Gene expression characteristics of breast cancer stem cells

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Summary

Purpose: Breast cancer stem cells have been found to be responsible for tumorigenic potential and resistance to therapy. This study aimed at comparing gene expression profiles in breast cancer, based on the differences of stem cells in their biological characteristics.

Methods: Four breast cancer cell lines with different molecular and biological characteristics were used to analyze 84 breast cancer-related gene expressions. These were the ductal human epithelial breast cancer cell line T47D (HTB-133) with metastatic origin, the invasive ductal human breast carcinoma cell line MDA-MB-231 (HTB-26), the ductal human epithelial breast cancer cell line BT-474 (HTB-20) and the human metastatic breast adenocarcinoma cell line MCF-7 (HTB-22).

Results: There were significant differences between the breast cancer cells and the stem cells, particularly in angiogenesis, migration, proliferation and the expression of the DNA repair genes.

Conclusion: These data indicated the absence of a general cancer stem cell in breast cancer. Our study supports the use of the term "breast cancer initiating cells" instead of breast cancer stem cells. All of these genetic differences should be taken into account in the planning of final therapeutic approach.

Key words: breast cancer, cancer initiating cell, cancer stem cell, gene expression

Introduction

Breast cancer in women is a most common and major public health problem both in developed and developing countries. One in ten of all new cancers diagnosed worldwide each year is a cancer of the female breast. It is also the principal cause of death from cancer among women worldwide [1].

Despite significant advances in breast cancer treatment over the years, relapse of this disease with time is a major roadblock to achieve cure of this disease. The only established reason behind this is the underlying presence of a small population of stem-like cells called cancer stem cells [2,3].

Cancer, being a stem cell disease, is not a new concept. In the last few years scientists have reassessed the biology of cancer using a stem cell-based hierarchical model. The basic arguments supporting the idea that tissue stem cells may be primary targets for transformation can be summarized as follows: (i) stem cells are long-lived, slowly dividing cells that persist in tissues long enough to accumulate multiple genetic alterations required for neoplastic transformation and are exposed to genotoxic insults much longer than are short-lived ones [4]; (ii) molecular pathways, playing a critical role in stem cell self-renewal are often deregulated in a number of tumors [5]; (iii) normal stem cells and tumor cells share a number of phenotypic features, such as a relatively undifferentiated state, the ability to self-renewal, the activation of cytoprotective mechanisms as well as a remarkable competence for migration. Definite evidence has been achieved recently that

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tumors arise from and are sustained by mutated stem/ progenitor cells in blood, brain and breast malignancies [6-10]. It was demonstrated that only a small proportion of cancer cells retains the ability to form new tumors after transplantation in immunodeficient mice which are termed 'tumor-initiating cells' or 'cancer stem cells' having competence for self-renewal and capacity to re-establish tumor heterogeneity.

The major goal of cancer treatment is to understand how many and which tumor cells must be eliminated. Therefore, elimination of cancer cells that have limited proliferation potential, while sparing cancer stem cells that have unlimited proliferation potential will unfortunately result in relapse or recurrence [11]. The surface marker proteins on membranes define cell types. Likewise, breast cancer stem cells (BCSCs) are mainly identified by the presence of the cell surface marker protein CD44, and low levels of CD24 which are also called adhesion molecules. BCSCs can be easily identified by their ability to grow in serum-free suspension cultures like the neural stem cell-forming aggregates called mammospheres. In breast tumors, the use of neoadjuvant regimens showed that conventional chemotherapy could lead to enrichment in CSCs in treated patients as well as in xenografted mice [12,13]. The radiation effect on CSCs was studied *in vitro*. In MCF7, CSC/progenitors isolated as mammospheres were more resistant to radiotherapy than cells in monolayer culture, and fractionated radiotherapy increased the proportion of BCSCs with the CD44+/CD24-/low phenotype [14]. These data reinforce the belief that CSCs resist many current therapies and that they are the actual targets to eliminate if treatment is to be curative. Interestingly, treatment of ERBB2-positive tumors with the EGFR/ERBB2 inhibitor lapatinib led to a small decrease in the percentage of BCSCs [12]. The characterization of BCSCs was an important step in order to clarify such issues like the recurrence and metastasis in breast cancer despite the developments in diagnosis, therapy and prognosis.

We therefore aimed to isolate CD44+CD24 low/- BCSCs from breast cancer cell lines which had different molecular and biological character-

istics and to reveal the gene expression profiles compared with ordinary breast cancer cells.

The ultimate common goal of research in this field should be the development of therapeutic strategies selectively targeting not only the breast cancer cells but also the breast cancer stem cells sparing normal stem cells.

Methods

Cell culture

Four breast cancer cell lines were cultured, which have different molecular and biological characteristics (Table 1). The ductal human epithelial breast cancer cell line T47D (HTB-133) with metastatic origin, expresses progesterone receptor (PR), estrogen receptor (ER) and has no amplification of ERBB2 (HER2). The invasive ductal human breast carcinoma cell line MDA-MB-231 (HTB-26) has a triple-negative molecular characteristic with PR negative, ER negative expressions and ERBB2 negative amplification. The ductal human epithelial breast cancer cell line BT-474 (HTB-20) with primary origin has a triple-positive molecular characteristic with PR positive, ER positive expressions and ERBB2 positive amplification. The human metastatic breast adenocarcinoma cell line MCF-7 (HTB-22) expresses both PR and ER, but has no amplification of ERBB2 (Figure 1). All cell lines were supplied from ATCC (Wesel, Germany).

These four cell lines were maintained in humidified incubator at 37°C with 5% CO_2 and cultured in complete medium consisting of Dulbecco's Eagle's : F12 medium (GibcoTM, Waltham, USA) supplemented with 10% fetal bovine serum (Biochrom, Cambridge, UK), 1 % L-glutamine (Gibco TM) and 1% penicillin/streptomycine (Gibco TM). The cell lines were proliferated until we obtained enough cells ($1x10^7$ per ml) for magnetic stem cell separation.

