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Original Research Article

The Integrative Analysis of Gene Expression Profile to Identify Gene Signature in Triple Negative Breast Cancer

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ABSTRACT

TNBC is defined pathologically as lacking expression of the estrogen and progesterone receptors (ER/PR) and amplification of the HER2/neu oncogenes. Due to its aggressive nature and overall poor prognosis, TNBC has gained a huge attention. Because of the absence of specific treatment guidelines for this group of patients, TNBC are managed with standard adjuvant chemotherapy. Hence, it is important to gain insight into the therapeutic targets for TNBC.

The present study used integrative analysis based approach to identify the candidate biomarkers associated with triple negative breast cancer. The microarray data of Affymetrix U133 Plus 2.0 Array and Affymetrix U133A Array platform from Gene expression Omnibus (GEO) and Array express was downloaded and analyzed in a platform/chip-specific manner using GeneSpring to identify gene/ meta-gene signatures.

The common pathways and gene ontology for the gene signatures/meta-gene signatures were predicted using PANTHER and protein-protein interactions were analyzed using STRING. The 12 genes (CHI3L1, COL9A3, EN1, IMPA2, KRT16, KRT81, LOC100129518, MMP1, MSLN, SOD2, TMEM158 and VGLL1) were up regulated, while 5 genes (CSAD, NEAT1, PIP, SCUBE2, SEMA3C) were down regulated in TNBC as compared to non-TNBC as well as reduction mammoplasty tissue. The integrative analysis done in the present study can be useful to find the gene signatures responsible for triple negative pathology of breast cancer.

Keywords: Triple negative breast cancer, Meta-analysis, Gene expression analysis, Microarray, meta-gene signature in TNBC

INTRODUCTION

Breast cancer is a heterogeneous disease, consisting of distinct molecular subtypes that have therapeutic and prognostic implications. Breast cancer can be divided into four subgroups based on estrogen receptor (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2) expressions into luminal A, luminal B, HER2 enriched and TNBC (triple negative breast cancer). TNBC, which accounts for approximately 15% of all breast cancers, is defined pathologically as lacking expression of the estrogen and progesterone receptors (ER/PR) and HER2/neu. [(1-3)] Patients of African descent more frequently develop TNBC at younger age, which offers a partial explanation for their worse clinical outcome after a breast cancer diagnosis [(2,4-9)] In India, majority TNBC patients are of early postmenopausal age (median age 52 years). Additionally, due to its unique biology, aggressive nature and overall poor prognosis, TNBC has gained a tremendous amount of attention. [(10)] Because of the

absence of specific treatment guidelines for this group of patients, TNBC are managed with standard adjuvant chemotherapy (including anthracyclines, taxanes, cyclophosphamide, and platinum salts), which, however, seems to be less effective in those cancers. [(11)] Hence, it is important to gain insight into the therapeutic targets for TNBC.

Gene expression profiling of TNBC could classify the characteristics of different subtypes as well as can verify the genes for novel therapeutic targets. [(12)] Many studies have been conducted on the TNBC gene expression profile. However, still there is a lack of adequate conclusions to understand the central mechanisms in TNBC. The integration of different datasets can help to overcome this.

The present study initially performed multiple class comparisons using the Gene expression Omnibus (GEO) and Array express data base to identify genes that were commonly deregulated in subgroups exemplifying aggressive clinical behaviour of TNBC v/s reduction mammoplasty; Non-TNBC v/s reduction mammoplasty; Adjacent normal v/s reduction mammoplasty and TNBC v/s non-TNBC. The sample of different subgroups of the sample was compared from different microarray chips using Venn diagram by means of GeneSpring to find out the meta-gene signatures. The common pathways and gene ontology for the gene signatures/meta-gene signatures were predicted using PANTHER and protein-protein interactions were analyzed using STRING.

This analysis revealed a list of 34 genes differentially expressed in adjacent normal tissues as compared to reduction mammoplasty tissues, 119 genes differentially expressed in TNBC as compared to non-TNBC subtype, 125 genes differentially expressed specifically in TNBC only, while 60 genes differentially expressed specifically in non-TNBC. Total 132 pathways altered in TNBC as compared to normal tissues and 134 altered in TNBC as compares to normal tissue. The 33 pathways showed alteration in TNBC sub types as compared to non-TNBC subtypes of breast cancer.

METHODS

Data mining:

To identify the gene expression mode of TNBC, microarray data were collected from the Array express (https://www.ebi.ac.uk/arrayexpress/) and Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/), which are freely available for users. Total 28 independent microarray data sets were analyzed among which 13 data sets were included in the present study. The selection was done based on the platform of the microarray performed. The data from two different platform were analyzed which are GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array (8 data set) and GPL96, [HG-U133A] Affymetrix Human Genome U133A Array (5 data set).

Data analysis using Gene Spring:

The raw data (.CEL) files used for the analyses were downloaded from GEO and array express and further analyzed using GeneSpring software [Agilent, California, USA]. The raw data was uploaded onto the GeneSpring and baseline transformation and normalization was done by Robust Multi-array Analysis (RMA). The sample files were classified into TNBC, Non-TNBC, adjacent normal and reduction mammoplasty. The reduction mammoplasty was taken as control group to find up/down regulated genes in various subgroups. The data from two different platforms were analyzed as separate experiment. The experimental data at gene level (arithmetic mean of all probes mapping to the same probe ID) was produced and the quality control has been carried out using Principal Component Analysis (PCA) using GeneSpring. The samples were analyzed for the fold change and Anova was performed to find significant gene entities. The gene entities with p-value <0.05 and fold change (FC) of > 2.0 were considered for further

analysis. The individual gene entity list from each technology were extracted from the GeneSpring software and exported to excel files. Similar approach was adopted to identify all the meta-gene signature and common pathways across the two platforms of HG-U133_Plus_2 and HG-U133A.

