Original Article

Integrated bioinformatic analysis of potential biomarkers of poor prognosis in triple-negative breast cancer

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Background: Triple-negative breast cancer (TNBC) is a heterogeneous disease associated with late-stage diagnosis and high metastatic rates. However, a gene signature for reliable TNBC biomarkers is not available yet. We aimed to identify potential key genes and their association with poor prognosis in TNBC through integrated bioinformatics.

Methods: Microarray datasets were downloaded from the Gene Expression Omnibus (GEO) database. Differentially expressed genes (DEGs) in TNBC *vs.* non-TNBC and TNBC *vs.* normal tissues were analyzed. Overlapping upregulated and downregulated DEGs were selected as inputs for Gene Ontology and pathway enrichment analyses using Metascape. Then, UALCAN and Kaplan-Meier plotter were employed to analyze the prognostic values of all overlapping DEGs.

Results: We identified 21 upregulated and 24 downregulated overlapping DEGs in TNBC *vs.* non-TNBC and TNBC *vs.* normal breast tissue. The upregulated overlapping DEGs were mainly enriched in various pathways including chromosome segregation, cell cycle phase transition, and cell division, whereas overlapping DEGs were significantly downregulated in pathways, such as multicellular organismal homeostasis, tissue homeostasis, and negative regulation of cell population proliferation. Key genes were identified by association with poor overall survival (OS). Our results showed that high expression of *CENPW* and *HORMAD1* was associated with poor OS of TNBC patients. Conversely, the low expression of *PIP*, *APOD*, and *ZNF703* indicated worse OS.

Conclusions: We identified key genes (*CENPW*, *HORMAD1*, *APOD*, *PIP*, and *ZNF703*) associated with poor OS. Thus, these genes might serve as candidate prognostic markers for TNBC.

Keywords: Triple-negative breast cancer (TNBC); bioinformatics; prognostic marker; overall survival (OS)

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Introduction

Triple-negative breast cancer (TNBC) comprises 10–20% of all breast cancers (BCs). TNBC is characterized by the lack of estrogen receptor (ER) and progesterone receptor (PR) expression and absence of human epidermal growth factor receptor 2 (*HER2*) gene amplification (1). Thus, hormone therapy and drugs that target HER2 cannot be used to treat TNBC. Chemotherapy, radiation therapy, and surgery remain the mainstays of TNBC treatment, although the outcomes are poor (2-4). Recurrence and metastases into other organs are common in TNBC patients and often lead to death from the disease (5). Patients with TNBC have worse survival than non-TNBC patients (6). TNBC tissues harbor mutations in TP53 (80%)—a tumor suppressor gene involved in tumor

occurrence and progression—more frequently than other BC subtype tissues (7,8). To date, there are no Food and Drug Administration-approved targeted therapies targeted at treating tumors harboring these mutations. Furthermore, TNBC is a highly heterogeneous tumor, and the absence of well-defined molecular targets results in difficulty in treatment (9). Therefore, it is critical to explore key genes and pathways that play an important role in the progression and prognosis of TNBC.

Integrating and reanalyzing the increasingly accumulating microarray data in many databases, could provide valuable information for the discovery of candidate genes and pathways involved in tumor progression, which in turn could lead to new hypotheses regarding cancer diagnosis, treatment, and prognosis. Nowadays, use of gene expression profile to define as prognostic biomarkers of TNBC patients have been still under-investigation. Although other studies found the genes related to poor prognosis, those finding have not applied in clinical practice (10-13). To develop the new prognostic indicators, more detailed research is required owing to the complex tumor heterogeneity and complicated molecular regulatory mechanism of TNBC. This might benefit to the clinical management of this disease. In this study, we identified overlapping DEGs which are unique gene expressions in both upregulated and downregulated and associated with poor TNBC prognosis through integrated bioinformatics. For this purpose, differentially expressed genes (DEGs) were analyzed in TNBC patients (compared with non-TNBC and normal breast tissues). Then, the overlapping DEGs across two comparisons were selected for Gene Ontology (GO) and pathway enrichment analyses. Expression and survival analysis of all overlapping DEGs was performed using UALCAN and Kaplan-Meier plotter. We present the following article in accordance with the REMARK reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-662/rc).