CD44+CD24 low/- BCSC magnetic isolation

The medium was discarded and the cells were washed with PBS, obtained by trypsinization and resuspended in magnetic separation buffer. CD24+ cells were removed by tagging them with biotinylated anti-human CD24 antibody followed by the addition of streptavidin-conjugated magnetic particles (MagCellect* Streptavidin Ferrofluid, Minneapolis, USA). The tubes with the cell suspension were then placed in a

Table 1. Characteristics of breast cancer cell lines

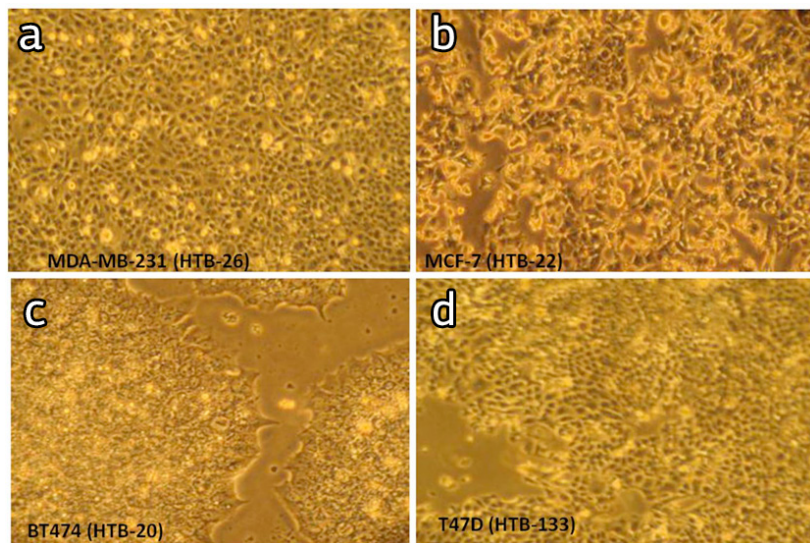| Cell line	ER	PR	Her2	Type	Tumor
MDA-MB-231	_	_	_	Basal B	Metastatic adenocarcinoma
MCF-7 (HTB-22)	+	+	-	Luminal	Metastatic adenocarcinoma
BT474 (HTB-20)	+	+	+	Luminal	Invasive ductal carcinoma
T47D (HTB-133)	+	+	-	Luminal	Invasive ductal carcinoma

Figure 1. Microscopic view of cell lines. **a:** invasive ductal human breast carcinoma cell line MDA-MB-231 (HTB-26), **b:** human metastatic breast adenocarcinoma cell line MCF-7 (HTB-22), **c:** ductal human epithelial breast cancer cell line BT-474 (HTB-20), **d:** ductal human epithelial breast cancer cell line T47D (HTB-133).

magnet. Magnetically tagged cells (CD24+) migrated toward the tube wall on the magnet side, leaving the untagged cells (CD24low/-) in suspension. The untagged CD24low/- cells were subsequently labeled with a biotinylated anti-human CD44 antibody and magnetically tagged with MagCellect Streptavidin Ferrofluid to select the CD44+ cells. The tubes with the cell suspension were then placed in a magnet. Magnetically tagged cells (CD44+) migrated toward the tube wall on the magnet side, leaving the untagged cells (CD-44low/-) in suspension. Then, we collected the suspension that included the untagged cells (CD44low/-) and resuspended the CD44+CD24 low/- BCSCs in the medium. We kept the cells in -196°C liquid nitrogen for 2 min and used for RNA isolation.

RNA isolation and RT-PCR assay

The four molecularly and biologically different breast cancer cells were grown to 80–90% confluence (1x10⁷), and were then harvested for total RNA. We used total RNA isolation kit (Macherey-Nagel, Duren, Germany) for RNA isolation.

cDNA was synthesized by using reverse transcriptase cDNA synthesis kit (QIAGEN, Venlo, Netherlands) according to the manufacturer's protocol. The cDNA samples were obtained and used for RT-PCR array analysis.

We used PAHS-131Z The Human Breast Cancer RT² Profile PCR Array (SABiosciences QIAGEN, Venlo, Netherlands) which profiles the expression of 84 key genes commonly involved in the dysregulation of signal transduction and other normal biological processes

during breast carcinogenesis and we analyzed the gene expression profiles of 4 molecularly and biologically different BCSCs and their CD44+CD24low/- stem cells.

