Defining heterogeneity of human non-metastatic breast cancer tumor by identifying individual cell types using cellular and molecular markers

Pravin D. Potdar, Sachin R. Chaugule

Department of Molecular medicine and Biology, Jaslok Hospital and Research Center, Mumbai, India

Abstract

Breast cancer is a complex and heterogeneous disease with a high degree of intratumor heterogeneity and diversity. In the present study, we isolated morphologically distinct cell types with tumorigenic potential from breast cancer tumor. The cells show epithelial, endothelial, mesenchymal and mixed cell types which were characterized by using specific molecular markers. Our data showed that these $CD44^+$ cells distinctly expressed mesenchymal phenotype with self-renewal potential and proliferative capacity. These cells also expressed oncogenic, cytokine and chemokine markers indicating their cancer initiating stem cell nature. Interestingly, these cancer-initiating cells exhibit an A3624G BRCA2 mutation. This study reports for the first time the heterogeneous population of malignant cells in the breast cancer tumor which are responsible for cancer development. It represents a suitable *in vitro* model to allow us to study the mechanism of breast cancer development, and to identify and design a specific molecular target for breast cancer therapies even at advanced stage of disease.

Introduction

Breast cancer is a leading cause of cancer-related deaths in women in all over world.[1] The availability of diseased tissue samples from biopsies and mastectomies provides a unique opportunity for studying human breast cancer. Breast tissue is a composite of breast glandular epithelial cells lining the breast ducts, other cells, and the surrounding collagen-rich stroma containing blood and lymph vessels.[2] Pece *et al.* have shown the heterogeneity of breast cancers by the number of cells displaying stem-like features contained within the tumor and also explained that poorly differentiated breast cancers are enriched in cancer-initiating cells.[3] Studies have shown that tumors are composed of a heterogeneous population of stem cells with tumorigenic and non-tumorigenic phenotypes with marked differences in their capacity to proliferate and differentiate.[4] In addition, it has been shown that $CD44^+/CD24^{-/low}$ cells within human breast tumors are breast cancer initiating cells[5] whereas CD44+ phenotypes have been correlated with breast, colon, prostrate and pancreatic cancer initiating cells.[5-8]

Recent studies have given impetus to the *cancer stem cell* (CSC) hypothesis that cancers arise from stem cells or contain a hierarchy of cells that drive tumorigenesis.[9] The heterogeneous cell population in the tumor is classified into a finite number of cell phenotypes based on their potential for growth and invasion. Breast cells belonging to the same tumor show considerable variation in their growth rate, ability to metastasize, immunogenicity, sensitivity to therapy, and phenotypic stability.[10-12] Therefore, the number of CSCs in human breast cancers can vary greatly, with a discernible impact on several clinical and pathological features which supports the concept of *cancer stem cell targeted therapy* to eradicate cancer.[3]

Cell surface markers have been used as a means of identification and isolation of stem cells. Most of the markers used to date are based on knowledge of tissue development or are derived from hematopoietic or embryonic stem cells. The most commonly used surface markers to identify CSCs are CD133, CD24 and CD44. CD44, a glycoprotein, has been used either alone or in combination with other surface markers to isolate cells with stem cell properties from multiple tumor types, including breast,[10] prostate,[11] colon,[12] pancreas,[8] and head and neck squamous cell carcinomas.[13] Several stem cell markers have been identified, such as CD105, CD13 and CD73 which are the molecular markers, expressed by mesenchymal stem cells (MSCs).[14] CD34 and CD45 are expressed by hematopoietic stem cells.[15,16] In order to understand pluripotency and self-renewal capacity, markers such as Oct4, Nanog and SOX2 have been used extensively by many researchers.[17] It has been established that there is a change in expression of molecular markers such as COX2, DAPK and LIF in cancerous cell types.[18-20] CD24 has recently been described as a negative regulator of CXCR4, a cytokine receptor important in facilitating breast cancer metastasis.[21] Cell surface molecular markers have introduced new methods to detect malignancies at an early stage and to provide a more accurate prognostic evaluation and better therapeutic management of breast cancer patients.