Methods

Microarray data collection

The overall workflow of this study is presented in *Figure 1*. The gene expression dataset analyzed in this study were obtained from the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/). A total of 141 samples were downloaded from the GSE65194 dataset, including 41 TNBC samples, 89 non-TNBC samples, and 11 normal breast tissue samples. All gene expression profiles were based on the GPL570 platform (Affymetrix Human Genome U133 Plus 2.0 Array) and were freely available online. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study has been approved by Human Research Ethics Committee (HREC), Faculty of Medicine, Prince of Songkla University, Thailand (No. REC.65-180-4-2).

Data preprocessing and identification of DEGs

To identify DEGs in TNBC, the microarray data were normalized and log-transformed using the Robust Multi-Array Average procedure (14). Then, DEGs associated with TNBC, non-TNBC, and normal breast tissues (TNBC *vs.* non-TNBC, TNBC *vs.* normal breast tissue) were analyzed using the "Limma" package (version 3.46.0) (15). Genes that met the following cutoff criteria: adjusted $P<0.05$ and $|logFC|>2$, were considered DEGs. Venn diagrams (Venny version 2.1 https://bioinfogp.cnb.csic.es/tools/venny/index.html) were generated to display the overlapping DEGs between two comparisons (16). Additionally, an online tool, Heatmapper (http://www.heatmapper.ca), was used to draw the heatmap of up- and downregulated overlapping DEGs (17).

Functional and pathway enrichment analysis

The Metascape software (http://metascape.org/) was then used for a functional enrichment analysis of the up- and downregulated overlapping DEGs. Functional enrichment was performed in three categories of GO terms, i.e., biological process, molecular function, and cellular component. Kyoto Encyclopedia of Genes and Genomes pathway enrichment was also performed. Terms with a $P<0.01$, a minimum count of 3, and an enrichment factor >1.5 (the ratio between the observed counts and the counts expected by chance) were collected and grouped into clusters based on their membership similarities. More specifically, P values were calculated based on the cumulative hypergeometric distribution. In addition, q-values were calculated using the Benjamini-Hochberg procedure to account for multiple testing. Kappa scores were used as the similarity metric when performing hierarchical clustering of the enriched terms; sub-trees with a similarity of >0.3 were considered clusters. The most significant term within a cluster was selected as the one representing the cluster (18).

