Journal of Clinical and Translational Research
10.18053/Jctres/07.202105.008

ORIGINAL ARTICLE

Supplementary: Evaluation of breast cancer stem cells in human primary breast carcinoma and their role in aggressive behavior of the disease

Ninjit Dhanota[1,2], Amanjit Bal[3], Gurpreet Singh[4] Sunil K Arora[1*]

1. Department of Immunopathology, Post Graduate Institute of Medical Education and Research, Chandigarh, India.
2. Department of Radiation Oncology, Weill Cornell Medicine, New York, USA.
3. Department of Histopathology, Post Graduate Institute of Medical Education and Research, Chandigarh, India.
4. Department of General Surgery, Post Graduate Institute of Medical Education and Research, Chandigarh, India.

*Corresponding author
Sunil K Arora
Department of Immunopathology, Post Graduate Institute of Medical Education and Research, Chandigarh. 160012, India.
Tel: +91-172-2755192
Fax: +91-172-2744401
E-mail: arora.sunilkumar@pgimer.edu.in

Article information:
Received: February 05, 2021
Revised: April 07, 2021
Accepted: September 07, 2021

Appendix 1:

1. Supplementary Materials and Methods

1.1 Specimen Collection

Total mastectomy and axillary clearance (TMAC) and lumpectomy specimens containing primary breast tumor and adjacent normal tissue (taken from the farthest distant site of the primary tumor without tampering with the resection limits of tissue) were collected. Specimens collected in DMEM medium supplemented with antibiotics and Fetal Bovine Serum (FBS) were transported to the Molecular Immunology Laboratory aseptically. Samples from reduction mammoplasties and other cosmetic surgeries were collected as normal control.

For studying migration markers on BCSCs, we collected tissue specimens at different tissue distances from the tumor (T- at the primary tumor site, T.A.1- at 3mm away from the tumor, T.A.2- at 1cm away from the tumor, T.A.3- at 2cm away from the tumor, T.D.- at 4 cm away from tumor margins) from 17 TMAC cases.

For flow cytometry (FCM) experiments, fresh specimens were processed for single-cell suspensions. Immunohistochemistry (IHC) and paraffin blocks were prepared from formalin-fixed samples from the primary tumor and adjacent normal tissue.

1.2 Characterization of BCSC:

1.2.1 Mammospheres forming assay

Sorted BCSCs (Lin^- $CD44^+$ & $CD24^-$) were characterized by mammosphere forming assay. Sorted BCSCs were seeded in a density of 40,000 cells per mL in Mammocult medium supplemented with growth factors (Stemcell Technologies, Canada) in ultra-low attachment condition plates (Corning, USA). Mammosphere formation was observed at alternative days till the 7^{th} day. Primary mammospheres were dissociated with 0.25% trypsin and seeded again for secondary mammosphere formation.

1.2.2 Stemness gene expression profiling

RNA was extracted from the sorted BCSCs population. cDNA was prepared using a Thermo verso cDNA preparation kit (Thermo Fisher Scientific, USA). Expression of classical stemness genes (SOX2,

Nanog, OCT4, KlF4, and ABCG2) was determined by end-point evaluation of the transcripts by reverse transcriptase-polymerase chain reaction (RT-PCR).

1.2.3 Identification of BCSCs expressing CXCR4 expression

Single-cell suspensions obtained from tissue specimens at different tissue intervals from tumor were stained with Lin-FITC, CD44-PE and CD24-APC H7, CXCR4-APC at 37°C for 15 minutes in a water bath. Cells were acquired on a flow cytometer (FACS Aria II, BD USA), and populations were analyzed using FACS Diva software (BD Biosciences). BCSCs showing CXCR4 expression were gated and analyzed.