Meta-gene signature prediction using cross chip analysis:

The different subgroups of the sample were compared from both the microarray chips using Venn diagram by means of GeneSpring to find out the gene signature for various subgroups. The comparison was between up/down regulated genes in TNBC; non-TNBC and adjacent normal tissue as compared to reduction mammoplasty between HG-U133_Plus_2 and HG-U133A. Present study also compared the differential gene expression in TNBC as compared to non-TNBC.

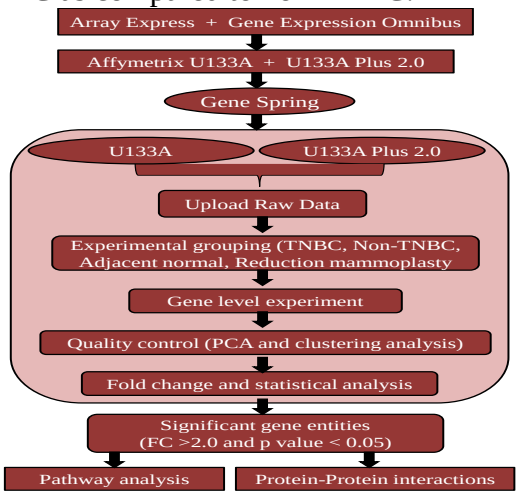

Figure 1: Analysis workflow for the integrated study

Functional Annotation:

The common pathways and gene ontology for the gene signature were predicted using PANTHER (Protein Annotation through Evolutionary Relationship) classification system (http://www.pantherdb.org/). [13] STRING (http://string-db.orgnewstring_cgi) was used to analyze the protein-protein interactions among the signature genes. [14] The list of genes was the input for PANTHER and STRING prediction. The analysis work flow of the present study is shown in **Figure 1.**

RESULTS

Data mining:

The data mining of the array express and gene expression omnibus identified total of 28 series (Affymetrix platform, Agilent platform and others) After further filtration 13 data sets from Affymetrix were included in the study. Total 1077 samples were included in the study among which, 456 were of TNBC, 540 Non-TNBC, 49 with reduction mammoplasty and 32 adjacent normal. The details of the data set included are shown in **Table 1.** Affymetrix Human Genome U133 Plus 2.0 Array (8 data set) included 98 were of TNBC, 153 Non-TNBC, 33 with reduction mammoplasty and 32 adjacent normal. The Affymetrix Human Genome U133A Array (5 data set) included 358 were of TNBC, 387 Non-TNBC and 16 with reduction mammoplasty.

Table 1: Details of the data set

GEO asession	Array Express	Population	TNBC	Non-TNBC	Adjacent Normal	Reduction mammoplasty	Reference
GPL96 [HG-U133A] Affymetrix Human Genome U133A Array							
GSE20437	E-GEOD-20437	USA	0	0	0	18	[15] Graham et al., 2010
GSE45255	NA	USA	15	114	0	0	[16] Nagalla et al., 2013
GSE32518	E-GEOD-32518	USA	16	39	0	0	[17] Gonzalez-Angulo et al., 2012
GSE31519	E-GEOD-31519	Germany	67	0	0	0	[18] Karn et al., 2014
GSE9574	NA	USA	0	0	0	15	[19] Tripathi et al., 2008
Total			98	153	32	33	
GPL570[HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array							
GSE43358	E-GEOD-43358	Belgium	17	40	0	0	[20] Fumagalli et al., 2014
GSE65216	E-GEOD-65216	France	55	98	0	11	[21] Maubant et al., 2015
GSE76275	E-GEOD-76275	USA	198	67	0	0	[22] den Hollander et al., 2016
GSE47389	E-GEOD-47389	Netherlands	47	0	0	0	[23] Zhang et al., 2013
GSE45827	E-GEOD-45827	France	41	89	0	0	[24] Gruosso et al., 2016
GSE48391	NA	Taiwan	0	64	0	0	[25] Huang et al., 2013
GSE25407	E-GEOD-25407	USA	0	0	0	5	[26] Latimer et al., 2010
GSE61304	E-GEOD-61304	Singapore	0	23	0	0	[27] Grinchuk et al., 2015
Total			358	387	0	16	

Data analysis using Gene Spring:

The data from different chips were analyzed as separate experiment in the GeneSpring. The PCA plot showed clear differentiation in gene expression pattern among different groups (TNBC, non-TNBC, adjacent normal and reduction mammoplasty). All of 1077 samples fit into the PCA as well clustering and considered for the further analysis. The PCA plot generated to analyze the behaviour and clustering of samples of different subgroup is shown in **Figure 2.** The statically significant gene entities having fold change value >2.0 and p-value <0.05 were considered for the further analysis.

Analysis of U133A Array chip showed more than 3000 genes differentially expressed in TNBC as compared to reduction mammoplasty among which 1783 were up regulated and 1834 were down regulated; more than 2500 genes differentially expressed in non-TNBC as compared to reduction mammoplasty among which 1144 were up regulated and 1632 were down regulated. The differential gene expression in adjacent normal tissue as compared to reduction mammoplasty showed total 39 gene entities among which 4 were up regulated and 35 were down regulated. The differential gene expression in TNBC as compared to non-TNBC showed total more than 200 gene entities among which 120 were up regulated and 100 were down regulated. Total 34 genes differentially expressed in adjacent normal tissue as compared to reduction mammoplasty tissue among which 4 were up regulated and 30 were down regulated (**Table 2**).

