Life Science Informatics Publications

Research Journal of Life Sciences, Bioinformatics, Pharmaceutical and Chemical Sciences

Journal Home page http://www.rjlbpcs.com/

RJLBPCS

ISSN 2454-6348

Original Research Article **DOI: 10.26479/2018.0406.12**

NETWORK-BASED META-ANALYSIS TO IDENTIFY THE KEY REGULATORS IN PROGRESSION OF BONE METASTATIC BREAST CANCERS

S Gudipati*, A Mankad, H A Pandya, Y T Jasrai

Department of Botany, Bioinformatics and Climate Change Impacts Management, School of Sciences, Gujarat University, Ahmedabad, India.

ABSTRACT: One of the most common types of cancers in the women is breast cancer. In spite of many modern therapeutic options the mortality associated with breast cancer is predominantly because of the metastasis and bone is the common metastatic site. Understanding the molecular mechanisms that aid cancer cells to survive at the metastatic site helps in developing biomarkers that would help in predicting and also developing novel strategies to prevent this organspecific tropism. In order to understand how breast cancer cells home and colonize in the bone metastatic niche after dissemination from the primary site, we performed meta-analysis of the gene expression profiles derived from the microarray data and tried to identify the hub genes that are involved in this process through network analysis. We propose FN1, NEDD4 and HDAC1 are hub genes that probably aid in promotion of breast cancer at metastatic site.

KEYWORDS: Breast cancer, Bone metastasis, Network Analysis, meta-analysis.

Corresponding Author: S Gudipati*

Department of Botany, Bioinformatics and Climate Change Impacts Management, School of Sciences, Gujarat University, Ahmedabad, India.

Email Address: sindhurag@yahoo.com

1. INTRODUCTION

One of the most common types of cancers in the women is breast cancer [1]. Morbidity and mortality associated with breast cancer are increasing and is attributed primarily to metastasis [2]. Since bone is frequent site of metastasis in breast cancer, associated pathological conditions include hypercalcemia, bone fractures, spinal cord compression, severe pain, coma and death. A deep understanding of the molecular mechanism underlying bone metastasis helps in identifying key

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components that drive cancer cells towards bone. Genetic transformations in the cancer cells aid these cells in their metastatic journey that begins by evading primary site followed by entering into circulation and finally reaching their destination that is bone. Cancer cells express certain genes and whose normal expression is otherwise restricted to bone cells, is referred to as osteomimicry, this attracts metastatic cancer cells towards bone. Some of the well characterized osteomimetic factors are CXCL12, Cadherin 11, ENPP1, BSP and osteoactivin [3]. Once inside the bone, breast cancer cells are known to interact with osteoblasts that in turn up-regulates RANKL which is known to induce the activation of osteoclasts. RANKL binds to RANK and activates various transcription factors like nuclear factor κB (NFκB), activator protein-1 (AP-1) and nuclear factor of activated T-cells cytoplasmic 1(NFATc1) these transcriptional factors upregulate molecules like TRAP, Cathespin K increasing the osteoclasts activity leading to osteolytic leisions [4]. Further extracellular matrix associated factors like MMP2 and MMP9 also cause degradation of bone resulting in the release of immobilized growth factors that aid in homing and growth of the tumor cells in bone [5]. Blood flow and adhesion molecules on the tumor cells also contribute to the survival of the breast cancer cells in the bone. Subtype of breast cancer and estrogen receptor status also show association with bone metastasis. Estrogen receptor positive breast cancer and especially luminal sub-type are prone to cause higher incidence of bone metastasis [6]. In essence changes in expression patterns of multiple genes, help in progression of breast cancer cells through metastasis and that their functional characterization simultaneously helps in, in-depth understanding of bone metastasis [7]. In this regard high throughput techniques help in identification of global expression patterns of genes, proteins and mRNA in various disease conditions. Many gene expression profiling studies were reported to identify genes that are associated with bone metastasis in breast cancer [8]. Various groups identified multiple gene signatures associated with cell cycle, DNA replication, proliferation, survival, angiogenesis, migration, osteoclastogenesis, extracellular matrix alterations and invasion which aid in progression of the metastatic cells towards bone [9] [10]. But the molecular mechanisms underlying the cancer cells that survive in the bone are not very clear. Therefore we ventured to understand such molecular mechanisms initially using microarray data and meta-analyzing it and subsequently constructing protein networks. Although microarray data helps in global gene expression profiles in various disease conditions, interactions among these genes cannot be interpreted. Systems level understanding of the diseases from the high throughput data can be obtained by constructing networks which consist of a set of genes or proteins called nodes and functional relationships among nodes is represented by the edges. Interactions among these genes or proteins result in particular biological processes [11]. Related pathways involved in a particular biological process can also be identified and further these can be visualized easily. Results are interpreted based on the topological features of the network. Our focus in this manuscript is based on the microarray data meta-analysis and resulting differentially expressed genes that were

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used as input to build protein interaction network. This exercise was carried to identify hub genes that are involved in the progression of bone metastasis.

