Localization and Molecular Characterization of human Breast Cancer Initiating Cells from heterogeneous population of Breast Cancer Mesenchymal Stem cells by Immunofluorescence Microscopy

Potdar P¹, Monteiro F¹

Breast Cancer (BC) is a heterogeneous disease and arises from breast cancer initiating stem cell population in the tumor and these cells are resistant to cancer therapies. Thus identifying this cell type within the tumor clone is an important area of research to understand the mechanism of breast cancer development. Recently, our laboratory has isolated and characterized Human breast cancer mesenchymal stem cells (hBCMSCs) from human breast cancer and showed the heterogeneity of these cells existing in the tumor. Therefore, our present objective is to use this model system to identify, localize and define specific breast cancer initiating cells (BCICs) from the heterogeneous population of hBCMSCs cell line developed in our laboratory. Localization of specific cell types can be done by using specific cancer marker antibodies using Immunofluorescence microscopy. In this study we have used FITC labeled specific cancer antibodies i.e. p53, Rb1, Hras, EGFR, GST, ETS1 and ATF2 to localize BCICs in this population of cells. Our results have demonstrated that few cells among many of the BC cells gave fluorescence with specific cancer antibody indicating that these cell types are BCICs that may be responsible for supporting the growth of other cell type to form tumors. The Phase Contrast Microscopy clearly showed giant cells with enlarged nucleus and scanty cytoplasm associated with many cytoplasmic granules. It also indicates that these cells are mainly responsible for supporting proliferation of surrounding cells that form a part of the BC tumor. We have further hypothesized that molecular profiling of these tumor cells will open a new avenue of molecular targeted therapies for Breast Cancer patients even at an advanced stage of disease.

Key Words: hBCMSCs, EGFR, Ki67, Hras, Rb1

Introduction

Breast cancer is the commonest cause of cancer deaths in women.¹ The incidence of breast cancer is increasing in the developing world and the majority of breast cancers that develop in low and middle income countries are diagnosed very late.¹ Breast cancer comprises almost 22.9% of all cancers.¹ 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.² These cells are defined as cancer stem cells (CSC). The CSC hypothesis suggests that the malignancies are associated with small population of tumor-initiating stem cells. These cancer-driving cells within the tumors have been hard to identify, separate and study in vitro. Recent studies from our laboratory have isolated and characterized Breast Cancer Mesenchymal Stem Cells from non-metastasized human breast cancer and also shown the heterogeneity of breast cancer tumor cells.³ It is less clear and hard to identify exactly as to which cells within the tumor clone are capable of initiating tumor due to the diversity of heterogeneous cell populations with different biological properties.

Several studied have reported the biomarkers for cancer cells which will allow us to define their malignant phenotypes. High Expression of tumor suppressor gene p53 is a common feature of many human neoplasias and is routinely used to monitor residual tumor cells.⁵⁶ Similarly high expression of retinoblastoma protein has been reported in the metastatic node in a breast cancer patient which evaluates its role as a marker for the presence of breast cancer metastasis.⁷ Yang et al 2003 have reported that Hras is involved in maintenance of tumor growth of human and further showed that retrovirus-mediated siRNA expression suppressed tumor growth.⁸ Proliferation is a key feature of the progression of tumors and is now widely estimated by the immunohistochemical assessment of the nuclear antigen Ki-67. The high expression of Ki-67 has been well correlated with poor prognosis associated with a good chance of clinical response to chemotherapy.⁹ Recent studies have shown that increased expression and activation of receptor tyrosine kinases occur frequently in human breast carcinomas. Several therapies targeting these receptors are currently in clinical trials. Trastuzumab is the first of these biologic therapies to be approved for patients with human epidermal growth factor receptor 2 (HER2) over expressing...
metastatic breast cancers and novel Trastuzumab-based combinations are being investigated in patients with advanced breast cancers. [12]

The glutathione S-transferases (GST) represent a major group of detoxification enzymes which are encoded by at least five distantly related gene families (designated class alpha, mu, pi, sigma, and theta GST). Hays and Pulford 1995 have shown that the level of expression of GST is a crucial factor in determining the sensitivity of cells to a broad spectrum of toxic chemicals. [13] Wang et al. 1999 have hypothesized that over expression of glutathione S-transferases (GST) in breast cancer cells is indicative of the multifactorial doxorubicin-resistant phenotype. [14]