Results

According to the data analysis results, the breast cancer carcinogenesis related gene expression profiles of 4 different molecularly and biologically characterized CD44+CD24low/- BCSCs showed significant increases and decreases when compared with their paired breast cancer cells. The transporter protein family related SLC39A6, epithelial cells mobility related TFF3, transcription factor XBP1, RB1, MAPK3 genes expressions increased significantly, while the cell matrix-related ADAM23, migration and proliferation related PLAU, antioxidant enzyme coding GSTP1, steroid hormone receptor related AR, anti-apoptotic BCL2, invasion and metastasis related MMP2, MMP9 and DNA damage repair related MGMT genes expressions decreased significantly in MCF-7 (HTB-22) breast cancer CD44+CD-24low/- stem cells when compared with its paired MCF-7 (HTB-22) breast cancer cells (Figure 2). The epithelial to mesenchymal transition related CTNBB1, DNA damage and apoptosis related CDKN1A and transcription factor JUN genes expressions increased significantly, while the nuclear steroid hormone receptor related PGR, epithelial cells mobility related TFF3, cell pro-
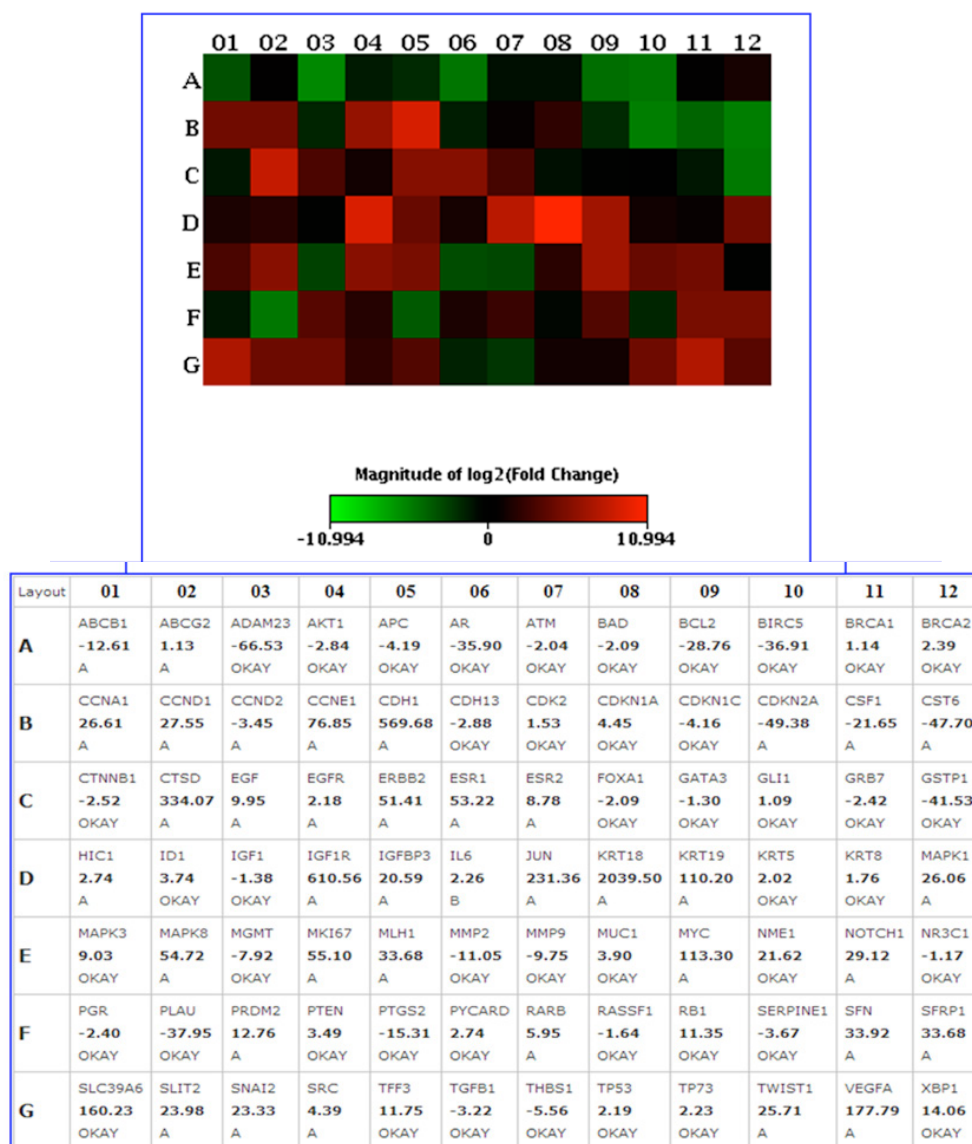
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B	CCNA1 26.61 A	CCND1 27.55 A	CCND2 -3.45 A	CCNE1 76.85 A	CDH1 569.68 A	CDH13 -2.88 OKAY	CDK2 1.53 OKAY	CDKN1A 4.45 OKAY	CDKN1C -4.16 OKAY	CDKN2A -49.38 A	CSF1 -21.65 A	CST6 -47.70 A
C	CTNNB1 -2.52 OKAY	CTSD 334.07 A	EGF 9.95 A	EGFR 2.18 A	ERBB2 51.41 A	ESR1 53.22 A	ESR2 8.78 A	FOXA1 -2.09 OKAY	GATA3 -1.30 OKAY	GLI1 1.09 OKAY	GRB7 -2.42 OKAY	GSTP1 -41.53 OKAY
D	HIC1 2.74 A	ID1 3.74 OKAY	IGF1 -1.38 OKAY	IGF1R 610.56 A	IGFBP3 20.59 A	IL6 2.26 B	JUN 231.36 A	KRT18 2039.50 A	KRT19 110.20 A	KRT5 2.02 OKAY	KRT8 1.76 OKAY	MAPK1 26.06 A
E	MAPK3 9.03 OKAY	MAPK8 54.72 A	MGMT -7.92 OKAY	MKI67 55.10 A	MLH1 33.68 A	MMP2 -11.05 OKAY	MMP9 -9.75 OKAY	MUC1 3.90 OKAY	MYC 113.30 A	NME1 21.62 OKAY	NOTCH1 29.12 A	NR3C1 -1.17 OKAY
F	PGR -2.40 OKAY	PLAU -37.95 A	PRDM2 12.76 A	PTEN 3.49 OKAY	PTGS2 -15.31 A	PYCARD 2.74 OKAY	RARB 5.95 A	RASSF1 -1.64 OKAY	RB1 11.35 OKAY	SERPINE1 -3.67 OKAY	SFN 33.92 A	SFRP1 33.68 A
G	SLC39A6 160.23 OKAY	SLIT2 23.98 A	SNAI2 23.33 A	SRC 4.39 A	TFF3 11.75 OKAY	TGFB1 -3.22 OKAY	THBS1 -5.56 OKAY	TP53 2.19 OKAY	TP73 2.23 OKAY	TWIST1 25.71 OKAY	VEGFA 177.79 A	XBP1 14.06 OKAY

Figure 2. Heatmap and expression Table of the genes in MCF7 cells compared with stem cells. The transporter protein family related SLC39A6, epithelial cells mobility related TFF3, transcription factor XBP1, RB1, MAPK3 gene expressions increased significantly, while the cell matrix-related ADAM23, migration and proliferation related PLAU, antioxidant enzyme coding GSTP1, steroid hormone receptor related AR, anti-apoptotic BCL2, invasion and metastasis related MMP2, MMP9 and DNA damage repair related MGMT gene expressions decreased significantly in MCF-7 (HTB-22) breast cancer CD44+CD24 low/- stem cells when compared with its paired MCF-7 (HTB-22) breast cancer cells.

liferation related MKI67 and nuclear steroid hormone receptor related ESR1 gene expressions decreased significantly in BT474 (HTB-20) breast cancer CD44+CD24low/- stem cells when compared with its paired BT474 (HTB-20) breast cancer cells (Figure 3). The angiogenesis related VEGFA gene expression increased significantly while the epithelial cells mobility related TFF3 gene expression decreased significantly in T47D (HTB-133) breast cancer CD44+CD24low/- stem cells when compared with its paired T47D (HTB-

