EJGO European Journal of Gynaecological Oncology

Screening crucial genes involved in triple-negative breast cancer through bioinformatics analysis of microarray data

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Summary

Purpose: Triple-negative breast cancer (TNBC) is breast cancer with estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) negative. TNBC patients have a high risk of distant recurrence and death. This study aimed to investigate the mechanisms of TNBC. *Materials and Methods:* GSE38959 downloaded from Gene Expression Omnibus database included 30 TNBC cells and 13 normal mammary gland ductal cells. The differentially expressed genes (DEGs) were identified using limma package in a bioconductor and then annotated. Using the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool, their functions were predicted through enrichment analysis. After the protein-protein interaction (PPI) interactions were searched using STRING database, PPI network and modules were constructed by Cytoscape software. *Results:* A total of 2,039 DEGs were identified in TNBC cells. Among the upregulated genes, there were 36 TFs and 78 TAGs. Among the downregulated genes, a total of 46 TFs and 89 TAGs were annotated. Three significant modules were identified from the PPI network for the DEGs. Enrichment analysis showed that CCNB1, CDK1, PLK1, BUB1, and BUB1B were enriched in cell cycle, meanwhile, *PSMD4* was enriched in the proteasome pathway. Genes might also function in TNBC through interacting with others (e.g. CDK1-CCNB1, PSMD14-UCHL5, BUB1-PLK1, and BUB1-BUB1B). *Conclusion:* In conclusion, CDK1 and CCNB1 were the key genes involved in the proliferation and apoptosis of TNBC cells. BUB1, BUBR1, and PLK1 might play crucial roles in the chromosomal instability in TNBC development. The interaction between UCHL5 and PSMD14 might be the pivotal mechanism in the degradation of ER in TNBC.

Key words: Triple-negative breast cancer; Differentially expressed genes; Enrichment analysis; Protein-protein interaction network.

Introduction

Breast cancer is increasingly regarded as a complex and heterogeneous disease, which can be classified into different molecular subtypes (luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-overexpression, and basal-like subtype) with prognostic significance [1]. Among them, triple-negative breast cancers (TNBC), mostly belonging to basal-like subtype, are characterized by a lack of expression of the estrogen receptor (ER), progesterone receptor (PR), and HER2 [2, 3]. TNBC accounts for 10-30% of all breast cancers. It is typically associated with poor prognosis, due to aggressive tumor phenotype, only partially responsive to chemotherapy and lacks clinically established targeted therapies [4]. TNBC patients have a high risk of distant recurrence and death, especially within three years of diagnosis [5], and they are generally younger than the overall population of breast cancer patients [6]. Due to the lack of HER2 and hormone-receptor expression, TNBC patients cannot be treated with hormonal therapy or HER2-targeted agents, leaving cytotoxic chemotherapy as the only choice for systemic therapy [7]. Consequently, it is necessary to investigate the pathogenesis of TNBC and establish a new treatment plan.

Currently, much attention has been paid on the biomarkers and genetic factors of TNBC. Previous studies have found that transcription factor (TF) checkpoint kinase 1 (Chk1) is highly expressed [8] and the level of DNA damage signaling kinase ataxia-telangiectasia mutated (ATM) is significantly reduced [9] in TNBC. Furthermore, it is estimated that 20% of women with TNBC are breast cancer 1 (BRCA1) mutation carriers, and have a four-fold higher risk in developing contralateral breast cancer, compared to non-BRCA1 mutation carriers [10]. Nevertheless, the pathogenesis of TNBC is still difficult to elucidate and targeted therapy for TNBC is still far from clinical application.

In 2013, Kosato *et al.* performed a gene expression profile analysis for 30 TNBC cells and 13 normal breast ductal cells, and identified 301 upregulated and 321 downregulated transcripts in TNBC cells [11]. In this study, to gain better insight into TNBC, the authors further screened the differentially expressed genes (DEGs) and

Revised manuscript accepted for publication July 26, 2016

Eur. J. Gynaecol. Oncol. - ISSN: 0392-2936
XXXVIX, n. 1, 2018
doi: 10.12892/ejgo3843.2018

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predicted their underlying functions using functional and pathway enrichment analyses. Moreover, the protein-protein interaction (PPI) network of the DEGs was constructed and module analysis was performed to further explore the molecular mechanisms of TNBC.

