendicular diameters. Number of mice (n=6) each mice one /cage.

3. Results

Our target to get close to the characterization of cell line CR4 (colorectal cancer stem cell) and if it is targeted with drug so, the tumor root is removed not just tumor shrinkage.

International Journal of Scientific & Engineering Research, Volume 9, Issue 9, september-2018
ISSN 2229-5518

3.1. Holoclone and spheroids forming capacity

These cells can be cultured in serum free media (Mesenchymal stem cell growth media (MSCGM)) and collagen coated rather than serum-containing media such other regular cells as shown in **Fig.(1A,1B,1C)**; show how holo clones were formed in collagen coated plate (2D) and then bubbling of sphere body on holoclone surface; once being transferred to ULA (Ultra low attachment) flask (3D), it grows in dense spheroids.

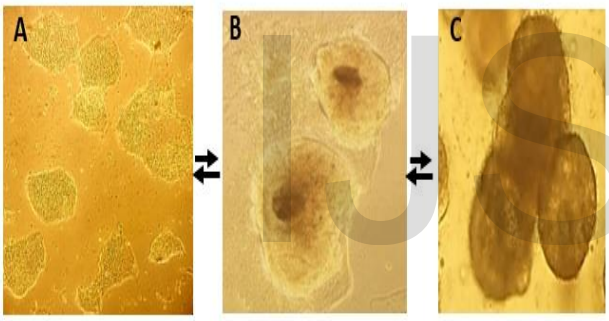

Figure 1: Behavior of CR4 in vitro and bubbling formation *(***A)⟶(c). (A)** holoclones was formed on collagen coated plate (2D culture), **(B)** Bubbling of spheroids above attached adherent cells **(C)** spheroid released from attached cell line and freely moving in culture media ready to be propagated in ultra-low attachment flask (3D). Two opposite arrow indicates that three processes of holoclones, bubbling of spheroids formation above adherent cells and spheroids in ULA can occur in different two ways.

By contrast this process can go in backward way, by transferring those spheroids from 3D flask to collagen coated dishes (2D), it started to attach within 24 hr and spheroids shape started to be dissociated to grow holoclones after attachment to collagen coated plate which we can called it "*in vitro* metastases". Both cells show unique properties of the CSC, such as self-renewal, drug resistance, both have a long-term survival as well as the ability to escape chemotherapy as shown in **[9].**

3.2. Characterization of the Tumor-Initiating Cells using Flow cytometry (FACS)

We have found that this cell line with the highest expression of CD133, CD44, CD 166, EPCAM and express CXCR4 as shown in **Table 1**

Table 1: Stemness markers expression for CR4 cell line.

CD MARKERS	General Function	% Expression (CR4)
CD133	Responsible for tumor initiation, metastasis, colony formation and resistance to treatment **(Sato et al., 2009).**	**82%**
CD+44	Hyaluronic acid receptor, tumor imitation, colony formation **(Sato et al., 2009).**	**65%**
CD+166	Responsible for tumor initiation, metastasis, colony formation **(Sato et al., 2009).**	**98%**
EPCAM	Colony formation **(Sato et al., 2009).**	**99.5%**
CXCR4	Cell migration and adhesion of CAFS cells which support CSC **(Burger et al., 2011).**	**19%**

The majority of CR4 cells express high levels of CD133 (82%), CD44 (65%), CD166 (98%), EpCAM (99.5%). Cells are positive for marker of metastatic activity, CXCR4. The expression of classical CSC markers e.g. CD 133+, 44+; is significantly expressed.

3.3. Tumorigenic capacity of CR4 cell line

The behavior of CR4 cells *in vitro* as in **Fig. (2), Fig.(2A)** show holoclones formed in collagen

International Journal of Scientific & Engineering Research, Volume 9, Issue 9, september-2018
ISSN 2229-5518

coated plate (2D) surrounded with CAFS (cancer-associated fibroblasts) which help CSC in the growth of tumor when are injected in mice.

To show clonogenic or sphere-forming capacities of the CR4 cells, a known number of cells were seeded on type I collagen-coated or ultra-low adherent plates, respectively. After one week of incubation, induced adherent clones or floating spheroids were counted and the clonogenic efficiency was calculated as the ratio of the number of seeded cells compared to the number of induced colonies or spheroids. cells have the capacity to form compacted sphere and holoclone as in **Fig.2B.**

Fig.(2B) show holoclone with empty center in collagen coated plate while a released spheroid even appeared in collagen coated plate as shown in **Fig.(2C).** Spheroids formation was observed for this cell line in ULA flask with free serum media (MSCBM) and finally compact spheroid is formed with necrotic center.