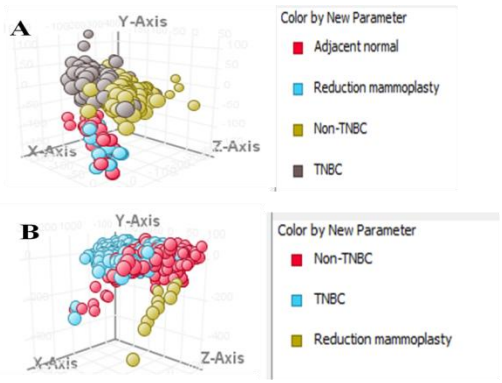

Figure 2: The PCA plot generated using GeneSpring. In figure A: PCA for U133A data set and B: PCA for U133A Plus 2.0 data set.

Table 2: Differential Gene expression in adjacent normal tissue as compared to reduction mammoplasty

Gene Symbol	Gene Title	Protein class	Pathway
Down regulated			
MAFF	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog F	Basic leucine zipper transcription factor Nucleic acid binding	
PTP4A1	protein tyrosine phosphatase type IVA, member 1		
DUSP1	dual specificity phosphatase 1		p38 MAPK pathway Oxidative stress response Gonadotropin releasing hormone receptor pathway
BTG2	BTG family, member 2		
JUN	jun proto-oncogene	Basic leucine zipper transcription factor Nucleic acid binding	Angiogenesis Apoptosis signaling pathway FAS signaling pathway TGF-beta signaling pathway T cell activation PDGF signaling pathway Oxidative stress response B cell activation Gonadotropin releasing hormone receptor pathway Huntington disease CCKR signaling map Inflammation mediated by chemokine and cytokine signaling pathway Toll receptor signaling pathway Ras pathway
ZFP36	ZFP36 ring finger protein	RNA binding protein	
IER3	immediate early response 3		CCKR signaling map
EGR1	early growth response 1	DNA binding protein Transcription cofactor Zinc finger transcription factor	Angiotensin II-stimulated signaling through G proteins and beta-arrestin Gonadotropin releasing hormone receptor pathway CCKR signaling map

Table 2 to be continued…			
IER2	immediate early response 2		
RGS2	regulator of G-protein signaling 2	G-protein modulator	Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway CCKR signaling map
ATF3	activating transcription factor 3	basic leucine zipper transcription factor	Apoptosis signaling pathway Gonadotropin releasing hormone receptor pathway
HIST2H2BE	histone cluster 2, H2be	Histone	
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	basic leucine zipper transcription factor	Gonadotropin releasing hormone receptor pathway
RGS1	regulator of G-protein signaling 1	G-protein modulator	Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway Inflammation mediated by chemokine and cytokine signaling pathway Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway
NR4A2	nuclear receptor subfamily 4, group A, member 2	C4 zinc finger nuclear receptor Receptor	
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	Oxygenase	Endothelin signaling pathway Inflammation mediated by chemokine and cytokine signaling pathway Toll receptor signaling pathway CCKR signaling map
DUSP2	dual specificity phosphatase 2		Oxidative stress response
TCN1	transcobalamin I (vitamin B12 binding protein, R binder family)		
SCGB2A1	secretoglobin, family 2A, member 1		
PTX3	pentraxin 3, long		
PROL1	proline rich, lacrimal 1		
LOC102724428	serine/threonine-protein kinase SIK1	non-receptor serine/threonine protein kinase	
SIK1	salt-inducible kinase 1	non-receptor serine/threonine protein kinase	
ADAM28	ADAM metallopeptidase domain 28	Metalloprotease	
FOS	FBJ murine osteosarcoma viral oncogene homolog	basic leucine zipper transcription factor	Angiogenesis Apoptosis signaling pathway Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade T cell activation PDGF signaling pathway B cell activation Gonadotropin releasing hormone receptor pathway Huntington disease Interleukin signaling pathway CCKR signaling map
CXCL2	chemokine (C-X-C motif) ligand 2	Chemokine	CCKR signaling map
CD69	CD69 molecule		
H3F3B	H3 histone, family 3B (H3.3B)		DNA replication
ZFAND5	zinc finger, AN1-type domain 5	Nucleic acid binding	
KLF4	Kruppel-like factor 4 (gut)	DNA binding protein Transcription cofactor Zinc finger transcription factor	CCKR signaling map
Up regulated			
PRDX2	peroxiredoxin 2	Peroxidase	
TAGLN	transgelin	Non-motor actin binding protein	
CSN1S1	casein alpha s1	Storage protein	
LTBP3	latent transforming growth factor beta binding protein 3	Annexin Calmodulin Cell adhesion molecule Extracellular matrix glycoprotein Extracellular matrix structural protein signalling molecule	

The analysis of U133 Plus 2.0 Array identified list >2500 differentially expressed genes in TNBC v/s reduction mammoplasty group among which 1898 were up regulated and 1062 were down regulated. Similarly >2500 genes observed in non-TNBC v/s reduction mammoplasty group among which 1917 were up regulated and 763 were down regulated. The differential gene expression in TNBC as compared to non-TNBC showed total more than 800 gene entities among which 333 were up regulated and 504 were down regulated.

Meta-gene signature prediction using cross chip analysis:

The meta-gene signature was identified by comparing different groups within two chips and between two chips. The meta-gene signature identifies for the following groups. **Group 1:** TNBC v/s reduction mammoplasty between two chips; **Group 2:** Non-TNBC v/s reduction mammoplasty between two chips; **Group 3:** TNBC v/s Non-TNBC between two chips and **Group 4:** differential gene expression in TNBC as compared to reduction mammoplasty v/s differential gene expression in non-TNBC as compared to reduction mammoplasty i.e. Group 1 v/s group 2.