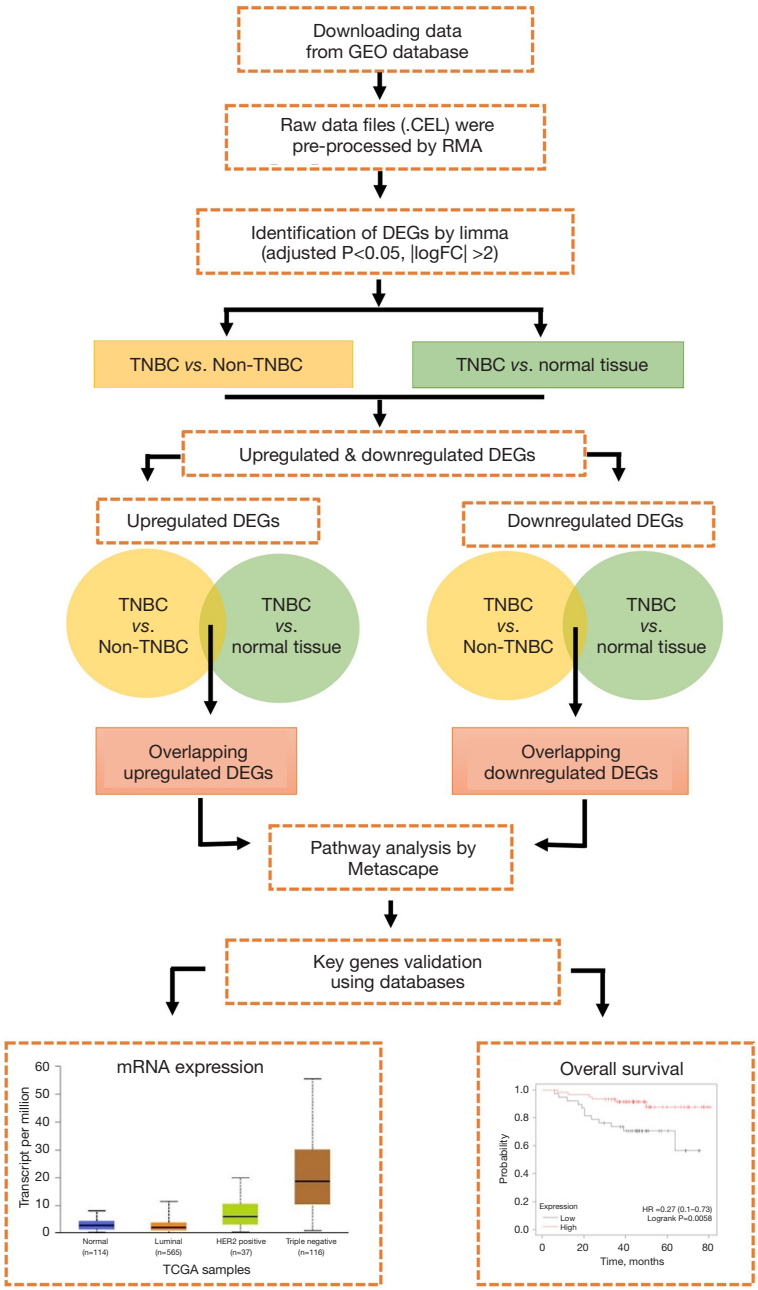

Figure 1 Workflow of the process for identifying microarray datasets for integrated analysis. GEO, Gene Expression Omnibus; RMA, Robust Multi-array Average; DEGs, differentially expressed genes; FC, fold change; TNBC, triple-negative breast cancer; TCGA, The Cancer Genome Atlas.

Construction of a protein-protein interaction (PPI) network

The interaction of PPI among the up- and downregulated DEGs were evaluated by the Search Tool for the Retrieval of Interacting Genes (STRING) database (STRING version 11; https://string-db.org/) under the default settings with a confidence of 0.4. (19).

Expression and survival analysis of overlapping DEGs

The expression levels of the up and downregulated

overlapping DEGs were validated in online clinical samples across tumor and normal samples. The University of ALabama at Birmingham CANcer data analysis Portal (UALCAN) (http://ualcan.path.uab.edu/index.html) is an interactive web portal that offers gene expression analysis and survival analysis based on clinical data from The Cancer Genome Atlas (TCGA) (20). The Kaplan-Meier plotter (http://kmplot.com/analysis) was used to validate survival analyses of TNBC patients (21). Overall survival (OS) was analyzed based on high gene expression and low gene expression. Log-rank P<0.05 was calculated and considered to indicate a statistically significant difference.

Statistical analysis

The results of mRNA expression are presented as mean ± standard error (SE). P<0.05 was considered a statistically significant difference.

Results

Identification of DEGs

For the purposes of this study, the discovery dataset (GSE65194) was analyzed to identify DEGs including 41 TNBC, 89 non-TNBC, and 11 normal tissues. Based on the criteria of adjusted P<0.05 and |logFC|>2, 117 DEGs were identified from the TNBC samples, compared with non-TNBC, including 43 upregulated genes and 74 downregulated genes; 599 DEGs were identified from comparison between TNBC samples and normal breast tissues, including 393 upregulated genes and 206 downregulated genes (available online: https://cdn.amegroups.cn/static/public/tcr-22-662-1.xlsx). Subsequently, Venn analysis was performed to obtain the intersection of the DEGs between the two comparisons. As shown in *Figure 2A*,*2B*, 21 upregulated genes and 24 downregulated genes overlapped between the two comparisons. We constructed an expression heat map for all overlapped DEGs (*Figure 2C*,*2D*).