2. MATERIALS AND METHODS

Data Sets

Publicly available microarray datasets were searched in NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/). Following key words and their combinations were used: "Breast cancer, bone metastasis, microarray and gene expression dataset". Following information was extracted from each identified data set, GEO accession number, sample type, platform, number of bone metastatic and other metastatic samples and references (**Table 1**). Human case studies, comparable conditions, untreated samples and availability of raw and processed data were set as criteria for inclusion and were strictly followed. Studies in other animals and integrated analysis of expression profiles were excluded.

Differential Expression of Genes

Differentially expressed (DE) genes can be identified based on meta-analysis of microarray datasets. We used Network Analyst program (http://www.networkanalyst.ca/faces/home.xhtml) a web interface for integrative microarray meta-analysis [12]. For the removal of the batch effect ComBat procedures utilizing empirical Bayes methods were employed [13]. Annotation of the datasets was performed after uploading the datasets into program by converting the gene symbols into respective Entrez IDs. Quantile normalization of the data was employed and the datasets were checked for data integrity before proceeding further [14]. For the statistical analysis we employed combined effect size and cochrans Q test was used and Random effects model (REM) was selected for the meta-analysis [15]. Using these DE genes as input, binary interactions were obtained based on curated protein-protein interaction databases downloaded from InnateDB [16]. To identify hub genes, topology analysis which considers entire network or module analysis which breaks entire network into highly connected sub-networks called modules can be used. Here we employed topology analysis to identify hub genes. Topological measures that are utilized for selection of hub genes are degree and betweenness. The hub genes with greater degree and betweenness are very important in signaling pathways and can either be utilized as biomarkers or therapeutic targets.

Enrichment Bioinformatics Analysis

The official genes symbols identified were uploaded to the Database for Annotation, Visualization and Integrated Discovery (DAVID: http://david.abcc.ncifcrf.gov) and the enrichment analyses of the GO terms for the biological process, molecular function and pathways involved was performed using the functional clustering annotation tools. The default options with high classification stringency were used. Finally, the cluster names were extracted from most biologically relevant GO term that was assigned to the cluster [17].

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Transcriptional Factor Analysis of Regulatory Networks

In order to understand upstream factors regulating DE genes we employed eXpression2Kinases (X2K) a web based tool. Significant transcriptional factors were extracted based on their *P* value. X2K web is freely available [18].

3. RESULTS AND DISCUSSION

Identifying Microarray Data Sets For Meta-Analysis

After applying inclusion criteria the data set GSE 14020 [19] was selected. This super-series consists of sub-series GSE14018 and GSE14017 and were considered for meta-analysis in Network Analyst, a network-based analytical tool for gene expression profiling, meta-analysis and interpretation. In GSE14017 metastatic breast cancer samples were analyzed using affymetrix (U133plus2) and in GSE14018 samples profiled on U133A platform were analyzed on affymetrix. The study included 58 breast cancer metastases from different organs like bone, brain, lung and liver, 29 samples were included in GSE14017 series in which seven are bone metastatic samples and in GSE14018 of the 35 samples 8 samples are bone metastatic,. Detailed dataset information is shown in **Table 1.**

Table 1: The table shows the data sets that are considered for the meta-analysis

	Data Set	Platform	No. of Samples
Data1	GSE14017	Affymetrix Human Genome U133 Plus 2.0 Array	29
Data2	GSE14018	Affymetrix Human Genome U133A Array	35

Batch Effect Adjustment

The primary goal of the study was to identify the differentially expressed genes (DEGs) in bone metastasis of breast cancer using selected datasets for meta-analysis. For effective integration of the data, batch effects must be removed prior to meta-analysis. There are many approaches that are useful in batch adjustment for the gene expression data. Some of them are Distance-weighted Discrimination (DWD), Surrogate Variable Analysis (SVA), Geometric ratio-based method (Ratio_G) and ComBat [20]. Here, data was subjected to the well-established ComBat procedures to remove the batch effects [21]. Intermixing of the samples from all the data sets after batch adjustment procedure was confirmed by visual inspection of using principal component analysis (PCA) as shown in the Figure 1.

