

Establishment and molecular characterization of breast cancer mesenchymal stem cell line derived from human non-metastasis breast cancer tumor

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ABSTRACT

Breast cancer remains a leading cause of morbidity and mortality in women mainly because of the propensity of primary breast tumors to metastasize. It is composed of heterogeneous cell populations with different biological properties. Breast cancer-initiating cells have been recently identified in breast carcinoma as CD44⁺/CD24^{-/low} cells, which display stem cell like properties. In the present study, we have isolated breast cancer stem cells from non-metastasis tumor tissue, which is presently at passage 18 and designated as human Breast Cancer Mesenchymal Stem Cells (hBCMSCs) line. These cells showed spindle shaped morphology and formed mammospheres as well as pluripotency clones indicating their stem cell nature. Molecular marker study confirmed mesenchymal nature as well as pluripotency, plasticity and oncogenicity of these cells. The hBCMSCs cell line may likely contain a heterogeneous population of malignant cells. Interestingly, we also found that these cells exhibit BRCA 2 mutation, which was found in Indian population. Overall, this study revealed that hBCMSCs cell line may represent a suitable *in vitro* model to study the mechanism of breast cancer which further leads to an identification of molecular targets for future breast cancer targeted therapy.

Keywords: Breast Cancer Stem Cells; Breast Cancer, BRCA2; Mesenchymal Stem Cells; Molecular Markers

1. INTRODUCTION

A fundamental problem in breast cancer research is to identify the cell type capable of sustaining the growth of

the neoplastic clone. It is less clear from most of the breast cancers studies that which cells within the tumor clone possess tumor-initiating cell function for maintaining tumor growth. The identification of potential breast cancer stem cells is the most important task for the study due to the characteristics of these stem cells which are resistant to conventional cancer therapy [1]. Pardal *et al.* [2003] have shown that a small subset of cells within a tumor, termed Cancer Stem Cells (CSCs) or tumor-initiating cells which are responsible for tumor initiation and tumor growth progression [2]. The concept about tumor containing heterogeneous populations of cells was demonstrated firstly by Lapidot and colleagues in leukemia [3]. Fidler and Kripke observed striking heterogeneity of dissociated tumor cells with respect to the clonogenic ability to form metastasis [4]. The existence of “cancer stem cells” or “cancer initiating cells” has been identified in a variety of solid tumors including gliomas, medulloblastoma, lung cancer, prostate cancer, colon cancer and breast cancer by various investigators [5-11]. Recent studies have suggested the existence of stem cells in the human mammary gland similar to those in the rodent and also shown that non-adherent mammospheres are highly enriched for cells with stem and progenitor cell properties [12,13]. The isolation and characterization of mammary cancer stem cells also has important implications for understanding mammary carcinogenesis. Significant progress has already been made in the identification of the stem cell in mammary gland of the mouse, though relatively little work has been carried out in humans [1]. It may help to design therapies targeted to the unique properties of the tumor stem cell to get selective killing. There is a need of comparing Breast cancer initiating cells phenotypes with non-breast cancer initiating cells phenotypes. The focus of future studies involves cell signaling pathways, molecular and cellular markers for normal and tumorigenic cells and drug development.

Molecular marker study is one of the important studies to characterize and establish any cell type. Recent studies on stem cell research have identified CD105, CD13 and CD73 as a Mesenchymal Stem Cells (MSCs) markers [14], CD34, CD45 as a Hematopoietic Stem Cells (HSCs) markers [15,16], Oct4, Nanog, SOX2 as a Pluripotency and self renewal capacity markers [17]. It has been established that there is change in expression of molecular markers like DAPK, COX2 and LIF in cancerous cell types [18-20]. CD44 is a receptor for haluronic acid, which has been shown to play a role in tumor migration and metastasis [21]. CD24 has recently been described as a negative regulator of CXCR4, a chemokine receptor important in facilitating breast cancer metastasis [22]. An understanding of the biology of the cells that drive tumorigenesis has the potential to lead to new therapeutic approaches for breast cancer.