In the present study, we isolated different cell types from non-metastatic breast tumor on the basis of their morphology and molecular stem cell marker expression to define their mesenchymal and hematopoietic phenotypes along with pluripotency and oncogenic properties. This study also investigated their cytokine and chemokine profile to differentiate their specific phenotypes. We are the first to report the heterogeneous population of distinct cell types, such as epithelial, endothelial, mesenchymal, transformed like cells and mixed cell type, from non-metastatic breast tumor using morphological observation and further identification by specific stem cell molecular markers. In addition, our laboratory has successfully established a *BRCA2* gene mutation in an Indian breast cancer population.[22] Our study confirmed this *BRCA2* gene mutation *in vivo* and *in vitro* breast tumor cells proving their specificity towards breast cancer development in humans. Collectively, our study confirms the heterogeneity of breast cancer tumor and also helps in designing specific targeted therapies for breast cancer patients at advanced stage of disease.

Correspondence: Pravin D. Potdar, Department of Molecular Medicine and Biology, Jaslok Hospital and Research Center, 15, Dr. G. Deshmukh road, Mumbai- 400 026, Maharashtra, India.
Tel. +91.22.66573445.
E-mail: ppotdar@jaslokhospital.net

Key words: breast cancer stem cells, molecular markers, BRCA2, mesenchymal stem cells, molecular targeted therapy.

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Contributions: PP concept and design of experimental studies, data analysis, and interpretation, wrote the manuscript and gave final approval. SC performed experiments, developed and maintained cell lines, gene expression study, data collection and wrote the manuscript.

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Materials and Methods

Collection and processing of human breast cancer tissue

Fresh specimen of human breast tumor was received in a sterile condition after surgery after obtaining prior consent according to the guidelines of the ethical committee of the Jaslok Hospital and Research Center, Mumbai, India. The tumor was cut into 2 mm pieces in sterile 1x Phosphate Buffer Saline (PBS) containing 1% Penstrep (HiMedia, Sigma Aldrich, St. Louis, MO, USA) and washed 2-3 times. The cleaned tissue fragments were then digested in 0.25% Trypsin-EDTA (HiMedia) at 37°C for 40 min. Tumor tissue explants were plated in 65 mm Nunc dishes. The tissues pieces were fed with freshly prepared DMEM (Dulbecco's Modified Eagle's Medium, HiMedia) supplemented with 10% FBS (Fetal Bovine Serum, Invitrogen, Life Technologies Ltd., Paisley, UK), 1% penicillin-streptomycin, 1 µL/mL insulin (Sigma), 2 µL/mL L-glutamine (HiMedia), 20 ng/mL EGF (Epidermal Growth Factor, Sigma) and dishes were incubated in a CO_2 incubator at 37°C with 5% CO_2. Explant cultures were observed daily under a phase contrast microscope for the outgrowth of cells from partially digested tissue fragments. After the 5th day, many cells were seen outgrowing from tissue, adhered to the bottom of the petridish. These adhered cells were fed with fresh growth media three times over a week. These adhered cells started to multiply and reached a confluent stage within 15-20 days. These cells were observed under phase contrast microscope for their differential morphology, such us: mesenchymal, epithelial, endothelial and transformed. The areas of these differential cell type colonies were located and marked, and the marked cells were isolated separately by using the puck cylinder method as described.[23]

Isolation of different cell types from primary cell cultures from breast tumor tissue explants by the puck cylinder method