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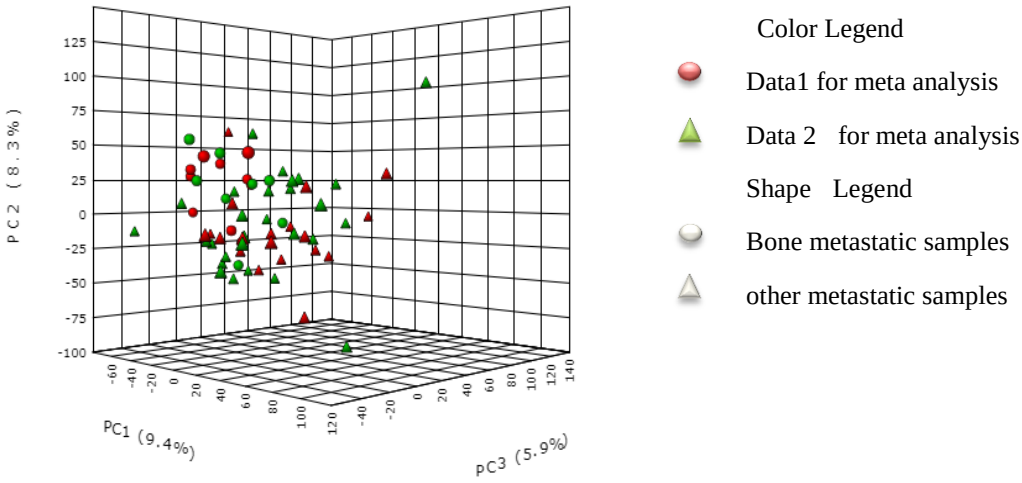

Figure 1: Principal component analysis (PCA) of the microarray dataset. Red indicates data1 and green indicates data2. Please refer table 2 for data1 and data2.

Identification of Differentially Expressed Genes Signatures by Meta-Analysis

To identify differentially expressed gene signatures in bone metastasis microarray data sets (Table 1) were analyzed using Network Analyst. Bone metastatic samples are compared with other distant metastatic samples corresponding to lung, liver and brain. The processed data are subjected to differential analysis. Combined effect size was employed to find the differentially expressed genes. There are two methods with which effect size can be calculated, FEM (Fixed effect model) and REM (Random Effect Model). Based on the values obtained from Cochrans Q test, statistical heterogeneities among the samples are estimated and method to calculate effect size was decided [22]. When the estimated Q values have approximate chi- squared distribution then FEM is selected and incase deviation from the chi- squared distribution exists then REM is selected. Based on our cochrans Q test analysis we selected REM which considers random effects due to non-biological heterogeneities like different microarray platforms to calculate the effect size [23]. From microarray meta-analysis we have identified a total of 837 DEGs across the datasets with a *P* value <0.05 of which 242 genes were underexpressed and 596 genes were overexpressed. The top up-regulated and down regulated differentially expressed genes are shown along with their combined size effect and *P* value in the **Table 2**. 455 genes were gained and 256 were lost and 126 DE genes were expressed in meta-data and data from our meta-analysis which is represented as Venn diagram in Figure 2(a). The heat map of the differentially expressed genes is shown in the Figure 2(b). Top few differentially expressed genes that are down-regulated are LAMC3, ITGB8, PQBP1, TMEM177, EXOSC4, GPR37, ZNF165, RIPK4, RALY and top up-regulated genes include LRRC15, MMP13, CTSK, COL8A2, FBN, OLFML2B and PCOLCE.

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Table 2: The table shows top 20 genes that are differentially expressed in the bone metastasis. Entrez Id's collected from NCBI; name indicates gene symbol and combined ES is effect size.

Up-regulated differentially expressed genes				
EntrezID	Gene Symbol	Name	CombinedES	*P* val
131578	LRRC15	Leucine Rich Repeat Containing 15	-3.7818	0.000885
4322	MMP13	Matrix Metallopeptidase 13	-3.1727	1.01E-07
1513	CTSK	Cathepsin K	-3.1011	4.95E-08
1296	COL8A2	Collagen Type VIII Alpha 2 Chain	-3.0397	1.96E-06
2200	FBN1	Fibrillin 1	-2.9091	0.000116
25903	OLFML2B	Olfactomedin Like 2B	-2.8695	4.15E-10
5118	PCOLCE	Procollagen C-Endopeptidase Enhancer	-2.8317	0.030017
8515	ITGA10	Integrin Subunit Alpha 10	-2.8186	4.15E-10
4958	OMD	Osteomodulin	-2.8049	4.15E-10
4286	MITF	Melanogenesis Associated Transcription Factor	-2.803	4.15E-10
11096	ADAMTS5	ADAM Metallopeptidase With Thrombospondin Type 1 Motif 5	-2.7649	0.000107
Down-regulated Differentially expressed genes				
EntrezID	Gene Symbol	Name	CombinedES	*P* val
10319	LAMC3	Laminin Subunit Gamma 3	1.715	0.001724
3696	ITGB8	Integrin Subunit Beta 8	1.4381	0.006725
10084	PQBP1	Polyglutamine Binding Protein 1	1.403	0.000519
80775	TMEM177	Transmembrane Protein 177	1.3618	0.00082
54512	EXOSC4	Exosome Component 4	1.334	0.002075
2861	GPR37	G Protein-Coupled Receptor 37	1.3253	0.001137
7718	ZNF165	Zinc Finger Protein 165	1.2881	0.013337
54101	RIPK4	Receptor Interacting Serine/Threonine Kinase 4	1.2835	0.006548
22913	RALY	RALY Heterogeneous Nuclear Ribonucleoprotein	1.2831	0.00162