In present study, we have mainly focused on isolation, characterization and propagation of *in vitro* breast cancer stem cells from non-metastasis human breast tumor. Moreover, our research further confined to the isolation of breast cancer stem cells and characterizes these cells for their mesenchymal or hematopoietic phenotypes as well as pluripotency, oncogenic, cytokines and chemokines markers. As our laboratory has already established Breast cancer mutation in BRCA2 gene in Indian population [23]. We have decided to study this mutation *in vivo* tumor tissue and *in vitro* isolated hBSCMSCs cell line to confirm the specificity of Breast cancer tumor cells. Overall, this study will develop *in vitro* model system to understanding mechanism of breast cancer development as well as to help in developing specific targeted therapies for breast cancer.

2. MATERIALS AND METHODS

2.1. Collection and Processing of Human Breast Cancer Tissue

Fresh specimen of human breast tumor was received in sterile condition within an hour after surgery with prior consent of patient as per the guidelines of ethical committee of Jaslok Hospital and Research Center, Mumbai, India and it was sent to the stem cell laboratory. The tumor was cut into 2 mm pieces in sterile 1X Phosphate Buffer Saline (PBS) containing 1% Penstrep (HiMedia, India) and washed 2 - 3 times. The cleaned tissue fragments, then digested in 0.25% Trypsin-EDTA (HiMedia, India) at 37°C for 40 min. Tumor tissue explants were plated in 65 mm Nunc dishes which were scratched with scarpel blade to adhere these tissue fragments. The tissues pieces were fed with freshly prepared DMEM (Dulbecco's Modified Eagle's Medium, HiMedia, India) supplemented with 10% FBS (Fetal Bovine

Serum, Invitrogen, Carlsbad, CA), 1% Penicillin-streptomycin, 1µl/ml Insulin (Sigma, USA), 2 µl/ml L-Glutamine (HiMedia, India), 20 ng/ml EGF (Epidermal Growth Factor, Sigma, USA) and dishes were incubated in a CO₂ incubator at 37°C with 5% CO₂. Explants cultures were observed daily under a phase contrast microscope for outgrowth of cells from partially digested tissue fragments. After 5th day many cells were seen outgrowing from tissue, which were adhered to the bottom of the petridish. These adhered cells were fed with fresh growth media three times in a week. These adhered cells started to multiply and reached confluent within 15 - 20 days. After attending confluency, the cells were passaged using 0.25% Trypsin-EDTA and plated in new 50 mm tissue culture flasks. The same procedure was repeated after every confluency and cells were cryopreserved at -85°C.

2.2. Phase Contrast Microscopy

The morphology of cultured cells was observed using phase contrast microscope equipped with TSview software (Tucsen Imaging, Fuzhou, PR China) for observing and capturing the images. Cells were regularly monitored and images were captured for analysis.

2.3. Cryopreservation and Revival

Semi-confluent cultures of isolated breast cancer stem cells were trypsinized with 0.25% Trypsin-EDTA for 3 - 5 minutes, centrifuged and washed with 1X PBS. The cell pellet was suspended slowly in ready-made cell freezing medium (HiMedia, India) and transferred into the cryovial and store at -85°C. For revival, vials were thawed and freezing medium was replaced by freshly prepared DMEM medium. These cells maintained 90% viability after revival.

2.4. Molecular Characterization of Breast Cancer Stem Cells

Total RNA was extracted from tumor tissue of breast cancer patient (*In vivo* study) and isolated breast cancer stem cells (*In vitro* study) by using Trizol Reagent (Invitrogen, Carlsbad, CA). RNA was transcribed to cDNA by using Applied Biosystems High Capacity cDNA Kit. (Applied Biosystem, USA). The molecular markers study was carried out by RT/PCR for Mesenchymal phenotypes (CD105, CD13, CD73), Hematopoietic phenotypes (CD34, CD45), Pluripotency and differentiating markers (Oct4, Nanog, SOX2, LIF, Keratin18 and Actin), Oncogenic markers (c-MYC, BCL2, COX2, DAPK, EGFR and CD44), Make Singular - Cytokine and Chemokine (IL6, TNFα, SDF1α and CXCR4) and BRCA2 gene mutation. PCR conditions and primer se-

quences were described previously by Potdar and Sutar 2010 [24] whereas, sequences and their respective PCR conditions for other than reported genes are listed in **Table 1**. The PCR products were checked for their respective amplification on 2% Agarose gel electrophoresis and photographed under UV light.