The cells outgrowing from explants were observed under phase contrast microscope. Cells showing different morphology such as epithelial, endothelial, mesenchymal and transformed-like cells were observed within 15-20 days of explant culture. The locations of colonies of particular cell types were first marked with a marking pen on the bottom of the plate. These marked colonies were examined under an inverted microscope. Those colonies which were sufficiently isolated from other cells were chosen for clone isolation. The medium was removed from the plate and cells were washed with 1xPBS. The sterile stainless steel cloning cylinder[23] was placed directly over the center of each colony. The cylinder was adhered to the bottom surface by applying a thin layer of sterile silicon grease and firmly placing it on the plate so as to enclose the respective colony. A few drops of Trypsin-EDTA were added to the cylinder and incubated for 2-3 min at room temperature. The trypsinized cells inside the cylinder were gently picked up by micropipette and were transfered to new 48-well plates containing growth medium. They were incubated in a CO_2 incubator at 37°C at 90% humidity. After confluency, each clone cell was trypsinized and plated in 65 mm petridishes and allowed to adhere for 45 min in a CO_2 incubator. After 45 min, supernatant was removed and suspended cells were replated in another 65 mm dish and allowed to grow. This procedure removes most of the fibroblasts which initially adhered to the 65 mm dish within 45 min. This procedure was repeated 2-3 times for each clone to ensure the cells were free from most of the fibroblast contamination. Theses clone cells were labeled and freeze-stored at -85°C till further experimentation. Thus, we have isolated 5 different cell lines from breast cancer explants and each was designated according to their morphological characteristics as breast cancer cells (BCC) mesenchymal (BCC MSC) Clone 1 and Clone 2, BCC epithelial, BCC endothelial and BCC transformed.

Molecular characterization of breast cancer stem cells

Total RNA was extracted from tumor tissue of breast cancer patients (*in vivo* study) and from different types of breast cancer cultured cells (*in vitro* study) by using Trizol Reagent (Invitrogen). RNA was transcribed to cDNA by using Applied Biosystems High Capacity cDNA Kit (Applied Biosystem, Life Technologies Ltd.). The molecular marker study was carried out by RT/PCR for mesenchymal phenotypes such as CD105, CD13, CD73, hematopoietic phenotypes such as CD34, CD45, pluripotency and differentiating markers such as Oct4, Nanog, SOX2, LIF, keratin18. Oncogenic markers such as C-MYC, BCL2, COX2, DAPK, IL6, EGFR and CD44. Cytokine and chemokine markers such as IL6, TNFα and CXCR4 and exon 11E *BRCA2* gene mutation were also studied in these cell types. Polymerase chain reaction (PCR) conditions and primer sequences for mesenchymal, pluripotency and differentiating genes were have been described previously by Potdar and Sutar[24] whereas sequences and their respective PCR conditions for other 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.

Table 1. Sequences of primers used for respective molecular markers.

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

DNA sequencing of *BRCA2* gene

Amplified product of exon 11E of the *BRCA2* gene was sequenced using ABI 3100 Genetic analyzer using Big Dye-Terminator reaction (Applied Biosystems). Details of DNA sequencing and analysis are provided elsewhere.[24]

Results

Isolation of morphologically different cell types of human breast cancer stem cells from human non-metastatic breast cancer tumor

Morphologically distinct breast cancer cell types were isolated and cultured by enzymatic and mechanical dissociation of breast cancer tumor tissue. Colonies of distinct cancer cell types were identified and cloned by the puck cylinder method.[23] These cell types showed endothelial, epithelial, mesenchymal and transformed cell like morphological phenotypes (Figure 1A-D). Colonies of respective cell types were cultured to generate cell lines and examined for gene expression. According to the morphological features of breast cancer cells, we were able to establish 5 distinct cancer stem cell lines. These cell lines were designated as BCC Endothelial, BCC Epithelial, BCC MSC Clone 1 and Clone 2 and BCC Transformed (Figure 1A-D), respectively. In particular, it was also observed that epithelial, endothelial, mesenchymal cells developed mammosphere (Figure 1F) whereas transformed cells showed formation of pluripotent clone (Figure 1G).