Materials and Methods

The gene expression profile of GSE38959 deposited by Kosato *et al.* [11] was downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo), which is based on the GPL4133 Agilent-014850 Whole Human Genome Microarray 4x44K G4112F (Feature Number version) platform. GSE38959 included 30 TNBC cells (30 females, mean age = 51.9 years) and 13 normal mammary gland ductal cells (13 females, mean age = 52.2 years). The study of Kosato *et al.* has been approved by the University of Tokushima's ethics committee, and informed consent was obtained from all participants [11].

After GSE38959 was downloaded, the Linear Models for Microarray Analysis (limma) package [12] (http://www.bioconductor.org/packages/release/bioc/html/limma.html) in Bioconductor was used to preprocess the data to obtain gene expression matrix. In brief, the preprocessing process included Robust Multichip Averaging (RMA) background correction, normalization, quantile normalization and probe summarization. Then, the *t*-test method in limma package [12] was applied to identify the DEGs between TNBC and normal samples. The adjusted p-value < 0.01 and |log2fold-change (FC)| > 1 were used as the cut-off criteria.

The functional annotation of DEGs was conducted to identify the TFs using the TRANSFAC database [13] (http://www.generegulation.com/pub/databases.html#transfac). In addition, the oncogenes and tumor suppressor genes in the DEGs were also annotated based on the tumor suppressor (TS) [14] and Tumor Associated Gene (TAG) [15] databases.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway [16] and Gene Ontology (GO) functional [17] enrichment analyses were performed to identify significantly enriched pathways and functions of the DEGs using the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/) online tool [18]. The p-value ≤ 0.05 was used as the threshold.

The DEGs were submitted to Search Tool for the Retrieval of Interacting Genes (STRING) 9.1 [19] database to search PPI pairs. Only the interaction pairs that had been recorded in the database, verified by experiments, and performed with co-expression analysis or text mining were selected. All interactions in STRING were provided with a probabilistic confidence score (combined score), and in the present analysis, only interactions with combined score > 0.9 were retained. Then, the PPI network was visualized by Cytoscape software [20]. The nodes in the network were proteins encoded by corresponding DEGs and the degree of a node corresponded to the number of interactions involved in it. The proteins with high degrees were considered as hub nodes.

Using the ClusterONE plug-in of Cytoscape [20], the authors further performed cluster analysis to identify the significant functional modules of the PPI network. By DAVID software [18], protein domain and KEGG pathway enrichment analyses were performed for the DEGs in each module based on the hypergeometric distribution algorithm. The p-value ≤ 0.05 was taken as the cut-off criterion.

Table 1. — *The DEGs between TNBC cells and normal mammary gland ductal cells.*

Gene types	Gene number	TF number (Part of TFs)	TAG number (part of TAGs)
Upregulated	808	36 (ZNF83, XBP1, TP63, THRB, THRA)	78 (THRA, RUNX1T1, PDGFRA, MYB, MME)
Downregulated	1,231	46 (ZHX1, TFDP1, TEAD4, TCEB3, TCEB2)	89 (WHSC1, VEGFA, VAV2, PTTG1, PIM1)
Total	2,039	82	167

DEGs: differentially expressed genes; TNBC: triple-negative breast cancer; TF: transcription factor; TAG: tumor associated gene.

Results

A total of 2,039 DEGs were identified in TNBC cells compared with normal mammary gland ductal cells, including 808 upregulated and 1,231 downregulated genes. There were more downregulated genes than up-regulated genes. Among the upregulated genes, there were 36 TFs and 78 TAGs (including 13 oncogenes, 55 TS genes, and ten other genes). Among the downregulated genes, total 46 TFs and 89 TAGs (including 20 oncogenes, 47 TS genes, and 22 other genes) were annotated (Table 1).

The upregulated genes were significantly enriched in 212 biological processes. The top ten biological processes are listed in Table 2A, including gland development (p-value = 1.84E-12), mammary gland development (p-value = 1.35E-08), and response to hormonal stimulus (p-value = 4.14E-08). Meanwhile, the downregulated genes were significantly enriched in 503 biological processes, including cell cycle (p-value = 4.16E-52), cell cycle process (p-value = 5.48E-48), and cell cycle phase (p-value = 2.70E-47) (Table 2A).

