European Journal of Molecular & Clinical Medicine
ISSN 2515-8260 Volume 7, Issue 11, 2020

Isolation and Characterization of Prostate Cancer Stem cells from Prostate Cancer Tissues

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Abstract**: Cancer, a metastatic disorder disruptsnormal growth and metabolic activities leads to Malignancy. Without signs and symptoms,prostate cancer is one increasing in men day by day and more than 1.1 million cases were recorded by 2012.It has been shown that basal epithelial cells are more potent in the conversion of PCaandsome of the luminal cells are castration resistant completely lost due to apoptotic cell death mechanism. After Androgen replacement therapy castration becomes a weapon for the identification of the altered mechanistic aversion of prostate cancer from stem cells. These cancer stem cells are the elusive therapeutic targets for present oncotherapies which are responsible for disease recurrence and metastases. The main aim of the present study is to isolate and characterize Prostate cancer stem cells based on prostate specific cell surface markers in prostate cancer tissues. In this study we have taken 6 prostate tissues and isolated CD 133 and CD 44 positive cells through Florescence staining method. Further isolation has done through MACS which has been cultured and taken from DMEM media. In conclusion confirmation of isolated cells as prostate cancer stem cells was done through western blot based on the identification of other markers OCT-4 and MDR-1.

Keywords: Malignancy; Prostate cancer; oncotherapy; recurrence; metastases

1. INTRODUCTION

Leading cause of cancer related mortality and most frequently diagnosed cancer among men is the Prostate cancer.Based on the studies done by section of cancer surveillance on prostate cancer in men, nearly 1.1 million cases were recorded by 2012 as asymptomatic even though prostate specific antigen is positive. Highest incidence has seen in martinique, Norway and France stood next followed by 68% can be seen in developed countries. In continents like Asia and Africa lowest incidence can be seen.

Even though, early detection of prostate cancer with much recent advancement has been developed, still there remains little effective therapy for patients with locally advanced and/or metastatic disease**(1)**. Basal, luminal and neuroendocrine regions of prostate epithelial cells expresses high and low molecular weight protein synthesis respectively. Luminal cells secrete prostate specific antigen and prostate specific alakaline phosphatase into glandular lumen through androgen dependant cascade. Secretory luminal and neuroendocrine cells express and secretes neuropeptides, synaptophysin and chromogranin, which are not androgen dependent.Most of the patients with advanced disease will initially respond due to the androgen-dependent nature of the vast majority of prostate cancer cellswith androgen ablation therapy. With very high frequency, androgen-independent cancers emerge and subsequently widespread metastases occur and thereby hormone resistant relapse will occur. Many studies elucidate the markers present in the prostate stem cells of three regions.

Multitude markers participate in the tumor progression, self renewal capacity, metastatic colonization & growth and recurrence and therapeutic resistance [2]. The common signaling pathways operates are NOTCH, HedgeHog and Wnt.

Normal prostate stem cells operates in the microenvironment of non-neoplastic activities like the cellular growth, signal response towards preapoptotic cell death mechanism, minimal chromosomal aberrations, controlling intensified cellular metabolic and genomic hyperactivity. Not necessarily, all the normal prostate stem cells need not to be converted into prostate cancer stem cells. Due to loss of response,conversion into primary PCa CSC's towards cellular preapoptotic signals, DNA repair splicing mechanisms, cell-cell contact inhibition,hypermutations due to chromosomal aberrations and so on. Not all prostate cancer stem cells express the markers present on prostate stem cells. Hence, origin and conversion into prostate cancer stem cell has become main conversation [3].

Malignancy development from normal stem cells is primary concept as in mouse models it has been shown that basal epithelial cells are more potent in the conversion of PCa even though both basal and luminal will participate. During castration, basal cells will be under control for the regular differentiation mechanism but luminal cells are completely lost due to apoptotic cell death mechanism. Hence castration becomes a weapon to identify altered mechanistic aversion of prostate cancer from stem cells. After Androgen replacement therapy, some of the luminal progenitor cells will survive and progressed towards prostate cancer which can be considered as castration resistant phenotypically [2&4-9].