Cells' holoclones containing small multinuclear cells, which can be either subpopulation of CSCs, or cells with mitotic catastrophe which it means the loss of the ability to divide after doubling of the cellular content, this cell line has many seed which able to grow many cells with high tumorigenic capacity **Fig. 2 (D, E),** this is confirmed by injection of those cells subcutaneous, huge tumor is formed and removed as shown in **Fig.2 (F)**.

CR4 showed unique features of CSCs; have a high tumor initiating capacity; as it gives rise a noticeable tumor within one week after cells transplantation, on the other hand it usually takes one month to give a remarkable tumor. We inject $5x10^5 – 6\ x10^6$ cells.

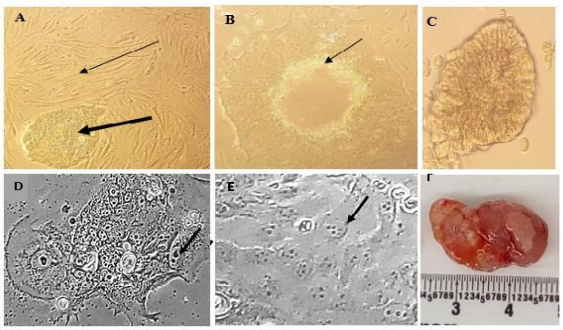

Figure 2: Holoclone-forming capacity of colorectal CSC CR4 cell line. **A**) holoclone indicated by heavy arrow and CAFS (cancer associated fibroblasts) indicated by light arrow formed in collagen coated plat and free serum media. **B**) Releasing of spheroids form holoclone leaving holoclone with empty center .**C**) Enlarged released spheroids. **D)** and **E)** Presence of multinucleated cells inside holoclones like **(A**), when injected into mice, tumor started to grow as in **(F).**

Although this number of cells is high, but our target to have tumor xenografts with high ratio of CSCs. After injection of our cells; tumor was growing rapidly and form measurable tumor (Around 100-200 mm^3) within the first week and volume of tumor reached to 1800 mm3 after one-month**,** however we notice that CR4 tumor volume in first 3 days growth was slow then suddenly increased in volume as in **fig.(3A)**, CR4 tumor volume for ^{1}st, ^{2}nd and ^{3}rd weeks was (123, 305 and 1381mm^3) respectively.

International Journal of Scientific & Engineering Research, Volume 9, Issue 9, september-2018
ISSN 2229-5518

We have determined that the CR4 cells possess high efficiency in induction of tumors in NOD/SCID mice **Fig.(3A)** after serial subcutaneous transplantations of the relatively low cell number 1x10^3 CR4 cells.

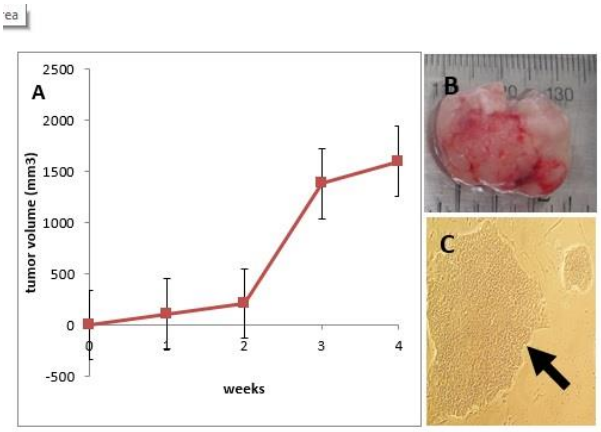

Figure 3: Tumorigenicity of colorectal CSC **CR4** cell line. **A.** shows tumor volume development after transplantation into subcutaneous of NOD/SCID mice, **(B)** colorectal CSC **CR4** removed tumor from Nod/SCID mice; (**C)** holoclones formed after dissociation of each tumors type indicated by dark arrow.

It shows increasing in tumor volume after transplantation; they induced large tumors formations all mice. Removed tumor from CR4 injected mice; as shown in **Fig.(3B)** tumor volume for CR4 is huge and full of blood vessels, we dissociated the tumor in serum-free media and collagen coated plates, after 24 hours plates show holoclones which it is one of characters of CSCs **Fig.(3C)**. This cell lines can create spheres once being cultured in ULA (Ultra Low Attachments) environment with serum-free medium, this phenomenon is called spheroid colony formation **[10]**.