2.5. DNA Sequencing of BRCA2 Gene

Exon 11E of BRCA2 gene was amplified by PCR using the primer combination shown in **Table 1**. The PCR product was sequenced using ABI 3100 Genetic analyzer using BigDye[®] Terminator reaction (Applied Biosystems, Warrington, UK). The sequenced obtained were analyzed by using Bioedit software and the mutation obtained was confirmed by repeating same sample for sequencing. Both sense and antisense strand sequencing were carried out to confirm this mutation reported in this study.

3. RESULTS

3.1. Isolation of Human Breast Cancer Mesenchymal Stem Cells (hBCMSCs) from Human Non-Metastasis Breast Cancer Tumor

The breast cancer stem cells were isolated and cul-

tured from non-metastasis breast cancer tumor is described above. Culture dishes were regularly monitored under phase contrast microscope for any out growth of the breast cancer cells from breast cancer explants. Out-growth of these cells was seen within 5 - 6 days of culture, from explant as shown in **Figure 1(a)**. These cells showed various morphological features from fibroblasts like appearance to epithelial and endothelial cells like appearance as shown in **Figures 1(b)-(d)**. The cells showed high rate of multiplication and observed many mitotic cells. These cells became confluent within 20 - 25 days as shown in **Figure 2(a)**. These cells were regularly washed and fed with usual growth medium described in material method. After confluency, cells were passaged by trypsinisation with 0.25% PBS/Trypsin and transferred into 50 mm culture flask. This process was repeated several times after cell reached to confluency and cryopreserved at different passages and kept at -85°C for future studies. Thus, this study has established breast cancer stem cell line and was designated as human Breast Cancer Mesenchymal Stem Cells (hBCMSCs) line after confirming their phenotypes by molecular markers described below. Presently, these cells are at passage 18 and still growing well (**Figure 2(a)**). It was also observed that several cells developed mammosphere formation and pluripotent clones as shown in **Figures 2(b)** and **(c)**.

Table 1. Shows sequences of primers used for respective molecular marker.

	Primer	Sequence 5'→3'	Annealing (°C)	Size (bp)
CD 44	Upstream	CAACCCTACTGATGATGACG	60	302
	Downstream	GGATGCCAAGATCATCAGCC		
EGFR	Upstream	TCTCAGCAACATGTCGATGG	60	473
	Downstream	TCGCACTTCTTACACTTGCG		
C-MYC	Upstream	GCCTCAGACTGCATCGAC	60	251
	Downstream	GTTGACCTTGGTCTGGTAGG		
COX 2	Upstream	TTCAAATGAGATTGTGGGAAAATTGCT	68	305
	Downstream	AGATCATCTCTGCCTGAGTATCTT		
BCL 2	Upstream	CATTCCACGTCAACAGAATTG	60	505
	Downstream	AGCACAGGATTGGATATTCCAT		
DAPK	Upstream	TGACAGTTTATCATGACCGTGTTCAG	60	231
	Downstream	GTGCTGGATCTCCTCAGGAT		
TNF alpha	Upstream	CGAGTGACAAGCCTGTAGCC	58	555
	Downstream	GTTGACCTTGGTCTGGTAGG		
IL 6	Upstream	GTCTCCTCATTGAATCCAGATTGG	58	328
	Downstream	AGCTCAGCTATGAACTCCTTCTC		
SDF1 alpha	Upstream	TGATCGTCTGACTGGTGTTA	57	188
	Downstream	CTTAGGGGATTGGAAGTTT		
CXCR4	Upstream	GGACCTGTGGCCAAGTTCTTAGTT	57	273
	Downstream	ACTGTAGGTGCTGAAATCAACCCA		
BRCA 2 11E	Upstream	CTGAACATAACATTAAGAAGAGC	60	473
	Downstream	GTCTACCTGACCAATCGATG		