The upregulated genes were significantly enriched in 15 pathways, including drug metabolism (p-value = 2.59E-04), focal adhesion (p-value = 8.83E-04), and prostate cancer (p-value = 1.34E-03) (Table 2B). Meanwhile, the downregulated genes were significantly enriched in 24 pathways. The top five pathways are listed in Table 2B, including cell cycle (p-value = 5.77E-21), systemic lupus erythematosus (p-value = 3.52E-08), and DNA replication (p-value = 3.54E-08).

The PPI network with 860 nodes and 3,782 interactions was constructed. The connectivity degree of each node in this PPI network was calculated. The top ten nodes with higher degrees were cyclin-dependent kinase 1 (CDK1, degree = 110), budding uninhibited by benomyl (BUB1, degree = 87), cyclin-A2 (CCNA2, degree = 84), polo-like kinase 1 (PLK1, degree = 79), kinetochore protein NDC80 homolog (NDC80, degree = 77), borealin (CDCA8, degree = 75), baculoviral IAP repeat-containing protein 5 (BIRC5,

Gene types	GO ID	Term	Gene number	*p*-value
Upregulated	GO:0048732	Gland development	28	1.84E-12
	GO:0030879	Mammary gland development	16	1.35E-08
	GO:0009725	Response to hormone stimulus	39	4.14E-08
	GO:0009719	Response to endogenous stimulus	40	1.90E-07
	GO:0042127	Regulation of cell proliferation	61	4.57E-07
	GO:0007167	Enzyme linked receptor protein signaling pathway	34	1.63E-06
	GO:0043434	Response to peptide hormone stimulus	21	2.37E-06
	GO:0051270	Regulation of cell motion	23	6.65E-06
	GO:0006936	Muscle contraction	20	8.20E-06
	GO:0003012	Muscle system process	21	9.07E-06
Downregulated				
	GO:0007049	Cell cycle	181	4.16E-52
	GO:0022402	Cell cycle process	147	5.48E-48
	GO:0022403	Cell cycle phase	124	2.70E-47
	GO:0000278	Mitotic cell cycle 1	16	2.15E-46
	GO:0000279	M phase	107	2.14E-44
	GO:0005694	Chromosome	121	3.55E-40
	GO:0044427	Chromosomal part	109	3.51E-39
	GO:0000087	M phase of mitotic cell cycle	81	2.94E-37
	GO:0007067	Mitosis	80	5.37E-37
	GO:0000280	Nuclear division	80	5.37E-37

(A)

Gene types	KEGG ID	Term	Gene number	*p*-value
Upregulated	hsa00982	Drug metabolism	11	2.59E-04
	hsa04510	Focal adhesion	20	8.83E-04
	hsa05215	Prostate cancer	12	1.34E-03
	hsa05218	Melanoma	10	3.04E-03
	hsa05200	Pathways in cancer	26	3.21E-03
	Down-regulated	hsa04110 cell cycle	48	5.77E-21
	hsa05322	Systemic lupus erythematosus	27	3.52E-08
	hsa03030	DNA replication	16	3.54E-08
	hsa04114	Oocyte meiosis	23	4.85E-05
	hsa03050	Proteasome	13	2.48E-04

(B)

Table 2. — *The significantly enriched functions and pathways for the differentially expressed genes (DEGs). (A) The top ten significantly enriched functions in biology process category for the DEGs. (B) The top five significantly enriched pathways for the DEGs.*

degree = 75), non-SMC condensin I complex subunit G (NCAPG, degree = 72), G2/mitotic-specific cyclin-B1 (CCNB1, degree = 69), mitotic spindle assembly checkpoint protein MAD2A (MAD2L1, degree = 68), which were considered as the hub nodes of this PPI network.