133) breast cancer cells (Figure 4). The invasion and metastasis related MMP9 and cell proliferation related MKI67 genes expressions increased significantly, while the transporter protein family related ABCB1 and SLC39A6 genes expressions decreased significantly in MDA-MB-231 (HTB-26) breast cancer CD44+CD24low/- stem cells when compared with its paired MDA-MB-231 (HTB-26) breast cancer cells (Figure 5). There were also significant differences in gene expression profiles of 4 breast cancer CD44+CD24low/- stem cells when
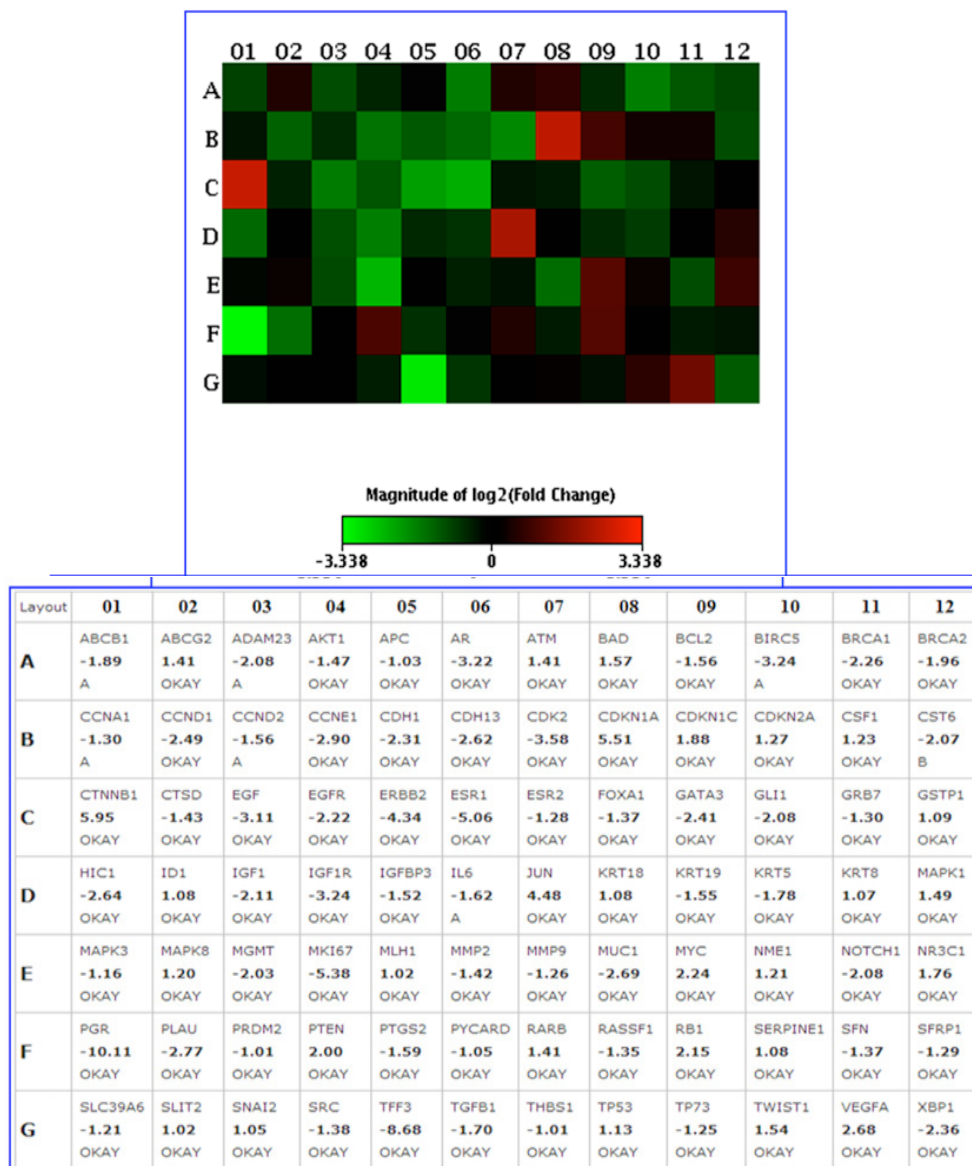
Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	ABCB1 -1.89 A	ABCG2 1.41 OKAY	ADAM23 -2.08 A	AKT1 -1.47 OKAY	APC -1.03 OKAY	AR -3.22 OKAY	ATM 1.41 OKAY	BAD 1.57 OKAY	BCL2 -1.56 OKAY	BIRC5 -3.24 A	BRCA1 -2.26 OKAY	BRCA2 -1.96 OKAY
B	CCNA1 -1.30 A	CCND1 -2.49 OKAY	CCND2 -1.56 A	CCNE1 -2.90 OKAY	CDH1 -2.31 OKAY	CDH13 -2.62 OKAY	CDK2 -3.58 OKAY	CDKN1A 5.51 OKAY	CDKN1C 1.88 OKAY	CDKN2A 1.27 OKAY	CSF1 1.23 OKAY	CST6 -2.07 B
C	CTNNB1 5.95 OKAY	CTSD -1.43 OKAY	EGF -3.11 OKAY	EGFR -2.22 OKAY	ERBB2 -4.34 OKAY	ESR1 -5.06 OKAY	ESR2 -1.28 OKAY	FOXA1 -1.37 OKAY	GATA3 -2.41 OKAY	GLI1 -2.08 OKAY	GRB7 -1.30 OKAY	GSTP1 1.09 OKAY
D	HIC1 -2.64 OKAY	ID1 1.08 OKAY	IGF1 -2.11 OKAY	IGF1R -3.24 OKAY	IGFBP3 -1.52 OKAY	IL6 -1.62 A	JUN 4.48 OKAY	KRT18 1.08 OKAY	KRT19 -1.55 OKAY	KRT5 -1.78 OKAY	KRT8 1.07 OKAY	MAPK1 1.49 OKAY
E	MAPK3 -1.16 OKAY	MAPK8 1.20 OKAY	MGMT -2.03 OKAY	MKI67 -5.38 OKAY	MLH1 1.02 OKAY	MMP2 -1.42 OKAY	MMP9 -1.26 OKAY	MUC1 -2.69 OKAY	MYC 2.24 OKAY	NME1 1.21 OKAY	NOTCH1 -2.08 OKAY	NR3C1 1.76 OKAY
F	PGR -10.11 OKAY	PLAU -2.77 OKAY	PRDM2 -1.01 OKAY	PTEN 2.00 OKAY	PTGS2 -1.59 OKAY	PYCARD -1.05 OKAY	RARB 1.41 OKAY	RASSF1 -1.35 OKAY	RB1 2.15 OKAY	SERPINE1 1.08 OKAY	SFN -1.37 OKAY	SFRP1 -1.29 OKAY
G	SLC39A6 -1.21 OKAY	SLIT2 1.02 OKAY	SNAI2 1.05 OKAY	SRC -1.38 OKAY	TFF3 -8.68 OKAY	TGFB1 -1.70 OKAY	THBS1 -1.01 OKAY	TP53 1.13 OKAY	TP73 -1.25 OKAY	TWIST1 1.54 OKAY	VEGFA 2.68 OKAY	XBP1 -2.36 OKAY