1.3 Identification of BCSC with Aldehyde Dehydrogenase 1A1 (ALDH1A1) expression by Immunohistochemistry (IHC) Staining

The paraffin sections from all tumors and adjacent normal tissues were stained with hematoxylin and eosin (H&E) for histopathological grading. Paraffin sections from the same tissue blocks were deparaffinized in xylene and rehydrated in ethanol for immunohistochemistry (IHC). Antigen retrieval was performed in heat-induced epitope retrieval in citrate buffer (pH 6.0) for 10 minutes. Endogenous peroxidase activity was blocked by incubation in 1% hydrogen peroxide (H_2O_2) for 10 minutes. Incubation with primary antibody (1:50) was carried out at room temperature (RT) for one hour. The secondary antibody (HRP Detector and Chromogen; Cell Marque, USA) was applied for 30 min at RT after washing with tris-buffered saline (TBS). Diaminobenzidine (DAB) solution was used for color detection, followed by counterstaining with hematoxylin. All staining runs were accompanied by appropriate control slides (normal human liver sections). We also have performed ALDH1A1 staining on non-metastatic/ metastatic lymph node sections of 12 breast carcinoma patients.

The positive to negative cellular profiles ratio was estimated as a percentage of all tumor cells in a slide. The intensity of ALDH1A1 expression was scored in tumor cells only. Stromal positivity (Leucocytes, Macrophages, Adipocytes, Mesenchymal cells present in stroma) was considered negative. Liver sections were used as a positive control for validating the ALDH1A1 staining on tumor sections. A histological score was obtained by counting the positive tumor cells with a score ranging from 0 to 4+.

In order to classify patients into ALDH1A1 (+) and ALDH1A1 (-) groups, ALDH1A1 (+) tumor sections were scored as 4+ (≥50% positive tumor cells), 3+ (≥ 10% - <50%), 2+ (≥5% - <10%), 1+ (1-5%) and 0 (Negative). For the analysis, all 1+, 2+, 3+ and 4+ were considered positive.

1.4 Custom-designed PCR Array

The expression profile of various genes for stromal factors in different sorted populations of cells from the tumor and adjacent tissue were evaluated using a custom-designed PCR array. The PCR array included 44 genes related to hypoxia, EMT, growth factors, cytokines, and stromal factors, selected based on their roles in various pathways leading to expansion/origin of cancer stem cells (Supplementary Table 1 & Table 2).

The custom PCR array was designed, and customized plates were received from Qiagen (RT^2 Custom Profile PCR array Human, Qiagen) (Supplementary Tables 1, 2 & 3). We used RT^2 SYBR Green Master Mix (Qiagen, Germany) for Real-Time PCR in a 96-well PCR plate format (48 genes, two samples per plate) using Light Cycler 480 II (Roche, Germany). Expression values of various genes were normalized against the house-keeping gene (GAPDH) of the same sample. Other controls like Positive PCR control (PPC), Genomic DNA contamination (GDC) control, and Reverse Transcriptase Control (RTC) were also used as per recommendations of the manufacturer. **1.5 Total RNA Extraction**

Total RNA was extracted from sorted cells using TRI Reagent (Sigma-Aldrich, USA). 1-Bromo-3-Chloropropane was added to the sample and vortexed for phase separation. The mixture was allowed to stand at room temperature for 15 minutes and centrifuged at 12,000xg for 15 minutes at 2-8^{0}C. The aqueous layer was carefully transferred into a new RNase-free microcentrifuge tube and processed using a standard protocol as provided in the RNeasy Mini kit. RNA from the spin column was eluted using 14µL of RNase-free water by adding directly onto the center of the membrane. The concentration and purity of RNA in the specimen were determined by reading the optical density at 260 nm and 280 nm (Nanodrop 2000).