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2(a)

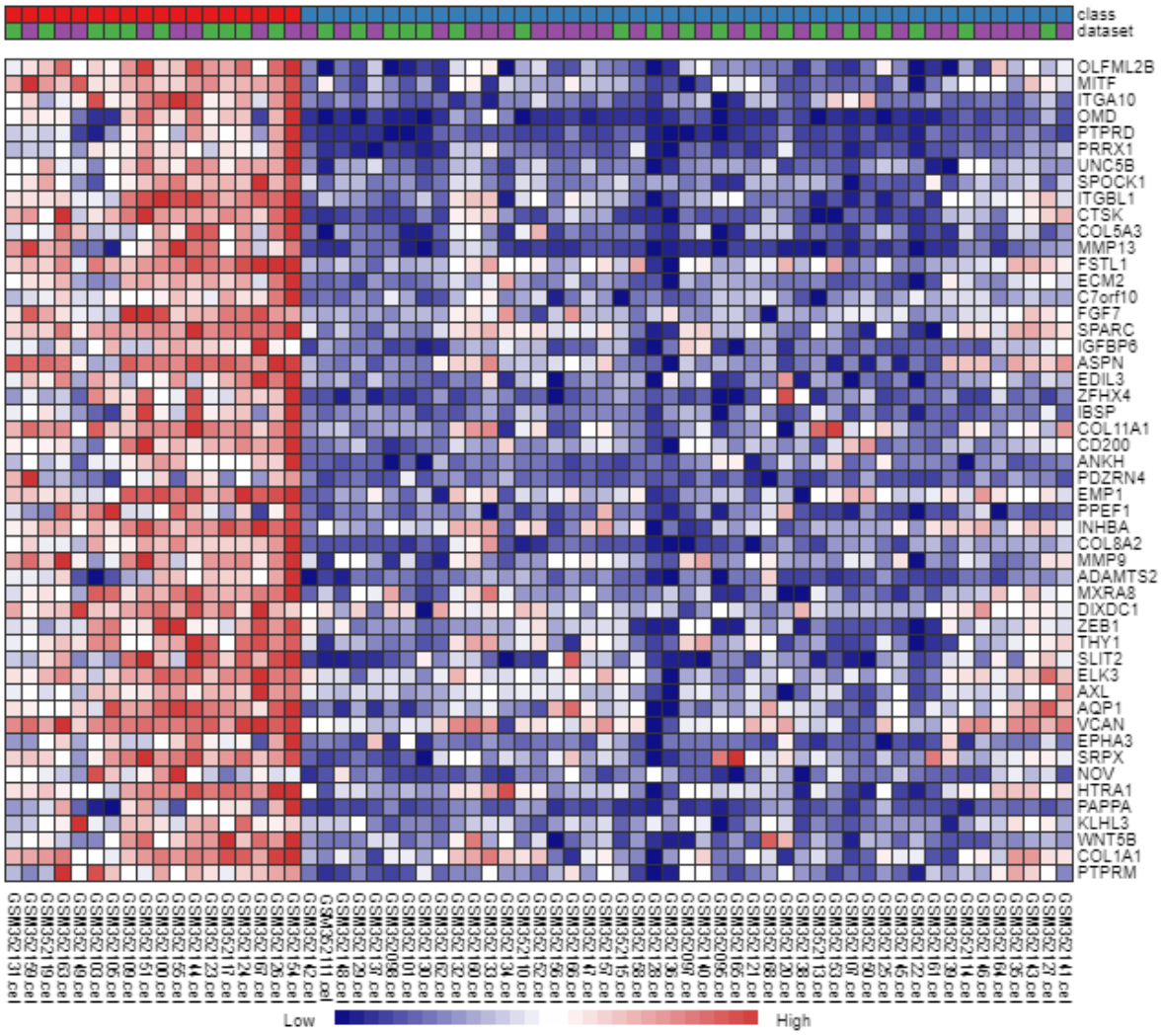

Figure 2: a) Venn diagram showing number of DE genes gained and numbers lost in meta-analysis b) Heat map generated for the differential expression of genes involved in the bone metastasis. Expression levels of genes are represented as intensity of the color. Blue is lower intensity of expression and red indicates high expression.