Figure 3. Heatmap and expression Table of the genes in BT474 (HTB20) cells compared with stem cells. The epithelial to mesenchymal transition related CTNBB1, DNA damage and apoptosis related CDKN1A and transcription factor JUN genes expressions increased significantly, while the nuclear steroid hormone receptor related PGR, epithelial cells mobility related TFF3, cell proliferation related MKI67 and nuclear steroid hormone receptor related ESR1 gene expressions decreased significantly in BT474 (HTB-20) breast cancer CD44+CD24 low/- stem cells when compared with its paired BT474 (HTB-20) breast cancer cells.

compared with each other. A marked difference between gene expression profiles of 4 breast cancer cells was also seen.

Five genes were highly expressed in 4 molecularly and biologically different breast cancer cells and their CD44+CD24low/- stem cells. These were the signal transduction and apoptosis related AKT1 gene, angiogenesis, epithelial to mesenchymal transition and steroid receptor-mediated signal transduction related CTNNB1 gene, adhesion and angiogenesis related THBS1 gene, cell cycle, DNA damage and apoptosis related Tp53 and transcription factor XBP1 gene.

Discussion

Breast cancer is a group of biologically different diseases rather than a single disease. Breast cancer related uncontrolled cell proliferation is usually associated with signs of genomic instability and changes such as disappearance of certain particular epithelial properties. Therefore, it
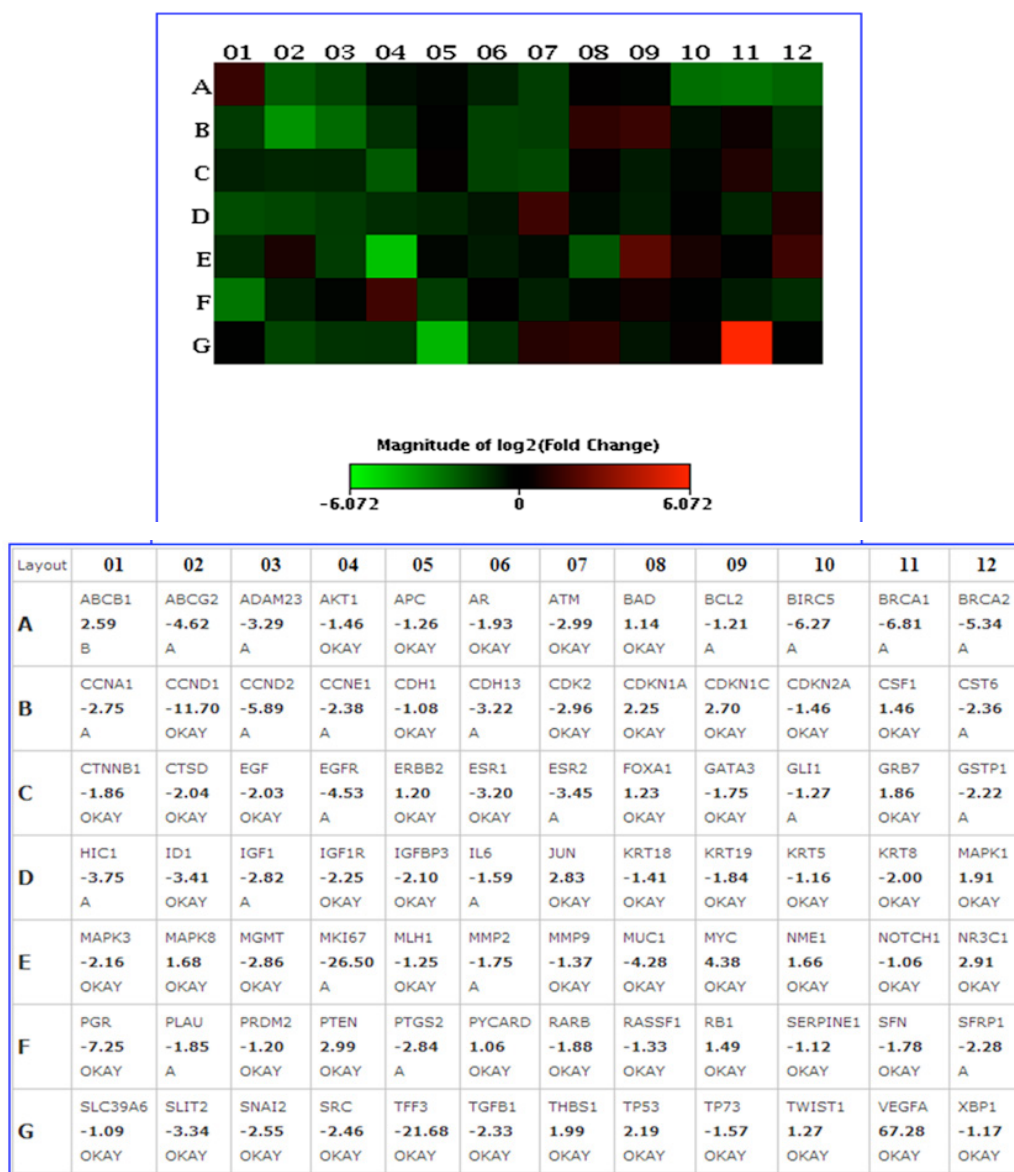
Figure 4. Heatmap and expression Table of the genes in HTB133 cells compared with stem cells. The angiogenesis related VEGFA gene expression increased significantly while the epithelial cells mobility related TFF3 gene expression decreased significantly in T47D (HTB-133) breast cancer CD44+CD24 low/- stem cells when compared with its paired T47D (HTB-133) breast cancer cells.

