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Identification of differentially expressed genes regulated by molecular signature in breast cancer-associated fibroblasts by bioinformatics analysis

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Abstract
Objective Breast cancer is a severe risk to public health and has adequately convoluted pathogenesis. Therefore, the description of key molecular markers and pathways is of much importance for clarifying the molecular mechanism of breast cancer-associated fibroblasts initiation and progression. Breast cancer-associated fibroblasts gene expression dataset was downloaded from Gene Expression Omnibus database.
Methods A total of nine samples, including three normal fibroblasts, three granulin-stimulated fibroblasts and three cancer-associated fibroblasts samples, were used to identify differentially expressed genes (DEGs) between normal fibroblasts, granulin-stimulated fibroblasts and cancer-associated fibroblasts samples. The gene ontology (GO) and pathway enrichment analysis was performed, and protein-protein interaction (PPI) network of the DEGs was constructed by NetworkAnalyst software.
Results Totally, 190 DEGs were identified, including 66 up-regulated and 124 down-regulated genes. GO analysis results showed that up-regulated DEGs were significantly enriched in biological processes (BP), including cell-cell signalling and negative regulation of cell proliferation; molecular function (MF), including insulin-like growth factor II binding and insulin-like growth factor I binding; cellular component (CC), including insulin-like growth factor binding protein complex and integral component of plasma membrane; the down-regulated DEGs were significantly enriched in BP, including cell adhesion and extracellular matrix organization; MF, including *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase activity and calcium ion binding; CC, including extracellular space and extracellular matrix. WIKIPATHWAYS analysis showed the up-regulated DEGs were enriched in myometrial relaxation and contraction pathways. WIKIPATHWAYS, REACTOME, PID_NCI and KEGG pathway analysis showed the down-regulated DEGs were enriched endochondral ossification, TGF beta signalling pathway, integrin cell surface interactions, beta1 integrin cell surface interactions, malaria and glycosaminoglycan biosynthesis—chondroitin sulfate/dermatan sulphate. The top 5 up-regulated hub genes, CDKN2A, MME, PBX1, IGFBP3, and TFAP2C and top 5 down-regulated hub genes VCAM1, KRT18, TGM2, ACTA2, and STAMBP were identified from the PPI network, and subnetworks revealed these genes were involved in significant pathways, including myometrial relaxation and contraction pathways, integrin cell surface interactions, beta1 integrin cell surface interaction. Besides, the target hsa-mirs for DEGs were identified. hsa-mir-759, hsa-mir-4446-5p, hsa-mir-219a-1-3p and hsa-mir-26a-5p were important miRNAs in this study.
Conclusions We pinpoint important key genes and pathways closely related with breast cancer-associated fibroblasts initiation and progression by a series of bioinformatics analysis on DEGs. These screened genes and pathways provided for a more detailed molecular mechanism underlying breast cancer-associated fibroblasts occurrence and progression, holding promise for acting as molecular markers and probable therapeutic targets.

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Introduction

Cancer is associated with fibroblasts at entire phases of disease progression, including metastasis, and they are a substantial component of the general host response to tissue damage induced by tumour cells [1]. Cancer-associated with fibroblasts become synthetic machines that produce diverse extracellular matrix (ECM) components, growth factors, cytokines, proteases, and hormones. Cancer-associated fibroblasts have generally been characterize as α-smooth muscle actin (α-SMA) [2]. Cancer-associated fibroblast expresses biomarkers such as such as vimentin, fibroblast specific protein, fibroblast activation protein (FAP), osteonectin, and desmin [3]. However, distinct biomarkers have been diagnosed in different tumour subtypes [4], and unique biomarkers that can perceive all of the cancer-associated fibroblasts in breast cancer remain inadequate to date.

Stromal cancer-associated fibroblasts of breast cancer have four specific cellular origins [5]. The first one is activated normal stromal fibroblasts, which is primary one and 80% of breast cancer-associated fibroblasts [6]. The second one is epithelial–mesenchymal transition (EMT) or endothelial cells, which undergo endothelial–mesenchymal transition [7]. The third one is bone marrow-derived mesenchymal stem cells (MSCs), which share to the regeneration of mesenchymal tissues, and are crucial in furnishing backing for the growth and differentiation of primitive hemopoietic cells within the bone marrow microenvironment [8]. The fourth one comprise trans differentiated cells in breast tissue, such as pericytes, adipocytes, or smooth muscle cells [9]. The significances of cancer-associated fibroblast in the clinical diagnosis and prognosis of breast cancer have been studied broadly, and the possible usage of some cancer-associated fibroblast biomarkers has been diagnosed. PDGF receptor β and FAP are the biomarkers significantly associated with breast cancer [10]. Alteration and/or loss of tumor suppressor genes are also critical features of breast cancer-associated fibroblast. Significantly alteration in tumor suppressor gene Cav-1 in breast cancer-associated fibroblast [11]. So triple negative breast cancer (TNBC) is comes under stromal cancer-associated fibroblasts.

Triple receptor-negative breast carcinoma (TNBC) is the most frequent and aggressive mammary gland malignancy in female adults, and is identified by absence of the estrogen receptor (ER) and progesterone receptor (PR) and lack of over expression of human epidermal growth factor receptor 2 (HER2) that are involved with advancement of the disease [12]. It is a rapidly fatal malignancy and the majority of patients with TNBC suffer from a poor quality of life [13]. Presently, the accepted clinical treatment is surgical resection of the malignant tissues, followed by radiotherapy and chemotherapy [14]. However, patients that receive these treatments may expeditiously advance resistance to chemotherapy [15]. Recent studies have focused on the recognition of candidate biomarkers of TNBC advancement, in order to produce a more efficient therapeutic strategy [16].

With the aim to explore the mechanisms of tumor initiation, progression and metastasis and develop new targeted therapies for TNBC, studies have focused on the signalling pathways deregulation and genes alternation related to TNBC in the past few years. Gene expression profiling is a beneficial tool to find differentially expressed genes (DEGs) in human TNBC so as to find possible critical genes or transcription factors that play crucial roles in the regulation of TNBC development and progression [13]. Numerous previous studies have identified some genes which may be used as diagnostic markers or therapy targets for TNBC. For example, FOXA1, KRT18, and XBP1 are found to be over-expressed in TNBC and involved in many important cell functions contribute to tumorigenesis [17]. FOXA1, a widely cytokeratin protein participated in many physiological and pathological processes such as lymphocyte homing and activation, cell survival and migration, and tumour growth and metastasis [18]. It is reported FOXA1, KRT18, and XBP1 are expressed at both high transcriptional and translational levels in TNBC and its expression is related to the degree of malignancy [19]. Furthermore, the up-regulation of FOXA1, KRT18, and XBP1 in malignant TNBC may be an indication of tumour cell growth and migration. ASNA1, NDUFS8, NDUFV1 and NDUFB7 [20] have all been studied and their possibilities to be used as targets for diagnosis and therapy of TNBC have also been evaluated. However these studies just noted a few DEGs and the interaction among these genes were still unknown.

In 2016, Marsh et al. [21] performed a microarray data analysis based on GSE75333 to identify gene expression changes responding to TNBC between normal fibroblasts, granulin-stimulated fibroblasts and cancer-associated fibroblasts. They obtained numerous differentially expressed genes (DEGs) between normal fibroblasts, granulin-stimulated fibroblasts and cancer-associated fibroblasts and found that the DEGs and their related function. This demonstrated there still existed distance between clinical application and the laboratory test, and more tests should be done to identify more candidates for therapy for TNBC.

In order to identify more genetic candidates for therapy for TNBC, expression microarray data GSE75333 deposited in Gene Expression Omnibus (GEO) by Marsh et al. [21]. DEGs in this dataset were analyze and their interaction relation were investigated by protein-protein interaction (PPI) network. Moreover, possible functions of DEGs involved in the PPI network were evaluated by gene and pathway

enrichment analyses. The functions of the significant DEGs involved in significant function terms would be argued and some of them might be treated as the candidates for TNBC therapy.

Materials and methods

Affymetrix microarray data

The gene expression profile data of GSE75333 based on theplatform of GPL570 (Affymetrix Human Genome U133_Plus_2 Array) (Affymetrix Inc., Santa Clara, CA, USA) were downloaded from Gene Expression Omnibus (GEO) database in National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/geo/), which was deposited by Marsh et al. [21]. The datasets available in this analysis contained nine samples, including three normal fibroblasts cultures, three granulin-stimulated fibroblasts cultures and three cancer-associated fibroblasts cultures.

Identification of DEGs

The raw data of the mRNA expression profiles were downloaded and investigated by R language software [22]. Background correction, quartile data normalization, and probe summarization were tested for the authentic data. The limma [23] technique in Bioconductor (http://www.bioconductor.org/) was used to analyze genes which were differentially expressed between normal fibroblasts, granulin-stimulated fibroblasts and cancer-associated fibroblasts; the significance of DEGs was determined by *t* test and was characterized by *p* value. To cut down the risk of false positives, *p* values were adjusted for multiple testing using the Benjamini–Hochberg False Discovery Rate (FDR) technique. The revised *p* value was characterized by FDR [24]. FDR < 0.05 were treated as the cut off values for DEG screening.

Gene ontology (GO) analysis of DEGs

Gene ontology is an effective tool for compiling an enormous number of gene annotation terms [25]. The Lynx tool for a database and knowledge extraction engine for integrative medicine [26], is bioinformatics resources consisting of an integrated biological knowledgebase and analytic tools aimed at consistently elicit biological functional annotation from enormous gene/protein lists, such as being derived from high-throughput genomic experiments. To hike the extensive sympathetic of the biological functions of DEGs, Lynx tool was used to obtain the enriched GO terms of DEGs based on the $p < 0.05$ was set as the threshold value.

Bio-pathway analysis of DEGs

WIKIPATHWAYS, REACTOME, NCI and KEGG are a database resource for understanding functions of genes list from molecular level [27–30]. Lynx is a valuable tool to functionally interpret results from experimental techniques in genomics [26]. This web-based application consolidate different sources of information for discovering groups of genes with similar biological meaning. The enrichment analysis of Lynx is crucial in the analysis of high-throughput experiments. In the study, Lynx software was used to test the statistical enrichment of DEGs in pathways. $p < 0.05$ was set as the threshold value.

Construction of protein-protein interaction (PPI) network

The online database resource InnateDB keeps individually broad coverage, and access to predicted and experimental interaction information [31]. Interactions in the InnateDB are administered with a confidence score [31]. In the current study, application of the InnateDB (http://innatedb.com/) was used to predict PPIs based on a confidence score > 0.4 and other default parameters. The PPI network was visualized using network analyst (http://www.networkanalyst.ca/) [32]. Subsequently, a PPI network was build up based on the analyzed DEGs. A hypergeometric algorithm was used, and $p < 0.05$ was treated to express statistically significant differences.

Prediction of target miRNAs for DEGs

DIANA-TarBasev7.0 (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=tarbase/index) [33] is an integrated system for exploring large sets of gene. DIANA-TarBase was used to identify hsa-mir s corresponding to DEGs. Hsa-mirs with DEGs number > 2 and raw $p < 0.05$ were identified as target hsa-mirs. The gene hsa-mir network was visualized with network analyst (http://www.networkanalyst.ca/).