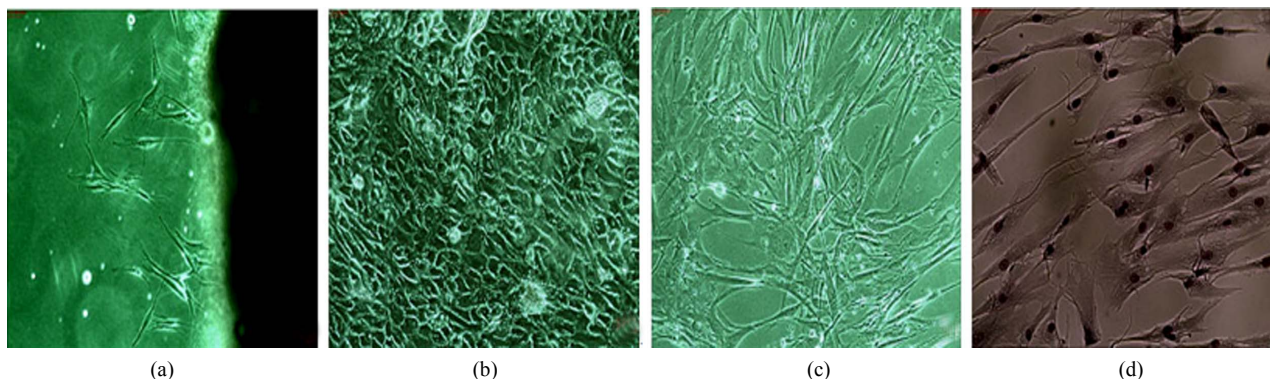


Figure 1. Shows human Breast Cancer Mesenchymal Stem Cells (hBCMSCs) derived from Breast cancer non-metastasis tumor outgrowing from explants on 5th day of culture (a), Epithelial (b), Endothelial (c) and Fibroblast like hBCMSCs cells (d).

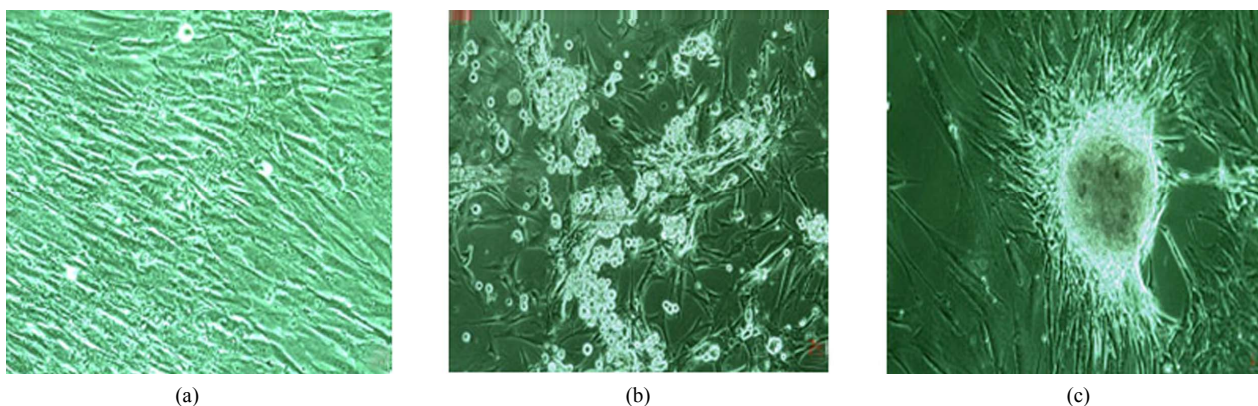


Figure 2. Shows confluent culture of hBCMSCs cell line grown as monolayer cells (a), mammospheres formation (b) and pluripotency clone (c).