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	ABCB1 2.59 B	ABCG2 -4.62 A	ADAM23 -3.29 A	AKT1 -1.46 OKAY	APC -1.26 OKAY	AR -1.93 OKAY	ATM -2.99 OKAY	BAD 1.14 OKAY	BCL2 -1.21 A	BIRC5 -6.27 A	BRCA1 -6.81 A	BRCA2 -5.34 A
B	CCNA1 -2.75 A	CCND1 -11.70 OKAY	CCND2 -5.89 A	CCNE1 -2.38 A	CDH1 -1.08 OKAY	CDH13 -3.22 A	CDK2 -2.96 OKAY	CDKN1A 2.25 OKAY	CDKN1C 2.70 OKAY	CDKN2A -1.46 OKAY	CSF1 1.46 OKAY	CST6 -2.36 A
C	CTNNB1 -1.86 OKAY	CTSD -2.04 OKAY	EGF -2.03 OKAY	EGFR -4.53 A	ERBB2 1.20 OKAY	ESR1 -3.20 OKAY	ESR2 -3.45 A	FOXA1 1.23 OKAY	GATA3 -1.75 OKAY	GLI1 -1.27 A	GRB7 1.86 OKAY	GSTP1 -2.22 A
D	HIC1 -3.75 A	ID1 -3.41 OKAY	IGF1 -2.82 A	IGF1R -2.25 OKAY	IGFBP3 -2.10 OKAY	IL6 -1.59 A	JUN 2.83 OKAY	KRT18 -1.41 OKAY	KRT19 -1.84 OKAY	KRT5 -1.16 OKAY	KRT8 -2.00 OKAY	MAPK1 1.91 OKAY
E	MAPK3 -2.16 OKAY	MAPK8 1.68 OKAY	MGMT -2.86 OKAY	MKI67 -26.50 A	MLH1 -1.25 OKAY	MMP2 -1.75 A	MMP9 -1.37 OKAY	MUC1 -4.28 OKAY	MYC 4.38 OKAY	NME1 1.66 OKAY	NOTCH1 -1.06 OKAY	NR3C1 2.91 OKAY
F	PGR -7.25 OKAY	PLAU -1.85 A	PRDM2 -1.20 OKAY	PTEN 2.99 OKAY	PTGS2 -2.84 A	PYCARD 1.06 OKAY	RARB -1.88 OKAY	RASSF1 -1.33 OKAY	RB1 1.49 OKAY	SERPINE1 -1.12 OKAY	SFN -1.78 OKAY	SFRP1 -2.28 A
G	SLC39A6 -1.09 OKAY	SLIT2 -3.34 OKAY	SNAI2 -2.55 OKAY	SRC -2.46 OKAY	TFF3 -21.68 OKAY	TGFB1 -2.33 OKAY	THBS1 1.99 OKAY	TP53 2.19 OKAY	TP73 -1.57 OKAY	TWIST1 1.27 OKAY	VEGFA 67.28 OKAY	XBP1 -1.17 OKAY

is important to understand the molecular mechanisms leading to cancer development and the properties of tumor in every patient and determining the appropriate treatment. The properties of breast cancer stem cell should also be understood to explain the reason of the recurrence and metastasis despite the diagnostic, therapeutic and prognostic improvements in breast cancer.

Four different breast cancer cell lines were used in our study and cell line-specific tumorigenic cells with CD44+CD24- phenotype were detected and the comparison of this group of breast cancer stem cells with the other breast cancer cells revealed significant differences in certain carcinogenesis-related gene expressions.