The proto-oncogene Ets-1 is a member of the Ets family of transcription factors which share a unique DNA binding Ets domain. Ets-1 regulates the expression of several angiogenic and extracellular matrix remodeling factors promoting an invasive phenotype. Lincoln et al. 2005 have shown that Ets1 may play a role in the disease progression of breast cancer and indicative of poorer prognosis. [15] Kars et al. 2010 have shown that over expression of ETS1 gene have contributed to the development of resistance in the breast cancer. [16] Park et al. 2008 have demonstrated that HER2-induced MMP-1 expression is positively regulated by Ets-1 in breast cancer cells. [17]

The transcription factor ATF2 is over expressed in various tumors. Recently, it was suggested that matrix metalloproteinase (MMP)-2 plays a role in the malignant progression of breast cancer cells and ATF 2 act as a transcription factor for MMP-2 gene expression through binding to the functional AP-1 site. Song et al. 2006 have shown that upregulation of MMP-2 provided an evidence for a direct role of ATF2 activation in human breast epithelial cells. [18] However, Knippen et al. 2009 have shown that they do not find any oncogenic role of active p-ATF2 in mammary carcinomas. [19]

The type I insulin-like growth factor receptor (IGF1R) and insulin receptor (IR) are structurally and functionally related to heterotetrameric receptors. Activation of IGF1R has been shown to regulate breast cancer cells and it has become an attractive therapeutic target. Zhang et al. 2007 have shown that down-regulation of Type I Insulin-like Growth Factor Receptor increases sensitivity of breast cancer cells to Insulin. [20] A role for Notch signaling in human breast cancer has been suggested following pathway activation and the loss of numb expression in a large proportion of breast carcinomas. Stylianou et al. 2006 have shown that attenuation of Notch signaling reverts the transformed phenotype of human breast cancer cell lines and further suggested that the inhibition of Notch signaling may be a therapeutic strategy for cure of this disease. [21]

In the present study, we have used an hBCMSCs cell line which is very well characterized and also reported to have heterogeneity of cells. [3, 4] We further thought of localizing specific breast cancer initiated cells which are resistant to chemotherapy as well as responsible for development of breast cancer tumor. We have selected 8 cancer specific antibodies playing the role of tumor suppressor genes, oncogenes, proliferation marker, growth factor receptor protein, detoxifying enzymes and transcription factors, to localize and confirm the breast cancer initiated stem cells among heterogeneous population of hBCMSCs cells by using Phase contrast microscopy and Immunofluorescence microscopy. Thus, by identification and molecular profiling of these specific cancer initiated cells, we will be able to find our specific target for killing these cells by using molecular targeted therapy for breast cancer even at an advanced stage of this diseases.

Materials and Methods

Maintenance of hBCMSCs cell line for Light and Immunofluorescence Microscopy

The established breast cancer stem cell line i.e. human breast cancer mesenchymal stem cells (hBCMScs) line was used for studying localization of specific cancer cells in heterogeneous population of these cells. [3, 4] This cell line was revived, cultured and maintained at various passages by feeding them with growth medium (DMEM + 10% FBS + 1% PenStrep + 1µg/ml Insulin + 2µg/ml Glutamine), thrice a week and after reaching confluency the cells were passaged by trypsinization with 0.25% PBS/ Trypsin. Coverslip cultures were set up for Light and Immunofluorescence microscopy, whereas, some of these cells were processed for RNA extraction for molecular analysis.