Potentially, all normal cells may not be converted into cancer cells. It is not necessary that all cancer cells to be converted into a subset of cells called cancer stem cells. The concept of these CSC was first proposed 45 years ago as complete analysis hasn't been done till the development of advanced research tools. The extraction of CSC evidence was depicted based on the hematological malignancies; its potentiality has been revealed based on the characteristic features of the embryonic stem cells. Hence, each organ has its own property based stem cells; hence different cancers have different CSCs thereby in different cancers has different CSCs. These CSCs can also be referred as progenitor cells that are the main possible cells for the tumor growth and relapse of the cancer [10-13]. The nature of the tumor is not the same as in tumors present in the same person as same type of different persons.

CSCs are self-renewing, totipotent cells resembles the characteristic feature of normal cells. These groups of subset of cells will show its propagation either through symmetric or asymmetric division. The descendants of these cancer stem cells progeny constitute the bulk of the heterogeneous tumor. This irregular symmetric or asymmetric cell division maintains the number of the CSCs within the tumors. Studying the nature of these tumors of different types will help us to understand the basic interactive neutral drift dynamic changes. These studies will also make us to understand the counteracting metastatic proclivity building with programmed clonal evolution sequence [14-18].In the present study we attempted to elucidate the cell surface markers for the isolation and characterization of cancer stem cells in prostate cancer tissues.

2. MATERIALS AND METHODS

The present study was conducted on the prostatic carcinoma extracted tissues collected from the patients who are already suffering with PCa. 6 PCa tissue samples collected through FNaC which is not required for further analysis has been collected from the hospital pathology Laboratory, AMC, Visakhapatnam. The study and sample collection protocols mentioned in the present study were duly approved by the Departmental Ethics Committee of GITAM University, Visakhapatnam, Andhra Pradesh.

1.1 Selection of the subjects for the present study

Selection Criteria for the collection of prostate tissues in the study
Inclusion criteria –

- Subjects should be having higher PSA
- Subjects should be diagnosed with confirmed PCa

Exclusion criteria –

- Expired/ long stored prostate cancer tissues
- Subjects diagnosed with Human Immunodeficiency Virus (HIV)

1.2 Subject selection based on acceptance

The samples are collected from the concerned laboratory with their consent after confirmation about the non usage of that tissue by them as collected.

1.3 Collection of Prostate tissue samples

Approximately 6 samples of PCa tissues have been collected from the laboratory for the investigations. All the samples were given with unique identification numbers and collected in normal saline solution without any further damage.

1.4 Isolation of CD44 positive cell fraction from PCa suspended samples

To isolate thecells from the tissues, magnetic cell sorter method was used with the following antibodies conjugated with magnetic microbeads.

Table 1: List of antibodies conjugated magnetic microbeads used for Magnetic Associate Cell Sorter (MACS)

Antibody conjugated magnetic microbeads	Dilution (volume/ cell number)	Make
CD 44 micro beads	20µl /1× 10^7	MACS MiltenyiBiotec
CD 133 micro beads	20µl /1× 10^7	MACS MiltenyiBiotec

1.5 Labeling of MNCs with CD44 magnetic microbeads

Prostate cancer tissues suspension was centrifuged at 1500 rpm for 5 minutes and the cell pellet was subjected to antigen labeling with CD44 antibody. The supernatant was discarded and the pellet was re-suspended in 80 µL of PBS. Based on the cell count 20 µl of CD 44 magnetic micro beads were added for every 1×10^7 cells. To label CD45 antigen, the cell suspension was mixed well and incubated for 15 min at 4°C to 8°C. After the incubation, the cells were washed twice with 1 – 2 mL of PBS. Cell suspension was centrifuged at 1500 rpm for 10 minutes. The supernatant was discarded and pellet was re-suspended in 500 µL of PBS. The cell pellet thus obtained was processed for magnetic cell separation using MS and LS columns.