Functional enrichment analyses of overlapping DEGs

The overlapping upregulated and downregulated DEGs were selected as the input for functional enrichment analysis using Metascape. The upregulated overlapping DEGs were significantly enriched in various biological processes, including the chromosome segregation, cell cycle phase transition, cell division, cell-substrate adhesion, and negative regulation of catalytic activity pathways (*Figure 2E*, available online: https://cdn.amegroups.cn/static/public/tcr-22-662-2.xlsx). The 24 downregulated overlapping DEGs were mainly involved in multicellular organismal homeostasis, tissue homeostasis, negative regulation of cell population proliferation, response to xenobiotic stimulus, and cell-substrate adhesion and gland development. In Reactome Gene Sets, the DEGs were enriched in signaling by nuclear receptors (*Figure 2F*, available online: https://cdn.amegroups.cn/static/public/tcr-22-662-2.xlsx).

Construction of a PPI network of overlapping DEGs

To better understand the role of both upregulated and downregulated overlapping DEGs in TNBC, PPI analysis was constructed with STRING tools. A total of 21 nodes and 19 edges were identified upregulated of DEGs in the PPI network with PPI-enrichment P<1.4e-09 which involved in chromosome segregation, mitotic cell cycle, and regulation of cell cycle process. According to downregulated of DEGs, 23 nodes and 18 edges were identified with PPI-enrichment P<1.0e-16 which protein involved in extracellular region and extracellular space (*Figure 3*, available online: https://cdn.amegroups.cn/static/public/tcr-22-662-3.xlsx).

mRNA expression and survival analysis of key genes

The UALCAN database, which contains BC patient data, was used to verify the expression levels of all up- and downregulated overlapping DEGs. The mRNA expression levels of all upregulated overlapping DEGs were all significantly higher in TNBC samples, compared with non-TNBC subtypes and normal samples (Figure S1). The upregulated *CENPW* and *HORMAD1* were significantly higher in TNBC samples compared with non-TNBC subtypes and normal samples (*Figure 4*). Moreover, the high expression of *CENPW* and *HORMAD1* were significantly increased in BC patients with *TP53* mutation compared with *TP53* non-mutation. *TP53* is a tumor suppressor gene and mutated in approximately 80% of TNBC (7). Thus, these genes might involve in tumor development, progression, and poor prognosis in TNBC. The mRNA expression levels of all downregulated overlapping DEGs were all significantly lower in TNBC samples, compared with non-TNBC subtypes and normal samples (Figure S2). In addition, the low expression of *APOD* and *ZNF703* were significantly decreased in patients with *TP53* mutation