Preparation of Coverslip Cultures for Light and Immunofluorescence Microscopy

Coverslips were cleaned by treating them in chromic acid solution overnight and then washed 2-3 times with distilled water followed by wash in 75% Ethyl alcohol. Under sterile conditions, individual coverslip was cleaned with dry cloth, kept in glass petri-dish, sterilized by autoclaving and used for this study. Confluent cells were trypsinised with 0.25% PBS-Trypsin for 3-5 minutes. Cells were suspended in growth medium at a density of 5x 10⁶ cells/100µl of growth media for light and Immunofluorescence staining. The100µl of cell suspension was placed on sterile coverslips, kept in 35mm petri-dishes and incubated for 1hour to adhere cells to the bottom of coverslip. After cells adhered, 1ml of additional growth media was added in each petri-dish and dishes were incubated further in CO₂ incubator at 37°C with 5% CO₂ for 24hours. After 24 hours coverslips were washed with 1X PBS and fixed for light Microscopy and Immunofluorescence staining as described below.

Giemsa, Alzarin Red and Oil Red Staining of hBCMScs cell line

The coverslip cultures were washed in 1X PBS and fixed with 50% Methanol for 30 minutes at room temperature. The coverslips were washed with fresh distilled water for complete removal of methanol. For Giemsa staining, the fixed and washed cells were stained with Giemsa stain for 10 minutes. For Alzarin Red staining, the fixed and washed cells were stained with 2% Alzarin Red stain at pH 4.2 for 30 minutes. For Oil Red staining, the fixed and washed cells were incubated with 60% isopropanol for 5 minutes and stained with 0.3% Oil Red stain for 5 minutes. After staining, the coverslips were washed 2-3 times with distilled water and
mounted in mounting media and observed under light microscopy and photographed.

**Immunofluorescence Microscopy of hBCMSCs cell line**

Freshly prepared coverslip cultures were washed twice with ice-cold PBS and then fixed with 4% paraformaldehyde for 20 minutes at 4°C. Immunofluorescence for various cancer genes such as p53 (Mouse Anti Human p53 protein, DAKO Cytomation, Denmark), RB1 (Anti Human Retinoblastoma gene, DAKO A/S, Denmark), EGFR (Mouse Anti Human EGFR H11, DAKO Corporation, CA,USA), Ki67 (Purified AntiHumanKi67, BD Pharmingen™, SanDiego CA), GST (Mouse Anti Human glutathione-s-transferase pi DAKO Corporation, CA,USA), ETS1 (ETS1 Rabbit Polyclonal IgG, Santacruz Biotechnology), ATF2 (ATF2 Rabbit Polyclonal IgG, Santacruz Biotechnology), have been carried out using respective specific antibodies present in cancer cells. These cancer antibodies were used to localize specific cancer cell types in the heterogeneous population of hBCMSCs cell line. Paraformaldehyde fixed coverslips were placed on 35mm petridishes, washed with 1XPBS twice and dried on a whatmann filter paper. Then 200µl of blocking buffer (500mg BSA in 50ml 1XPBS) was added and coverslips were incubated for 30minutes at room temperature in humid chamber. The blocking buffer was decanted off and the coverslips were incubated with 200µl of 1:10 diluted respective primary antibody made in blocking buffer for 2 hours at room temperature and thereafter incubated with 200µl of 1:50 diluted in 1 XPBS, FITC labeled goat anti-mouse IgG, secondary antibody for 2 hours at room temperature under dark humid conditions. After quick wash with 1XPBS, the coverslips were mounted on a glass slide with Fluromount mounting media (Fluromount, Sigma, USA) and sealed with quick dry nail polish. After complete drying, the slides were observed under Carl Zeiss Phase Contrast Microscope using FITC filter for fluorescence microscopy. The TSview software was used to capture images. The coverslips without incubated with primary antibodies served as negative controls. All above experiments were repeated thrice to show high reproducibility of these findings.

**Molecular Marker Expression**

Total RNA was extracted from hBCMSCs cells by Trizol method (Invitrogen, Carlsbad, CA). RNA was transcribed to cDNA by using Applied Biosystems High Capacity cDNA Kit (Applied Biosystem, USA). The hBCMSCs cells were reconfirmed for their specific characteristic by studying for mesenchymal (CD105, CD13, CD73) & hematopoietic (CD34) phenotypes, pluripotency genes (OCT4, NANOG, and SOX2), differentiating markers (LIF, Keratin 18) and cytokines (IL6) markers by using RT/PCR technology. The primers for these genes are already been described earlier. In addition to this, we have also studied 2 new genes such as IGFR and Notch2 which may be supportive for further confirmation of cancer phenotypes of the hBCMSCs cells. The PCR primers for these genes are given in Table1. The PCR products obtained were checked for their respective amplification on 2% agarose gel and photographed by gel documentation system.