3.2. Phase Contrast Microscopy of hBCMSCs Cell Line

Phase contrast microscopy of hBCMSCs cell line was shown in **Figures 1(a)-(d)** and **Figures 2(a)-(c)**. There are different cell types were seen emerging from explants confirming the heterogeneous population of breast cancer cells associated with breast cancer development. **Figure 1(b)** shown epithelial like cells emerging from explants with epithelial sheet like structure having large nuclei and dense cytoplasm whereas, **Figure 1(c)** shown endothelial like cells, which are elongated and randomly scatter cells with scanty cytoplasm having large nucleus with 1 - 2 nucleoli. **Figure 1(d)** showed Geimisa stained typical fibroblast like mesenchymal cells having scanty cytoplasm, large nucleus and nucleoli with cytoplasmic granules. **Figure 2(a)** showed a confluent culture with elongated morphology. **Figure 2(b)** showed cells have mammosphere formation during growth of these cells whereas; **Figure 2(c)** showed formation of pluripotency clone in growing culture.

3.3. Molecular Marker Analysis of Human Breast Cancer Tumor and hBCMSCs Cell Line

The present study was undertaken to find out molecular characterization of human breast cancer tumor (*in vivo*) and hBCMSCs cell line (*in vitro*) to confirmed specific phenotypes of these cells by using specific set of stem cell markers such as mesenchymal stem cell markers, hematopoietic stem cell markers, pluripotency markers, oncogenic markers, cytokines and chemokines markers.

3.4. Selection of Positive Controls

RNA from CML, Normal blood cells and SCAT cell line developed in our laboratory (24) were use as positive control for (CD105, CD45, CD34), (SOX2, Oct4, Nanog, LIF, Keratin18, and DAPK) and (CD13, CD73, COX2 and IL-6) respectively [24]. K562 cells were use for positive control for c-MYC gene. Amplified PCR products were sequenced for confirmation of required

gene sequences using specific primers. The sequences obtained for each gene were confirmed from NCBI database using BLAST software [data not shown] and shown 100% match for these specific genes reported here. On the basis of this, positive controls were selected for this study.

3.4.1. Mesenchymal and Hematopoietic Markers in Human Breast Tumor Cells and hBCMSCs Cell Line

Mesenchymal and hematopoietic markers expression in human breast tumor cells and hBCMSC cells is shown in **Figure 3**. It was showed that tumor cells did not express CD105, CD13 and CD73 genes whereas, hBCMSCs cell line showed higher expression of all three genes, thus confirming their mesenchymal stem cell phenotypes. These cells also mildly expressed CD34 but showed negative expression of CD45 gene indicating that hBCMSCs cells may have hematopoietic phenotypes. Both these genes were not expressed in tumor tissue.

3.4.2. Pluripotency Markers in Human Breast Tumor Cells and hBCMSCs Cell Line

We have studied expression of Oct4, Nanog, SOX2, LIF and Keratin18 genes as markers for pluripotency and differentiation in tumor cells and in hBCMSCs cell line respectively as shown in **Figure 4**. We have observed that Oct 4, Nanog and SOX2 were expressed at in vivo cells whereas, in hBCMSCs cell line Oct 4, Nanog were mildly expressed and SOX2 was shown to be down regulated (**Figure 4**) indicate that SOX2 plays some role in pathogenesis of Breast cancer and needs further investigation. Differentiation markers LIF and keratin18 were expressed equally *in vivo* and *in vitro* cells (**Figure 4**).