The list of genes commonly identified between the two chips showed a total of 691 gene entities ($p<0.05$ and FC > 2.0) of which 495 were up regulated and 196 were down regulated in TNBC as compared to reduction mammoplasty (Group 1). The differential gene expression among non-TNBC as compared to reduction mammoplasty (Group 2) showed total 538 gene entities, 383 up regulated and 155 down regulated. The differential gene expression among TNBC as compared to non-TNBC (Group 3) showed total 119 gene entities, 52 up regulated and 67 down regulated.

The comparison between TNBC v/s mammoplasty and non-TNBC v/s mammoplasty (Group 4) showed total 322 common gene entities among which 240 were up regulated (in both TNBC and non-TNBC) and 82 (in both TNBC and non-TNBC) were down. Total 101 genes were found specifically up regulated and 24 were down regulated in TNBC, while 38 genes were up regulated and 22 down regulated in non-TNBC only. The Venn diagram generated to identify meta-gene signature is shown in **Figure 3.**

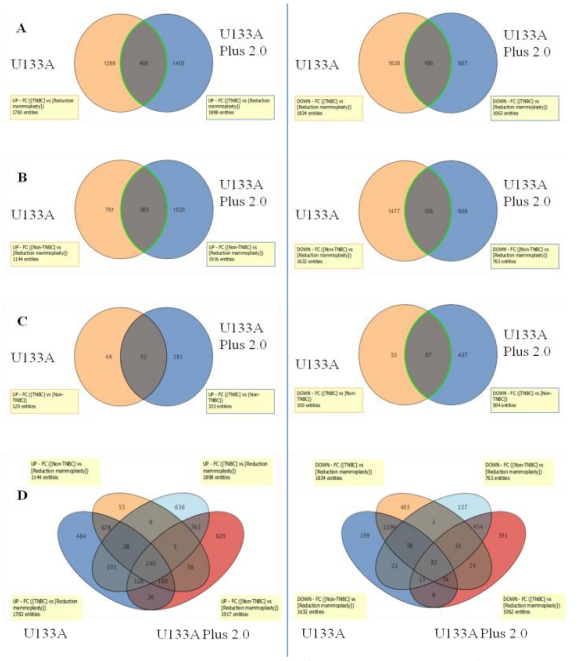

Figure 3: The cross chip analysis for the prediction of meta-gene signature in various sub-groups. In figure, A: group 1 i.e. TNBC v/s reduction mammoplasty; B: group 2 i.e. non-TNBC v/s reduction mammoplasty; C: group 3 i.e. TNBC v/s non-TNBC; D: group 4 i.e. TNBC to reduction mammoplasty v/s non-TNBC to reduction mammoplasty

Functional Annotation:

The common pathways and gene ontology for the meta-gene signature were predicted using PANTHER classification system. Total 96 pathways were up regulated and 36 pathways down regulated in TNBC as compared to reduction mammoplasty (Group 1). Most of the up regulated meta-gene signatures were predicted to be involved in integrin signalling pathway (COL9A3, FN1, COL10A1, RAP2B, ARF1, COL11A1, COL5A2, PTK2, CDC42, RAP2A, MAPK1, ITGB2, ARPC5L, ARPC5 and GRB2); CCKR signaling map (CDH1, MMP9, ELAVL1, PDK1, CREB1, CALM1, PTK2, CDC42, MCL1, MAPK1, PLAU,

GNB1 and GRB2); inflammation mediated by chemokine and cytokine signaling pathway (ALOX5AP, CXCR4, PDK1, CCL5, GNAI3, CCL18, STAT1, FPR3, IFNGR1, CDC42, CXCL10, MAPK1, PAK2, ITGB2, CCL2, ARPC5L, ARPC5, GRB2); T cell activation (HLA-DQA1, HLA-DRA, LCK, HLA-DQA2/HLA-DQA1, TRBV19, CALM1, CD74, HLA-DMA, CALM2, CDC42, CD86, LCP2, HLA-DPA1, MAPK1, PAK2, PTPRC, NCK1 and GRB2); gonadotropin releasing hormone receptor pathway (PTK2, CDC42, PITX1, MAPK1, GNAS, GNAI3, GUCY1B3, CREB1, INHBA, GNB1 and GRB2); Parkinson disease (CSNK2A1, HCK, MAPK1, YWHAZ, LCK, MCM5, HSPA9, SFN and SEPT2); angiogenesis (HN1, PTK2, BIRC5, MAPK1, PAK2, STAT1, NCK1 and GRB2); apoptosis signaling pathway (BCL2A1, M6PR, MCL1, CFLAR, MAPK1, MCM5, LTB, GZMB and CREB1); Huntington disease (CYC1, GAPDH, ARF1, CDC42, ACTR2, TRAC, ARPC5L, ARPC5 and ARF4) and heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway (CALM2, GNAS, GNAI3, PRKAR1A, CREB1, SSR1, GNB1 and CALM1). The down regulated meta-gene signatures were involved in angiogenesis (F3, PDGFRA, TCF7L2, FGF1, PKD2, PRKD1, SFRP1, DLK1, PDGFD and FGFR1); gonadotropin releasing hormone receptor pathway (NR3C1, IGF1R, IRS2, TGFBR3, IRS1, PTGER3, AR and IGF1) etc.