Results

Preprocessing of data

After preprocessing and data integration, we obtained 9 samples, including expression data matrix of 52,238 gene probes. Through comparing the quality of expression values of microarray before and after normalization, we found

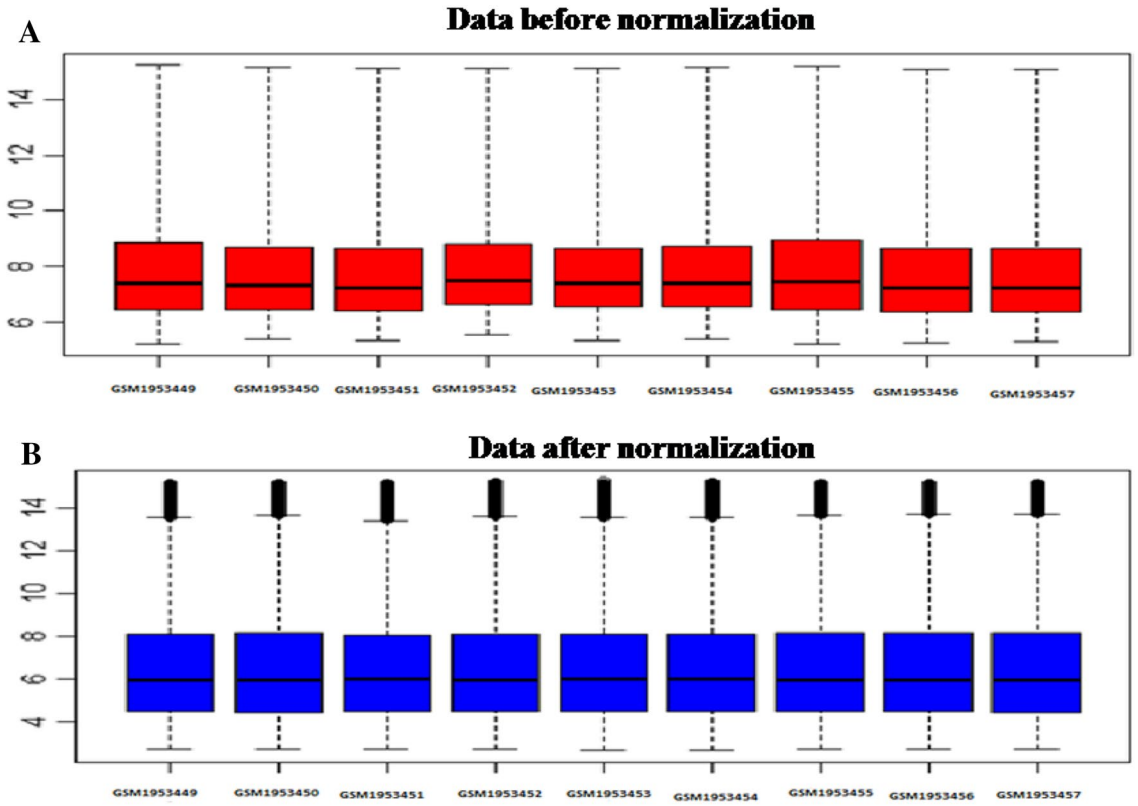

Fig. 1 Box plots of the gene expression data before and after normalization. Horizontal axis represents the sample symbol and the vertical axis represents the gene expression values. The black line in the box plot represents the median value of gene expression

that the medians of expression value of nine samples were in a straight line (Fig. 1).

Identification of DEGs

Among the 52,238 genes identified from the gene expression profile microarray analysis, 190 differentially expressed genes were screened out ($|\log FC| \geq 1$ and $p < 0.05$) (Table 1). Among these, 66 genes were up-regulated, and 124 genes were down-regulated. A "volcano plot" was constructed by plotting the differentially expressed genes between the p values and fold changes (Fig. 2). The corresponding Venn diagrams are shown in Fig. 3. The overlapping part represents the number of overlapping DEGs between groups, whereas the non-overlapping part represents the number of unique DEGs between groups.

Hierarchical clustering analysis of DEGs

After extracting the expression values of the DEGs, hierarchical clustering analysis was conducted for the DEGs. As shown in Fig. 4, the DEGs could clearly distinguish the normal fibroblasts samples from the granulin-stimulated fibroblasts and normal fibroblasts samples from the cancer-associated fibroblasts samples. In granulin-stimulated fibroblasts and cancer-associated fibroblasts samples, there were more down-regulated genes than up-regulated genes (Fig. 4).

GO enrichment analysis

After the GO enrichment analysis, the DEGs in the PPI network of up-regulated genes were mainly associated with the GO BP categories of cell-cell signalling(including ADM, EFNB2, NOV, SSTR1, TFAP2C and TRHDE, $p = 0.0000562$) and negative regulation of cell proliferation (including ADM, BCHE, CDKN2A, CDKN2B, IGFBP3, SSTR1, and TWIST2, $p = 0.0000733$), GO MF categories of insulin-like growth factor II binding (including IGFBP3 and IGFBP5, $p = 0.000201$) and insulin-like growth factor I binding (including IGFBP3 and IGFBP5, $p = 0.000258$) and GO CC categories of insulin-like growth factor binding protein complex (including IGFBP3and IGFBP5, $p = 0.0000217$) and integral component of plasma membrane (including EFNB2, ENPP2, EPHA5, FLRT3, KCND2, KCNJ8, MME, NLGN1, PTGER3, SLC38A5, SSTR1 and TRHDE, $p = 0.000258$) (Table 2).

Table 1 The statistical metrics for key differentially expressed genes (DEGs)

Affy ID	Gene symbol	logFC	*p* value	FDR	Up/down regulation	Gene name
226824_at	CPXM2	4.419478	1.036085e−14	2.814808e−10	Up	Carboxypeptidase X, M14 family member 2
1563933_a_at	PLD5	5.169100	2.059302e−14	2.814808e−10	Up	Phospholipase D family member 5
209392_at	ENPP2	3.602643	7.596058e−14	5.802871e−10	Up	Ectonucleotide pyrophosphatase/phosphodiesterase 2
236313_at	CDKN2B	4.954639	2.767753e−13	1.035589e−09	Up	Cyclin dependent kinase inhibitor 2B
202157_s_at	CELF2	3.391369	3.486476e−13	1.081265e−09	Up	CUGBP, Elav-like family member 2
206163_at	MAB21L1	2.335038	3.559721e−13	1.081265e−09	Up	Mab-21 like 1
230925_at	APBB1IP	3.012620	4.035109e−13	1.161156e−09	Up	Amyloid beta precursor protein binding family B member 1 interacting protein
228218_at	LSAMP	4.166242	4.401999e−13	1.181044e−09	Up	Limbic system-associated membrane protein
219304_s_at	PDGFD	2.309779	4.536247e−13	1.181044e−09	Up	Platelet derived growth factor D
202156_s_at	CELF2	3.501838	5.837152e−13	1.240400e−09	Up	CUGBP, Elav-like family member 2
205935_at	FOXF1	2.515980	6.496570e−13	1.275255e−09	Up	Forkhead box F1
234973_at	SLC38A5	2.284512	1.144279e−12	1.971236e−09	Up	Solute carrier family 38 member 5
212143_s_at	IGFBP3	2.452483	1.416744e−12	2.278249e−09	Up	Insulin like growth factor binding protein 3
228335_at	CLDN11	2.534948	2.125667e−12	3.228356e−09	Up	Claudin 11
210839_s_at	ENPP2	3.271573	2.654275e−12	3.631680e−09	Up	Ectonucleotide pyrophosphatase/phosphodiesterase 2
205304_s_at	KCNJ8	3.654705	3.080445e−12	3.827803e−09	Up	Potassium voltage-gated channel subfamily J member 8
204457_s_at	GAS1	2.412123	3.313650e−12	3.961086e−09	Up	Growth arrest specific 1
226415_at	VAT1L	3.234308	4.398106e−12	4.715028e−09	Up	Vesicle amine transport 1 lik
229158_at	WNK4	2.126206	5.377380e−12	5.547326e−09	Up	WNK lysine deficient protein kinase 4
229404_at	TWIST2	1.921715	6.316769e−12	6.167309e−09	Up	Twist family bHLH transcription factor 2
235591_at	SSTR1	3.665891	6.569058e−12	6.301110e−09	Up	Somatostatin receptor 1
219049_at	CSGALNACT1	1.885919	1.162467e−11	8.951816e−09	Up	Chondroitin sulfate *N*-acetylgalactosaminyltransferase 1
203435_s_at	MME	2.314352	1.260938e−11	9.448783e−09	Up	Membrane metalloendopeptidase
205286_at	TFAP2C	2.724768	1.278213e−11	9.448783e−09	Up	Transcription factor AP-2 gamma
219025_at	CD248	1.941032	1.642488e−11	1.197374e−08	Up	CD248 molecule
212148_at	PBX1	2.180438	2.010431e−11	1.411056e−08	Up	PBX homeobox 1
202668_at	EFNB2	2.378833	2.023008e−11	1.411056e−08	Up	Ephrin B2
231984_at	MTAP	1.816560	2.486855e−11	1.613776e−08	Up	Methylthioadenosine phosphorylase
210095_s_at	IGFBP3	1.647021	2.598848e−11	1.633242e−08	Up	Insulin like growth factor binding protein 3
213438_at	NFASC	2.608772	3.600960e−11	2.072447e−08	Up	Neurofascin
207039_at	CDKN2A	2.660221	4.182215e−11	2.263986e−08	Up	Cyclin dependent kinase inhibitor 2A
209276_s_at	GLRX	1.836998	5.863012e−11	2.921865e−08	Up	Glutaredoxin
202912_at	ADM	1.748182	6.334498e−11	3.038059e−08	Up	Adrenomedullin
224458_at	TMEM246	2.188037	7.206844e−11	3.399058e−08	Up	Transmembrane protein 246
237939_at	EPHA5	1.997188	8.589734e−11	3.946586e−08	Up	EPH receptor A5
219937_at	TRHDE	4.591826	1.039320e−10	4.578413e−08	Up	thyrotropin releasing hormone degrading enzyme
204501_at	NOV	1.420382	1.046734e−10	4.578413e−08	Up	Nephroblastoma overexpressed
209708_at	MOXD1	1.444732	1.086308e−10	4.676684e−08	Up	Monooxygenase DBH like 1
202158_s_at	CELF2	3.554425	1.101523e−10	4.705138e−08	Up	CUGBP, Elav-like family member 2
231736_x_at	MGST1	1.315594	1.140863e−10	4.798208e−08	Up	Microsomal glutathione *S*-transferase 1
212151_at	PBX1	1.859123	1.344917e−10	5.528820e−08	Up	PBX homeobox 1
207103_at	KCND2	2.848144	1.402643e−10	5.680704e−08	Up	Potassium voltage-gated channel subfamily D member 2
231361_at	NLGN1	1.795555	1.417126e−10	5.697160e−08	Up	Neuroligin 1