1.6 First-strand complementary DNA synthesis

RNA was reverse transcribed to make complementary DNA (cDNA) using the RT^2 First Strand kit (Qiagen, Germany) according to the manufacturer's protocol. Each RNA sample's genomic DNA elimination mix was briefly prepared by adding RNA (25ng-5µg), buffer GE (2ul), and RNase-free water to make the volume up to 10µL. The mixture was incubated for 5 minutes at 42^{o}C and immediately placed on ice for 1 minute. Next, the reverse-transcription mix (20µL) was prepared by adding 5X buffer BC3 (8µL), Control P2 (2µL), RE3 Reverse Transcriptase mix (4µL), and RNase-free water (6µL). Ten µL of reverse transcriptase mix was added to each tube containing 10µL genomic DNA elimination mix and mixed gently by pipetting up and down. The mixture was incubated at 42^{o}C for 15 minutes and the reaction immediately terminated by incubating at 95^{o}C for 5 minutes. The volume of the mix was made to 111 µL by adding RNase-free water. The cDNA prepared was kept at -20^{o}C till use.

1.7 Conventional GAPDH PCR for checking sample quality

The RNA concentration and purity were assessed with Nanodrop 2000. Samples showing high concentrations and good quality were further processed for cDNA library construction. Before running the samples for RT-PCR, we performed conventional PCR for a housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Samples showing good expression of GAPDH were processed further for gene expression analysis.

1.8 Real-Time PCR

For a 48-well array format, a total of 1350µL of PCR mix was prepared by adding 675µL 2X RT^2 SYBR green master mix, 51µL cDNA, and 624µL RNase-free water. The reaction mixture was mixed well by gentle pipetting. 25µL/well was dispensed into 48 wells (A1-A6, B1-B6, C1-C6, D1-D6, E1-E6, F1-F6, G1-G6, and H1-H6) of customized RT^2 profiler PCR array. The plate was sealed carefully with optical adhesive film, centrifuged briefly, and placed in the real-time cycler programmed for:

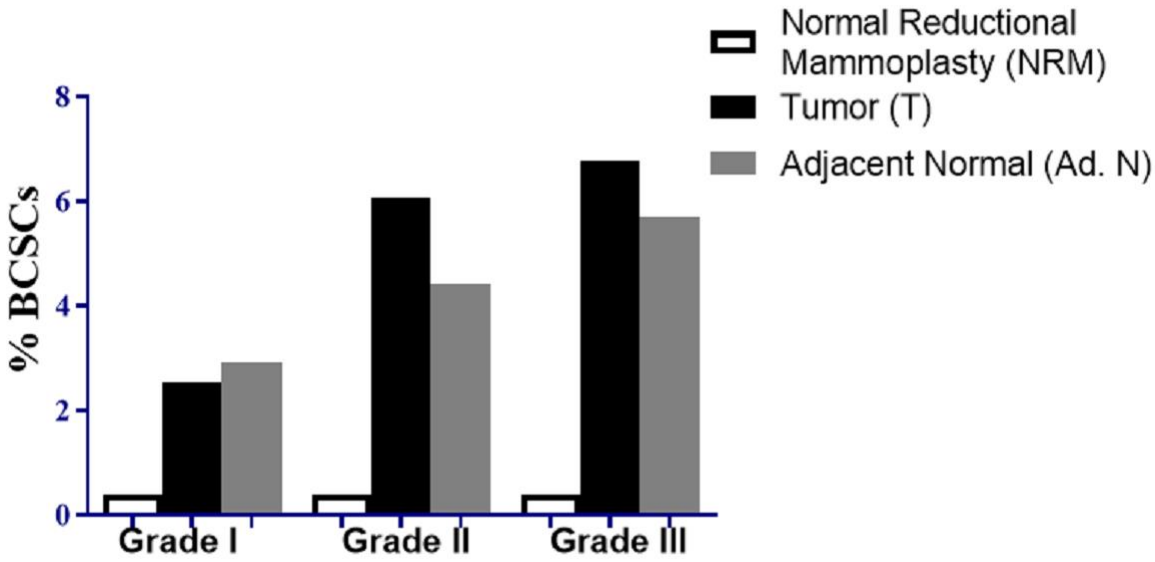
Program	Cycles	Duration	Temp.
Pre-incubation	1	10 min	95^{o}C
Denaturation	45	15 sec	95^{o}C

Amplification	45	1 min	60ºC
Melting curve analysis	1	5 sec	95ºC
Melting curve analysis	1	1 min	65ºC
Melting curve analysis	1	-	95ºC

The threshold cycle (Ct) for each well was calculated, and the data were analyzed using advanced online software RT^2 Profiler PCR array data analysis version 3.5.