1.6 Magnetic separation with MS or LS column

To isolate CD positive and negative selected cell population from the PCa tissues, Mini-Midi MACS system (manufactured by MiltenyiBiotec, Singapore) was used. MS/LS columns were designed for positive selection of cells. They were also suitable for depletion of magnetically labeled cells. The column matrix of the MS/LS Columns was composed of ferromagnetic spheres, which were covered with a cell-friendly coating allowing fast and gentle separation of cells. When placed in the magnetic field of a MACS® separator, the spheres amplified the magnetic field by 10,000-fold, thus inducing a high gradient within the column. This was crucial for isolation of cells which were only minimally labeled with MACS®microbeads, leaving enough epitopes free for concurrent antibody staining. The space between the spheres

was several times larger than primary and cultured cells. This allowed the cells to freely flow through the column. Magnetically labeled cells were held in suspension within the column and did not actually "bind" to the column matrix. This suspension minimized stress on the cells and allowed for efficient sterile washing by avoiding cell aggregation. The columns were placed in the magnetic field of a Mini-Midi MACS system.

1.7 The column was prepared by rinsing with appropriate amount of buffer. For MS: 500 µl and LS: 3ml volumes were used as washing buffers.

The cells suspension with magnetic microbeads was added to the column. The unlabeled cells which pass through the column were collected at the bottom of the column in a sterile tube. The column was washed with PBS for three times. MS Column with 3 X 500 µl ; LS column with 3 X 3 ml volumes of PBS was used during the column washing. The unlabeled cells referred to as CD 44negative fraction was collected.

After collection of unlabelled cells, column was removed from the separator and placed it on a suitable collection tube and added appropriate amount of buffer onto the column. Immediately flush out the magnetic labeled cell fraction by firmly applying the plunger. These labeled cells referred to CD44 positive cells.

1.8 Isolation of CD133 positive / negative from CD44 positive / negative fraction

CD 133 positive / negative were isolated from CD44 positive / negative fraction of cell type by repeated labeling and magnetic separation steps with anti-CD133 magnetic microbeads.

1.9 Characterization of cells using flow cytometry

The above isolated cell types were to be analyzed using flow cytometry. The antigens of CD44+/-, CD133+/- and CD44 and CD 133+/- cultured cells were analysed using flow cytometer as described by Bianchi et al., 1990.

To perform flow cytometry of the above cell types, 2 X 10^4 cells were stained from freshly isolated cells from PCa tissues, by incubating with 20 µl of diluted with antibodies at 4^oC for 45 minutes. The cells were washed with PBS for 4 minutes and fixed with 0.2% paraformaldehyde for 30 minutes at room temperature. The antibodies used in the study were Mouse monoclonal FITC labeled CD44 (MiltenyiBiotec, Singapore), FITC labeled CD133 (MiltenyiBiotec, Singapore), PE labeled CD (MiltenyiBiotec, Singapore), Rabbit anti-human CD235A / Glycophorin A (Pharmingen, BD Biosciences, USA), FITC labeled monoclonal anti-human α1, PE labeled monoclonal anti-human β1, FITC labeled monoclonal anti-human αvβ3, FITC labeled monoclonal anti-human αvβ5, FITC labeled monoclonal anti-human α2β1, PE labeled monoclonal anti-human α1β1, PE labeled monoclonal anti-human β2, PE labeled monoclonal anti-human α4β1, PE labeled monoclonal anti-human α5β1, PE labeled monoclonal anti-human α6β1, PE labeled monoclonal anti-human αIIbβ3. All the above integrin antibodies were purchased from Pharmingen, BD Biosciences, USA. Cells were analyzed on a customized BD Accuri C6 flow cytometer (BD Biosciences, USA) using filters of 533/30 BP for FITC and 585/40 BP for PE with a 488-nm argon laser to excite both PE and FITC. The cells were analyzed by flow cytometer or covered with foil and stored at 4°C until analysis. Analysis of the samples was done using WinMDI^{TM} (Stanford University, U.S.A).