Table 1 (continued)

Affy ID	Gene symbol	logFC	*p* value	FDR	Up/down regulation	Gene name
236144_at	CPXM2	2.974055	1.567697e−10	6.131230e−08	Up	Carboxypeptidase X, M14 family member 2
205828_at	MMP3	2.519694	1.592382e−10	6.131230e−08	Up	Matrix metallopeptidase 3
228399_at	OSR1	1.486319	1.657064e−10	6.250104e−08	Up	Odd-skipped related transcription factor 1
1555997_s_at	IGFBP5	1.383062	1.657549e−10	6.250104e−08	Up	Insulin like growth factor binding protein 5
229831_at	CNTN3	1.841815	1.787648e−10	6.694497e−08	Up	Contactin 3
205433_at	BCHE	4.404753	2.087281e−10	7.585256e−08	Up	Butyrylcholinesterase
203434_s_at	MME	2.392716	2.156761e−10	7.757956e−08	Up	Membrane metalloendopeptidase
206969_at	KRT34	1.413432	2.783268e−10	9.451874e−08	Up	Keratin 34
236420_s_at	ANO4	2.455334	2.826477e−10	9.539362e−08	Up	Anoctamin 4
213933_at	PTGER3	1.660288	3.234101e−10	1.063279e−07	Up	Prostaglandin E receptor 3
220115_s_at	CDH10	4.180496	3.247694e−10	1.063279e−07	Up	Cadherin 10
224918_x_at	MGST1	1.234774	3.494179e−10	1.130439e−07	Up	Microsomal glutathione *S*-transferase 1
203184_at	FBN2	3.387293	3.618209e−10	1.163680e−07	Up	Fibrillin 2
214460_at	LSAMP	3.319523	4.372702e−10	1.340445e−07	Up	Limbic system-associated membrane protein
233533_at	KRTAP1-5	1.804071	4.753684e−10	1.428064e−07	Up	Keratin-associated protein 1–5
203401_at	PRPS2	1.465685	5.478297e−10	1.619059e−07	Up	Phosphoribosyl pyrophosphate synthetase 2
235494_at	LSAMP	1.683050	5.686397e−10	1.639400e−07	Up	Limbic system-associated membrane protein
222853_at	FLRT3	2.610121	5.751433e−10	1.639400e−07	Up	Fibronectin leucine rich transmembrane protein 3
209738_x_at	PSG6	1.203199	5.861929e−10	1.652067e−07	Up	Pregnancy specific beta-1-glycoprotein 6
212713_at	MFAP4	2.352389	5.916013e−10	1.658759e−07	Up	Microfibrillar-associated protein 4
222862_s_at	AK5	1.228954	6.305752e−10	1.741247e−07	Up	Adenylate kinase 5
204998_s_at	ATF5	1.460538	6.361424e−10	1.747793e−07	Up	Activating transcription factor 5
204830_x_at	PSG5	1.302785	6.453668e−10	1.760654e−07	Up	Pregnancy specific beta-1-glycoprotein 5
219529_at	CLIC3	− 4.264790	2.020892e−15	1.104923e−10	Down	Chloride intracellular channel 3
220979_s_at	ST6GALNAC5	− 3.449405	1.816938e−14	2.814808e−10	Down	ST6 *N*-acetylgalactosaminide alpha-2,6-sialyltransferase 5
223475_at	CRISPLD1	− 5.453766	3.184113e−14	3.221353e−10	Down	Cysteine rich secretory protein LCCL domain containing 1
202016_at	MEST	− 4.200287	3.535092e−14	3.221353e−10	Down	Mesoderm specific transcript
37892_at	COL11A1	− 4.471196	8.490712e−14	5.802871e−10	Down	Collagen type XI alpha 1 chain
206373_at	ZIC1	− 3.579856	9.859103e−14	5.989405e−10	Down	Zic family member 1
225525_at	KIAA1671	− 4.379022	1.292303e−13	7.065667e−10	Down	KIAA1671
202411_at	IFI27	− 4.614614	1.850483e−13	8.621529e−10	Down	Interferon alpha inducible protein 27
201809_s_at	ENG	− 2.859304	1.892242e−13	8.621529e−10	Down	Endoglin
201147_s_at	TIMP3	− 3.155272	2.796441e−13	1.035589e−09	Down	TIMP metallopeptidase inhibitor 3
202291_s_at	MGP	− 3.969597	2.841122e−13	1.035589e−09	Down	Matrix Gla protein
209386_at	TM4SF1	− 2.657547	3.225877e−13	1.081265e−09	Down	Transmembrane 4 L six family member 1
204967_at	SHROOM2	− 3.450298	5.075817e−13	1.240400e−09	Down	Shroom family member 2
203666_at	CXCL12	− 2.847854	5.315601e−13	1.240400e−09	Down	C-X-C motif chemokine ligand 12
204570_at	COX7A1	− 4.358774	5.619148e−13	1.240400e−09	Down	Cytochrome c oxidase subunit 7A1
203868_s_at	VCAM1	− 3.204453	5.898564e−13	1.240400e−09	Down	Vascular cell adhesion molecule 1
204198_s_at	RUNX3	− 4.261677	6.530799e−13	1.275255e−09	Down	Runt related transcription factor 3
201148_s_at	TIMP3	− 2.853555	7.147742e−13	1.347596e−09	Down	TIMP metallopeptidase inhibitor 3
200704_at	LITAF	− 3.732391	1.157190e−12	1.971236e−09	Down	Lipopolysaccharide induced TNF factor
213122_at	TSPYL5	− 4.533827	1.189772e−12	1.971236e−09	Down	TSPY like 5
41660_at	CELSR1	− 4.130121	2.122567e−12	3.228356e−09	Down	Cadherin EGF LAG seven-pass G-type receptor 1
215034_s_at	TM4SF1	− 2.643134	2.276622e−12	3.285539e−09	Down	Transmembrane 4 L six family member 1
210869_s_at	MCAM	− 2.569089	2.283502e−12	3.285539e−09	Down	Melanoma cell adhesion molecule

Table 1 (continued)

Affy ID	Gene symbol	logFC	*p* value	FDR	Up/down regulation	Gene name
211071 _s_at	MLLT11	− 2.018537	2.656922e−12	3.631680e−09	Down	Myeloid/lymphoid or mixed-lineage leukemia; translocated to, 11
201150_s_at	TIMP3	− 2.548024	2.857268e−12	3.733044e−09	Down	TIMP metallopeptidase inhibitor 3
202811_at	STAMBP	− 4.383529	2.867633e−12	3.733044e−09	Down	STAM binding protein
213711_at	KRT81	− 3.979391	3.076577e−12	3.827803e−09	Down	Keratin 81
217428_s_at	COL10A1	− 3.110195	3.332601e−12	3.961086e−09	Down	Collagen type X alpha 1 chain
201721_s_at	LAPTM5	− 3.412420	3.946919e−12	4.517983e−09	Down	Lysosomal protein transmembrane 5
215177_s_at	ITGA6	− 3.227442	4.017400e−12	4.517983e−09	Down	Integrin subunit alpha 6
212950_at	ADGRF5	− 2.498394	4.051579e−12	4.517983e−09	Down	Adhesion G protein-coupled receptor F5
228098_s_at	MYLIP	− 2.931012	4.131671e−12	4.517983e−09	Down	Myosin regulatory light chain interacting protein
227236_at	TSPAN2	− 3.648334	5.077291e−12	5.338479e−09	Down	Tetraspanin 2
219602_s_at	PIEZO2	− 5.358974	5.749376e−12	5.752858e−09	Down	Piezo type mechanosensitive ion channel component 2
204320_at	COL11A1	− 3.171753	5.787054e−12	5.752858e−09	Down	Collagen type XI alpha 1 chain
216598_s_at	CCL2	− 2.874133	6.715801e−12	6.330800e−09	Down	C-C motif chemokine ligand 2
209583_s_at	CD200	− 3.340673	7.408415e−12	6.759213e−09	Down	CD200 molecule
215704_at	FLG	− 3.187103	7.541143e−12	6.759213e−09	Down	Filaggrin
203066_at	CHST15	− 2.450131	8.155619e−12	7.192072e−09	Down	Carbohydrate sulfotransferase 15
206018_at	FOXG1	− 4.902242	8.769169e−12	7.610386e−09	Down	Forkhead box G1
209687_at	CXCL12	− 1.894754	9.057542e−12	7.737830e−09	Down	C-X-C motif chemokine ligand 12
201596_x_at	KRT18	− 3.234290	9.582511e−12	8.060366e−09	Down	Keratin 18
222908_at	PIEZO2	− 4.212262	9.964231e−12	8.244900e−09	Down	Piezo type mechanosensitive ion channel component 2
209081_s_at	COL18A1	− 2.068758	1.010349e−11	8.244900e−09	Down	Collagen type XVIII alpha 1 chain
205713_s_at	COMP	− 2.676925	1.047703e−11	8.423991e−09	Down	Cartilage oligomeric matrix protein
205080_at	RARB	− 3.646181	1.063271e−11	8.425264e−09	Down	Retinoic acid receptor beta
212843_at	NCAM1	− 2.613789	1.133583e−11	8.854093e−09	Down	Neural cell adhesion molecule 1
201042_at	TGM2	− 3.344269	1.278848e−11	9.448783e−09	Down	Transglutaminase 2
230482_at	ST6GALNAC5	− 3.223812	2.069344e−11	1.411056e−08	Down	ST6 *N*-acetylgalactosaminide alpha-2,6-sialyltransferase 5
209909_s_at	TGFB2	− 2.193456	2.076769e−11	1.411056e−08	Down	Transforming growth factor beta 2
218499_at	STK26	− 4.272665	2.086764e−11	1.411056e−08	Down	Serine/threonine protein kinase 26
201348_at	GPX3	− 1.757731	2.090453e−11	1.411056e−08	Down	Glutathione peroxidase 3
204671_s_at	ANKRD6	− 4.379536	2.205722e−11	1.468139e−08	Down	Ankyrin repeat domain 6
242100_at	CHSY3	− 1.945082	2.228726e−11	1.468139e−08	Down	Chondroitin sulfate synthase 3
214077_x_at	MEIS3P1	− 2.266315	2.508843e−11	1.613776e−08	Down	Meishomeobox 3 pseudogene 2
236044_at	PLPP4	− 1.652698	2.565381e−11	1.630956e−08	Down	Phospholipid phosphatase 4
222446_s_at	BACE2	− 2.258061	2.639951e−11	1.640220e−08	Down	Beta-site APP-cleaving enzyme 2
211518_s_at	BMP4	− 3.021027	2.705512e−11	1.662066e−08	Down	Bone morphogenetic protein 4
220356_at	CORIN	− 2.004016	3.050175e−11	1.827521e−08	Down	Corin, serine peptidase
201656_at	ITGA6	− 2.659241	3.086385e−11	1.827521e−08	Down	Integrin subunit alpha 6
212543_at	AIM1	− 3.204883	3.108541e−11	1.827521e−08	Down	Absent in melanoma 1
225728_at	SORBS2	− 2.362214	3.290812e−11	1.914097e−08	Down	Sorbin and SH3 domain containing 2
230836_at	ST8SIA4	− 3.177619	3.706796e−11	2.109747e−08	Down	ST8 alpha-*N*-acetyl-neuraminide alpha-2,8-sialyltransferase 4
221558_s_at	LEF1	− 2.638352	3.742945e−11	2.109747e−08	Down	Lymphoid enhancer binding factor 1
205381_at	LRRC17	− 2.198976	3.884960e−11	2.167451e−08	Down	Leucine rich repeat containing 17
1552701_a_at	CARD16	− 2.540640	4.030675e−11	2.222570e−08	Down	Caspase recruitment domain family member 16
202838_at	FUCA1	− 1.968928	4.312906e−11	2.311844e−08	Down	Fucosidase, alpha-L-1, tissue