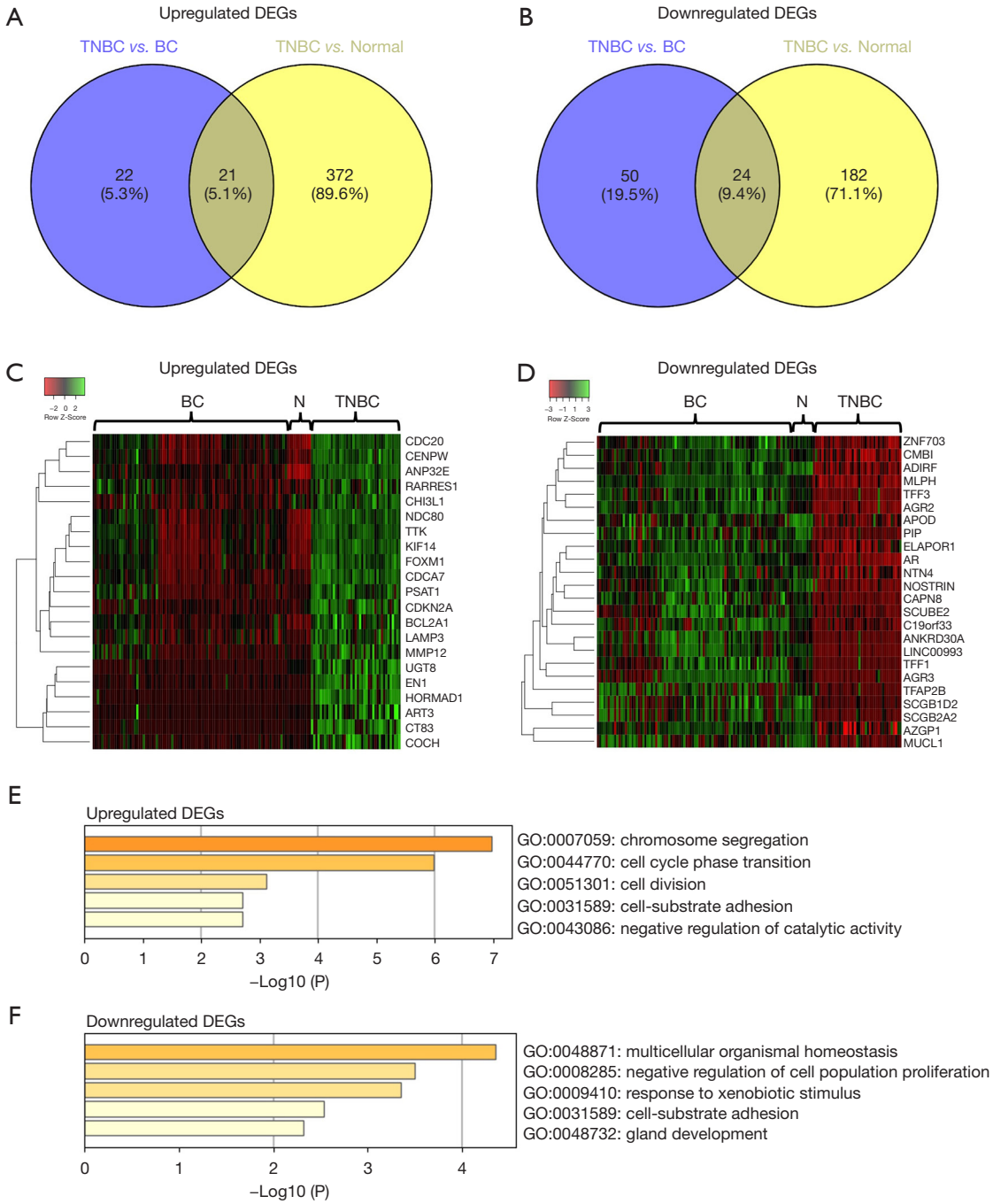

Figure 2 Identification of DEGs. Venn diagrams of the DEGs upregulated (A) and downregulated (B) in TNBC *vs.* non TNBC and TNBC vs. normal breast tissue. Heat map of the upregulated (C) and downregulated (D) overlapping DEGs. Each column represents samples, and each row represents one gene. The color spectrum ranging from green to red represents the range of downregulation or upregulation. Functional enrichment analysis of the upregulated (E) and downregulated (F) overlapping DEGs. DEGs, differentially expressed genes; TNBC, triple-negative breast cancer; BC, breast cancer.

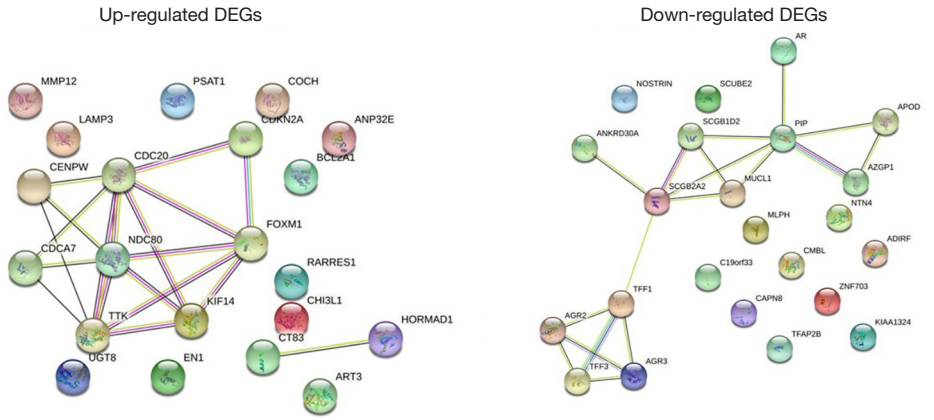

Figure 3 The protein-protein interaction network of the proteins encoded by the 21 upregulated overlapping DEGs and 24 downregulated overlapping DEGs. The nodes represent proteins, and the lines between nodes represent protein interactions. DEGs, differentially expressed genes.