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Functional Annotation of the differentially expressed genes

The differentially expressed genes were subjected to the DAVID analysis in order to identify the enriched GO terms and biological pathways. The most significant biological process that was enriched belonged to GO category extra cellular matrix organization (GO:0030198) with a *P* value 6.58E-39, other significant terms that were enriched categories are cell adhesion (GO:0007155) with a *P* value of 2.08E-30, angiogenesis (GO:0001525) with *P* value 1.44E-17, osteoblast differentiation (GO:0001649) with *P* value 6.86E-09, skeletal system development (GO:0001501), with a *P* value of 3.87E-13, collagen catabolic process (GO:0030574) with a *P* value of 7.60E-19, collagen fibril organization (GO:0030199) with a *P* value of 2.26E-13 and extracellular matrix disassembly (GO:0022617) with a *P* value of 1.18E-11are shown in the figure. The molecular functions pertaining to protein binding (GO:0005515) with a *P* value 3.94E-28, integrin binding (GO:0005178) with a *P* value 8.14E-12, collagen binding (GO:0005518) with a *P* value 3.74E-11 are among the most significantly enriched categories. In order to understand the significant pathways that were enriched the DE genes were mapped to KEGG database. The top pathways that were significantly enriched include focal adhesion pathway (hsa04510) with a *P* value 2.07E-11, ECM-receptor interaction pathway (hsa04512) with a *P* value 1.47E-08 and pathways in cancer (hsa05200) with a *P* value 1.06E-07. The enriched biological processes, molecular functions and pathways are shown in Figure 3. The significantly enriched cellular components to which the DE genes belonged to were extracellular matrix, extracellular space and also extracellular exosomes.

Hub Genes Identification

Network based meta-analysis was conducted to find out the key hub genes among the DEGs obtained from the meta-analysis of different datasets. NetworkAnalyst, a web based tool was used to generate a protein-protein interaction (PPI) network by integrating the InnateDB interactome with the original seed of 838 DEGs. A large network was generated with 6975 nodes, and 17957 edges. For the better visualization of the networks minimally connected sub graph containing all seeds was constructed using zero order networks option. The top hub genes with their respective centrality scores are as follows ELAVL1 (Degree = 102, betweenness = 39979.03), FN1 (Degree = 49, betweenness = 18297.78), HDAC1(Degree = 31, betweenness = 7754.37), CTNNB1 (Degree = 26, betweenness = 6691.09), FYN(Degree = 23, betweenness = 5351.3), NEDD4 (Degree = 23, betweenness = 5081.17), JUN (Degree = 23, betweenness = 4485.1), CDH1 (Degree = 20, betweenness = 2304.3) as shown in the Figure 4.

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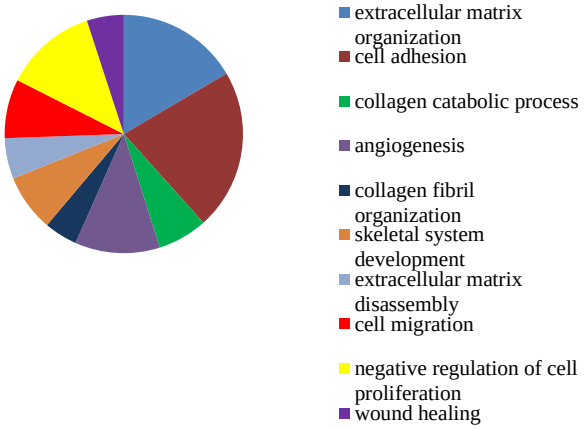

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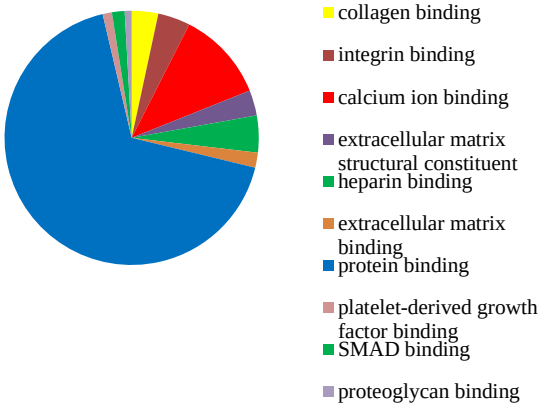