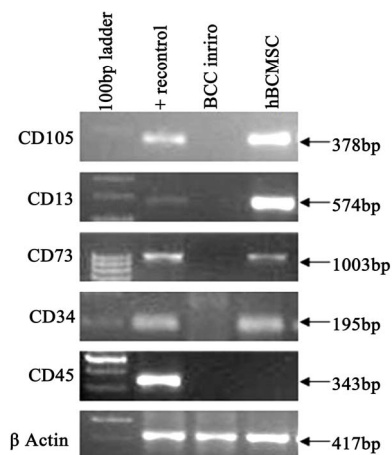


Figure 3. Shows expression of mesenchymal and hematopoietic stem cell markers in Breast cancer tumor tissue (*in vivo*) and in hBCMSCs cell line (*in vitro*).

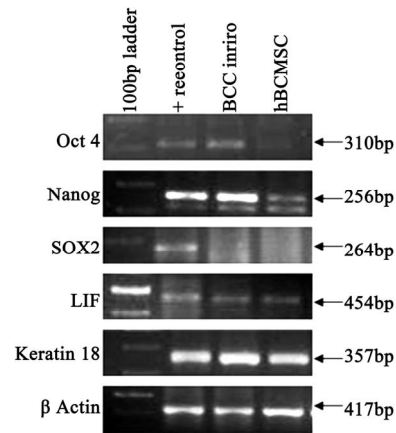


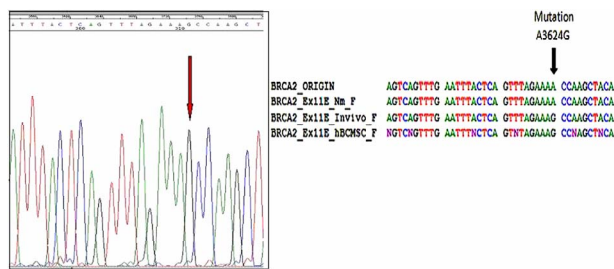
Figure 4. Shows expression of pluripotency stem cell markers in Breast cancer tumor tissue (*in vivo*) and in hBCMSCs cell line (*in vitro*).

3.4.3. Oncogenic Markers in Human Breast Tumor Cells and hBCMSCs Cell Line

As hBCMSCs cell line was derived from non-metastasis tumor cells, we thought of studying oncogenic markers expression in these cells. We studied c-MYC, COX2, BCL2, CD44, EGFR, and DAPK genes in tumor as well as hBCMSCs cells as shown in **Figure 5**. It was observed that c-MYC, EGFR, CD44, DAPK and BCL2 genes were expressed in hBCMSCs cell line whereas only BCL2 and COX2 genes were expressed by tumor tissue (**Figure 5**). Within these expressions, CD44 was shown to be highly expressed in hBCMSCs cell line confirming, the presence of initiating breast cancer tumor cells. Expression of DAPK in tumor cells and hBCMSCs cell line also confirmed their premalignant status, as DAPK, a tumor suppressor gene expressed in normal and initiated cells (**Figure 5**). These results were further confirmed by studying chemokine receptor CXCR4 expression in tumor and hBCMSCs cells. It was also observed that CXCR4 gene was prominently expressed in tumor but mildly expressed in hBCMSCs cell line indicating that the hBCMSCs cells isolated from tumor were not metastatic but they are breast cancer initiated cells. We also observed expression of SDF1 α in tumor and hBCMSCs cell line (**Figure 6(b)**). As cytokines play very important role in mammary carcinogenesis, we have studied expression of IL6 and TNF α in tumor and hBCMSCs cell line (**Figure 6(a)**). We observed that TNF α was expressed prominently in tumor but shown mild expression in hBCMSCs cell line. Similarly tumor tissue cells expressed mild expression of IL6 whereas, hBCMSCs cells showed significantly higher expression of IL6 indicating specific role of IL6 in breast carcinogenesis as reported by various investigator [25].