In another study on cells endowed with stem cell properties which were considered to derive from tumor cells with different characteristics according to the molecular classification of breast cancer it was found that when breast cancer cells were ER+, the cells endowed with stem cell properties were also ER+ [15]. It has been demonstrated that the differentiation of the mammary tissue begins from ER- cells and these cells transform into the ER+ cells under the influence of BRCA1 during the terminal period of differentiation [16].
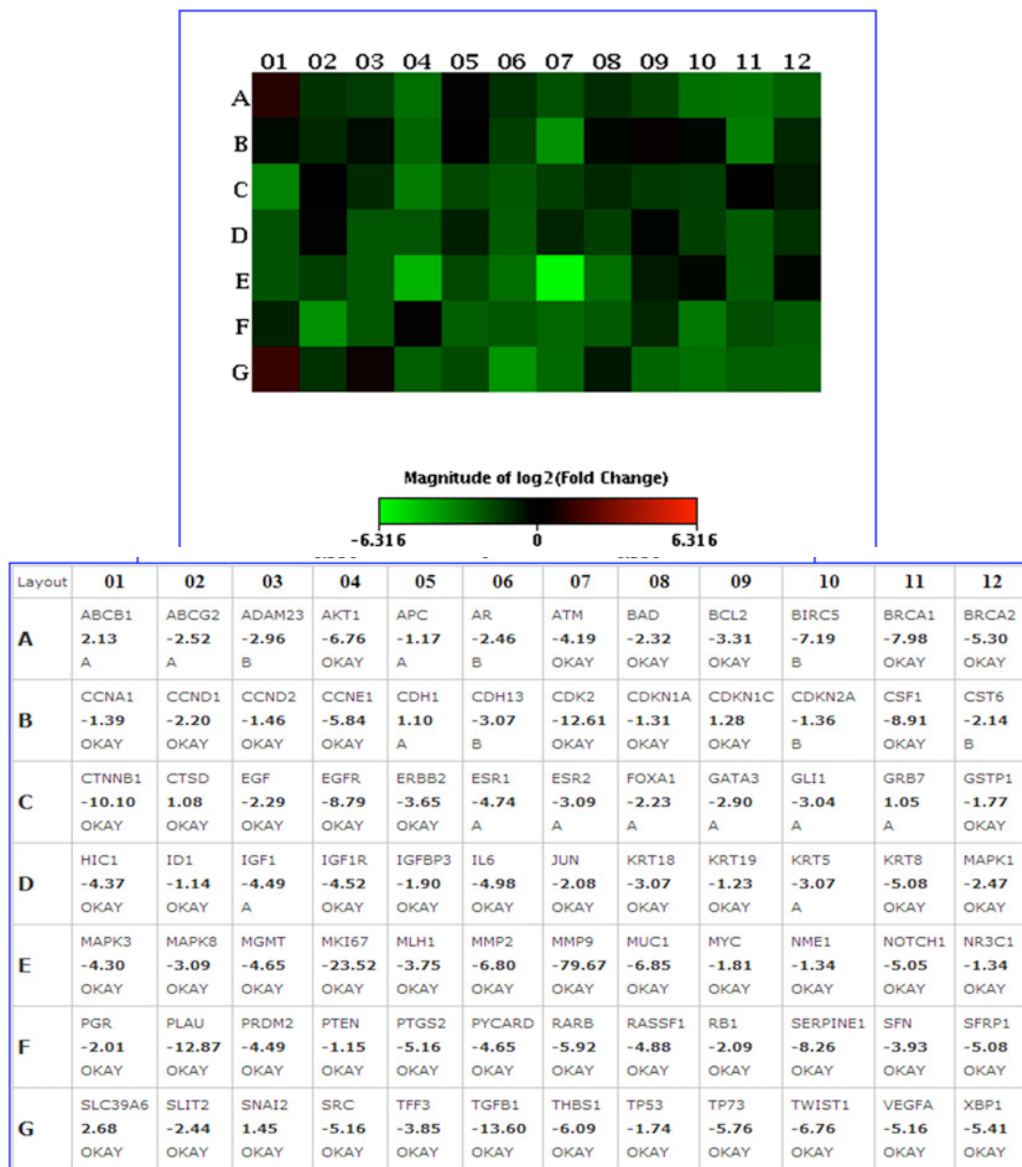
Layout	01	02	03	04	05	06	07	08	09	10	11	12
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B	CCNA1 -1.39 OKAY	CCND1 -2.20 OKAY	CCND2 -1.46 OKAY	CCNE1 -5.84 OKAY	CDH1 1.10 A	CDH13 -3.07 B	CDK2 -12.61 OKAY	CDKN1A -1.31 OKAY	CDKN1C 1.28 OKAY	CDKN2A -1.36 B	CSF1 -8.91 OKAY	CST6 -2.14 B
C	CTNNB1 -10.10 OKAY	CTSD 1.08 OKAY	EGF -2.29 OKAY	EGFR -8.79 OKAY	ERBB2 -3.65 OKAY	ESR1 -4.74 A	ESR2 -3.09 A	FOXA1 -2.23 A	GATA3 -2.90 A	GLI1 -3.04 A	GRB7 1.05 A	GSTP1 -1.77 OKAY
D	HIC1 -4.37 OKAY	ID1 -1.14 OKAY	IGF1 -4.49 A	IGF1R -4.52 OKAY	IGFBP3 -1.90 OKAY	IL6 -4.98 OKAY	JUN -2.08 OKAY	KRT18 -3.07 OKAY	KRT19 -1.23 OKAY	KRT5 -3.07 A	KRT8 -5.08 OKAY	MAPK1 -2.47 OKAY
E	MAPK3 -4.30 OKAY	MAPK8 -3.09 OKAY	MGMT -4.65 OKAY	MKI67 -23.52 OKAY	MLH1 -3.75 OKAY	MMP2 -6.80 OKAY	MMP9 -79.67 OKAY	MUC1 -6.85 OKAY	MYC -1.81 OKAY	NME1 -1.34 OKAY	NOTCH1 -5.05 OKAY	NR3C1 -1.34 OKAY
F	PGR -2.01 OKAY	PLAU -12.87 OKAY	PRDM2 -4.49 OKAY	PTEN -1.15 OKAY	PTGS2 -5.16 OKAY	PYCARD -4.65 OKAY	RARB -5.92 OKAY	RASSF1 -4.88 OKAY	RB1 -2.09 OKAY	SERPINE1 -8.26 OKAY	SFN -3.93 OKAY	SFRP1 -5.08 OKAY
G	SLC39A6 2.68 OKAY	SLIT2 -2.44 OKAY	SNAI2 1.45 OKAY	SRC -5.16 OKAY	TFF3 -3.85 OKAY	TGFB1 -13.60 OKAY	THBS1 -6.09 OKAY	TP53 -1.74 OKAY	TP73 -5.76 OKAY	TWIST1 -6.76 OKAY	VEGFA -5.16 OKAY	XBP1 -5.41 OKAY

Figure 5. Heatmap and expression Table of the genes in triple negative MDA-MB-231 cells compared with stem cells. The invasion and metastasis related MMP9 and cell proliferation related MKI67 gene expressions increased significantly, while the transporter protein family related ABCB1 and SLC39A6 gene expressions decreased significantly in MDA-MB-231 (HTB-26) breast cancer CD44+CD24 low/- stem cells when compared with its paired MDA-MB-231 (HTB-26) breast cancer cells.

Three out of the 4 cell lines used in our study expressed ER while one cell-line did not. In the analysis of either cancer cell lines or gene expressions in the stem cells of these cell lines, different gene variations were observed in each group. These variations were also observed in the HTB22 and HTB 133 cell lines which show identical ER, PR and HER2 gene expressions. For example TFF3 gene expression increases in the stem cells of the HTB 22 cell line, while this expression decreases in the HTB cell lines. When considering the research mentioned above, one may say that tumors with identical ER, PR and HER expressions may comprise stem cell-like cells with different genetic properties.

The cell cycle regulator "cyclin dependent kinase inhibitor 1" (CDKI1) was found to be increased both in the primary tumor and metastasis [17-22]. This gene expression was found to be significantly increased in the stem-cell like cells isolated from the HTB 20 cell line which was derived from the human primary tumor. This gene expression was found to be similar in stem cell-like cells and cancer cells in the other 3 cell-lines derived from metastatic tumors.

Vascular endothelial growth factor A is a

protein encoded by the VEGFA gene. This protein stimulates angiogenesis and cell migration and inhibits apoptosis. The VEGFA blocker Bevacizumab is still used in the treatment of many different types of metastatic cancer [23,24]. In our study, in the HTB 133 cell line (derived from metastatic breast cancer), the expression of VEGFA was significantly increased in stem cell-like cells compared to the tumor cells. The increased expression of VEGFA in the breast cancer stem cell-like in a metastatic environment suggests that VEGFA may be a treatment target.