Table 1 (continued)

Affy ID	Gene symbol	logFC	*p* value	FDR	Up/down regulation	Gene name
214954_at	SUSD5	− 4.168527	4.356892e−11	2.312748e−08	Down	Sushi domain containing 5
230493_at	SHISA2	− 5.155491	4.558999e−11	2.396762e−08	Down	Shisa family member 2
218312_s_at	ZSCAN18	− 1.867703	5.195433e−11	2.705336e−08	Down	Zinc finger and SCAN domain containing 18
221111_at	IL26	− 2.546202	5.291144e−11	2.729182e−08	Down	Interleukin 26
222557_at	STMN3	− 1.681310	5.638767e−11	2.881304e−08	Down	Stathmin 3
228407_at	SCUBE3	− 3.620062	5.857143e−11	2.921865e−08	Down	Signal peptide, CUB domain and EGF like domain containing 3
231789_at	PCDHB15	− 1.797653	5.917959e−11	2.921865e−08	Down	Protocadherin beta 15
209087_x_at	MCAM	− 2.485227	5.946642e−11	2.921865e−08	Down	Melanoma cell adhesion molecule
209369_at	ANXA3	− 3.928589	5.985347e−11	2.921865e−08	Down	Annexin A3
217867_x_at	BACE2	− 2.252919	7.211537e−11	3.399058e−08	Down	Beta-site APP-cleaving enzyme 2
232914_s_at	SYTL2	− 1.862426	7.292694e−11	3.407932e−08	Down	Synaptotagmin like 2
209032_s_at	CADM1	− 2.091452	7.821157e−11	3.623913e−08	Down	Cell adhesion molecule 1
229357_at	ADAMTS5	− 2.541169	8.822972e−11	4.019967e−e−08	Down	ADAM metallopeptidase with thrombospondin type 1 motif 5
203397_s_at	GALNT3	− 2.429144	9.939276e−11	4.491157e−08	Down	Polypeptide *N*-acetylgalactosaminyltransferase 3
229498_at	MBNL3	− 2.528113	1.010068e−10	4.526678e−08	Down	Muscleblind like splicing regulator 3
209031_at	CADM1	− 3.847247	1.038851e−10	4.578413e−08	Down	Cell adhesion molecule 1
214803_at	CDH6	− 1.702792	1.059892e−10	4.599175e−08	Down	Cadherin 6
227498_at	SOX6	− 3.164997	1.127542e−10	4.778941e−08	Down	SRY-box 6
226301_at	SLC18B1	− 1.605550	1.150978e−10	4.803798e−08	Down	Solute carrier family 18 member B1
1562736_at	LHX9	− 2.638738	1.206855e−10	4.998849e−08	Down	LIM homeobox 9
200974_at	ACTA2	− 1.428172	1.366756e−10	5.576670e−08	Down	Actin, alpha 2, smooth muscle, aorta
205479_s_at	PLAU	− 2.708964	1.436906e−10	5.734512e−08	Down	Plasminogen activator, urokinase
219874_at	SLC12A8	− 2.682319	1.537259e−10	6.090555e−08	Down	Solute carrier family 12 member 8
209840_s_at	LRRN3	− 3.596643	1.582521e−10	6.131230e−08	Down	Leucine rich repeat neuronal 3
215856_at	SIGLEC15	− 2.101512	1.586390e−10	6.131230e−08	Down	Sialic acid binding Ig like lectin 15
204401_at	KCNN4	− 2.168128	2.027717e−10	7.541864e−08	Down	Potassium calcium-activated channel subfamily N member 4
214587_at	COL8A1	− 2.937927	2.074186e−10	7.585256e−08	Down	Collagen type VIII alpha 1 chain
209082_s_at	COL18A1	− 1.797901	2.090193e−10	7.585256e−08	Down	Collagen type XVIII alpha 1 chain
201149_s_at	TIMP3	− 3.120352	2.250222e−10	8.041234e−08	Down	TIMP metallopeptidase inhibitor 3
209550_at	NDN	− 1.404076	2.340185e−10	8.308416e−08	Down	Necdin, MAGE family member
214298_x_at	SEPT6	− 1.489261	2.404714e−10	8.482434e−08	Down	Septin 6
201578_at	PODXL	− 2.096916	2.461333e−10	8.626499e−08	Down	Podocalyxin like
210302_s_at	MAB21L2	− 3.081215	2.545884e−10	8.866000e−08	Down	Mab-21 like 2
209387_s_at	TM4SF1	− 2.554463	2.601970e−10	8.952299e−08	Down	Transmembrane 4 L six family member 1
209841_s_at	LRRN3	− 3.446559	2.603412e−10	8.952299e−08	Down	Leucine rich repeat neuronal 3
202458_at	PRSS23	− 1.334742	2.712644e−10	9.269614e−08	Down	Protease, serine 23
226372_at	CHST11	− 1.951456	3.048322e−10	1.016262e−07	Down	Carbohydrate sulfotransferase 11
225314_at	OCIAD2	− 2.693849	3.081460e−10	1.021084e−07	Down	OCIA domain containing 2
203180_at	ALDH1A3	− 2.754089	3.412934e−10	1.110727e−07	Down	Aldehyde dehydrogenase 1 family member A3
231726_at	PCDHB14	− 1.586856	3.696484e−10	1.181902e−07	Down	Protocadherin beta 14
205533_s_at	CDH6	− 1.582963	3.798708e−10	1.207525e−07	Down	Cadherin 6
212909_at	LYPD1	− 1.648163	3.862414e−10	1.220679e−07	Down	LY6/PLAUR domain containing 1
200706_s_at	LITAF	− 3.779163	4.173324e−10	1.311359e−07	Down	Lipopolysaccharide induced TNF factor
228121_at	TGFB2	− 1.698335	4.257556e−10	1.330182e−07	Down	Transforming growth factor beta 2
223093_at	ANKH	− 1.289004	4.312648e−10	1.339739e−07	Down	ANKH inorganic pyrophosphate transport regulator

Table 1 (continued)

Affy ID	Gene symbol	logFC	*p* value	FDR	Up/down regulation	Gene name
223349_s_at	BOK	− 2.477976	4.350992e−10	1.340445e−07	Down	BOK, BCL2 family apoptosis regulator
235542_at	TET3	− 2.408419	4.388471e−10	1.340445e−07	Down	Tet methylcytosine dioxygenase 3
202720_at	TES	− 1.212527	4.583632e−10	1.392278e−07	Down	Testin LIM domain protein
201842_s_at	EFEMP1	− 1.292132	4.649108e−10	1.404365e−07	Down	EGF containing fibulin like extracellular matrix protein 1
209099_x_at	JAG1	− 1.553905	5.075516e−10	1.516414e−07	Down	Jagged 1
228802_at	RBPMS2	− 1.649178	5.541944e−10	1.629063e−07	Down	RNA binding protein with multiple splicing 2
227337_at	ANKRD37	− 1.563917	5.605372e−10	1.636119e−07	Down	Ankyrin repeat domain 37
204197_s_at	RUNX3	− 3.196364	5.625796e−10	1.636119e−07	Down	Runt related transcription factor 3
219935_at	ADAMTS5	− 2.587473	5.731712e−10	1.639400e−07	Down	ADAM metallopeptidase with thrombospondin type 1 motif 5
225864_at	FAM84B	− 3.333123	5.797697e−10	1.642431e−07	Down	Family with sequence similarity 84 member B
236562_at	ZNF439	− 1.698490	5.995016e−10	1.672334e−07	Down	Zinc finger protein 439
227566_at	NTM	− 1.310032	6.174443e−10	1.713643e−07	Down	Neurotrimin

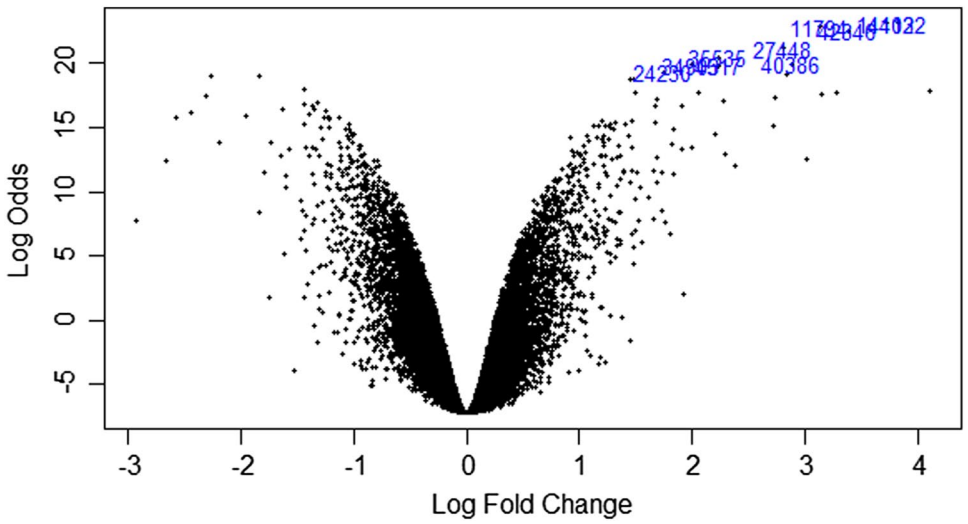

Fig. 2 Volcano plot of differentially expressed genes. Genes with a significant change of more than twofold were selected

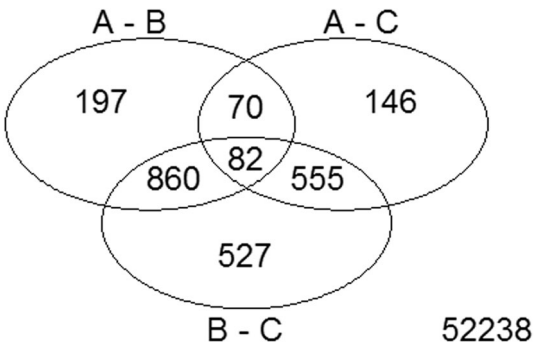

Fig. 3 Venn diagram of differentially expressed genes (DEGs) in three groups. A vs. B represents DEGs between A and B; A vs. C represents DEGs between A and C; B vs. C represents DEGs between B and C. *A* normal fibroblasts group, *B* granulin-stimulated fibroblasts group, *C* cancer-associated fibroblasts group