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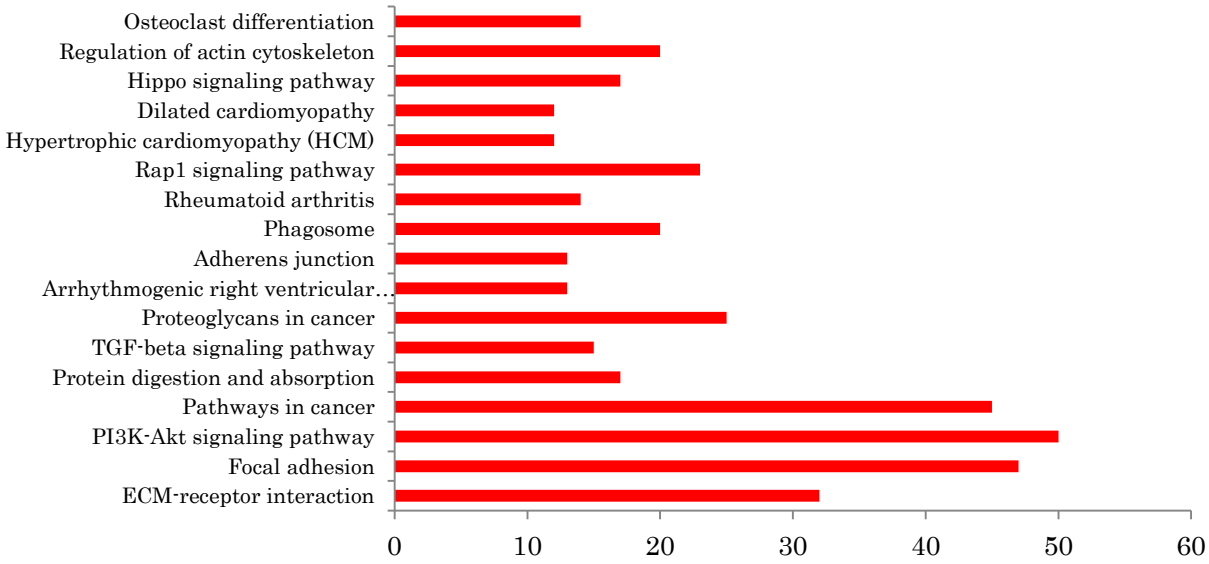

3c

Figure 3: Functional annotations of differentially expressed genes a) Biological processes that are enriched. b) Molecular functions enriched that are enriched c) The pathways in which the differentially genes are involved

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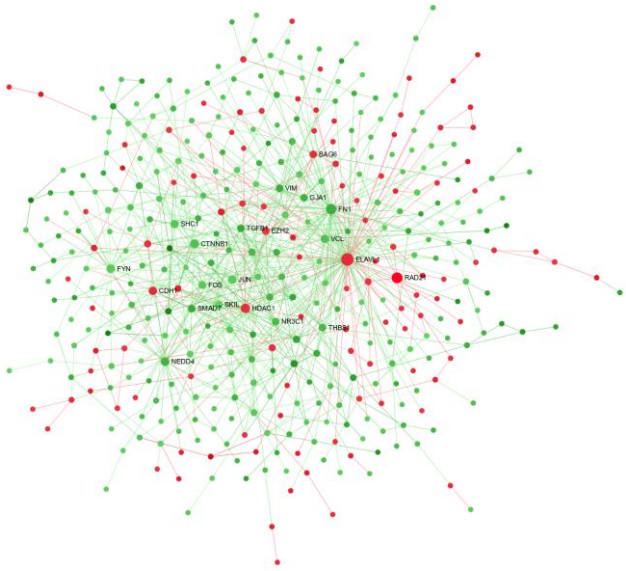

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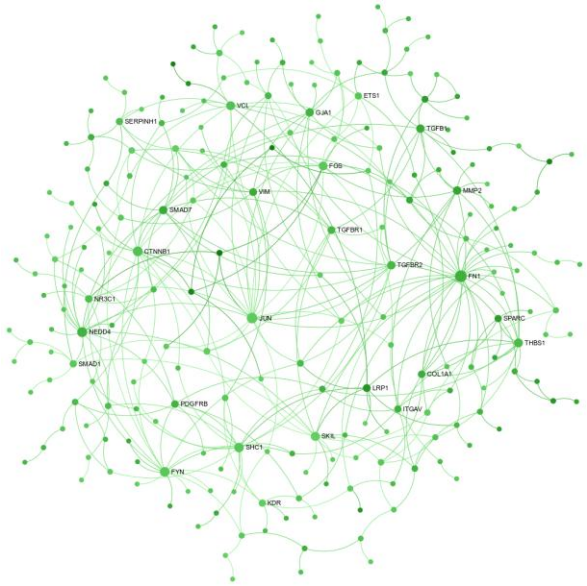

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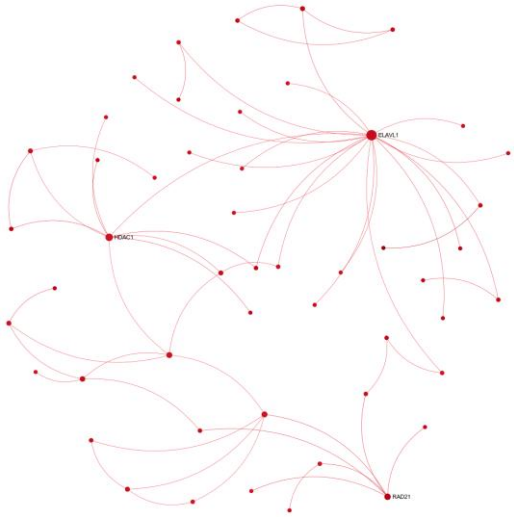

4c

Figure 4. Network based meta-analysis of hub genes. (A) Zero-order interaction network of shared DEGs obtained from meta-analysis; red nodes represents overexpressed and green nodes represents underexpressed DEGs. (B) PPI Subnetwork of most significant DEG that are over expressed with its interacting partners. (C) PPI Subnetwork of most significant DEG that are under expressed with its interacting partners.