**Results:**

**Morphological characteristic of Human Breast Cancer Mesenchymal Stem Cell (hBCMSCs) Line**

The Human Breast Cancer Cell line has been established in our laboratory and was designated as human breast cancer mesenchymal stem cell line (hBCMSCs) after confirming their phenotypes by molecular markers. Passage 12 cells were revived and used for this study. The cells were maintained by wash and feed with 1XPBS and cell growth medium respectively as described in materials & methods. The adhered hBCMSCs cells showed proper growth rate and confluency within a week and their morphological features remain the same as shown in figure 1a & 1b. These cells also have properties of forming pluripotency clones and mammosphere formation as shown in figure 1c &1d. The cells also showed differentiation into adipocytes differentiation which is confirmed by Phase contrast microscopy and Oil Red stained respectively as shown in Figure 2a, 2b.

| Primer | Sequence 5’→ 3’ | Annealing (°C) | Size (bp) |
|--------|----------------|----------------|-----------|
| IGFR   | GCCCGAAGGTTCTGTTGAGGA | 60            | 554       |
| NOTCH 2| GGTACCGGTTGCCAGGGTA | 60            | 345       |

Table1: shows sequence of primers used for respective molecular markers.
These cells when stained with Giemsa Stain for looking at general morphology, it was seen that these cells look like typical fibroblast like cells having large nucleus and nucleoli with scanty cytoplasm associated with large number cytoplasmic granules as shown in Figure 2c. Whereas, Alzarin staining clearly indicated that these cells did not differentiate into bone cells such as chondrocytes or osteoblasts as shown in figure 2d where there is no staining observed for these cells by Alzarin Red stain.

**Figure 1**: hBCMSCs cells at passage 12 (a) cells shows proper growth rate (b) cells after confluency (c) formation of pluripotency clones and (d) formation of mammospheres

**Figure 2**: (a) shows phase contrast microscopy of differentiation of hBCMSCs cells into adipocyte cells (b) Confirmation of adipocyte cells by Oil Red staining (c) Giemsa stain showed fibroblast like cells hBCMSCs cells with large nucleus and nucleoli and scanty cytoplasm and (d) hBCMSCs cells showed negative staining for Alzarin Red stain indicating an absence of differentiation into bone cells.

**Figure 3**: shows Molecular Characterization of hBCMSCs cell line at passage 12

### Molecular Characterization of hBCMSCs cell Line

We have used passage 12 hBCMSCs cells for this study. It was shown that the morphological characteristic of these cells remain the same at this passage as described above. We have studied the molecular characterization of these cells to show that these cells phenotype remain the same after passaging of these cells. Figure 3 showed the molecular profiling of hBCMSCs cells by using 10 stem cell molecular markers. It was seen in figure 3 that these cells very well retained Mesenchymal and hematopoietic phenotypes by expression of CD105, CD13, and CD73 & CD34 respectively. These cells also expressed pluripotency and Differentiating genes such as OCT4, NANOG & LIF and Keratin 18 respectively. Being cancer cells, these cells did not express SOX2 gene. High expression of cytokine IL6 was observed in these cells. So it was shown that the phenotypes of hBCMSCs cells remain same even after passage 12. As we are looking for localization of cancer initiated cells in heterogeneous population of hBCMSCs cells, we have additionally studied two important genes, IGFR and Notch 2 involved in breast cancer development. Figure 4 showed high expression of IGFR and Notch 2 in hBCMSCs cell line indicating that these cells have Breast Cancer initiating phenotypes.
Localization of specific Breast Cancer initiated cells from heterogeneous population of hBCMSCs cells by Immunofluorescence Microscopy