3.4. Rate of holoclone formation for CR4

Cancer stem cell able to form holoclones, following the holoclones formation process, both has high capacity to form, indicating high tumorigenic capacity **Fig. (4A:4B)**.

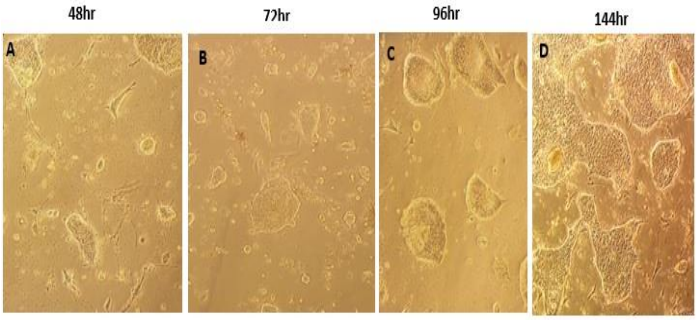

Figure 4: holoclones Formation Capacity **A**)-**D**) After single cell suspension of CR4 cell line being transferred to collagen-coated plate.

Colorectal cells CR4 show stages of holoclones formation, it takes 6 days to form 70% confluent holoclone. The formation of holoclone indicate how much tumorigenic and self- renewal capacity which they have.

We notice that CR4 growth rate is slow after thawing at the beginning, during trypsinization, CR4 takes longer time to be detached, it means that despite cells are CSC, but it is not easy to digest extracellular matrix which hold CR4 cells (high resistance to digestive enzyme). During cells storage, gradually reducing temperature is optimum way to keep cells viable but when we tried to do store cells without gradually freezing; CR4 lost their viability.

International Journal of Scientific & Engineering Research, Volume 9, Issue 9, september-2018
ISSN 2229-5518