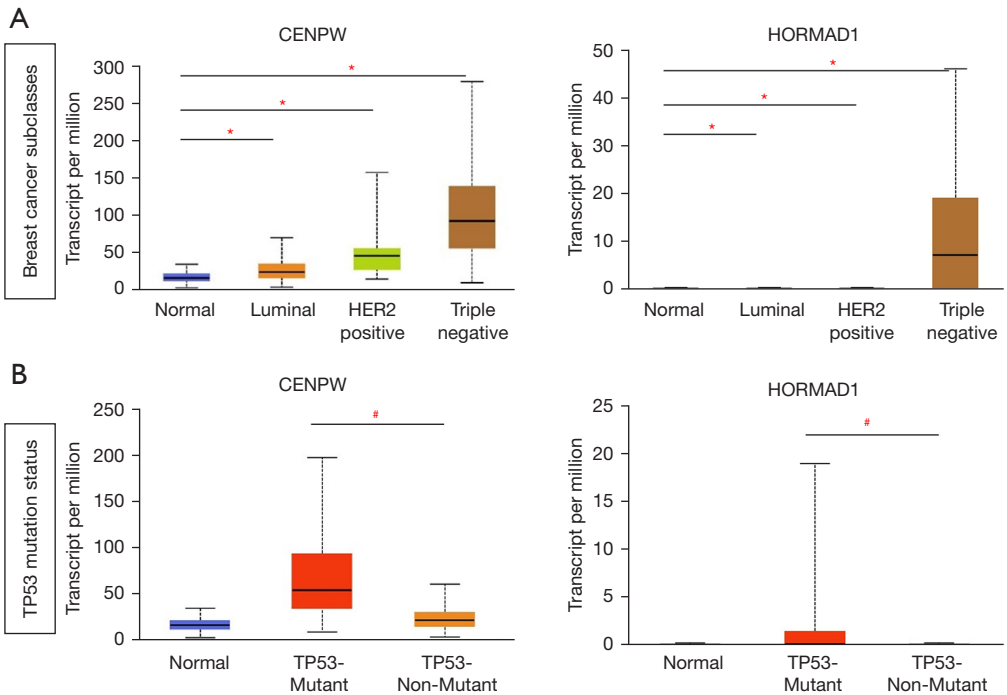

Figure 4 mRNA expression of the upregulated key genes. The expression of *CENPW* and *HORMAD1* genes in normal tissues and breast cancer tissues based on subclasses (A), and *TP53* mutation status (B). Data represent means ± SE. *, P<0.05 between each subtype and normal; #, P<0.05 between *TP53* mutant and *TP53* non-mutant status.

(*Figure 5*).

Then, survival analysis based on gene expression levels was performed using the KM plotter to predict the prognostic value of all key genes. The available data of TNBC samples on the KM plotter platform was divided into high and low expression groups according to the median mRNA level of each gene. Key genes, including *CENPW*, *HORMAD1*, *APOD*, *PIP*, and *ZNF703*, were identified by association with poor OS. Our results showed that high expression of *CENPW* and *HORMAD1* was