Furthermore, the down-regulated genes were essentially involved in the GO BP categories of cell adhesion (including CCL2, CDH6, COL18A1, COL8A1, COMP, CXCL12, ENG, ITGA6, MCAM, NCAM1, NTM, PCDHB15, PODXL, SORBS2, SUSD5, and VCAM1, p = 9.03e−10) and extracellular matrix organization (including ADAMTS5, BMP4, COL10A1, COL11A1, COL18A1, COL8A1, COMP, EFEMP1, ITGA6, NCAM1, TGFB2 and VCAM1, p = 4.65e−8), GO MP categories of *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase activity (including CHST11and CHST15, p = 0.0000243) and calcium ion binding (including ANXA3, CDH6, CELSR1, COMP, EFEMP1, FLG, GALNT3, JAG1, MGP, PCDHB14, PCDHB15, SCUBE3 and SYTL2, p = 0.0000335) and GO CC categories of extracellular space (including ACTA2,

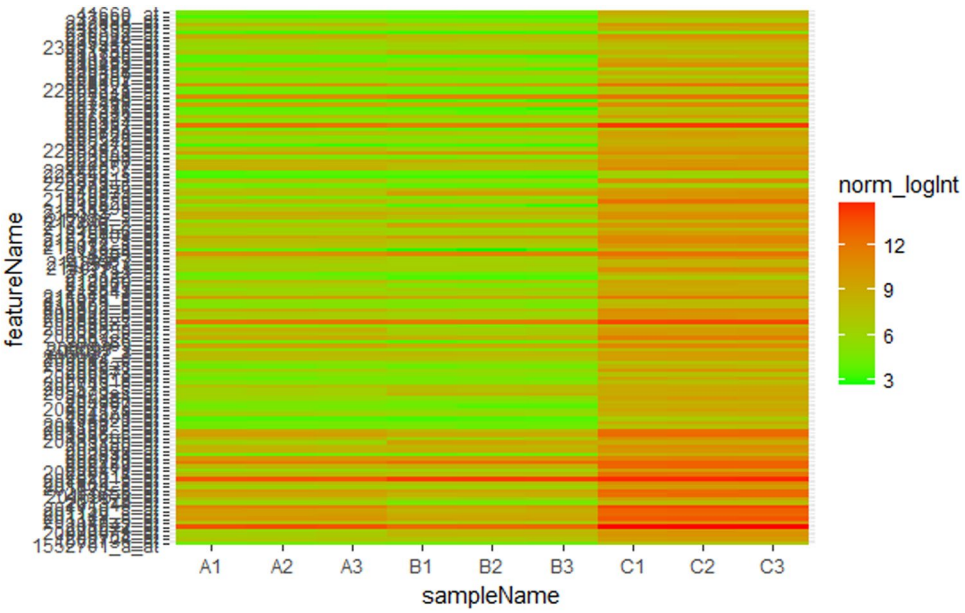

Fig. 4 Heat map of differentially expressed genes. The red colour represents down-regulated gene and the green represents up-regulated gene in TNBC samples. Legend on the top left indicate log fold change of genes (A1, A2, A3: normal fibroblasts group; B1, B2, B3: granulin-stimulated fibroblasts group; C1, C2, C3: cancer-associated fibroblasts group)

BMP4, CCL2, COL18A1, COMP, CXCL12, EFEMP1, ENG, GPX3, IL26, KRT81, LRRC17, MCAM, PLAU, PODXL, TGFB2, TIMP3 and VCAM1, $p = 0.000258$) and extracellular matrix (including COL18A1, COL8A1, COMP, EFEMP1, MGP, TGFB2 and TIMP3, $p = 0.000258$) (Table 2).

Pathway enrichment analysis

After the pathway enrichment analysis, up-regulated genes were mainly associated with the WIKIPATHWAYS categories of myometrial relaxation and contraction pathways (including ADM, ATF5, IGFBP3 and IGFBP5, $p = 0.001$) (Table 3).

Furthermore, the down-regulated genes were essentially involved in the WIKIPATHWAYS categories of endochondral ossification (ADAMTS5, CHST11, COL10A1, MGP, PLAU, RUNX3, SOX6, TGFB2, TIMP3, $p = 4.27e{-}11$) and TGF beta signalling pathway (including BMP4, ENG, LEF1 and RUNX3, $p = 0.00016$), REACTOME categories of extracellular matrix organization (including ADAMTS5, CHST11, COL10A1, MGP, PLAU, RUNX3, SOX6, TGFB2 and TIMP3, $p = 9.35e{-}9$) and integrin cell surface interactions (including COL10A1, COL18A1, COL8A1, COMP, ITGA6 and VCAM1, $p = 0.00000408$) and PID_NCI categories of beta1 integrin cell surface interactions (including COL11A1, COL18A1, ITGA6, PLAU, TGM2 and VCAM1, $p = 8.35e{-}7$), and KEGG categories of malaria (including CCL2, COMP, TGFB2 and VCAM1, $p = 0.000102$) and glycosaminoglycan biosynthesis—chondroitin sulfate/dermatansulfate (including CHST11, CHST15 and CHSY3, $p = 0.000127$) (Table 3).

PPI network analysis

The PPI networks of up- and down-regulated genes are shown in Figs. 5a and 6a, respectively. The up-regulated network was constructed with 472 nodes and 529 edges (A). The proteins cyclin dependent kinase inhibitor 2A (CDKN2A, degree = 148), membrane matalloendopeptidase (MME, degree = 78), PBX homebox 1 (PBX1, degree = 39), insulin like growth factor binding protein 3 (IGFBP3, degree = 25) and transcription factor AP-2 gamma (TFAP2C, degree = 23) were hub nodes in this network. The distribution of node degrees complied with exponential distribution. R squared and correlation coefficient are 0.618 and 0.898, respectively (Fig. 7a). The down-regulated PPI network was constructed with 1143 nodes and 1374 edges (B). The proteins vascular cell adhesion molecule (VCAM1, degree = 426), keratin 18 (KRT18, degree = 89), transglutaminase 2 (TGM2, degree = 69), actin, alpha 22, smooth muscle, aorta (ACTA2, degree = 55) and STAM binding protein (STAMBP, degree = 44) were hub proteins in this network. The distribution of node degrees complied with exponential distribution. *R* squared and correlation coefficient are 0.684 and 0.914, respectively (Fig. 7b). After the connectivity degree analysis, the top 20 nodes with high degrees for the up- and down-regulated PPI network were screened (Table 4). The connectivity degrees of the top 20 nodes in the up-regulated and down-regulated PPI were all higher than 5.

Moreover, in the four subnetworks screened from up-regulated genes, several hub genes were also identified, such as MMP3 (Fig. 5b), CDH10 (Fig. 5c), LSAMP (Fig. 5d), CSGALNACT1 (Fig. 5e) and four subnetworks for down-regulated genes, such as CARD16 (Fig. 6b),

Table 2 Enriched functions of DEGs

GO ID	GO term	Count	*p* value	Genes
Up-regulation				
Biological process				
GO:0007267	Cell-cell signaling	06	0.0000562	ADM, EFNB2, NOV, SSTR1, TFAP2C, TRHDE
GO:0008285	Negative regulation of cell proliferation	07	0.0000733	ADM, BCHE, CDKN2A, CDKN2B, IGFBP3, SSTR1, TWIST2
GO:0071493	Cellular response to UV-B	02	0.000201	MFAP4, MME
GO:0044342	Type B pancreatic cell proliferation	02	0.000201	IGFBP3, IGFBP5
GO:0006139	Nucleobase-containing compound metabolic process	03	0.000265	AK5, MTAP, PRPS2
Molecular function				
GO:0031995	Insulin-like growth factor II binding	02	0.000201	IGFBP3, IGFBP5
GO:0031994	Insulin-like growth factor I binding	02	0.000258	IGFBP3, IGFBP5
GO:0004861	Cyclin-dependent protein serine/threonine kinase inhibitor activity	02	0.00047	CDKN2A, CDKN2B
Cellular component				
GO:0016942	Insulin-like growth factor binding protein complex	02	0.0000217	IGFBP3, IGFBP5
GO:0005887	Integral component of plasma membrane	12	0.000143	EFNB2, ENPP2, EPHA5, FLRT3, KCND2, KCNJ8, MME, NLGN1, PTGER3, SLC38A5, SSTR1, TRHDE
GO:0001527	Microfibril	02	0.000322	FBN2, MFAP4
GO:0005578	Proteinaceous extracellular matrix	05	0.00044	CD248, FBN2, FLRT3, MMP3, NOV
Down regulation				
Biological process				
GO:0007155	Cell adhesion	16	9.03e−10	CCL2, CDH6, COL18A1, COL8A1, COMP, CXCL12, ENG, ITGA6, MCAM, NCAM1, NTM, PCDHB15, PODXL, SORBS2, SUSD5, VCAM1
GO:0030198	Extracellular matrix organization	12	4.65e−8	ADAMTS5, BMP4, COL10A1, COL11A1, COL18A1, COL8A1, COMP, EFEMP1, ITGA6, NCAM1, TGFB2, VCAM1
GO:0042472	Inner ear morphogenesis	05	0.000011	ALDH1A3, CELSR1, COL11A1, FOXG1, ZIC1
GO:0060326	Cell chemotaxis	05	0.0000152	CCL2, CXCL12, ENG, LEF1, VCAM1
GO:0022617	Extracellular matrix disassembly	06	0.0000296	ADAMTS5, COL10A1, COL11A1, COL18A1, COL8A1, ENG
Molecular function				
GO:0050659	*N*-Acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase activity	02	0.0000243	CHST11, CHST15
GO:0005509	Calcium ion binding	13	0.0000335	ANXA3, CDH6, CELSR1, COMP, EFEMP1, FLG, GALNT3, JAG1, MGP, PCDHB14, PCDHB15, SCUBE3, SYTL2
Cellular component				
GO:0005615	Extracellular space	18	0.0000225	ACTA2, BMP4, CCL2, COL18A1, COMP, CXCL12, EFEMP1, ENG, GPX3, IL26, KRT81, LRRC17, MCAM, PLAU, PODXL, TGFB2, TIMP3, VCAM1
GO:0031012	Extracellular matrix	07	0.0000452	COL18A1, COL8A1, COMP, EFEMP1, MGP, TGFB2, TIMP3
GO:0005578	Proteinaceous extracellular matrix	07	0.000171	ADAMTS5, BMP4, COL10A1, COMP, EFEMP1, MGP, TIMP3
GO:0030175	Filopodium	04	0.000288	ACTA2, ITGA6, PODXL, VCAM1
GO:0009897	External side of plasma membrane	06	0.000402	CXCL12, ENG, ITGA6, MCAM, NCAM1, VCAM1