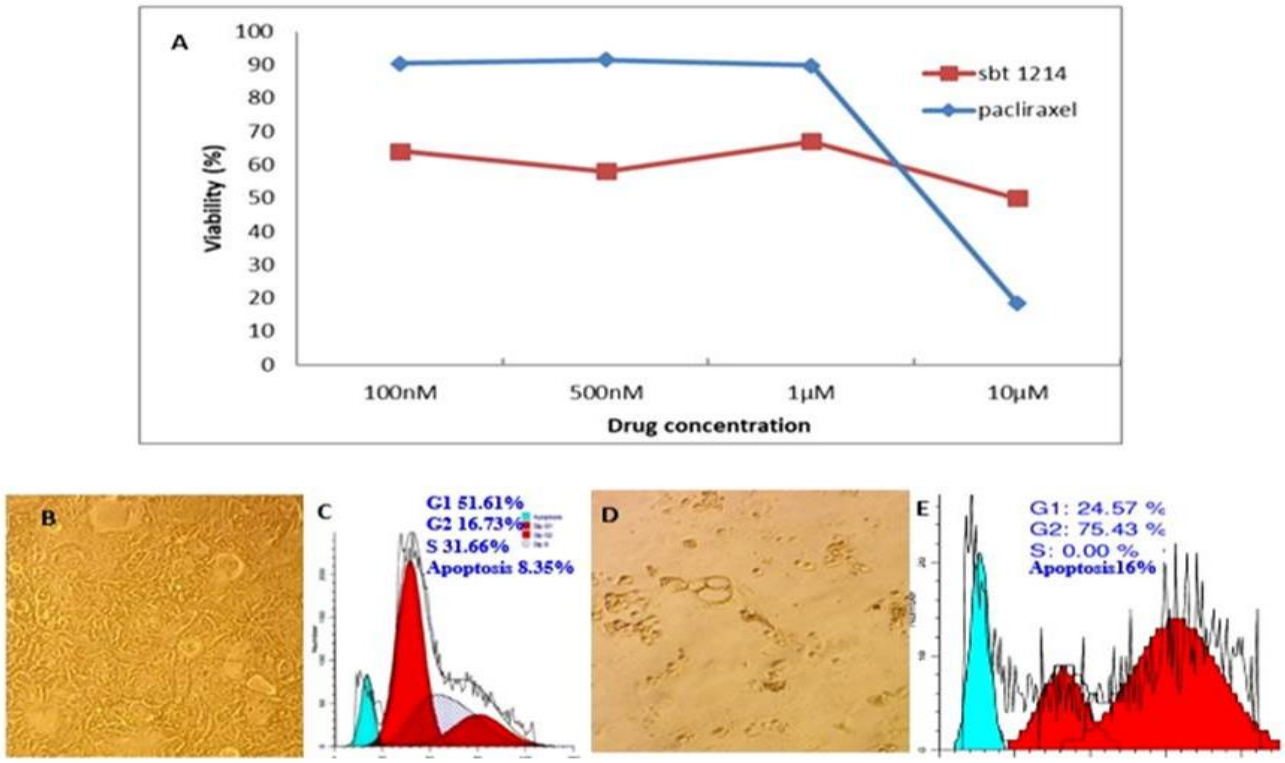

Figure 5: CR4 cell line are highly resistant to treatment with cytotoxic drugs. (MTT assay after drug treatment for 48 h). Using common used anticancer drug (paclitaxel) in treatment of CR4 show high resistance while using new generation of taxoids (**SB-T-1214)** is more effective. **B**) Morphology of CR4 cells as control. **C)** Cell cycle analysis of CR4 cells before treatment. **D)** Morphology of CR4 after treatment using SBT-1214. **E)** Cell cycle analysis of CR4 after treatment using SBT-1214.

3.5. CR4 resistance against antitumor drug

The effect of SB-T- 1214 on cell cycle distribution was next assessed by flow cytometry. cells were found to be either in the G0/ G1 and S phase, indicating Cells increase in size in S phase, DNA replication occurs without control and so cells continue growth and cause resistant treated tumor and no or low percent of apoptosis percent **Fig. (5A, B, E and F).**

From this result; CR4 cells have stemness characters and are highly resistance and should be targeted during any drug synthesis.

Significant alterations in the percentage of cells were observed in nearly G2/M phases of both CR4 cells and we used a new generation of taxoids sb-t-1214 **[11]**, for treatment of both cell lines *in vitro with 1 µM.* Treatment *in vitro* shows that this cell line has a high resistance response against the most common anti-cancer drugs "paclitaxel". CR4 cells has a high resistance capacity to treatment with commonly used anticancer drugs e.g. Paclitaxel or Taxoids **Fig.(5A**).

International Journal of Scientific & Engineering Research, Volume 9, Issue 9, september-2018
ISSN 2229-5518

After 48-hour treatment in concentration range from 100 nM up to 10 μM using (MTT assay), CR4 cells have shown little or no cytotoxicity at concentration lower than 10 μm **Fig.(5A)**; moreover, SBT-1214. **Fig.(5A)** is more cytotoxic against CR4 cell line. SB-T-1214 is more efficient than paclitaxel as it is CSC-targeted drug reduce viability to 65% for CR4 compared with paclitaxel reduce viability to 90%.

In **Fig.(5B) and Fig. (5C) CR4** cells as control show their viable shiny morphology; cell cycle analysis shows **51.61%** in G1/G0 and apoptosis is **8.35 %. Fig.(5D)** cells started to give rise to apoptosis but still some cells are attached as in **Fig.(5D)** compared with CR4 as control. **(5E)** CR4 showed a decrease in the percentage of cells in the G1/G0 phase which increased in G2/M arrest after treatment to **75.5 %** after 24 hr.**;** cells started to give rise to low percent of apoptosis as bubbling of cells, but it did not lose their attachment .CR4 Cells has a high resistance as it showed no apoptosis. The drug made arrest of both cells' cell cycle at G2 phase.

Scientific research should focus on treatment of CSC, it shows that CSC is the real enemy which is the best target of any drug. The preparation of any promising drug should be CSC-targeted to kill it which is the root of disease. CSC has unique properties like self- renewal, highly therapy-resistant and quiescent. Tumor consist of CSC and differentiated tumor after mainstream drug treatment differentiated cell in tumor died and tumor shrink but the root still there (**CSC**) so tumor regain its volume so when using CSC-targeted drug so, tumor shrink forever.

4. Discussion

It is widely known that the tumor-initiating cells (TICs) or cancer stem cells (CSCs), are not only highly resistant to conventional therapeutic strategies, but may promote cancer progression due to the drug-induced compensatory increase in their self-renewal **[12; 13;15]**. Therefore, the search for effective therapeutic interventions should be based on the evaluation of the post-treatment status of the tumor-initiating CSCs, and not only on tumor shrinkage. Cancer should be defined as cancer stem cell disease. Within tumor a niche of CSC which it responsible for the growth and metastasis of tumor **[16]**. This cell is responsible for survival of malignant cells and escaping from conventional therapy.