Morphological identification of cancer cell types by phase contrast microscopy

Distinct cancer cell types obtained from explants confirmed the heterogeneity of breast cancer tumor tissue in breast cancer development. Breast cancer epithelial cells (BCC epithelial) have large nuclei and dense cytoplasm with cytoplasmic connection (Figure 1B) whereas endothelial cells (BCC endothelial) showed elongated flattened morphology and scattered cell types (Figure 1A). Typical fibroblast-like mesenchymal stem cells (BCC MSC) have scanty cytoplasm, large nucleus and nucleoli with cytoplasmic granules (Figure 1C). Transformed cells showed several patches of aggregated cells at a focal point with high proliferative rate (Figure 1D).

Molecular profiling of human breast cancer tumor cells (*in vivo*) and cultured breast cancer cells lines (*in vitro*)

The present study was undertaken to obtain molecular characterization of human breast cancer tumor cells (*in vivo*) and cultured heterogeneous cells (*in vitro*) to confirm specific phenotypes using molecular stem cell markers such as mesenchymal and hematopoietic stem cell markers, pluripotency, oncogenic, cytokine and chemokine markers.

Mesenchymal and hematopoietic phenotypes of in vivo and *in vitro* breast cancer cells

Figure 2 shows the expression of mesenchymal (CD105, CD13, CD73) and hematopoietic (CD34, CD45) stem cell markers in different types of cells associated with breast cancer tumor development. BCC tumor cells did not express CD105, CD13, CD73, CD34 and CD45, indicating that at *in vivo* status, these cells were not expressing mesenchymal or hematopoietic phenotypes (Figure 2). However, all *in vitro* cultured cell types distinctly expressed CD105, CD13 and CD73, confirming their mesenchymal phenotypes whereas all these cultured cell lines only expressed hematopoietic stem cell marker CD34, indicating the presence of hematopoietic phenotypes (Figure 2).

Pluripotency and differentiating markers in *in vivo* and *in vitro* breast cancer cells

Figure 3 shows the expression of pluripotency markers (Oct4, Nanog, and SOX2) and differentiating markers (LIF and keratin) *in vivo* BCC cells and 5 *in vitro* BCC cell lines. It was shown that Oct4 was mildly expressed in BCC mixed, epithelial, endothelial and transformed cells whereas it was absent in the BCC MSC cell line indicating a different pluripotency marker expression from the other 4 cell types (Figure 3). Nanog was well expressed in both *in vivo* and *in vitro* cell lines. However, SOX2 showed complete down-regulation in *in vivo* cells as well as in all 5 BCC cell lines, indicating its essential role in breast carcinogenesis (Figure 3). LIF also showed mild expression in all *in vivo* and *in vitro* cell types, except BCC Epithelial cells in which it was completely down-regulated. Keratin 18 was prominently expressed in all *in vivo* and *in vitro* cell lines indicating their epithelial origin (Figure 3).

Oncogenic phenotypes of breast cancer cell tumor cells and breast cancer cell cell lines

Figure 4 shows the oncogenic phenotypes of BCC tumor cells and cultured cell lines. Breast cancer tumor cells (*in vivo*) did not express *CD 44*, *EGFR*, *DAPK* and *C-MYC* genes but, interestingly, all *in vitro* cell lines highly expressed *CD 44*, *EGFR*, and *DAPK* genes. BCC tumor cells highly expressed the *BCL2* gene whereas in all BCC cell lines it was significantly down-regulated. *COX2*, a gene involved in tumor invasion and metastasis, was expressed in breast cancer cells and also showed mild expression in BCC MSC, BCC endothelial and BCC transformed cell lines, indicating that these cells may reduce its invasive and metastasis properties in culture (Figure 4).