A total of three significant modules (Modules 1, 2, and 3) were identified from the PPI network. Module 1 had 68 nodes and 1,091 interactions. The top five nodes with higher degrees were CDK1 (which interacted with CCNB1, degree = 110), BUB1 (degree = 87), CCNA2 (degree = 84), PLK1 (degree = 79), and NDC80 (degree = 77). In Module 2, there were 43 nodes and 604 interactions. The top five nodes with higher degrees were BUB1 (degree = 87), PLK1 (degree = 79), NDC80 (degree = 77), CDCA8 (degree = 75), and BIRC5 (degree = 75). Furthermore, BUB1 could interact with both PLK1 and Budding Uninhibited by Benzimidazole 1B (BUB1B). Module 3 had 25 nodes and 160 interactions. The top five nodes with higher degrees were 26S proteasome non-ATPase regulatory subunit 14 (PSMD14, degree = 28), (proteasome 26S subunit ATPase 3 (PSMC3, degree = 27), 26S proteasome non-ATPase regulatory subunit 8 (PSMD8, degree = 25), 26S proteasome non-ATPase regulatory subunit 12 (PSMD12, degree = 24), and transcription elongation factor B polypeptide 1 (TCEB1, degree = 24) (Figure 1). In addition, PSMD14 had interaction with ubiquitin carboxyl-terminal hydrolase L5 (UCHL5).

For Module 1, there were 16 significantly enriched protein domains. Genes such as CDK1 and PLK1 were significantly enriched in four protein domains (protein kinase, ATP binding site, *p*-value = 3.37E-06, serine/threonine protein kinase, active site, *p*-value = 3.39E-06, protein kinase, core, *p*-value = 3.61E-05, and serine/threonine protein kinase-related, *p*-value = 3.22E-05). Meanwhile, CCNB1 and CDK1 were simultaneously enriched in the four significantly enriched pathways, including cell cycle (*p*-value =

8.14E-14), oocyte meiosis (*p*-value = 7.45E-08), progesterone-mediated oocyte maturation (*p*-value = 4.47E-07), and p53 signaling pathway (*p*-value = 9.75E-05). For Module 2, a total of five protein domains and two pathways were significantly enriched. PLK1, BUB1, and BUB1B were enriched in the cell cycle pathway. For Module 3, there were five significantly enriched protein domains and three significantly enriched pathways. A total of 13 DEGs (including *PSMD4*) were enriched in the proteasome pathway (Table 3).

Discussion

In this study, a total of 2,039 DEGs, including 808 upregulated and 1231 downregulated genes, were identified in TNBC cells. Among the upregulated genes, there were 36 TFs and 78 TAGs (including 13 oncogenes, 55 TS genes, and ten other genes). Among the downregulated genes, a total of 46 TFs and 89 TAGs (including 20 oncogenes, 47 TS genes, and 22 other genes) were annotated. Furthermore, three significant modules were identified from the

Gene types	Protein domain ID	Term	Gene number	*p*-value
Module 1	IPR017441	Protein kinase, ATP binding site	11	3.37E-06
	IPR008271	Serine/threonine protein kinase, active site	10	3.39E-06
	IPR000719	Protein kinase, core	10	3.61E-05
	IPR017442	Serine/threonine protein kinase-related	9	3.22E-05
	IPR001752	Kinesin, motor region	8	1.05E-10
	IPR019821	Kinesin, motor region, conserved site	8	1.05E-10
	IPR002290	Serine/threonine protein kinase	8	3.10E-05
	IPR014400	Cyclin, A/B/D/E	3	7.69E-04
	IPR004367	Cyclin, C-terminal	3	1.06E-03
	IPR006671	Cyclin, N-terminal	3	5.87E-03
	IPR013763	Cyclin-related	3	7.34E-03
	IPR006670	Cyclin	3	8.54E-03
	IPR015661	Mitotic checkpoint serine/threonine protein kinase, Bub1	2	6.95E-03
	IPR015454	G2/mitotic-specific cyclin B	2	6.95E-03
	IPR013212	Mad3/BUB1 homology region	1	2 6.95E-03
	IPR000959	POLO box duplicated region	2	1.39E-02
Module 2	IPR019821	Kinesin, motor region, conserved site	4	7.02E-05
	IPR001752	Kinesin, motor region	4	7.02E-05
	IPR015661	Mitotic checkpoint serine/threonine protein kinase, Bub1	2	3.84E-03
	IPR013212	Mad3/BUB1 homology region	1	2 3.84E-03
	IPR011516	Shugoshin, N-terminal	2	3.84E-03
Module 3	IPR001353	Proteasome, subunit alpha/beta	5	1.27E-08
	IPR001353	Proteasome, alpha and beta subunits	4	9.46E-07
	IPR000426	Proteasome, alpha-subunit, conserved site	3	5.54E-05
	IPR016050	Proteasome, beta-type subunit, conserved site	3	1.30E-04
	IPR011991	Winged helix repressor DNA-binding	3	2.94E-02