Table 3: Altered pathways in TNBC subtype as compared to non-TNBC

Pathway	Genes involved
Up regulated	
Alzheimer disease-presenilin pathway	CDH3,MMP1,MMP7
Angiogenesis	SFRP1,CRYAB
Arginine biosynthesis	ASS1
CCKR signaling map	ODC1,MMP7
Cadherin signaling pathway	EGFR,CDH3,
EGF receptor signaling pathway	EGFR
GABA-B receptor II signaling	SLC6A14
Gonadotropin releasing hormone receptor pathway	EGFR
Integrin signaling pathway	COL9A3
Muscarinic acetylcholine receptor 2 and 4 signaling pathway	SLC6A14
Ornithine degradation	ODC1
PDGF signaling pathway	ELF5
Plasminogen activating cascade	MMP1
Serine glycine biosynthesis	PHGDH
VEGF signaling pathway	CRYAB
Wnt signaling pathway	SFRP1,MMP7,CDH3,EN1
P53 pathway	SERPINB5
Down regulated	
Adrenaline and noradrenaline biosynthesis	NAT1
Alzheimer disease-presenilin pathway	BRBB4,
Aminobutyrate degradation	ABAT
B cell activation	VAV3
Bupropion degradation	CYP2B6
CCKR signaling map	TFF1
Cadherin signaling pathway	CELSR1,BRBB4,ERBB2
EGF receptor signaling pathway	BRBB4,ERBB2,AREG
Gamma-aminobutyric acid synthesis	CSAD,ABAT
Intigrin signaling pathway	COL4A5
Oxidative stress response	DUSP4
PDGF signaling pathway	VAV3,SPDEF
Pyrimidine metabolism	ABAT
Synaptic vesicle trafficking	SYT1
T cell activation	VAV3
Wnt signaling pathway	CELSR1

Eighty eight pathways showed upregulated and 46 pathways down regulated in non-TNBC as compared to reduction mammoplasty (Group 2). Total 17 pathways showed upregulated and 16 pathways down regulated in TNBC sub types as compared

to non-TNBC subtypes of breast cancer (Group 3). The up regulated meta-gene signatures involved in Wnt signaling pathway (SFRP1, MMP7, CDH3 and EN1); Alzheimer disease-presenilin pathway (CDH3, MMP1 and MMP7); Angiogenesis (SFRP1 and CRYAB); CCKR signaling map (ODC1 and MMP7); Cadherin signaling pathway (EGFR and CDH3) etc. The down regulated meta-gene signatures involved in Cadherin signaling pathway (CELSR1, BRBB4 and ERBB2); EGF receptor signaling pathway (BRBB4, ERBB2 and AREG); Gamma-aminobutyric acid synthesis (CSAD and ABAT); PDGF signaling pathway (VAV3 and SPDEF) etc. (**Table 3**).

Table 4: Altered pathways specifically in TNBC subtype of breast cancer

Pathway	**Genes**
Up regulated	
Angiogenesis	MAPK1,NCK1
TGF-beta signaling pathway	MAPK1
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	MAPK1
De novo purine biosynthesis	AK2
p53 pathway	SUMO3,PDK1
Heme biosynthesis	EPRS
PI3 kinase pathway	PDK1
p53 pathway feedback loops 2	CCNA2,PDK1
Salvage pyrimidine ribonucleotides	UCK2
PDGF signaling pathway	MAPK1,PDK1,NCK1
Angiotensin II-stimulated signaling through G proteins and beta-arrestin	MAPK1
PLP biosynthesis	PSAT1
Plasminogen activating cascase	MMP1
Interleukin signaling pathway	MAPK1,PDK1
Integrin signaling pathway	COL9A3,RAP2B,RAP2A,MAPK1,ARPC5L
CCKR signaling map	MAPK1,PDK1
Endothelin signaling pathway	MAPK1
Mannose metabolism	GMDS
Inflammation mediated by chemokine and cytokine signaling pathway	MAPK1,PDK1,CCL18,ARPC5L
Alzheimer disease-presenilin pathway	MMP1,MMP12
FGF signaling pathway	MAPK1
Ras pathway	MAPK1,PDK1
Serine glycine biosynthesis	PSAT1
EGF receptor signaling pathway	MAPK1
Vitamin B6 metabolism	PSAT1
Interferon-gamma signaling pathway	MAPK1
Alzheimer disease-amyloid secretase pathway	MAPK1
DNA replication	RFC3,RNASEH2A
B cell activation	MAPK1
Parkinson disease	MAPK1,MCM5
Huntington disease	ARPC5L
VEGF signaling pathway	MAPK1
Gonadotropin releasing hormone receptor pathway	MAPK1
Apoptosis signaling pathway	CFLAR,MAPK1,MCM5
FAS signaling pathway	LMNB2,CFLAR
Wnt signaling pathway	EN1
T cell activation	MAPK1,NCK1
Insulin/IGF pathway-protein kinase B signaling cascade	PDK1
Down regulated	
Nicotine pharmacodynamics pathway	EPB41L1
Alzheimer disease-presenilin pathway	TRPC1,APBB2
TGF-beta signaling pathway	CITED1
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	IGF1R
Gonadotropin releasing hormone receptor pathway	IGF1R,AR
Insulin/IGF pathway-protein kinase B signaling cascade	IGF1R
Interleukin signaling pathway	IL6ST
Dopamine receptor mediated signaling pathway	EPB41L1
Gamma-aminobutyric acid synthesis	CSAD