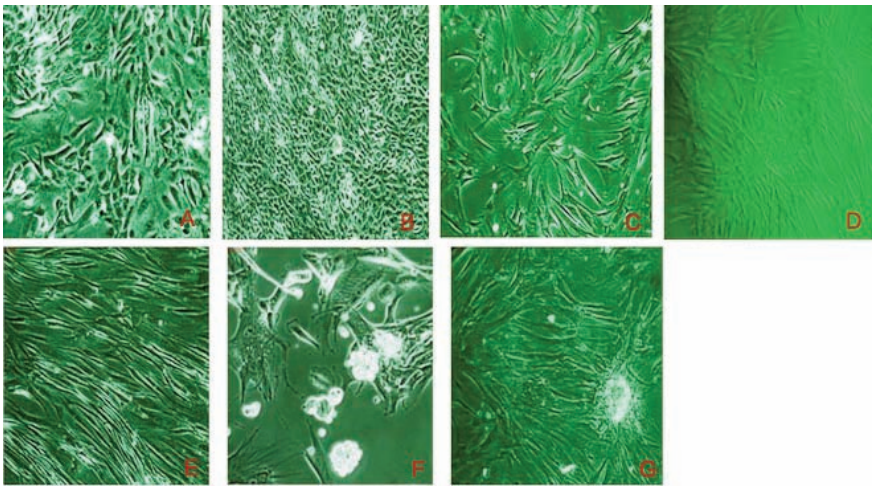

Figure 1. Phase contrast microphotographs of isolated breast cancer cell (BCC) endothelial (A), BCC epithelial (B), BCC MSC (C), BCC transformed (D) and BCC mixed (E) cell types from breast cancer non-metastatic tumor. (F) BCC MSC cells formed mammospheres in culture. (G) Pluripotency clone formation in BCC transformed cell line.

However, the *COX2* gene was strongly expressed in the BCC epithelial cell line (Figure 4). Similarly, we have analyzed variability of *EGFR* gene status in which it was strongly expressed in BCC Mixed, BCC endothelial and BCC transformed cells and mildly expressed in BCC epithelial cell line (Figure 4). In the present study, we observed normal expression of DAPK in BCC MSC and BCC transformed cell lines whereas it was down-regulated in BCC tumor cells (*in vivo*), BCC mixed, BCC epithelial and BCC endothelial cells (Figure 4). C-MYC was shown to be completely down-regulated in BCC tumor cells and BCC epithelial cells whereas it was normally expressed in BCC mixed, BCC MSC, BCC endothelial and BCC transformed cell lines (Figure 4). Overall, this study revealed that BCC mixed and BCC epithelial cell lines have retained their malignant phenotypes in culture and have shown good correlation with BCC tumor cell phenotypes whereas the other 3 cell lines showed mildly transformed phenotypes.

Cytokine and chemokine phenotypes of *in vivo* and *in vitro* breast cancer cells

Cytokine and chemokine phenotypes of breast cancer tumor cells and BCC clones were studied to find out whether they differ in different clones. It was shown that TNFα was highly expressed in *in vivo* breast cancer cells. However, all cultured cell types showed very mild expression of TNFα indicating a reduction in the invasive and transformative properties of these cells (Figure 5). IL6 was highly expressed in all 5 cell lines except in *in vivo* cells (Figure 5). CXCR4 was prominently expressed in *in vivo* tumor cells but was mildly expressed in all cell lines indicating and confirming the previous observation on its reduction and transformative properties in culture (Figure 5).

Expression of *BRCA2* gene mutation in *in vivo* and *in vitro* breast cancer cells

We investigated *BRCA2* gene mutation in tumor tissue as well as in all 5 breast cancer cell lines by DNA sequencing to confirm their breast cancer phenotypes. We evaluated the mutation at exon 11E of the *BRCA2* gene in tumor cells as well as all 5 BCC cell lines. This study revealed that the cells isolated from tumor cells have an 11E *BRCA* mutation indicating their breast cancer origin and also confirming that this gene is responsible for causing breast cancer in humans (Figure 6). Thus, we can conclude that the cells isolated from non-metastatic breast cancer tumor are Initiated breast cancer stem cells.