(A)

Gene types	KEGG ID	Term	Gene number	*p*-value
Module 1	hsa04110	Cell cycle	12	8.14E-14
	hsa04114	Oocyte meiosis	8	7.45E-08
	hsa04914	Progesterone-mediated oocyte maturation	7	4.47E-07
	hsa04115	p53 signaling pathway	5	9.75E-05
Module 2	hsa04110	Cell cycle	5	1.15E-05
	hsa04114	Oocyte meiosis	4	3.24E-04
Module 3	hsa03050	Proteasome	13	2.34E-20
	hsa04120	Ubiquitin mediated proteolysis	5	2.11E-03
	hsa05211	Renal cell carcinoma	4	2.78E-03

(B)

Table 3. — *The significantly enriched protein domains and pathways for genes in Modules 1, 2, and 3. (A) The significantly enriched protein domains for genes in Modules 1, 2, and 3. (B) The significantly enriched pathways for genes in Modules 1, 2, and 3.*

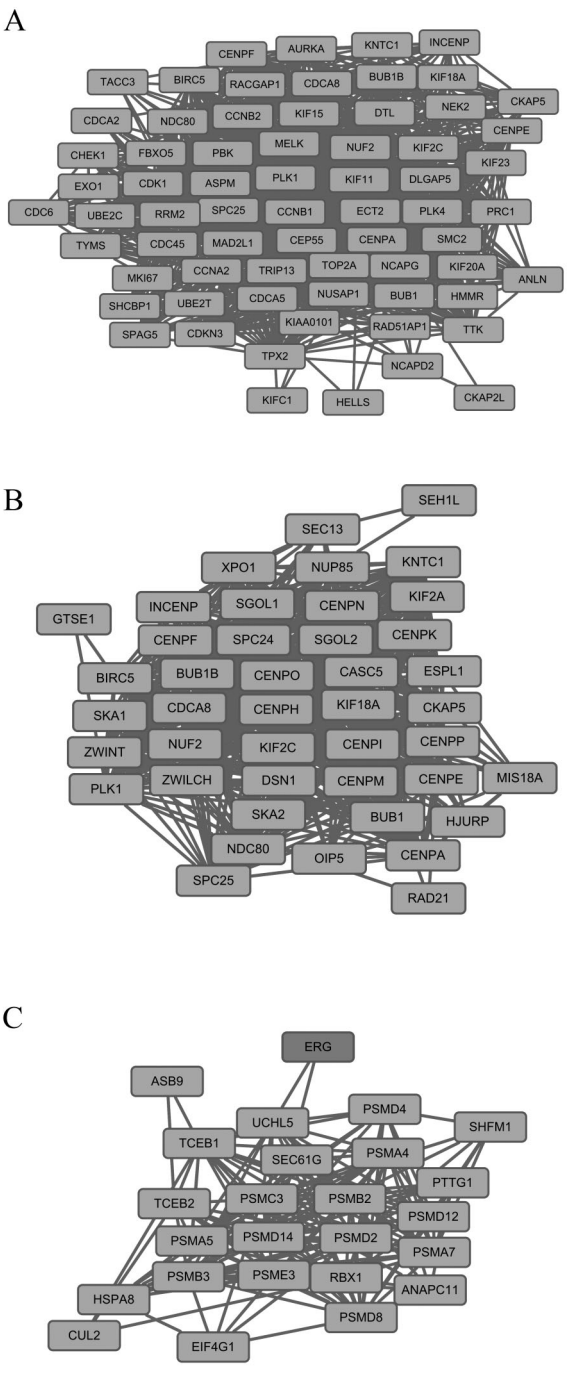

Figure 1. — The three significant modules (Modules 1, 2, and 3) identified from the protein-protein interaction (PPI) network for the differentially expressed genes (DEGs). A) Module 1 identified from the PPI network for the DEGs. B) Module 2 identified from the PPI network for the DEGs. C) Module 3 identified from the PPI network for the DEGs. The green nodes represent downregulated genes, and the red nodes represent upregulated genes. The grey lines represent the interaction between two proteins.