Before the depletion of CD 44 cells from the cultured Pca cells of the tissue, the flow cytometry analysis was carried out to evaluate the percentile of occurrence of CD44, CD133 and both CD44 and CD 133 positive and negative cells of the PCa tissue.

1.10 Western blot analysis for other markers oct 4, MDR 1/ABCB 1, beta actin

The freshly isolated cells and cultured cellssamples were lysed by adding SDS sample buffer containing 1 mMorthovanadate, and the samples were processed for western blotting as described by Hempelmann*et al.,* in 1987. CD44 and CD 133 or both positive PCa cells are used for Western blot analysis. For the confirmation of CSC's in PCa tissues oct 4, MDR 1/ABCB 1, beta actin antibodies were used.

3. RESULTS:

Subjects who are having higher PSA prostate tissues has been collected and subjected for the quantification of cell surface markers to determine CSC. In 6 prostate cancer tissue samples on average, most of the cellsare CD 44 negative marker associated cells (2.8×10^4). Remaining pool contains CD 133 negative cells (2.2×10^4) followed by the cells that contains both markers (2.05×10^4) as shown in the table 2.

Prostate tissue samples	CD 133 Negative	CD 44 Negative	CD 133/44 Positive
Pca 1	2.8×10^4	2.9×10^4	2.4×10^4
Pca 2	1.6×10^4	3.7×10^4	1.5×10^4
Pca 3	2.25×10^4	2.65×10^4	2.2×10^4
Pca 4	2.6×10^4	2.8×10^4	2.4×10^4
Pca 5	1.05×10^4	1.9×10^4	1.0×10^4
Pca 6	2.9×10^4	2.7×10^4	2.8×10^4

Table: 2 Number of cells isolated from six prostate cancer tissues

Further identification of CD133 marker associated specific prostate cancer stem cells was done through 4′, 6-diamidino-2-phenylindole (DAPI staining) which is used to stain the nucleus of Fluorescence stained FITC cells.

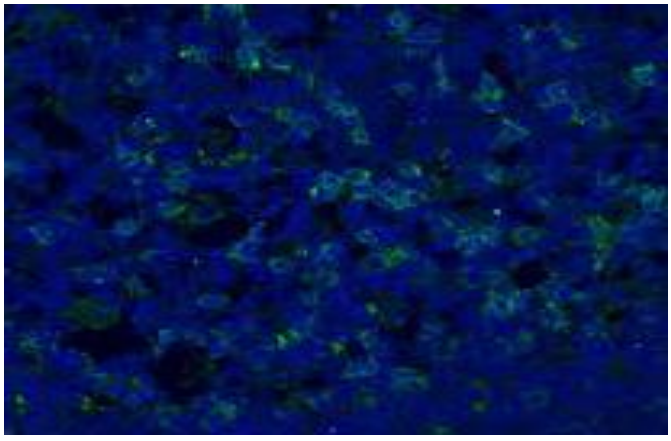

Figure: 1 Florescence staining of CD133 in prostate cancer stem cells

During the identification of cells with specific marker through IgG, cells cultured in Poly-ethylene Teripthalate and fluorescence attached cells detection showing very poor detection. But specific cells with CD 133 and CD44 markers through cultured Poly-ethylene Teripthalate stain against FITC labeled can be observed very prominently as shown in the diagram.