GO gene ontology

Table 3 Enriched pathways of DEGs

Path ID	Path terms	Count	*p* value	Genes
Up-regulation				
WIKIPATHWAYS				
WP289	Myometrial relaxation and contraction pathways	04	0.001	ADM, ATF5, IGFBP3, IGFBP5
Down-regulation				
WIKIPATHWAYS				
WP474	Endochondral ossification	09	4.27e−11	ADAMTS5, CHST11, COL10A1, MGP, PLAU, RUNX3, SOX6, TGFB2, TIMP3
WP560	TGF beta signaling pathway	04	0.00016	BMP4, ENG, LEF1, RUNX3
REACTOME				
REACT_118779	Extracellular matrix organization	12	9.35e−9	ADAMTS5, CHST11, COL10A1, MGP, PLAU, RUNX3, SOX6, TGFB2, TIMP3
REACT_13552	Integrin cell surface interactions	06	0.00000408	COL10A1, COL18A1, COL8A1, COMP, ITGA6, VCAM1
REACT_150180	Assembly of collagen fibrils and other multimeric structures	05	0.00000775	COL10A1, COL11A1, COL18A1, COL8A1, ITGA6
REACT_120729	Collagen formation	05	0.0000687	COL10A1, COL11A1, COL18A1, COL8A1, ITGA6
REACT_120989	Chondroitin sulfate biosynthesis	03	0.00016	BMP4, ENG, LEF1, RUNX3
PID_NCI				
200016	Beta1 integrin cell surface interactions	06	8.35e−7	COL11A1, COL18A1, ITGA6, PLAU, TGM2, VCAM1
KEGG				
path:hsa05144	Malaria	04	0.000102	CCL2, COMP, TGFB2, VCAM1
path:hsa00532	Glycosaminoglycan biosynthesis—chondroitin sulfate/dermatansulfate	03	0.000127	CHST11, CHST15, CHSY3

KEGG Kyoto Encyclopedia of Genes and Genomes

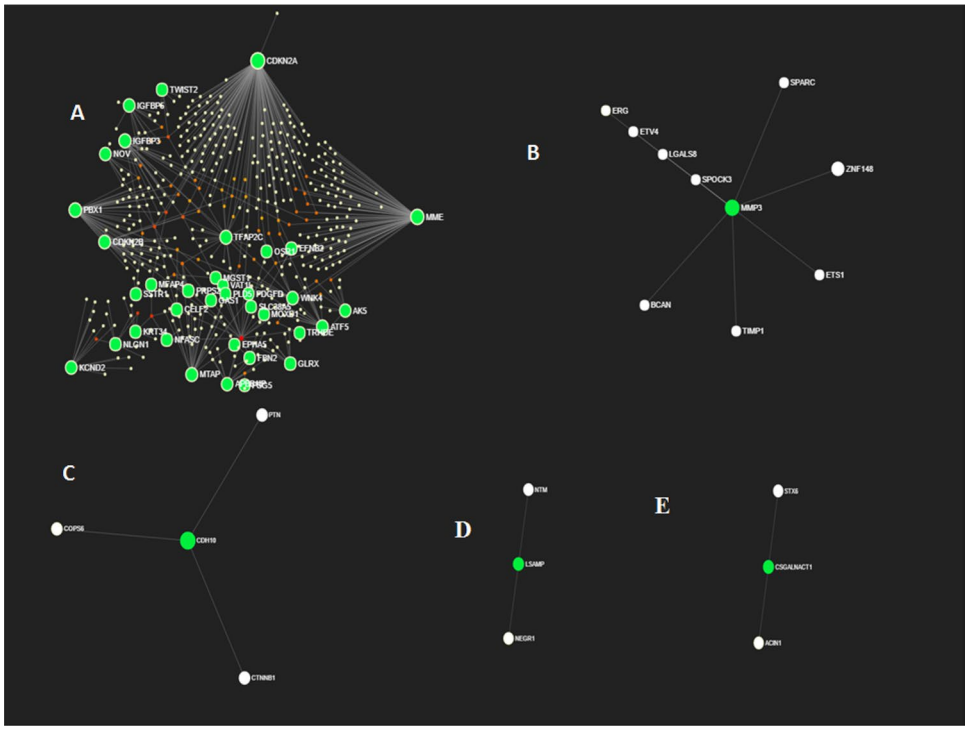

Fig. 5 Protein-protein interaction network of the DEGs (**a**). Top four PPI subnetworks in co-expression module (**b–e**) drawn from the protein-protein interaction network of the differentially expressed genes. The green circles represent for the up-regulated genes. The PPI pairs were identified with the required confidence (combined score) > 0.9 as a threshold, and the PPI network of these connections were constructed using NetworkAnalyst software

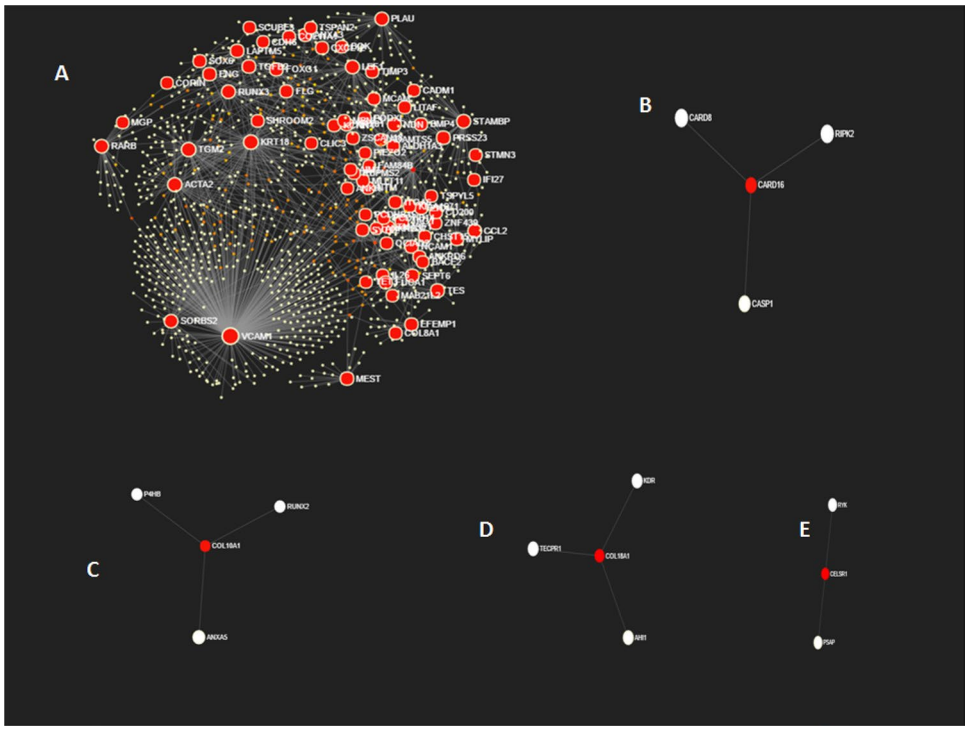

Fig. 6 Protein-protein interaction network of the DEGs (**a**). Top four PPI subnetworks in co-expression module (**b**–**e**) drawn from the protein-protein interaction network of the differentially expressed genes. The red circles represent for the up-regulated genes. The PPI pairs were identified with the required confidence (combined score) > 0.9 as a threshold, and the PPI network of these connections were constructed using NetworkAnalyst software

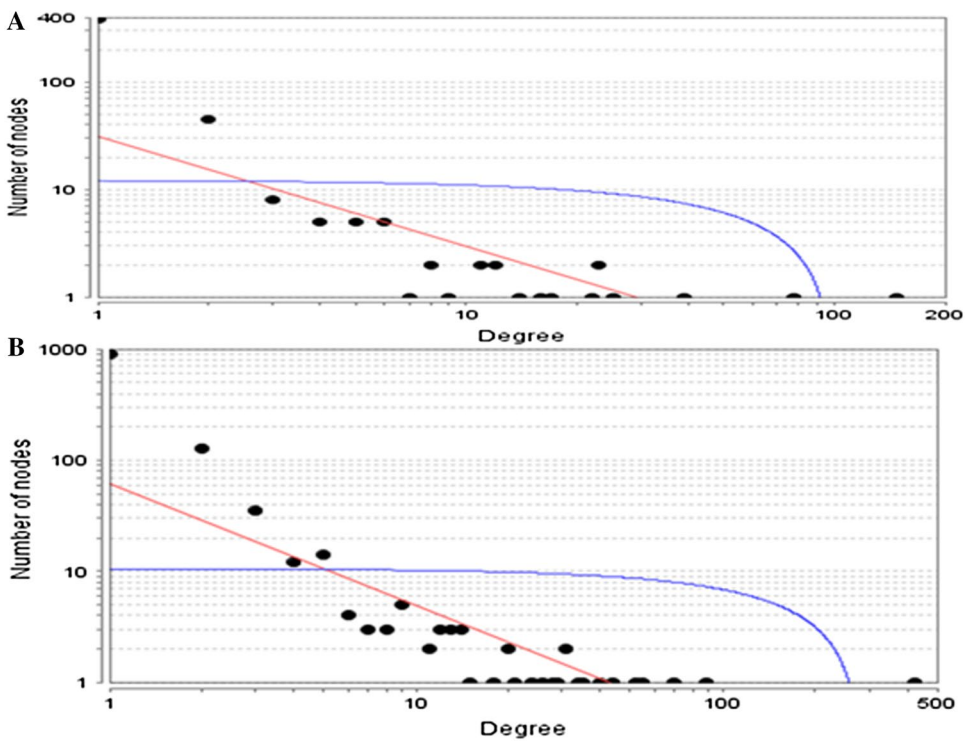

Fig. 7 **a** Scatter plot of node degree distribution for up-regulated genes; **b** scatter plot of node degree distribution for down-regulated genes

Table 4 Top 20 nodes with higher connectivity degrees in the protein-protein interaction network of the up-regulated and down-regulated differentially expressed genes

Category	Gene	Degree
Up	CDKN2A	148
Up	MME	78
Up	PBX1	39
Up	IGFBP3	25
Up	TFAP2C	23
Up	MTAP	22
Up	CDKN2B	17
Up	ATF5	16
Up	WNK4	14
Up	KCND2	12
Up	IGFBP5	12
Up	NOV	11
Up	AK5	9
Up	PRPS2	8
Up	GLRX	8
Up	NLGN1	7
Up	FBN2	6
Up	NFASC	6
Up	EFNB2	6
Up	CELF2	6
Down	VCAM1	426
Down	KRT18	89
Down	TGM2	69
Down	ACTA2	55
Down	STAMBP	44
Down	LEF1	40
Down	RUNX3	35
Down	RARB	34
Down	NDN	31
Down	PLAU	31
Down	CCL2	29
Down	SORBS2	28
Down	PRSS23	26
Down	TES	24
Down	LAPTM5	21
Down	TGFB2	20
Down	ENG	20
Down	MEST	18
Down	SEPT6	15
Down	EFEMP1	14

COL10A1 (Fig. 6c), COL18A1 (Fig. 6d), and CELSR1 (Fig. 6e).