The comparison between TNBC v/s mammoplasty and non-TNBC v/s mammoplasty (Group 4) showed 38 pathways specifically upregulated and 9 pathways were down regulated in TNBC only. The up regulated gene signatures in TNBC type involved in Integrin signalling

pathway (COL9A3, RAP2B, RAP2A, MAPK1 and ARPC5L); Inflammation mediated by chemokine and cytokine signaling pathway (MAPK1, PDK1, CCL18 and ARPC5L); PDGF signaling pathway (MAPK1, PDK1 and NCK1); Apoptosis signaling pathway (CFLAR, MAPK1 and MCM5) etc. The down regulated gene signatures involved in Alzheimer disease-presenilin pathway (TRPC1 and APBB2); Gonadotropin releasing hormone receptor pathway (IGF1R and AR) etc. (**Table 4**). Total 10 pathways were predicted to be up regulated and 9 down regulated in non-TNBC only (**Table 5**). Total 76 pathways were up regulated and 20 were down regulated commonly in both TNBC and non-TNBC. Total 22 pathways showed down regulation in adjacent normal tissue as compared to reduction mammoplasty (**Table 2**).

Table 5: Altered pathways specifically in non-TNBC subtypes

Pathway	**Genes**
Up regulated	
Angiogenesis	GRB7
CCKR signaling map	TFF1
Ubiquitin proteasome pathway	WWP1
PDGF signaling pathway	SPDEF
TGF-beta signaling pathway	BMPR1B
Gonadotropin releasing hormone receptor pathway	AR
Wnt signaling pathway	BMPR1B
Adrenaline and noradrenaline biosynthesis	NAT1
EGF receptor signaling pathway	ERBB2
Cadherin signaling pathway	ERBB2
Down Regulated	
Integrin signalling pathway	RND3
Inflammation mediated by chemokine and cytokine signaling pathway	CX3CL1
Alzheimer disease-presenilin pathway	TCF7L1
Axon guidance mediated by semaphorins	DPYSL2
Gonadotropin releasing hormone receptor pathway	EGFR, TCF7L1
Wnt signaling pathway	TCF7L1
Cadherin signaling pathway	EGFR, TCF7L1
EGF receptor signaling pathway	EGFR
Pyrimidine Metabolism	DPYSL2

The protein-protein interactions among the signature genes/ meta-gene signatures were analyzed using STRING. The results are shown in **Figure 4-7.** The down regulated gene signature of adjacent normal tissue as compared to reduction mammoplasty showed the predicted reactome pathways of estrogen-dependent gene expression (4 of 118 gene set count), activation of the AP-1 family of transcription factors (2 of 10 gene set count), senescence-associated secretory phenotype (3 of 78 gene set count), oxidative stress induced senescence (3 of 92 gene set count) and RAF-independent MAPK1/3 activation (2 of 22 gene set count). The up regulated gene signatures did not predicted any reactome pathway involved.

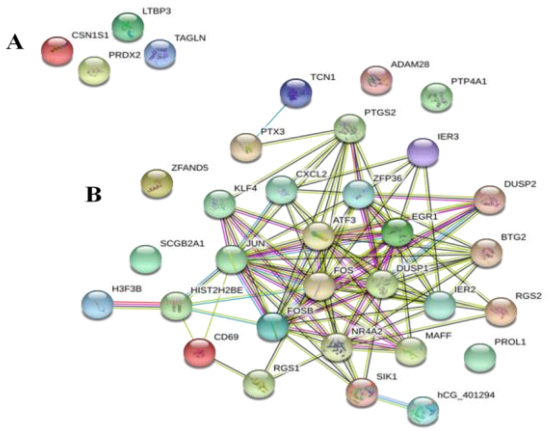

Figure 4: Protein-protein interactions of differentially expressed gene signature of adjacent normal tissue as compared to reduction mammoplasty. **A:** up regulated; **B:** down regulated

The reactome pathways predicted for TNBC v/s non-TNBC up regulated meta-gene signatures showed formation of the cornified envelope (8 of 128 gene set count), developmental biology (10 of 1023 gene set count), type I hemidesmosome assembly (2 of 11 gene set count) and

collagen degradation (3 of 64 gene set count). However, down regulated meta-gene signature did not predict any reactome pathway involved.

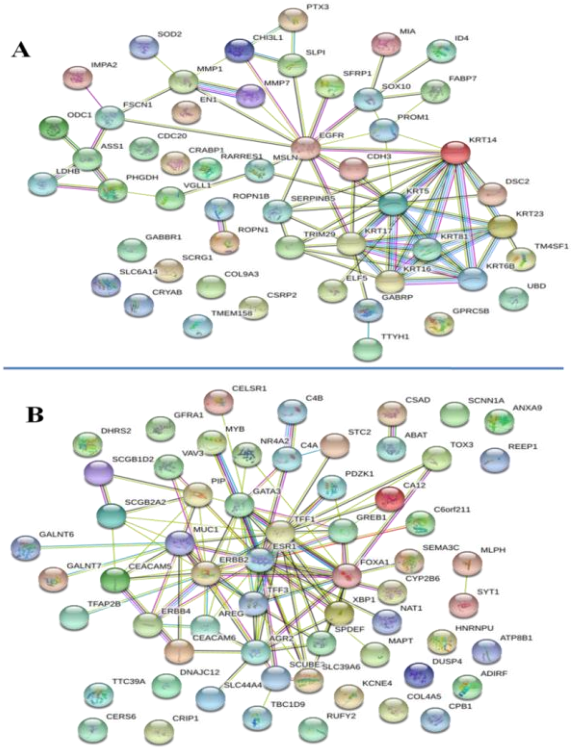

Figure 5: Protein-protein interactions of meta-gene signature of TNBC as compared to non-TNBC. **A:** up regulated; **B:** down regulated