Immunofluorescence technology is a simple and specific technique to localize any specific cell types by using specific antibodies associated with these cells. In the present study, we have used 8 cancer related antibodies to define breast cancer initiated cells form heterogeneous population of hBCMSCs cell line developed in our laboratory. We have used tumor suppressor gene protein p53 and Rb1 for localization of these cells as these proteins are well expressed in malignant cells as well as in breast cancer cells. Further, we have used Hras as an oncogene and Ki67 as a proliferative marker, which have also shown great role in breast cancer development. We have used EGFR as a surface protein receptor which plays important role in diagnosis and therapies of breast cancer. Two transcriptional factors such as ETS 1, ATF2 have recently shown important role in breast cancer development and metastatic potential of breast cancer cells. GST protein has also shown important role in detoxification mechanism during breast cancer development. The results of individual protein localization in hBCMSCs cell line are described below.

Localization of p53 and Rb1 proteins in hBCMSCs cell line

p53 proteins are undetectable or are present at a low level in normal cells. On the other hand, they are found in large amounts in a wide variety of transformed cells. Figure 5a showed mild expression of p53 in some of the giant cells of hBCMSCs indicating that these large cells among all other cells are breast cancer initiated cells. These cells are larger than other surrounding cells. Rb1 is expressed ubiquitously in mammary epithelial cells and it typically shows a nuclear staining pattern in normal human breast tissue. Figure 5c showed the high expression of Rb1 in some of the cytoplasmic region of the giant cells of hBCMSCs cell line indicating that these cells are mainly the breast cancer initiating cells. Looking at the morphology, it was seen that these giant cells have very large nucleolus and nucleoli and scanty cytoplasm. The phase constrast and Immunofluorescence immunofluorescence studies have shown that the cells which are positive for p53 and Rb1 proteins are giant cells with different morphology than other surrounding cells. It was also been seen that this giant cell is probably enhancing the growth of surrounding cells to form breast cancer tumor. Figures 5b and 5d show the phase contrast microscopy of same cells with and without fluorescence.
Localization of EGFR and GST proteins in hBCMSCs cell line

Several studies have shown high expression and activation of EGFR protein in human breast carcinomas. Figure 7a showed the expression of EGFR in hBCMSCs cells line. It was seen that the cells localized by EGFR antibody is very giant cells with large nucleolus and nucleoli and there are very few cells shown in whole population of hBCMSCs cells indicating that these large cells may be Breast cancer initiating cells which promote other cell growth to form the breast cancer tumor in human. Similarly it was hypothesized that over expression of glutathione S-transferases (GST) in breast cancer cells is indicative of the multifactorial doxorubicin-resistant phenotype. Figure 7c clearly showed high expression of GST in few large cells of hBCMSCs cell lines indicating that the stained cells may be breast cancer initiating cells which may be resistant to chemotherapy of breast cancer and helps in further growth of breast cancer cells to form tumor in human. Figures 7b and 7d show the phase contrast microscopy of same cells without fluorescence.

Localization of ETS1 and ATF2 proteins in hBCMSCs cell line

The ETS-1 and ATF2 are important transcription factors which regulate the expression of several angiogenic and extracellular matrix remodeling factors promoting an invasive phenotype. In the present study, we have shown that both these protein are over expressed in giant cells of hBCMSCs cell line indicating clearly that these cell types may be the breast cancer initiating cells (Figure 8a & 8c). It was also seen like in other above studied that these cells were large and strongly expressed ETS1 &EST2 proteins but many of surrounding cells were negative for these proteins indicating the premalignant phenotypes of these cells which are promoted by these giant positive cells (Figure 8a, 8c).