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Identification of Transcriptional Factors Associated With Hub Genes

In order to understand the upstream factors associated with differentially expressed genes transcriptional factors that are associated are understood. The significant transcriptional factors that are associated with the differentially expressed genes are EP300 (E1A Binding Protein P300), WT1(Wilms Tumor 1), SOX2(Sex Determining Region Y-Box 2), EGR1(Early Growth Response 1), PPARγ(Peroxisome Proliferator Activated Receptor Gamma), NRF2 (Nuclear Factor, Erythroid 2-Like 2), CLOCK (Clock Circadian Regulator), HNF4A (Hepatocyte Nuclear Factor 4 Alpha), SUZ12 (Suppressor Of Zeste 12 Protein Homolog). The transcriptional factors are shown along with the P value, Z score and combined score in the given in **Table 3.**

Table 3: Transcriptional Factors That Are Involved Regulation Of DE Genes

Transcription Factor	*P* value	Z-score	Combined Score
EP300	1.34E-28	-2.0125	129.1624673
WT1	5.87E-30	-1.63001	109.711959
SOX2	6.08E-30	-1.40196	94.31256988
EGR1	5.49E-42	-0.92071	87.47340544
PPARG	1.20E-34	-1.04427	81.56682644
NRF2	1.78E-24	-1.48819	81.38061813
NFE2L2	1.78E-24	-1.48819	81.38061813
CLOCK	8.17E-21	-1.72625	79.84579411
HNF4A	3.82E-43	-0.73275	71.56853191
SUZ12	7.73E-36	-0.8376	67.7181651
SOX2	7.97E-25	-1.20253	66.72674905

DISCUSSION

Primary aim of our work is to identify hub genes. Hub genes are known to play a critical role in organization of the network and are considered to be important in many biological processes. We hypothesize that the identified hub genes play a significant role in homing and colonization of breast cancer cells in the bone metastatic niche when compared to the non-hub genes. As discussed in the methods section ‘degree’ and ‘betweenness’ are the topological measures that can be used in the selection of these genes. Degree centrality refers to the number of edges to which the node is connected and betweenness centrality is the number edges that are passing through the particular node. As shown in the Figure 4(b) and 4(c) nodes with high degree centrality obtained from zero

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order PPI network option are considered as hub genes. Although CTNNB, FYN and ELVAL1 have high degree centrality and are discussed elsewhere we restricted our discussion to the following hub genes FN1 with degree = 49, betweenness = 18297.78, HDAC1 with degree = 31, betweenness = 7754.37 and NEDD4 with a degree = 23, betweenness = 5081.17. FN1 (Fibronectin 1) is a 250 kDa extracellular matrix associated glycoprotein and is a ligand for many integrins. Upon interaction with integrins many genes are activated resulting in their involvement in important biological process. Our enrichment analysis suggests that FN1 might have a key role in biological processes such as cell adhesion, extra cellular matrix organization, skeletal system development and angiogenesis at the bone microenvironment. Though FN1 functions are well understood at the primary breast cancer tissue but its implications at the metastatic site are not clearly understood (23, 24). Probable role of FN1 in promoting osteolytic lesions could be by activating osteoclasts [26]. Further we found that it is associated in regulating multiple pathways like EMT-interaction pathway, focal adhesion pathway and also PI3K-Akt signaling pathway. Another interesting hub gene that we identified is NEDD4 (Neural Precursor Cell Expressed, Developmentally Down-Regulated 4). It is an E3 Ubiquitin protein ligase and is involved in the degradation of membrane proteins such as ion channels and transporters thus regulating their availability. Expression of NEDD4 in tumors is tissue specific, high expression is seen in prostate, colon, bladder and also breast cancers [28] and reduced expression is observed in neuroblastoma [29]. NEDD4 promotes tumor progression through multiple pathways like activating PI3K/AKT pathway by regulating PTEN (phosphatase and tensin homolog depleted on chromosome 10), suppressing the activity of p53 [30] and also by modulating RTK (receptor tyrosine kinases) signaling [31]. Interestingly, in breast cancers NEDD4 acts in PTEN independent pathways in tumor progression [32]. Our pathway analysis identified that NEDD4 is involved in endocytosis in the breast cancer cells at bone metastatic niche. One probable mechanism by which NEDD4 could increase RTK signaling and tumor progression is by reducing endocytosis of RTK [31]. HDAC1 functions by deacetylation of histones and chromatin condensation leading to epigenetic silencing. Transcription factors such as E2f, Stat3, p53, the retinoblastoma protein, NF-kB, TFIIE regulates cell homeostasis and are known to interact with HDAC1 [27]. Varied expression pattern of HDAC1 is seen in different cancers [28] and is overexpressed in prostate [29] and breast cancers [30]. Multiple studies have demonstrated the important role of HDAC1 in cancers, through transcriptional inhibition of tumor suppressor genes and thereby influencing cell cycle events [30]. Our results indicate that HDAC1 is down regulated in bone metastatic niche in contrast to its high expression in primary breast cancer tissues. We identified that from our enrichment analysis the key biological processes with which HDAC1 is associated is negative regulation of cell proliferation. Cancer cells tend to enter a state of dormancy for certain period after reaching the metastatic site through cell cycle arrest [31]. Therefore probable role of HDAC1 at the metastatic site could be to aid in cancer cell dormancy, by negatively