Prediction of target hsa-mir for DEGs

The target hsa-mir for up- and down-regulated genes are presented in Figs. 8 and 9, respectively. In the up-regulated gene hsa-mir network, hsa-mir-759 ($n = 3$) and hsa-mir-4446-5p ($n = 4$) regulated the most up-regulated genes. Insulin like growth factor binding protein 5 (IGFBP5) was regulated by 143 hsa-mirs, such as hsa-mir-219a-1-3p and hsa-mir-4446-5p. Besides, hsa-mir-26a-5p ($n = 5$) and hsa-mir- 301a-3p ($n = 5$) families regulated the most down-regulated gene hsa-mir network. Gene myosin regulatory light chain interacting proteins (MYLIP) was the hub node and was regulated by 113 hsa-mirs, including hsa-mir-26a-5p and hsa-mir- 301a-3p.

Discussion

Firstly, total 66 up-regulated and 124 down-regulated genes were identified between three normal fibroblasts cultures, three granulin-stimulated fibroblasts cultures and three cancer-associated fibroblasts cultures. The result indicates that the common DEGs might play important roles during TNBC development and progression. In order to explore the potential roles of these DEGs, we performed functional analyses to them. The GO term analysis showed that up-regulated DEGs were mainly involved in cell-cell signalling and negative regulation of cell proliferation with high significant p value. The previous study shows that cell-cell signaling is mainly involved in TNBC development [34]. ADM, EFNB2, NOV, SSTR1, TFAP2C and TRHDE are biomarkers enriched in cell-cell signalling. Smirnov et al., 2006 [35] showed that ADM (adrenomedullin) was a potent vasodilator and a hypotensive agent and mainly associated with metastatic carcinomas. Higher expression of EFNB2 (ephrinB2) controls the arterial/venous specialization and vessel branching in TNBC [36]. NOV (nephroblastoma overexpressed) is a connective tissue growth factor is highly expressed in TNBC [37]. Hormone receptor gene SSTR1 (somatostatin receptor 1) play an important role in breast cancer [38]. Most of the breast cancers expressing estrogen receptor-α (ERα). TFAP2C (transcription factor AP-2 gamma) control the expression of ERα directly by binding to the ERα promoter region [39]. TRHDE (thyrotropin releasing hormone degrading enzyme) gene might increase the risk of TNBC development [40]. The key role of negative regulation of cell proliferation is associated with cancer metastasis and angiogenesis [41]. ADM, BCHE, CDKN2A, CDKN2B, IGFBP3, SSTR1 and TWIST2 are the biomarkers enriched in negative regulation of cell proliferation. Increased expression of BCHE (butyrylcholinesterase) was found in TNBC [42]. Increased risk of cancer with germline CDKN2A (cyclin-dependent kinase inhibitor 2 A) and CDKN2B (cyclin-dependent kinase inhibitor 2 B) mutation [43, 44]. High expression of mutated version of CDKN2A and CDKN2B gene in TNBC [42, 45]. Activation of altered IGFBP3(insulin like growth factor binding protein

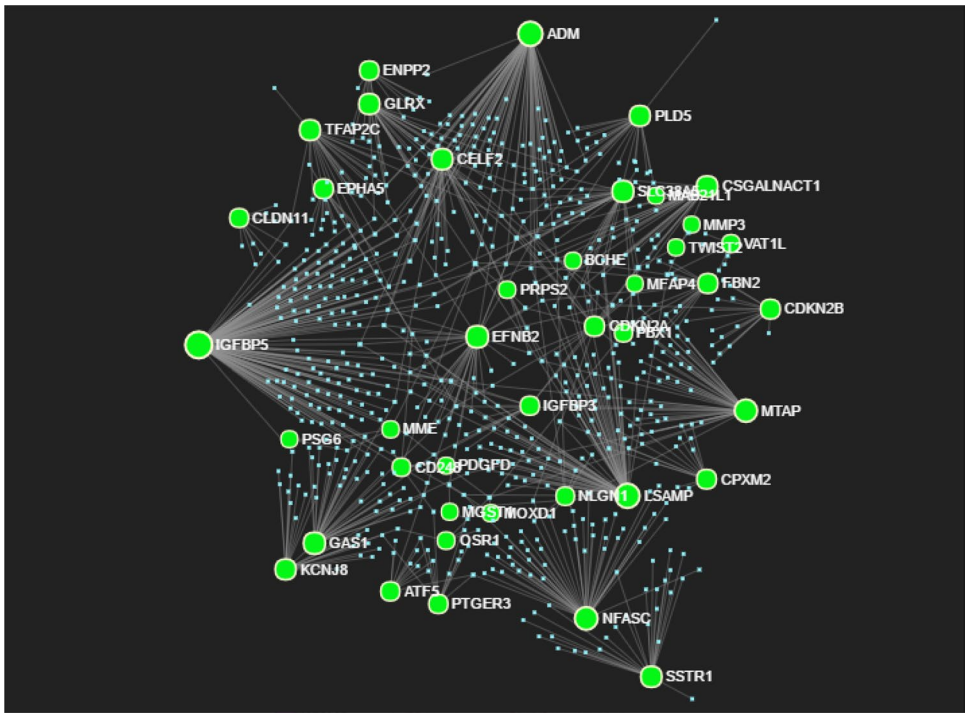

Fig. 8 Target gene-miRNA networks of Down-regulated genes. Green node stands for up-regulated genes, blue diamond stands for miRNA. The network was constructed and visualized using NetworkAnalyst software

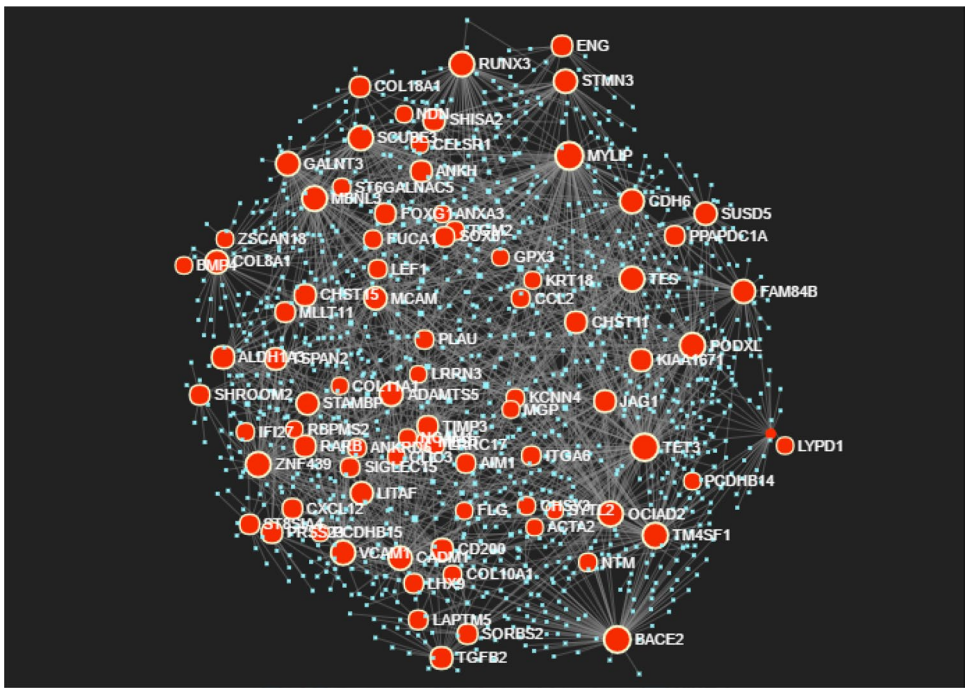

Fig. 9 Target gene-miRNA networks of down-regulated genes. Red node stands for up-regulated genes, blue diamond stands for miRNA. The network was constructed and visualized using NetworkAnalyst software

3) stimulates mitosis and inhibits apoptosis [46]. And Key et al. [47] found the increased expression of IGFBP3 was correlated with prognosis in patients with breast cancer. Expression of TWIST2 (twist family bHLH transcription factor 2) is a highly conserved basic helix-loop-helix transcription factor that play in embryogenesis and promotes cancer invasion [48]. Accumulating evidence showed that over-expression of TWIST2 was involved in breast cancer development [49]. Similarly, GO term analysis showed that down-regulated DEGs were mainly involved in cell adhesion and extracellular matrix organization with high significant *p* value. Cell-to-cell and cell-to-extracellular matrix adhesion controls the social behavior of cells in tumour development [50, 51]. Carey et al., 2010 [52] reported that cell adhesion

is primarily involved in TNBC development. Molecular markers such as CCL2, CDH6, COL18A1, COL8A1, COMP, CXCL12, ENG, ITGA6, MCAM, NCAM1, NTM, PCDHB15, PODXL, SORBS2, SUSD5 and VCAM1 enriched in cell adhesion. CCL2 (C-C motif chemokine ligand 2) negotiate between cancer cells and stromal fibroblasts that control cancer progression [53]. Expression of CCL2 is involved TNBC development [54]. Expression of CDH6 (cadherin 6) in the metastatic progression of many cancer types [55]. Polymorphism of the COL18A1 (collagen type XVIII alpha 1 chain) gene in breast cancer [56]. High molecular weight keratin COL8A1 (collagen type VIII alpha 1 chain) and variable elaboration that is diagnose in ~ 10–15% of breast cancers [57]. COMP (cartilage oligomeric matrix protein) is one of the most auspicious serologic markers with regard to an ability to prognose development of cancer and also play key roles in chondrogenesis and cartilage development in cancer [58]. CXCL12 (C-X-C motif chemokine ligand 12) and its specialized receptor, CXCR4, have newly been shown to be involved in tumorigenesis, proliferation and angiogenesis [59]. CXCL12 play a critical role in the progression in breast cancer through participating in cell adhesion [60]. ENG (Endoglin) is highly expressed in the tumor-associated vascular endothelium and is an component receptor for TGF-β that has been implicated in cancer cell detachment, migration, and invasiveness [61]. Molecular marker ENG play critical role in breast cancer progression [62]. ITGA6 (integrin subunit alpha 6) is tumour suppresser genes and mutation of this gene results in cancer progression [63]. Biomarker ITGA6 is found to be strongly associated with the TNBC [64]. MCAM (melanoma cell adhesion molecule) is a cell-surface glycoprotein molecule that is actively expressed on leading human carcinoma [65]. MCAM protein is confirmed to induce cell adhesion and induces TNBC [66]. NCAM1 (neuronal cellular adhesion molecule) expression has been corresponded with the existence of perineural invasion in specimens from a variety of tumours [67]. NCAM1 highly expressed in advanced breast cancer [68]. NTM (neurotrimin) is novel cell adhesion biomarkers expressed in many cancer types [69]. It has been reported in TNBC development [70]. Mutated PCDHB15 (protocadherin beta 15) expression was associated TNBC [71]. Molecular markers PODXL (podocalyxin like) is involved in the arrangement of both adhesion and cell morphology and cancer development [72]. Castro et al. [73] showed PODXL involvement in TNBC progression. SORBS2 (sorbin and SH3 domain containing 2) is a adapter protein that plays a key role in the assembling of signalling complexes, being a link between ABL kinases and actin cytoskeleton and also express in most of cancer development [74]. SUSD5 (sushi domain containing 5) is involved in metastatic colonization of most of the cancers [75]. SUSD5 is a one of the prognostic marker in breast cancer [76]. VCAM1 (vascular cell adhesion molecule-1) is an endothelial cell membrane glycoprotein that has been implicated in leukocyte/endothelial cell interactions in cancer cell metastasis [77]. VCAM1 was a pivotal contributor to TNBC progression [78]. ADAMTS5, BMP4, COL10A1, COL11A1, COL18A1, COL8A1, COMP, EFEMP1, ITGA6, NCAM1, TGFB2 and VCAM1 are the gene highly enriched in extracellular matrix organization. Extracellular matrix enzyme ADAMTS5 (ADAM metallopeptidase with thrombospondin type 1 motif) has emerged as key players in angiogenesis and cancer development [79]. BMP4 (bone morphogenetic protein 4) controls distinct cellular processes, such as proliferation, differentiation, and apoptosis [80]. Expression of BMP4 plays a positive role in progression of breast cancer [81]. COL10A1 (collagen type X alpha 1 chain) promotes cell proliferation in cancer development [82]. Accumulating evidence has demonstrated that expression of COL10A1 is involved in TNBC development [83]. COL11A1 (collagen type XI alpha 1 chain) important in cell invasiveness and tumour formation [84]. Molecular marker COL11A1 involves in the TNBC progression [85]. The polymorphisms of EFEMP1 (EGF containing fibulin like extracellular matrix protein 1) gene were identified with breast cancer and might share to the susceptibility of the progression of TNBC [86]. A polymorphism in the promoter of TGFB2 (transforming growth factor beta 2) that intensify expression of the protein was related with lymph node metastasis in TNBCpatients, pointing to a role of TGFB2 in the process of invasion [87].