3.4.4. Expression of BRCA2 Gene Mutation in Human Breast Tumor Cells and hBCMSCs Cell Line

As our laboratory has established a founder mutation in BRCA2 gene in Indian breast cancer patients [23], we thought of evaluated this BRCA2 mutation in tumor tissue as well as in hBCMSCs cell line by DNA sequencing (Figure 6(c)). We did find the mutation at exon 11E of BRCA 2 gene in tumor as well as hBCMSCs cells indicating the cells isolated from tumor cells have similar characteristic, as it appeared *in vivo*. Thus, we can conclude that the cell line isolated from non-metastasis breast cancer tumor is none other than Initiated breast cancer stem cells causing tumor in humans.



(c)

Figure 6. (a) Shows expression of cytokine markers in Breast cancer tumor tissue (*in vivo*) and in hBCMSCs cell line (*in vitro*); (b) shows expression of chemokine markers in Breast cancer tumor tissue (*in vivo*) and in hBCMSCs cell line (*in vitro*); (c) shows electropherograms of exon 11E of BRCA2 gene mutation (A3624G) observed in tumor tissue as well as in hBCMSCs cell line.

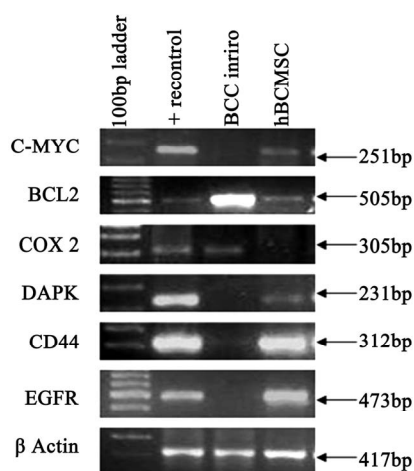
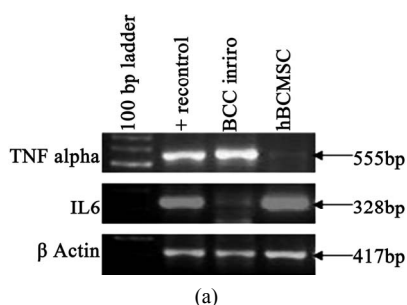
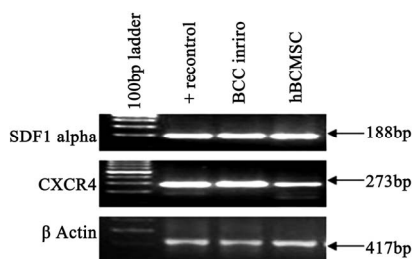


Figure 5. Shows expression of oncogenic cell markers in Breast cancer tumor tissue (*in vivo*) and in hBCMSCs cell line (*in vitro*).



(a)



(b)

4. DISCUSSION

Breast cancer is a complex disease, with multiple genetic and environmental factors involved in its aetiology. Recent advances in stem cell research have demonstrated that the cancers originally develop from normal cells, which gain the ability to proliferate aberrantly and eventually turn malignant [1]. These malignant cells have been defined as cancer stem cells. These cells have exclusive ability to self-renewal and to differentiate into the heterogeneous lineages of cancer cells that comprise the tumor [1]. Two of major challenges in breast cancer studies are 1) Isolation of Breast cancer stem cells from breast tumor tissue and 2) identify breast cancer stem cells using specific stem cell markers. The technology for isolating Breast cancer stem cells from breast tumor has been shown that CD24^{low/-} CD44⁺ are a define population of cells of potential breast cancer stem cells [5]. In present study, we have examined non-metastasis breast tumor for the presence of Breast cancer stem cells and characterized them using molecular markers. We are the first to isolate human breast cancer mesenchymal stem cells from non-metastasis breast tumor. We have designated this cell line as human Breast Cancer Mesenchymal Stem Cells (hBCMSCs), which is presently at passage 18. Recent studies have suggested the existence of stem cells in the human mammary gland and shown non-adherent mammospheres secretory cancer stem cells with progenitor cell properties [12,13]. In our study, we have observed similar phenomenon of formation of mammospheres by hBCMSCs cells indicating clearly that the cells isolated from non- metastatic tumor are Breast cancer initiated stem cells.