Discussion

Recent advances in cancer research have shown the existence of breast cancer stem cells and their significant role in tumorigenesis.[25-27] Wendy *et al.* have shown that breast cancer tumors have a uniform population or heterogeneous populations of cancer stem cells and these stem cell populations differ among different tumor types.[28] Similarly, Groner *et al.* have suggested that a cell population with distinct genetic and epigenetic changes gives rise to the cellular heterogeneity of breast tumors.[26] Zucchia *et al.* have characterized and isolated 3 distinct types of cells from rat mammary tumors that differ in morphology, immunophenotype and their ability to

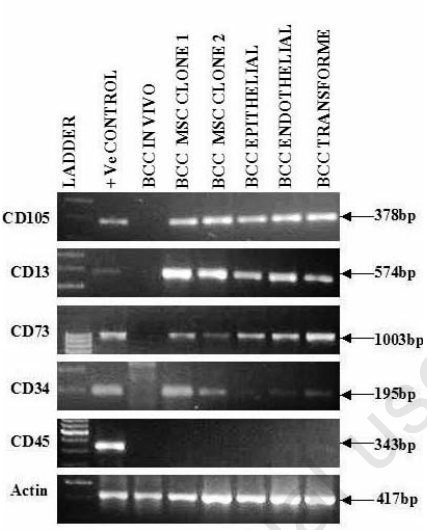

Figure 2. Expression of mesenchymal (CD105, CD13, CD73) and hematopoietic (CD34, CD45) stem cell markers in breast cancer tumor tissue (*in vivo*) and 5 breast cancer cell lines isolated from the same tumor (*in vitro*).

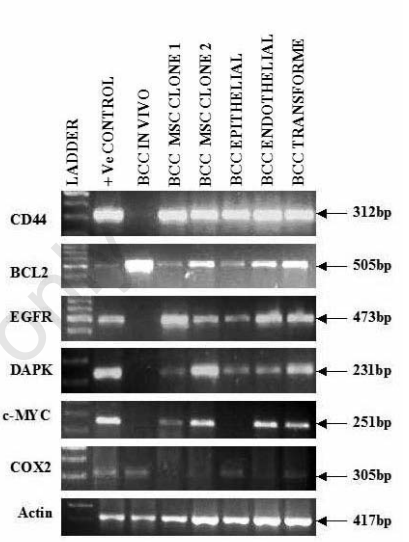

Figure 4. Expression of oncogenic markers (CD44, BCL2, EGFR, DAPK, C-MYC, COX2) in breast cancer tumor tissue (*in vivo*) and 5 breast cancer cell lines isolated from the same tumor (*in vitro*).

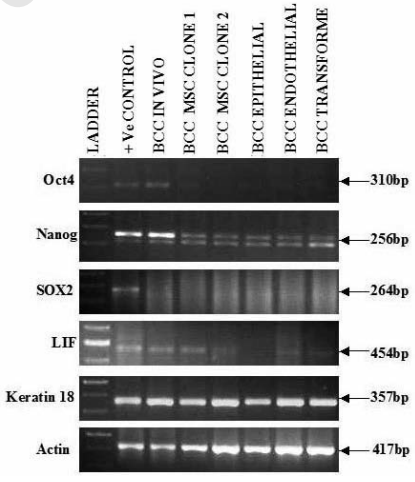

Figure 3. Expression of pluripotency (Oct4, NANOG, SOX2) and differentiating stem cell markers (LIF, keratin 18) in breast cancer tumor tissue *(in vivo)* and 5 breast cancer cell lines isolated from the same tumor (*in vitro*).