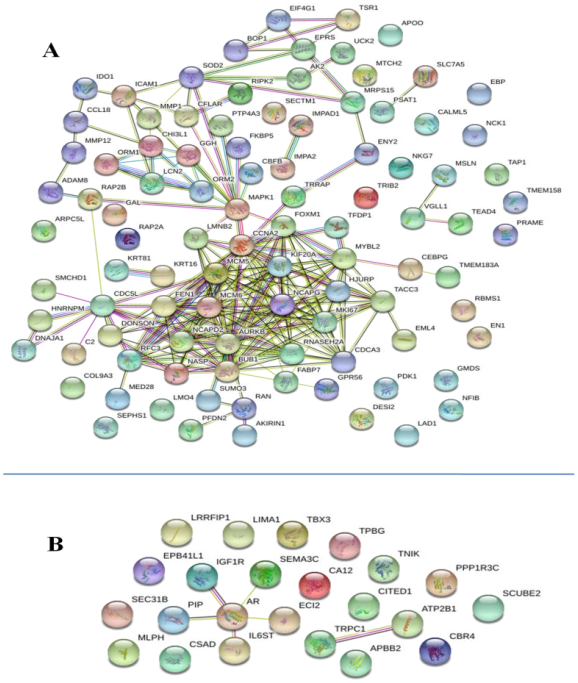

Figure 6: Protein-protein interactions of the gene signatures differentially expressed specifically in TNBC. **A:** up regulated; **B:** down regulated

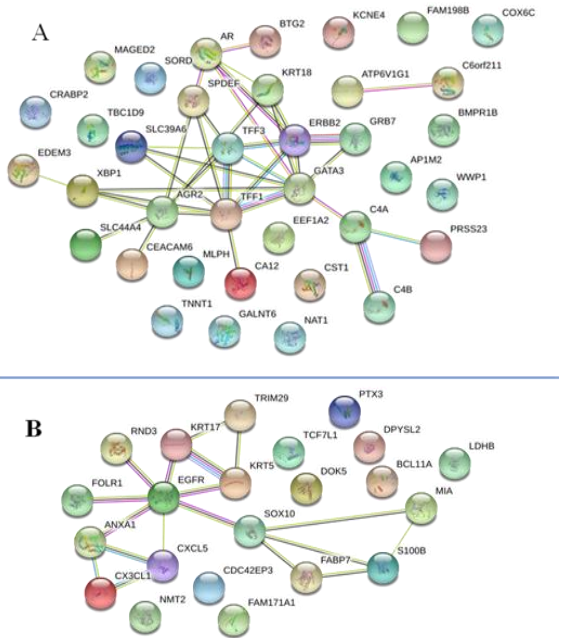

Figure 7: Protein-protein interactions of the gene signatures specifically differentially expressed in non-TNBC. **A:** up regulated meta-gene signature; **B:** down regulated meta-gene signatures

In case of up regulated meta-gene signature specifically in TNBC subtype, the reactome pathways predicted were mitotic cell cycle (14 of 483 gene set count), cell cycle (15 of 856 gene set count), DNA strand elongation (4 of 31 gene set count), transcription of E2F targets under negative control by p107 (RBL1) and p130 (RBL2) in complex with HDAC1 (3 of 16 gene set count) and S phase (6 of 156 gene set count). The down regulated meta-gene signature did not predicted any reactome pathway involved. The up regulated meta-gene signature specifically in non-TNBC subtype, the predicted reactome pathways shows GRB7 events in ERBB2 signaling (2 of 4 gene set count), activation of C3 and C5 (2 of 7 gene set count), signalling by nuclear receptors (4 of 167 gene set count), initial triggering of complement (2 of 21 gene set count) and estrogen-dependent gene expression (3 of 118 gene set count). The down regulated meta-gene signature did not predicted any reactome pathway involved.

DISCUSSION

The use of molecular approach in the clinical practice is rising now-a-days. The

international guidelines for the breast cancer endorse the implementation of gene signatures derived from microarray supporting the clinicians in the treatment decision making process. [28] The present study determined alteration in gene expression among adjacent normal tissue v/s reduction mammoplasty tissue, TNBC/ non-TNBC tumor tissue v/s reduction mammoplasty tissue, as well as TNBC v/s non-TNBC tumor type of breast cancer.

The analysis of differential gene expression in adjacent normal tissue in comparison to reduction mammoplasty showed up regulation of PRDX2, TAGLN, CSN1S1 and LTBP3 genes and down regulation of MAFF, PTP4A1, DUSP1, BTG2, JUN, ZFP36, IER3, EGR1, IER2, RGS2, ATF3, HIST2H2BE, FOSB, RGS1, NR4A2, PTGS2, DUSP2, TCN1, SCGB2A1, PTX3, PROL1, LOC102724428, SIK1, ADAM28, FOS, CXCL2, CD69, H3F3B, ZFAND5 and KLF4 genes. The up regulated genes are involved in CCKR signaling map, gonadotropin releasing hormone receptor pathway, inflammation mediated by chemokine and cytokine signaling pathway, oxidative stress response, apoptosis signaling pathway etc. **(Table 2).**