Appendix 2:

2. Supplementary Figure 1



Appendix 3:

3. Supplementary Tables

Table 1: List of selected genes based on their involvement in the expansion of CSCs for custom PCR array.

S. No.	Various factors/ mechanisms involved in the expansion of CSCs	Genes
1.	Stromal factors/ growth factors/cytokines involved in CSC self-renewal, proliferation, maintenance, migration	**IL-6, IL-8, TGF-β1, VEGFA, EGF, FGF2, CXCL12, PDGFD, IGF2, BMI1, HGF, TNF-α**
2.	Genes related to Hypoxia and EMT- the mechanism involved in CSC expansion	**HIF1A, ARNT, EPAS1, TAZ, SIAH1, SNAI1, SNAI2, TWIST1, SOX9, ZEB1, CDH1, CDH2, VIM**

3.	Extracellular proteins involved in CSC expansion, self-renewal, maintenance of CSC niche	**HAS1, HAS2, TNC, SPP1, LUM, SPARC, POSTN, COL6A3, S100A4, SDC1**
4.	Signaling molecules involved in CSC expansion, self-renewal, maintenance	**SMAD2, SMAD3, SMAD4, WNT3A, WNT5A, JAG1**
5.	Chemokines/ chemokine receptors involved in CSC self-renewal, maintenance	**CXCR2, CXCR1, PPBP**

Table 2: Gene symbols and their official full names.

Gene Symbol	Official Full Name
HIF1A	Hypoxia Inducible Factor 1, Alpha Subunit
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
EPAS1	Endothelial PAS Domain Protein 1
TAZ	Tafazzin
SIAH1	Seven In Absentia Homolog 1
IL-6	Interleukin 6
IL-8	Interleukin 8
TGF-β1	Transforming Growth Factor- Beta 1
VEGFA	Vascular Endothelial Growth Factor A
EGF	Epidermal Growth Factor
FGF2	Fibroblast Growth Factor 2
CXCL12	Chemokine (C-X-C) ligand 12
PDGFD	Platelet Derived Growth Factor D
IGF2	Insulin-Like Growth Factor 2
BMI1	BMI1 Polycomb Ring Finger Oncogene
HGF	Hepatocyte growth factor
TNF-α	Tumor Necrosis Factor-Alpha
SNAI1	Snail Homolog 1
SNAI2	Snail Homolog 2
TWIST1	Twist Homolog 1
SOX9	SRY (Sex Determining Region Y)-Box 9
ZEB1	Zinc Finger E-Box Binding Homeobox 1
CDH1	Cadherin 1, Type 1, E-Cadherin
CDH2	Cadherin 2, Type 1, N-Cadherin
VIM	Vimentin
HAS1	Hyaluronan Synthase 1
HAS2	Hyaluronan Synthase 2
TNC	Tenascin C
SPP1	Secreted Phosphoprotein 1
LUM	Lumican
SPARC	Secreted Protein, Acidic, Cysteine-Rich
POSTN	Periostin
COL6A3	Collagen, Type VI, Alpha 3
S100A4	S100 Calcium Binding Protein A4
SDC1	Syndecan 1
SMAD2	SMAD Family Member 2
SMAD3	SMAD Family Member 3
SMAD4	SMAD Family Member 4
WNT3A	Wingless-Type MMTV Integration Site Family, Member 3A
WNT5A	Wingless-Type MMTV Integration Site Family, Member 5A

Gene Symbol	Official Full Name
CXCR2	Chemokine (C-X-C) Receptor 2
PPBP	Pro-Platelet Basic Protein (Chemokine (C-X-C) Ligand 7)
CXCR1	Chemokine (C-X-C) Receptor 1
JAG1	Jagged 1
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDC	Genomic DNA Control
RTC	Reverse Transcriptase Control
PPC	Positive PCR Control

Table 3: Custom PCR Array plate design and format.