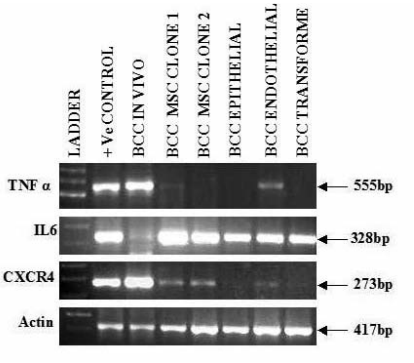

Figure 5. Expression of cytokine (IL6, TNFα) and chemokine (CXCR4) markers in breast cancer tumor tissue (*in vivo*) and 5 breast cancer cell lines isolated from the same tumor (*in vitro*).

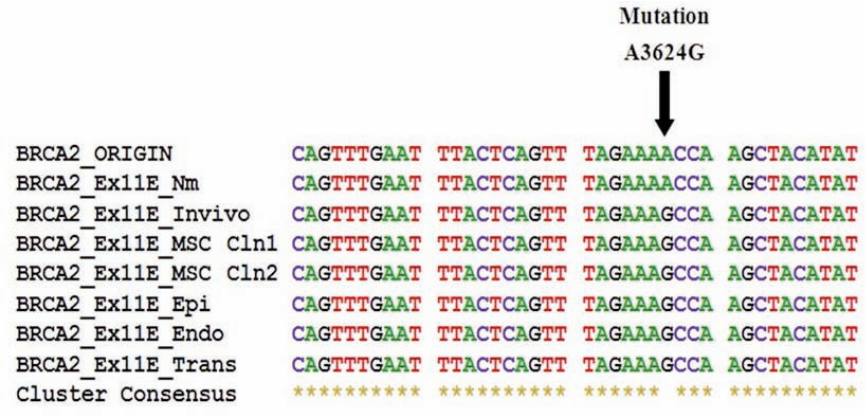

Figure 6. Bioedit of exon 11 E of *BRCA2* gene mutation (A3624G) in breast cancer tumor tissues (*in vivo*) and 5 breast cancer cell lines isolated from the same tumor (*in vitro*), respectively.

sustain mammosphere and tumor formation.[29] The main challenges in human breast cancer studies are to isolate the breast cancer stem cells that are responsible for causing this disease and to define its molecular profile in order to identify their malignant phenotypes by using stem cell molecular markers. Technologies for the isolation of breast cancer stem cells from breast tumor have been developed by many investigators and have shown that $CD44^{+}/CD24^{-/low}$ or $ALDH^{+}$ are a well-defined population of cells of potential breast cancer stem cells.[5,28] However, no study has tried to define specific markers which can be used to target breast cancer cells. In the present study, we cultured non-metastatic breast cancer tumor cells to identify a heterogeneous population of tumor cells involved in breast cancer development. Here, we have shown that non-metastatic breast tumor contains a hierarchy of cells with different capabilities of generating mammosphere which is one of the properties of breast cancer stem cells. In this study, we have further characterized and isolated 5 distinct types of cells from breast tumor that have clearly shown different morphological features, such as epithelial, endothelial, mesenchymal and transformed stem cells. This is the first study in which we have isolated morphologically different cell types from non-metastatic human breast cancer tumor characterized by specific molecular markers. The mesenchymal characteristics of these cells were confirmed by CD 105, CD 13 and CD73 markers which are putative markers for mesenchymal stem cells.[14] These cell types also showed partial phenotype for hematopoietic stem cells by expressing the *CD34* gene.[15] Pluripotency and differentiation markers were also expressed in these cell types confirming their self renewal and differential potency, as shown by Olamura *et al*.[30] Interestingly, it was shown that all breast cancer cell lines consistently expressed pluripotency marker Oct4 except BCC MSC Clone 2 in which Oct4 was down-regulated. Expression of keratin[18] in all breast cancer cell lines confirmed their epithelial origin.[31,32]