In our studies, a high combined expression of CD133 and CD44 was selected as the first criterion for the isolation and initial enrichment of colorectal CSCs. It is known that recurrent cancer is associated with more malignant phenotype, increased intrinsic or acquired drug resistance and high mortality rates **[17]**. That is why, it would be beneficial to develop drugs targeting CSCs using the most aggressive tumor types or cell lines, because such drugs can potentially have a larger spectrum of mechanisms of action, and therefore, broader anti-cancer implications. There is a growing body of evidence

International Journal of Scientific & Engineering Research, Volume 9, Issue 9, september-2018
ISSN 2229-5518

that tumorigenic cells with a CD133+ phenotype are present in many human cancer types, and isolation of CSCs based on CD133-positivity seems promising, since it was demonstrated that the expression of this marker correlates with both resistance to treatment **[18];[20]; [21]** and tumor aggressiveness and poor prognosis **[18].**

CSCs are quiescent and non-cycling through cell cycle and this the reason why CSCs is chemotherapeutic resistance and any treatment just was working on cycling cells **[22].** CSCs niche includes fibroblasts and endothelial cells which secrete factors that participate in the regulation of CSCs **[23]; [25]**. Colorectal cells CR4 show that they are in quiescent state through cell cycle analysis; majority of CR4 are in G1 was 51.61% and S phase was 31.66% respectively and cells showed a low percent in G2 phase 16.73% respectively. Cell cycle analysis show that cell cycle arrest at cell proliferation and keep away from error checking phase G1. CR4 cells had no or low percent of apoptosis where CR4 8.35% so, as shown from cell cycle analysis, we are dealing with quiescent and non-cycling cells.

Using one of the new generation of taxoids SB-T-1214 in treatment of cells, this drug made shift of cells to G2 and this was higher G2 arrest for CR4, indicating that CR4 is highly drug resistance. Treatment of CR4 cell line using SB-T-1214 allow cells to be easily killed as it is CSC-targeted drug it able to reduce viability than paclitaxel. The new generation of taxoid, SBT-1214 shows better activity than paclitaxel against anti-tumor resistant cancer cells **[26].** This new-generation taxoid SBT-1214 induced around 167 days relapse and tumor formation inhibition of drug-resistant colon tumor xenografts **[27]**.

Despite slower growth rate, spheroids formed by budding or aggregation initially showed sensitivity to cytotoxic drugs that was equivalent to monolayers. This was followed by progressive acquisition of drug resistance during several cycles of spheroid passaging. This lead to drug resistance **[28]**; our cells show spheroid forming shape in ultra-low attachment environment and serum free media. This model of 3D spheroid model is the best model for *invitro* drug screening. Our cells CR4 show spheroids formation in the same environments indicating how cells were resistant to drug treatment.

Clonogenicity is one of the characteristic of stem cells to ensure their proliferation and differentiation **[29]**. Stem cells have high proliferative rate, self-renewal nature, and multi-lineage differentiation ability **[30]**. The clonogenicity nature of the stem cells is effectively represented at a restricted plating efficiency; our cells show holoclone forming capacity and formation of tumor after transplantation in NOD/SCID mice.

Cancer stem cells have Stemness markers which are responsible for regulating the tumorigenesis, proliferation, aggressiveness, chemo-radio resistance, recurrence and metastasis. Expression of various stem cell markers (CD44, CD133 and ALDH1) and transcription factors e.g. Oct4, Sox2, Nanog play a significant role in

International Journal of Scientific & Engineering Research, Volume 9, Issue 9, september-2018
ISSN 2229-5518

clonal proliferation; all of them are a functional characteristics of stem cells in the tumor micro-environment and the reason of resistance- response to therapy **[31]**. Our cells show high expression of stemness markers (CD 133; CD 44) significantly and low expression of CXCR4 so, those cells gained aggressive and drug-resistance properties.

5. Conclusion

CR4 are cancer stem cells, highly tumorigenic and drug-resistant. Cells shows a positive attitude towards stemness characters so, CR4 are perfect models for anti-tumor drug screening. CR4 has a high resistance ability against treatment using antitumor drugs.

6. Acknowledgement:

Thanks to Dr. Ahmed A. Attallah, postdoctoral associate, department of medicine – nephrology, Stony Brook University for his helpful cooperation in writing and his scientific opinion.

7. Funding:

This work was also supported by a Joint Supervision Fellowship to Rana El sadda funded by the Cultural Affairs and Mission sectors of the Egyptian Ministry of Higher Education.

8. Declaration of interest

Authors declare that there is no conflict of interest

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