Discussion

There is accumulating evidence that breast cancer may arise from mutated mammary stem/progenitor cells which have been termed as a breast cancer-initiating cells (BCIC). Findings obtained in the last few years indicate that breast cancers contain a small population of cells with stem-cell-like properties, which may arise from mutated breast stem/progenitor cells that retain the ability to form new tumors. These breast cancer stem/progenitor cells are also designated as breast cancer-initiating cells (BCIC). The objective of our research was to identify, localize and characterize this small population of human breast cancer mesenchymal stem cells (hBCMSCs) cell line developed and characterized in our laboratory. Initially, In this study, we have shown that the hBCMSCs cell even at passage 12 retained all their morphological as well as molecular features. These cells maintained their Mesenchymal, hematopoietic phenotypes by expressing CD105, CD13, and CD73 & CD34 respectively. These cells also retained their pluripotency and downregulating status except down regulation SOX2 gene observed due to its malignant phenotypes of these cells. As IGFR and NOTCH involved in breast cancer development, we have introduced these genes for confirming malignant phenotypes of hBCMSCs cell line. This study has clearly shown that both these genes were up-regulated in hBCMSCs cell lines as reported earlier by various authors. Immunofluorescence study helps in localization of specific cell types using Immunofluorescence Microscopy. In the present study, we have selected 8 antibodies which are up-regulated in cancer cells.
The high protein expression in cells confirms the malignant phenotypes of these cells. Several studies have shown that p53 and Rb1, tumor suppressor genes, are up-regulated in breast cancer cells. In our study, it has clearly seen that p53 and Rb1 proteins were expressed in specific cells of hBCMScs indicating these cell types are malignant cells in the whole population of hBCMScs and assumed to be breast cancer initiated cells. The phase contrast microscopy of these cells showed a very large cell type with large nucleolus and nuclei and scanty cytoplasm with several granules. This is the first study showing the morphological structure of breast cancer initiated cells in breast cancer tumor cell line. Mild expression of Hras and high expression of Ki67 were observed in few cells of hBCMScs cells. The stained cells also showed large cell morphology similar to observed in p53 and Rb1 expressing cells.

EGFR expression in breast cancer has been extensively studied and very useful in monitoring therapies of cancer patients. In the present study, EGFR expression was studied in hBCMScs cell line in a view to localize breast cancer initiated cells. It was shown that the localized cells with high expression of EGFR showed very large cells or giant cells having nucleus, nucleoli with scanty cytoplasm and plenty of granules. It was also observed that these cancer cells supported proliferation of other non cancerous cells which were stained negative but certainly supporting to form tumors in due course. Wang et al. has hypothesized that high expression of GST indicative of doxorubicin resistant. In the present study very few cells of hBCMScs showed very high expression of GST clearly indicating that these cell type localized by GST expression were cancer initiating cells and it seems to be resistant to cancer therapy. These resistant cells promote the proliferation of surrounding cells to form breast cancer tumors. Observation with ETS1 and ATF2 showed high expression of their protein in few large cells of hBCMScs. ETS1 and ATF2, transcriptional factors, were associated respectively with MMP1 & MMP2 which are major genes involved in metastasis process. So high expression of ETS1 and ATF2 confirmed breast cancer initiated cells which are resistant to chemotherapies and also showed metastatic potential.

Overall, this study has clearly localized and demonstrated the presence of breast cancer initiated cells in heterogeneous population of hBCMScs which is responsible for supporting the growth of surrounding cells. The cancer markers which were used in this study have clearly indicated the nature of breast cancer initiating cells among heterogeneous population of hBCMScs cell line. It was clearly shown that these cells were resistant to chemotherapy. High expression of GST clearly shown the detoxification mechanism involved in breast cancer development. We further hypothesized that molecular profiling of these cells will give us a specific molecule to target breast cancer initiated cells which are resistant to chemotherapy as well as helping surrounding cells to grow infinitely to form breast cancer tumor. This study will open up a new avenue to develop molecular targeted therapies for the treatment of breast cancer even at advance stage of disease.

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Abbreviations:

Breast Cancer - BC
Human breast cancer mesenchymal stem cells - hBCMSCs
Breast cancer initiating cells- BCICs
Human Epidermal growth factor receptor 2- HER2
Cancer stem cells- CSC
Epidermal growth factor receptor - EGFR
Glutathione S-transferase - GST
Insulin like growth factor receptor - IGF1R

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Potential Conflict of Interests:

None

Correspondence to be addressed to:

Dr. Pravin D. Potdar., Head, Department of Molecular Medicine & Biology, Jaslok Hospital& Research Centre, 15, Dr. G. Deshmukh Marg, Mumbai- 400026, Maharashtra, India, Phone-91-22-66573445, Mobile - 91-9820833530, Fax - 91-22-66573000, Email ID ppotdar@jaslokhospital.net