The comparison of TNBC with non-TNBC subtype and/or reduction mammoplasty tissue showed up regulation of 12 genes (CHI3L1, COL9A3, EN1, IMPA2, KRT16, KRT81, LOC100129518, MMP1, MSLN, SOD2, TMEM158 and VGLL1) and down regulation of 5 genes (CSAD, NEAT1, PIP, SCUBE2 and SEMA3C). These suggesting that these gene signatures may be responsible to triple negative pathology of breast cancer. The up regulated genes were predicted to be involved in integrin signalling pathway, Wnt signalling pathway, plasminogen activating cascade and Alzheimer disease-presenilin pathway. The CSAD gene was predicted to be involved in Gamma-aminobutyric acid synthesis, while rests of the down regulated genes are not involved in any pathways. Previous studies showed association of CHI3L1 suggesting that elevated level of serum glycoprotein CHI3L1 (chitinase-3 like-protein-1) is associated with shorter recurrence free intervals, poor prognosis and reduced survival in patients with metastatic breast cancer. [29-31] Bender and Mac Gabhann analyzed the expression VEGF and semaphorin genes in triple negative breast cancer. The authors suggested that TNBC is highly associated with dysregulation of VEGF and semaphorin genes. Higher expression of VEGF and low expression of secreted semaphorins (SEMA3B, SEMA3C, SEMA3E, SEMA3F, and SEMA3G) found to be associated with 60% of the TNBCs. [32] The present study also observed the down regulation of secreted semaphorins SEMA3C in TNBC as compared to non-TNBC as well as reduction mammoplasty tissue. den Hollander et al. observed the over expression of IMPA2 gene in TNBC in comparison to ER positive breast tumours. Present study also observed the up regulation of IMPA2 in TNBC subtype. [22]

EGFR, KRT17, KRT5, LDHB, MIA, PTX3, SOX10 and TRIM29 showed up regulation in TNBC in comparison to non-TNBC but not in comparison to normal breast tissue as they observed to be down regulated specifically in non-TNBC subtype. These genes were predicted to be involved in EGF receptor signalling pathway, cadherin signalling pathway and gonadotropin releasing hormone receptor pathway. Dutta and co-workers observed EGFR as central hub gene in triple negative breast cancer. [33] KRT17 was also previously reported to be associated with TNBC. [34] The current study also observed the similar expression. The results of McCleland and co-workers suggested LDHB as an important gene for TNBC. [35] Moreover, Dennison et al., observed that LDHB can predict the prognosis of basal-like subtype among the HER2 negative and TNBC groups with high degree of power. The authors suggested that breast cancer having higher LDHB showed most response to neoadjuvant chemotherapy as an

independent of established prognostic factor and molecular markers. [(36)]

AGR2, ARMT1, C4A, C4B, C4B_2, CEACAM6, ERBB2, GALNT6, GATA3, KCNE4, NAT1, SLC39A6, SLC44A4, SPDEF, TBC1D9, TFF1, TFF3 and XBP1 down regulated in TNBC in comparison to non-TNBC but not in comparison to normal breast tissue suggesting gene signatures be up regulated in specifically non-TNBC subtype. These genes were predicted to be involved in CCKR signalling map, Adrenaline and noradrenalin biosynthesis, PDGF signalling pathway, EGF receptor signalling pathway and cadherin signalling pathway. SOX10, a transcriptional factor, supports stem-like properties in normal and cancer cells. In normal cells SOX10 keep stem cells in their undifferentiated forms by controlling differentiation. [(37-39)] SOX10 is reported as a marker for basal like breast cancer TNBC. [(40-41)] Two of the meta-gene signatures of the present study that are COL9A3 and MIA have been reported to co-function with SOX10 gene and negative co-relation of SOX10 with GATA3, XBP, MLPH and AGR2 in basal like breast cancer. [(41)] Present study also observed similar expression as COL9A3, MIA and SOX10 genes found up regulated and GATA3, XBP, MLPH and AGR2 found under expressed in TNBC subtype. COL9A3 has been previously reported to bind EGFR (epidermal growth factor receptor), which is commonly recognized marker for basal like breast cancer and regulator. [(42-43)] Dutta et al. observed that ESR1 gene plays central role in ER positive breast cancer and under expressed in TNBC as compared to non-TNBC. [(33)] ESR1 gene encodes for the ERα which transcriptionally target TFF1 gene. Moreover, they also observed over expression of XBP1 gene in ER positive breast cancer and ERBB2 and GRB7 over expression in Her2 positive breast cancer. Present study also observed up regulation of TFF1, XBP1 and GRB7 in non-TNBC subtype as well as down regulation of ESR1 gene in TNBC as compared to non-TNBC subtype of breast cancer.

The present study observed the up regulation of FABP7 in TNBC in comparison to non-TNBC and normal breast tissue as well as observed to be down regulated in non-TNBC. FABP7 was previously reported as marker of poor prognosis in basal like breast cancer. [(41)] In present study also FABP7 was observed up regulated in TNBC and CA12 and MLPH down regulated in TNBC in comparison to non-TNBC and normal breast tissue as well as observed to be up regulated in non-TNBC. Thakkar et al. also observed that ERα (+) breast tumors significantly over express MLPH in comparison to ERα (-) breast tumors. [(44)]

The results of the integrative analysis of gene expression pattern of TNBC and non-TNBC subtypes of breast cancer to identify gene signature in triple negative breast cancer suggested that CHI3L1, COL9A3, EN1, IMPA2, KRT16, KRT81, LOC100129518, MMP1, MSLN, SOD2, TMEM158, VGLL1, CSAD, NEAT1, PIP, SCUBE2 and SEMA3C can be most significant gene signatures for TNBC subtype of breast cancer.

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Conflict of interest:

Authors declares that they have no conflict of interest

Abbreviations:

TNBC: triple negative breast cancer; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor

2; GEO: gene expression omnibus; PCA: principal component analysis; FC: fold change; PANTHER: protein annotation through evolutionary relationship

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