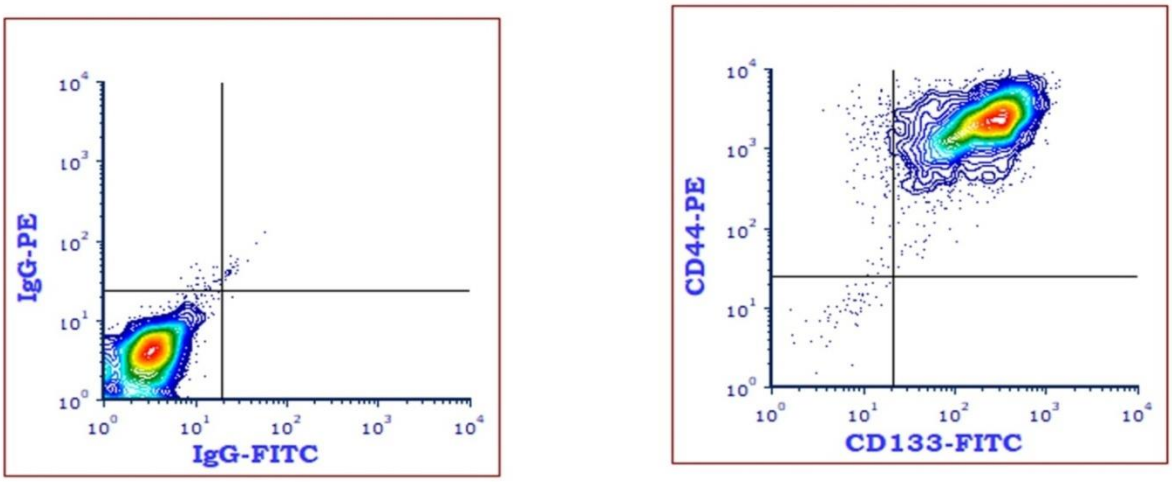

Figure: 2 Determination of CD133/44 positive CSC's through flow cytometry analysis

Further confirmation of these prostate cancer stem cells from prostate tissues apart from the marker associated cells (CD 133/44 positive), the samples are subjected to western blot analysis. These stem cells contains along with CD133 and CD 44 also exhibits other specific markers like octamer-binding transcription factor 4 (OCT 4) and ATP Binding Cassette Subfamily B Member ***1/*** *Multi Drug Resistance 1(ABCB 1/MDR 1)as shown in the Figure 3.*

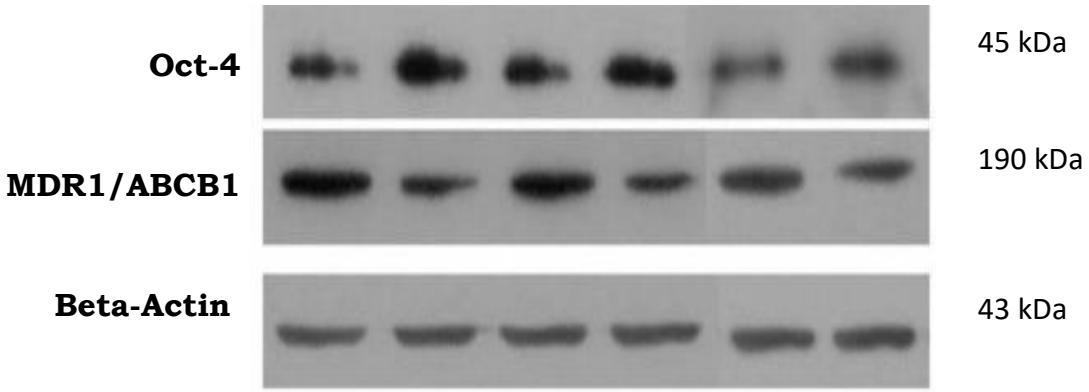

Figure: 3 Western blot analyses of Prostate cancer stem cells

It is clearly evident that all the isolated prostate cancer stem cells from prostate tissues exhibit Oct-4 and MDR 1 which was prominently extrapolated against the standard beta actin protein.

4. DISCUSSION

Cancer Stem Cells (CSC), makes an oncologist for non effective therapy is due to their self renewal with asymmetrical targets on exposure to treatment becomes resistant. CSC's are present with distinct anatomical niches that are not randomly distributed within a tumor for self renewal mechanism. CSCs and their niches are dynamic activates regulatory niche formation and maintenance. These contain nutrients, oxygen, and physical and soluble interactions that maintain CSC self-renewal. Present therapies by inducing stem cell state generate stresses and shows impact on tumor microenvironment.