Beta-catenin is a protein regulating the coordination of cell-cell adhesion and gene transcription and is encoded by the CTNNB1 gene [25,26]. Mutation and overexpression of beta-catenin are associated with a number of cancers including hepatocellular carcinoma, colorectal carcinoma, malignant breast tumors, ovarian and endometrial cancers [27]. In addition, beta-catenin takes part in the WNT signaling pathway and the increased expression of beta-catenin in particular cell groups was found to be effective in maintaining their pluripotency [28]. In our study, the increased CTNNB1 expression in stem-cell-like cells in the primary tumor-derived HTB 20 cell line was consistent with the results of previous studies [27,28]. No significant change of the CTNNB1 expression was observed in the other three metastasis-derived cell-lines.

In a previous study on HTB 22 cells, increased expression of c-Jun contributed to a more aggressive tumor behavior by increasing the motility of cancer cells and their invading ability. In the same study, overexpression of c-Jun was found to be associated with the development of estrogen and tamoxifen resistance and breast cancer cells acquired an estrogen-independent phenotype [29]. Another study reported that increased expression of c-Jun was associated with metastasis and this expression was found to be increased in breast cancers metastatic to liver [30]. Moreover, another study reported that c-Jun expression maintained the invasiveness of cancer by regulating BCSCs [31]. In our study the expression of c-Jun was found to be increased in the stem-cell-like cells isolated from the HTB 20 cancer cell line and this data was consistent with the previous results. [29,30]. Increased expression of XBP1 HIF1 alpha, which has been reported to be increased in triple negative breast cancers along with the increased expression of c-Jun, plays an effective role in the metabolism and angiogenesis of cancer and this increase in the expression has been reported as

a poor prognostic indicator and gives rise to aggressive and treatment-resistant tumors [32]. In our study, this gene expression was found to be significantly increased in the stem cell-like cells of the HTB 22 breast cancer cell line. In addition, this gene expression was found to be increased in all studied cancer cells and stem cell-like cells. This data is consistent with the medical literature. Analysis of XBP1 expression in aggressive and treatment-resistant tumors may be considered to be effective in determining the target for treatment.

Extracellular signal-regulated kinases (ERKs) are involve in cell proliferation, differentiation and the progression of the cell cycle. MAPK3 expression [33] which belongs to the ERKs family, was found to be increased in the stem cell-like cells of the HTB 22 cell line. Considering the role of this gene product, one may expect that the level of this expression may be higher in incompletely differentiated stem cell-like cells compared to cancer cells.

In a study on the methylation profiles of tumor cells in breast cancer patients, the expression of ADAM 23 gene was found to be lower in tumor cells compared the control group and this property gave rise to an increased ability of the tumor to metastasize [34]. In another study, the increased methylation in ER+ breast cancer was found to be associated with a more aggressive behavior of the tumor, but no correlation was found between tumor behavior and the expression level [35]. In our study, these two genes' expressions were found lower in the stem cell-like cells isolated from the metastasis-derived HTB 22 tumor cell line, compared to cancer cells of the same line.

MMP2 and MMP9 gene products that are involved in the degradation of extracellular matrix in the physiologic events such as proliferation, wound healing and angiogenesis in normal tissues, have significant effects on cancer invasion and metastasis. For example, it has been reported that their expressions are increased in metastatic breast cancer [36]. Furthermore, they have been found to be associated with tumor invasion and metastasis in certain types of human cancers and to be responsible for the increased aggressiveness of the tumor [37]. In our study, these two genes' expressions were found lower in the stem cell-like cells isolated from metastasis-derived HTB 22 tumor cell line compared to cancer cells of the same line, as occurred with ADAM 23 and GSTP1. Although this result appears to be inconsistent with the data in the literature, it may sug-

gest a potential differentiation of the stem cell-like cells in a metastatic tumor.

AKT1 plays an important role in the development of normal breast tissue while it also plays an important role in cancer initiation and progression. AKT1 is one of the most frequent genes with increased expressions in human cancer. Therefore, the role of AKT1 should be explored in depth in order to better understand the development of cancer and to develop new treatment strategies. As a result of the increased AKT1 activation, the cell cycle omits the control points at G1 and G2 phases and is led to an uncontrolled growth [38]. This process is important in the development of cancer. In line with these data, AKT1 gene expression was found to be increased in all cancer cell lines and related stem cell-like cells which were included in the study.

THBS1 gene products contribute to the inhibition of angiogenesis and the prevention of tumor growth. Reduced THBS1 gene products levels indicate a poor prognosis in colorectal, pancreatic, cervical and oral squamous cell cancers. Although the data are inadequate in breast and prostate cancer, increased THBS1 expression has no protective effect due to the increased VEGF level in breast cancer [39]. The increased THBS1 gene expression in all cancer cell lines and related stem cell-like cells in our study, is inconsistent with these reports.

Conclusion and suggestions

This study showed that, in comparison to the "normal" cancer cell line, cancer stem cells revealed significant changes in the expression of certain genes that play a role in breast car-

cinogenesis. These changes were particularly observed in the genes that are involved in cell-cell interactions, angiogenesis, tumor migration and proliferation and DNA damage repair. In addition to gene expressions, differences between cancer cells and cancer stem cells in relation to AKT1, CTNNB1, THBS1, TP53 and XBP1 gene expression were increased in both cancer cells and cancer stem cell-like cells. Our results show that all these genetic differences should be taken into consideration in the planning of final therapeutic goal. Furthermore, the differences of gene expressions between cancer cell lines with different phenotypic and genetic structures and stem cell-like cells obtained from these cell lines, suggest that cancer stem cells which are known to divide asymmetrically cannot be identified based on the stem cell surface markers CD44 and CD24. Therefore, we propose the use of the term "breast cancer initiating cells" instead of "breast cancer stem cells".

Authors' contributions

Drs Dogan and Ercetin, carried out cell culture studies. Drs Dogan, Ercetin and Aktas, carried out stem cell isolation. Drs Altun and Dursun carried out PCR array test. Dr Aktas performed bioinformatic analysis and heat maps. Dr Dogan wrote the draft manuscript. All authors read and approved the final manuscript.

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