PPI network for the DEGs. CDK1, BUB1, and PLK1 had higher degrees both in Modules 1 and 2, meanwhile, PSMD14 was hub node in Module 3.

In Module 1, CDK1 interacted with CCNB1. CDK1, as one of the cyclin-dependent kinases (CDKs), plays a pivotal role in cell cycle regulation and cell proliferation in breast cancers [21-24]. CCNB1 is essential for the control of cell cycle at the G2/M (mitosis) transition and is involved in the genesis or progression of breast carcinomas [25]. Several studies have indicated that the suppression of CDK1 expression by roscovitine (small-molecule inhibitor of CDK1) can induce efficient apoptosis and inhibit tumor proliferation in breast epithelial cell [26, 27]. In addition, recent studies have revealed that aggressive TNBC with elevated c-Myc is uniquely sensitive to CDK inhibitors [28, 29]. Liu *et al.* have demonstrated that delivery of siRNA targeting CDK1 with the nanoparticle carrier (NPsiCDK1) induces cell viability decreasing and cell apoptosis through RNAi-mediated CDK1 expression inhibition in c-Myc overexpressed TNBC cells in mice [30]. On the other hand, overexpression of CCNB1 is responsible for G_2/M transition of the cell cycle in patients with breast cancer [31]. Moreover, the CCNB1 promoter is directly regulated by c-Myc and the overexpression of c-Myc can cause upregulation of CCNB1, thus providing additional mitotic thrust [32]. Enrichment analysis showed that CCNB1 and CDK1 in Module 1 were simultaneously enriched in cell cycle, oocyte meiosis, progesterone-mediated oocyte maturation, and p53 signaling pathway. These suggested that CDK1 and CCNB1 might play key roles in proliferation and apoptosis of TNBC cells.

Pathway enrichment indicated that PLK1, BUB1, and BUB1B in Module 2 were enriched in the cell cycle pathway. In Module 2, BUB1 was a hub node with highest degree and interacted with both BUB1B (also known as BUBR1) and PLK1. Some studies have shown that the physical interaction of BUB1 and BUBR1 is linked to the kinetochore–microtubule network in the mitotic checkpoints mechanisms [33-35]. Changes in expression levels of BUB1 and BUBR1 are often encountered in cancer cells and result in the impairment of mitotic checkpoint function [35], which is associated with the chromosomal instability in breast cancers [36]. In addition, BUB1B could suppress centrosome amplification via regulating PLK1 activity [37], meanwhile, centrosome amplification could drive chromosomal instability in breast tumor development [38]. Therefore, these interactions might play important roles in TNBC development through chromosomal instability.

In addition, PSMD14 was a hub node with highest degree and interacted with UCHL5 in Module 3. Enrichment analysis showed that PSMD4 in Module 3 was enriched in the proteasome pathway. The ubiquitin-proteasome pathway is the major mechanism for the targeted degradation of proteins with short half-lives, including ER [39]. It has been reported that estrogen receptor-α (ERα) is downregulated

by ubiquitin-proteasome pathways, which are switched by ligand binding to ERα [40]. Moreover, the existence of a proteasome-dependent mechanism is utilized to maintain a steady-state level of ERα by human breast cancer MCF-7 cells [41]. In this study, the expression of proteasome family was downregulated, which might be the reason for lacking of ER expression. It was also reported that the 26S proteasome is required for ERα and co-activator turnover and for efficient transactivation of ERα [42]. PSMD14, as one of the subunits of 26S proteasome, plays an important role in the deubiquitination and degradation induced by the 26S proteasome [43]. UCHL5 is the major deubiquitinating enzyme associated with the 26S proteasome in ubiquitin-proteasome pathway [44]. Thus, the interaction between UCHL5 and PSMD14 might contribute to the degradation of ER in TNBC.

In conclusion, through bioinformatics analysis, a total of 2,039 DEGs were identified in TNBC cells. Furthermore, CDK1 and CCNB1 might be key genes involved in proliferation and apoptosis of TNBC cells. BUB1, BUBR1, and PLK1 might play crucial roles on the chromosomal instability in TNBC development. The interaction between UCHL5 and PSMD14 might be the pivotal mechanism in the degradation of ER in TNBC. However, further studies are still needed to confirm these results.

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