In the tumor associated cancers like prostate, the stem cells are centrally surrounded by normal cells. During the oncotherapy targets either by chemo or radio, killing of these non cancer associated stem cells occurs, as the progression of tumor growth expanding cells areleft over. These cells further exhibits altered characteristic representations likeself-renewal, metastasis, apoptosis, heterogeneity, immune resistance, and properties related to

radioresistance and/or chemoresistance.The above said might be one of the metastatic characteristic spread to other organs by cancer stem cells.

The classic epithelial–mesenchymal (EMT) model of metastasis (top) posits that the dissemination of cancer cells requires loss of epithelial cell traits commensurate with gain of mesenchymal cell traits, which enables the cells to detach from the primary tumour and invade surrounding tissue, intravasate and survive in circulation and, finally, extravasate and localize to a distant metastatic site. Several genes like TGF-B, Vimentin, TWIST 1, Sna 1 and ZEB 1 have been shown to drive EMT and their expression serves as a marker of the process as gene signatures. Expression of EMT gene signature is seen interestingly during the enrichment of cancer stem cells (CSCs) in disseminated tumour cells. Furthermore, the capacity for tumour propagation, which is required for establishment of a tumour at a distant site, is a salient feature of stem cells. The parallels between EMT cells and CSCs raise the possibility that they represent overlapping concepts (19-24).

Studies on hypoxic niche are not well defined structurally, characterized by low oxygen tension and increased acidity. This brings alterations in pH, oxygen, or nutrient supply. In spite of several adaptive mechanism alterations, CSC niche survives that becomes more clinical evaluation for the development of therapeutic inhibitors. Many studies have been conducted on multiple niches that include perivascular, proliferative, hypoxic and perinecrotic niches. With the ablation of the vasculature leading to tumor regression, Proximity to vascular endothelial cells has been shown to regulate directly CSC growth. In every type of cancer, specific group population of cells called cancer stem cells are present which can be recognized with specific cell surface markers as shown in below Figure 5 **[25].**

Cell surface markers associated with cancer stem cells

Tumor type	Cell surface markers	Tumorigenic
Acute myeloid leukemia	$CD34^{+}$ $CD38^{-}$	In SCID mice
Acute myeloid leukemia	$CD34^{+}$ $CD38^{-}$	In NOD/SCID mice
Breast tumors	$CD44^{+}$ $CD24^{-/low}$ $Lineage^{-}$	In NOD/SCID mice
Brain tumors	$CD133^{+}$	In NOD/SCID mice
Bone sarcomas	Stro-1^{+} $CD105^{+}$ $CD44^{+}$	Self-renewal and colony formation capacity *in vitro*
Lung adenocarcinomas	Sca-1^{+} $CD45^{-}$ $Pecam^{-}$ $CD34^{+}$	Self-renewal, colony formation and multilineage differentiation capacity *in vitro*
Metastatic melanomas	$CD20^{+}$	In SCID mice
Prostate cancers	$CD44^{+}$ alpha2beta1^{hi} $CD133^{+}$	Self-renewal and extensive proliferation capacity *in vitro*

Figure:5 Makers of Prostate cancer stem cells

Hence, ablation of these CSC acquired their prominence in the present oncotherapy. In the present study prostate cancer stem cells has shown their significance interms of having specific cell surface markers of CD 133/44 positive. It was further confirmed based on the

metastatic progression due to the presence of the other tumor relapse markers like OCT 4 and MDR 1.

5. CONCLUSION

Tumor relapse is due to the presence of specific cell subpopulation. Potent oncotherapies are those at which targeting cells which are self-renewal, totipotent and having specific cell surface markers considered to be as cancer stem cells will makes to stop the progression of both intra and inter tumor heterogeneity. Based on these onco-targets tumor niche is also disturbed and thereby perivascular niche progression has been obstructed thereby inhibits metastatic progression.

6. CONFLICTSOF INTEREST:

- The authors declare that they have no conflicts of interest.

7. ACKNOWLEDGEMENTS

Data presented in this article is a part of Science and Engineering Research Board sponsored project from Department of Science and Technology, Govt of India with file no. SB/FTP/ETA-0234/2013.

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