HIF1A 1	ARNT 2	EPAS1 3	TAZ 4	SIAH1 5	IL-6 6
IL-8 7	TGF-β1 8	VEGFA 9	EGF 10	FGF2 11	CXCL12 12
PDGFD 13	IGF2 14	BMI1 15	HGF 16	TNF-α 17	SNAI1 18
SNAI2 19	TWIST1 20	SOX9 21	ZEB1 22	CDH1 23	CDH2 24
VIM 25	HAS1 26	HAS2 27	TNC 28	SPP1 29	LUM 30
SPARC 31	POSTN 32	COL6A3 33	S100A4 34	SDC1 35	SMAD2 36
SMAD3 37	SMAD4 38	WNT3A 39	WNT5A 40	CXCR2 41	PPBP 42
CXCR1 43	JAG1 44	GAPDH 45	GDC 46	RTC 47	PPC 48

Table 4A: Gene symbols and their up-regulated expression (fold change) in stromal cells Hi-BCSCs_SC group (Test) as compared to Lo-BCSCs_SC group (Control).

Gene Symbol	Fold Regulation	Gene Symbol	Fold Regulation

HIF1A	2.61	TWIST1	3.61
ARNT	2.55	SOX9	3.4
EPAS1	2.36	CDH1	3.35
TAZ	2.56	VIM	5.33
IL-6	9.42	HAS2	2.21
IL-8	22.1	LUM	10.84
TGF-β1	2.03	SPARC	5.71
VEGFA	9.67	POSTN	3.35
FGF2	8.55	COL6A3	2.86
CXCL12	4.29	SMAD2	2.06
PDGFD	2.48	SMAD4	5.06
HGF	3.54	PPBP	9.65
TNF-α	6.01	JAG1	3.64

Table 4B: Gene symbols and their up-regulated expression (fold change) in cancer cells Hi-BCSCs_CC group (Test) as compared to Lo-BCSCs_CC group (Control).

Gene Symbol	Fold Regulation	Gene Symbol	Fold Regulation
IL-8	27.72	TWIST1	3.54
IL-6	22.18	ZEB1	3.08
LUM	20.36	HAS2	3.06
COL6A3	11.00	SMAD4	3.00
VIM	9.16	SIAH1	2.73
POSTN	8.28	SPARC	2.64
HIF1A	5.94	BMI1	2.55
CXCL12	5.83	PDGFD	2.51
SPP1	5.20	TNF-α	2.50
HGF	4.49	CDH2	2.44
VEGFA	4.45	ARNT	2.33
FGF2	3.68	TNC	2.26
S100A4	3.65	SMAD2	2.25

Table 5: Spearman's rank correlation coefficients of differentially expressed genes (fold change) and BCSCs percentage.

	Spearman's Rank Correlation Coefficient(ρ)	p-value
HIF1α	0.279	0.124
ARNT	0.313	0.096
EPAS1	0.068	0.390
TAZ	0.014	0.477
SIAH1	0.111	0.326
LUM	0.365	0.062

COL6A3	0.279	0.124
POSTN	0.361	0.064
SPP1	0.135	0.291
HAS2	0.235	0.166
SPARC	0.239	0.163
TNC	0.292	0.112
HGF	0.207	0.395
VEGFA*	0.552	0.014
FGF2	0.335	0.08
PDGFD	0.274	0.257
CXCL12*	0.453	0.026
PPBP	0.100	0.342
IL-6*	0.509	0.026