Mesenchymal characteristic of these cells were confirmed by examination of CD105, CD13 and CD73 markers, which are putative markers for Mesenchymal Stem Cells [14]. These cells also expressed hematopoi-

etic marker represented partial phenotype of hematopoietic stem cells [15]. Shi *et al.* (2006) have shown that transcription factors Oct-3/4, Nanog and SOX2 maintain the expression of Pluripotent factors at a steady state [26] whereas; Wilson *et al.* (1988) have shown that the LIF maintains the developmental potential of embryonic stem cells, which can be regarded as a pluripotency marker [20]. In present study Breast cancer tumor cells and hBCMSCs cells expressed Oct4, Nanog and LIF showing their pluripotency. Expression of keratin 18 in tumor cells and hBCMSCs cell line confirmed the epithelial origin of these cells [27].

After establishment of breast cancer stem cell line, it was very essential to know whether, the cell line possesses normal or malignant phenotypes so we have utilized oncogenic markers such as CD44, EGFR, c-MYC, DAPK, COX2, BCL2, and SOX2 to study the malignancy nature. Oncogenes overexpression is a common phenomenon in the development and progression of many human cancers hence it provide potential target for cancer gene therapy [28]. Breast cancer cells were heterogeneous with respect to expression of a variety of cell surface markers including CD44 and CD24. CD44 is a complex transmembrane glycoprotein whose expression is associated with drug resistance and metastatic progression in malignancy [21]. In present study, we have shown that significant expression of CD44 in hBCMSCs cell line and mild expression in tumor cells indicating that the cells isolated from heterogeneous population of tumor cells are breast cancer initiated cells. Studies have shown that oncogenes c-MYC, COX2, EGFR and DAPK plays important role in development of breast cancer whereas, their overexpression or loss of expression is useful to study correlation with invasive potential and metastasis in aggressive tumors and neoplastic tissues [18,19,29,30]. Similarly expression of BCL2 and SOX2 may be associated with metastasis, proliferation and tumorigenesis of breast cancer cells [31,32]. Here in the present study, there was significant expression of c-MYC and EGFR in hBCMSCs cell line whereas, *in vivo* study of tumor cells showed mild expression of COX2 and down regulation of DAP Kinase gene suggesting that the hBCMSCs cell lines contains initiated breast cancer stem cells which may become transformed to metastatic cancer cells when triggered by endogenous factors.

Cytokines, such as IL-6 and Tumor Necrosis Factor (TNF α), have an important role in regulating estrogen synthesis and shown *in vitro* expression by aggressive mesenchymal/basal-like breast cancer cell lines, tissues and xenograft which promote malignant features in breast cancer cells [25,33]. In present study, we found low expression of IL6 in tumor cells whereas, significantly high expression in hBCMSCs cell line which con-

firmed with the finding of other investigators [25,33]. Chemokine receptor CXCR4 showed significant high expression in many solid tumors and also involved in cell migration and invasion, as well as in angiogenesis [34]. In present study, hBCMSCs cell line showed low expression of CXCR4 indicating non-metastatic nature of hBCMSCs cells isolated from tumor cells, which showed significant high expression of CXCR4 gene.

We further thought of confirming cancer-initiated cells by studying gene mutation in BRCA2 gene. Our laboratory recently reported a specific founder mutation in Exon 11E of BRCA2 gene in Indian population as an early prognostic marker for Breast cancer [23]. In the present study, we have sequenced DNA samples of breast cancer tumor tissue and hBCMSCs cell line. Our study showed that this founder mutation was at *in vivo* and *in vitro* level indicating that the cells isolated from non-metastasis breast cancer tumor are not but Breast cancer initiated cells which are responsible for development of breast cancer.

Overall, this study establishes a technology for development of breast cancer stem cell lines from non-metastasis breast cancer tumor and represents an *in vitro* model to understand the mechanism of Breast carcinogenesis. The identification of specific molecular markers in these cells allows us to plan targeted therapies for breast cancer.

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