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regulating cell cycle. We have also identified transcriptional upstream factors regulating these DE genes that include EP300, WT1, SOX2, EGR1, PPARG, NRF2. We are in the process of experimentally validating the proposed genes that would help in utilizing them as biomarkers for bone metastasis. Our microarray meta-analysis identified 838 differentially expressed genes. The top up regulated gene that was differentially expressed is LRRC15 and has a combined effect size score of -3.7818 with an adjusted P value of 0.00088461. LRRC15 (Leucine Rich Repeat Containing 15) a membrane protein belongs to leucine rich repeat superfamily and is known to play an important role in cell-cell adhesion, trafficking and hormone receptor interactions [38]. High level of LLRC15 expression patterns are seen in the breast cancer tissues when compared with the normal tissues [39]. Our meta-analysis showed a comparable results to that of Klein et al., who also identified LLRC15 was one of the top differentially expressed gene in bone metastasis [40]. Therefore LLRC15 probably helps cancer cells in homing at the metastatic site. The top DE gene that was down regulated was LAMC3 (Laminin Subunit Gamma 3) is extracellular glycoprotein. It is involved in cell adhesion, differentiation, signaling, and metastasis. LAMC3 is known to be highly methylated in breast cancers [32]. Probable mechanism by which down regulation of LAMC3 promotes cancer progression at the bone could involve inhibiting DNA repair [33] or by promoting osteoclastogenesis and osteolytic leisions [34]. Further, this study highlights various biological processes, molecular functions, pathways that are associated with differentially expressed genes so as to understand their contribution towards progression of breast cancer at bone metastatic site. The highly enriched biological process was extracellular matrix organization. Extracellular matrix is composed of number of complex biomolecules that regulate many biological processes and also developmental processes. Extracellular matrix is associated with progression of tumors by promoting epethilial mesenchymal transition, dysregulate the behavior of the stromal cells, promote angiogenesis [35] and in essence it influences all the hall marks of the cancer described by Hanahan [36]. Cell adhesion, angiogenesis are also highly enriched terms that are associated with our DE genes which aid the cancer cells to home at the novel bone environment and promote neovascularization respectively. Collagen catabolic process is associated with breakdown of collagen in extracellular matrix. Large part of the extracellular matrix is made up of collagen and research indicates that collagen is involved in promoting tumor progression. Collagen changes are as associated with certain biomechanical signals that are sensed by both tumor and stromal cells that trigger cascade of biological events aiding tumor progression and also escaping immune surveillance [37]. DE genes are also associated with skeletal system development, these probably involve in osteomimicry that aid cancer cells to survive in the new metastatic niche and also cause osteolysis providing space as well as nutrition for the cancer cells. In a nutshell, cancer cells once enter into the bone acquire certain genetic alterations that aid them in performing novel biological processes by involving in various pathways so as to adapt and establish themselves in the bone.

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4. CONCLUSION

In summary we identified differentially expressed genes between bone metastasis and other metastatic conditions. These genes were used to construct protein-protein interaction network to identify hub genes that are associated with progression of the breast cancer cells in the bone metastatic niche. Our analysis predicted the hub genes that are involved in various biological processes like cell adhesion, extracellular matrix regulation, skeletal development etc., and aid cancer cells in adapting and growing in the new microenvironment. Till date there are no proper biomarkers that indicate bone metastasis. Through our network analysis we nominate FN1, NEDD4 and HDAC1 as promising hub genes and further in vitro and in vivo analysis of these markers are required to validate their potential as biomarkers in bone metastasis.

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