The enriched Wikipathways pathways of up-regulated DEGs is myometrial relaxation and contraction pathways. Previous studies have shown that myometrial Relaxation and contraction pathways up-regulated genes in TNBC development can predict the overall survival of TNBC patients [88]. Several common genes enriched in these above pathways (ADM, ATF5, IGFBP3 and IGFBP5) showed up regulation. Biomarker ATF5 (activating transcription factor 5) encourage invasion by activating the expression of integrin-alpha2 and integrin-beta1 in several human cancer cell [89]. Accumulating evidence showed that over-expression of ATF5 was involved in TNBC [90]. Reticence of IGFBP3 (insulin like growth factor binding protein 3) and IGFBP5 (insulin like growth factor binding protein 5) imbalance their proliferative action and programmed cell death in breast cancer [91, 92]. The Wikipathways results showed that the down-regulated genes were significantly enriched in two pathways, which included endochondral ossification and TGF beta signalling pathway. Endochondral ossification play key role in vascularization particularly in cancer development [93]. TGF-beta signalling reinforce tumour recurrence through IL-8–dependent expansion of cancer stem-like cells (CSCs) [94]. Genes such as ADAMTS5, CHST11, COL10A1, MGP, PLAU, RUNX3, SOX6, TGFB2 and TIMP3, were significantly enriched in endochondral

ossification. Enzyme CHST11 (carbohydrate sulfotransferase 11) catalyzes the transfer of sulfate to position 4 of the *N*-acetylgalactosamine (GalNAc) residue of chondroitin [95]. CHST11 may play a explicit role in advancement of breast cancer and that its expression is restrained by DNA methylation [96]. Among the proteins involved in vascular calcium metabolism, the vitamin K-dependent MGP (matrix Gla-protein) plays a dominant role in breast cancer development [97]. PLAU (plasminogen activator, urokinase) play essential roles in tumour invasion and metastasis [98]. PLAU genes have been reported participate in the breast cancer development [99]. RUNX3 (Runt-related transcription factor 3) is a contender tumour suppressor gene and that is down-regulated in diverse cancers [100] and also activation of Wnt/β-catenin signalling in TNBC [101]. SOX6 (SRY-box 6) is tumor-suppressive function and its inactivation results in cancer progression [102]. Pinto et al. [103] found that low expression of SOX6 results in breast cancer development. Clinical exercise found that TIMP3(TIMP metallopeptidase inhibitor 3) was silenced in a number of cancer types [104]. Yuan et al. [105] revealed that TIMP3 silencing results in TNBC development. BMP4, ENG, LEF1 and RUNX3 are the down-regulated genes enriched in TGF beta signalling pathway. LEF1 (lymphoid enhancer binding factor 1) aberrantly controlled signalling pathways in cancer the WNT/β-catenin pathway plays an dominant role, since it was shown to be perilously involved in a wide range of cancer developments [106]. Delaunay and colleagues [107] demonstrated that LEF1 gene play important role breast cancer development. And for reactome pathway results showed that the down-regulated genes were most significantly enriched in extracellular matrix organization and integrin cell surface interactions. ADAMTS5, CHST11, COL10A1, MGP, PLAU, RUNX3, SOX6, TGFB2 and TIMP3 are the gene enriched in included extracellular matrix organization. Modification of extracellular matrix organization results in TNBC progression [108]. Integrin cell surface interactions including 23 alterations in integrin, laminin and collagen genes results in TNBC progression [109]. COL10A1, COL18A1, COL8A1, COMP, ITGA6 and VCAM1 are the gene enriched in integrin cell surface interactions. PID_NCI pathway results showed that the down-regulated genes were most significantly enriched in beta1 integrin cell surface interactions. Beta1 integrin cell surface interactions induces cellular proliferation results in breast cancer development [110]. COL11A1, COL18A1, ITGA6, PLAU, TGM2 and VCAM1 are gene enriched in beta1 integrin cell surface interactions. Finally KEGG pathway results showed that the down-regulated genes were significantly enriched in malaria and glycosaminoglycan biosynthesis—chondroitin sulfate/dermatansulfate. Glycosaminoglycan biosynthesis is crucial in TNBC development [111]. CHST11, CHST15 and CHSY3 are the gene enriched in glycosaminoglycan biosynthesis. Therefore, these results were consistent with previous studies and we identified critical genes involved in the PPI network.

We constructed the PPI network with up-regulated DEGs and list the top degree hub genes: CDKN2A, MME, PBX1, IGFBP3 and TFAP2C. CDKN2A was identified as one of the hub genes exhibiting the highest degree of connectivity. Over expression of MME (membrane metalloendopeptidase) plays a key role in the pathogenesis of TNBC [112]. Another hub gene PBX1 (PBX homeobox 1) is a TALE homeodomain protein and a proto-oncogene involved in the development of different types of cancers [113]. Moreover, proto oncogene PBX1 promotes the breast cancer development [114]. TFAP2C (transcription factor AP-2 gamma) is a transcription factor, which plays a very important role in the control of both estrogen receptor-alpha (ERα) and c-ErbB2/HER2 (Her2) and also promotes breast cancer development [115]. We also constructed PPI network for down-regulated genes and VCAM1, KRT18, TGM2, ACTA2 and STAMBP are the top five hub genes. VCAM1 was identified as one of the hub genes exhibiting the highest degree of connectivity. KRT18 (keratin 81) is a epithelial differentiation marker and it encodes the type I intermediate filament chain keratin 18 in breast cancer [116]. TGM2 (Transglutaminase 2) plays a crucial role in cancer cell growth and endurance through the antiapoptosis signalling pathway [117]. Down regulation of TGM2 in cancer cells is an important pathogenic factor in breast cancer [118]. Down regulation of epithelial–mesenchymal transition-associated (EMT-associated) gene ACTA2 (actin, alpha 2, smooth muscle, aorta) was correlated to invasion in TNBC [119]. Mutation of STAMBP (STAM binding protein) gene results in cancer development [120].

Subnetwork analysis of the PPI network for up-regulated genes revealed that the development of TNBC was associated with MMP3, CDH10, LSAMP and CSGALNACT1 genes. MMP3 (matrix metallopeptidase 3) is class of matrix metalloproteinase enzyme and this gene is associated with tumour cell invasion and metastasis with their promoter polymorphisms regulating the level of transcription [121]. MMP3 was a pivotal contributor to TNBC progression and could function as a potential therapeutic target [122]. CDH10 (cadherin 10) gene encodes a member of the cadherin family of calcium-dependent glycoproteins that mediate cell adhesion and controls many cellular events during cancer development [123]. CDH10 is important biomarker in maintenance of cell adhesion and polarity, alterations of which contribute to TNBC development [124]. LSAMP (limbic system-associated membrane protein) is a tumour suppressor gene and mutation of this gene results in cancer development [125]. Evidence reflected that inactivation of LSAMP gene may result in TNBC progression [126]. CSGALNACT1 (chondroitin sulfate *N*-acetylgalactosaminyltransferase 1) gene

for enzymes generating chondroitin sulfate glycosaminoglycans and altered expression of this gene results in breast cancer development [127]. We also extracted subnetwork form PPI network of down-regulated genes and CARD16, COL10A1, COL18A1 and CELSR1 hub genes in this subnetwork with highest degree of connectivity. Mutation of CARD16 (caspase recruitment domain family member 16) gene and harbour this specific genes relevant to breast cancer development [128]. The CELSR1 (cadherin EGF LAG seven-pass G-type receptor 1) gene express a biomarker that is a member of the flamingo subfamily, which is factor of the cadherin superfamily and is main factor for breast cancer development [129].

Micro RNA play essential role in cancer progression [130]. Aberrant microRNA expression profiles have been identified in breast cancer [131]. Apart from DEGs and their functions, hsa-mirs such as hsa-mir-759 and hsa-mir-4446-5p for up-regulated genes and hsa-mir-26a-5p and hsa-mir-301a-3p for down-regulated genes may be important for the progression of TNBC. Recently, it has been reported that the expression of the fibrinogen alpha gene regulated by hsa-mir-759 was associated with susceptibility to TNBC [132]. Micro RNA (hsa-mir-4446-5p) is binding site for SHANK2(SH3 and multiple ankyrin repeat domains protein 2) and is responsible for breast cancer progression [133]. Micro RNA (hsa-miR-26a-5p) act as tumour suppressors in several types of cancers targeting oncogenic genes, such as breast cancer [134], nasopharyngeal carcinoma [135], and hepatocellular carcinoma [136]. Previuos report said that hsa-miR-301a-3p acts as an oncogene in TNBC [137].

In conclusion, our data provide a comprehensive bioinformatics analysis of DEGs, which may be involved in the progress of TNBC. As a result of this preliminary study, we confirm that these DEGs, including CDKN2A, MME, PBX1, IGFBP3, TFAP2C, VCAM1, KRT18, TGM2, ACTA2, and STAMBP, may play a role in the TNBC development and could be candidate molecular targets for the treatment of TNBC. In addition, extracellular matrix organization and cell adhesion may play important roles in promoting development of TNBC. The study provides a set of useful targets for future investigation into the molecular mechanisms and biomarkers. However, further molecular biological experiments are required to confirm the function of the identified genes in TNBC.

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Author contributions BV carried out the design of this study, performed the statistical analysis and drafted the manuscript. CV collected important background information and software. AKT and SI participated in its design and coordination, visualizations and also helped to draft the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent No informed consent because this study does not contain human or animals participants.

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