After establishing different cell types from breast cancer tumor, it was essential to know whether these cell types possesse normal or malignant phenotypes, So we have studied oncogenic markers, such as CD44, EGFR, C-MYC, DAPK, COX2, BCL2, which are known markers for malignant transformation.[11,18,19,33-35] We detected varying patterns of oncogenic marker expression in different cell types of breast cancer. Overexpression of the *CD44* gene was observed in all breast cancer cell lines confirming that these cell types are breast cancer-initiating cells.[11] According to Patrawala *et al.,* CD44 expression is associated with drug resistance and metastatic progression in malignancy.[11] Studies have shown that oncogenes C-MYC, COX2, EGFR, DAPK and BCL2 play an essential role in the development and tumorigenesis of breast cancer cells whereas their overexpression or loss of expression is useful to study invasive potential and metastatic profile in aggressive tumors and neoplastic tissues.[18,19,33-35] Here, we report that there is a definite change at cellular and molecular levels in all 5 types of breast cancer cell lines which formed breast cancer tumor. Our study further showed that C-MYC and DAPK, which were expressed in normal cells, were down-regulated in BCC tumor cells (*in vivo*) and BCC epithelial cells (*in vitro*), indicating that BCC epithelial cells retained their original characteristics of malignant transformation of BCC tumor cells *in vivo.* In contrast, it was shown that BCC MSC Clone 1 and Clone 2, BCC endothelial and BCC transformed cell lines mildly expressed C-MYC and DAPK. EGFR and BCL2 have been shown to be over-expressed in breast cancer.[34,35] In our study, EGFR and BCL2 were highly expressed in all 5 cell lines whereas no expression of EGFR was shown in BCC tumor cells *in vivo*. *COX2* is one of the oncogenic genes involved in invasion and metastasis of tumor.[18] In the present study, we observed expression of COX2 in BCC tumor cells *in vivo* as well as in BCC Epithelial cells indicating that BCC Epithelial cells more closely resemble *in vivo* tumor cells and may be more invasive than other cell lines isolated from the same tumor. *COX2* gene expression in all other cell lines was either mild or inexistent. Several authors have shown that TNF activates NF-KB in most cancer cells.[36,37] Our study is well correlated with their findings and have shown that TNF is over-expressed in BCC tumor cells and may be one of the causative agents for breast cancer development. However, all breast cancer cell lines showed mild or no expression of TNF indicating that these cell lines may have less malignant property than *in vivo* tumor cells and thus they can be designated as initiated breast cancer cell lines. Therefore, further study is essential to confirm the initiated phenotypes of all these cell lines by using *in vivo* and *in vitro* transformation assays. We observed that IL6 was expressed in all cell lines and CXCR4 was highly expressed in BCC tumor cells indicating a probable role for CXCR4 in invasion and metastatic phenotypes of *in vivo* cells. IL6 was down-regulated in all breast cancer cell lines suggesting that these cells may have reduced invasive and metastatic properties when grown in culture.[28] We further confirmed breast cancer-initiated cells by studying gene mutation in the *BRCA2* gene. Our laboratory reported a founder mutation in exon 11E of the *BRCA2* gene in an Indian population as an early prognostic marker for breast cancer.[22] In the present study, we have sequenced DNA samples of breast cancer tumor tissue and different types of breast cancer cell lines. Our study showed that the presence of this founder mutation in *in vivo* and *in vitro* BCC tumor cell lines confirmed that the cells isolated from non-metastatic breast cancer tumor were breast cancer-initiated cells which may be responsible for the development of breast cancer in humans. In summary, our comprehensive molecular and phenotypic analysis of breast cancer cell hierarchy derived from breast carcinomas revealed that they represent defined cell populations with distinct gene

expression and genetic profiles, as supported by the concept of *tumor heterogeneity* in breast cancer.[27] Such gene expression and molecular profiling of tumor cell types will be useful in identifying possible ways to target each cancer stem cell to cure breast cancer. Overall, this study provides a better understanding of breast cancer development and the involvement of various populations of cells. It can, therefore, be used as an *in vitro* model system to help understand the mechanism of human breast carcinogenesis and to design targeted therapies for breast cancer patients even at advanced stage of disease.

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