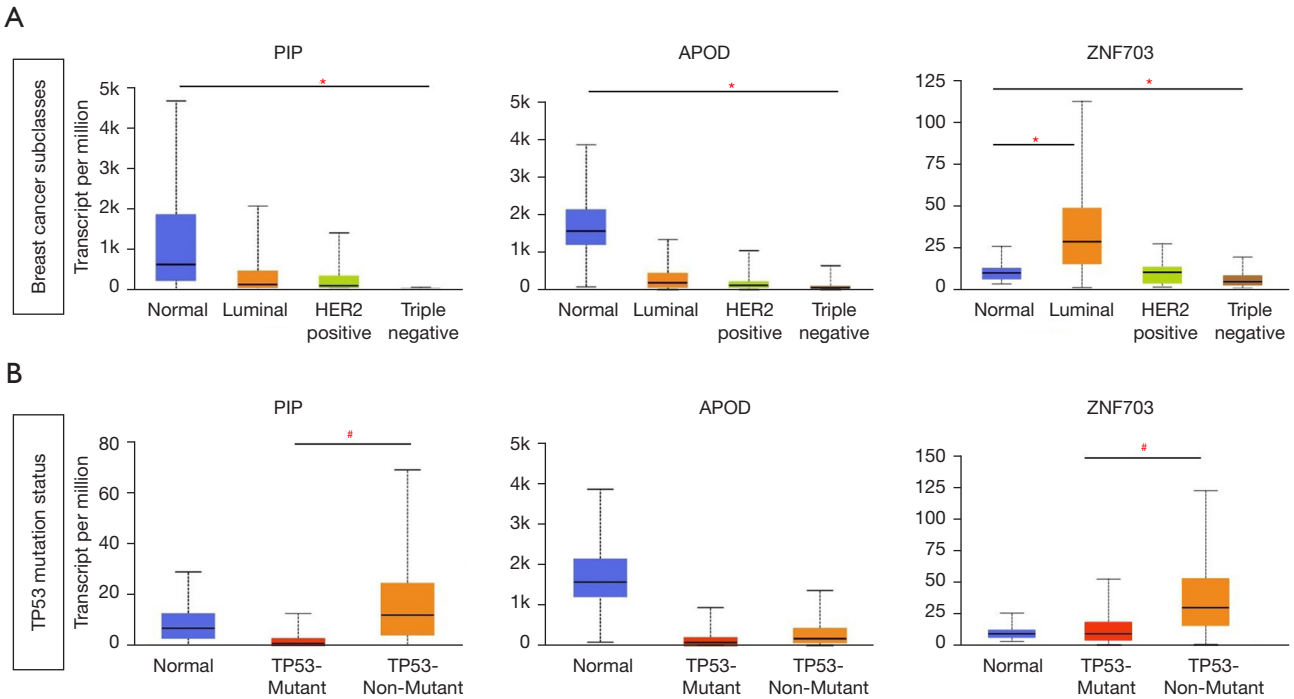

Figure 5 mRNA expression of downregulated key genes. The expression of *APOD*, *PIP*, and *ZNF703* genes in normal tissues and breast cancer tissues based on subclasses (A) and *TP53* mutation status (B). Data represent means ± SE. *, P<0.05 between each subtype and normal; #, P<0.05 between *TP53* mutant and *TP53* non-mutant status. SE, standard error.

associated with unfavorable OS of TNBC patients. Low expression of *PIP*, *APOD*, and *ZNF703* was related to worse OS in TNBC patients (log-rank P<0.05; *Figure 6*). Therefore, we speculate that these key genes could be potential biomarkers for TNBC patient prognosis.

Discussion

TNBC is the most clinically challenging subtype of all BCs owing to its poor OS rates, and high invasion and rate of metastasis. In the absence of hormone receptors in TNBC, chemotherapy is still the main adjuvant treatment (9). Considering the limited therapeutic options for TNBC and its heterogeneous pattern, it remains a very challenging disease with the poorest prognosis among the different molecular subtypes of BC (6). Thus, exploring new genes and pathways associated with TNBC may help to understand potential molecular mechanisms and to develop medical approaches with optimal precision for TNBC patients. Recent advancements in microarray and computational analysis tools have provided valuable information for the development of reliable biomarkers or gene signatures for TNBC diagnosis and prognosis. However, the precise gene expression signatures of TNBC have not been elucidated.

In the present study, we identified DEGs in TNBC (compared with non-TNBC and normal tissues), to improve the understanding of disease development, progression, and prognosis. The functional enrichment analyses of all upregulated overlapping DEGs revealed that they were significantly enriched in the chromosome segregation, cell cycle phase transition, cell division, cell-substrate adhesion, and negative regulation of catalytic activity pathways. The overexpression of genes associated with cell cycle regulation is closely related to the proliferation, recurrence, and metastasis of cancer. Hence, the pathways involving upregulated overlapping DEGs may play an important role in TNBC patients. We also analyzed the functional enrichment of all downregulated overlapping DEGs. Our results demonstrated that downregulation of genes closely associated with tissue homeostasis and negative regulation of cell population proliferation. They may involve important pathways that promote cancer progression.

Survival analysis revealed that overexpression of *CENPW*

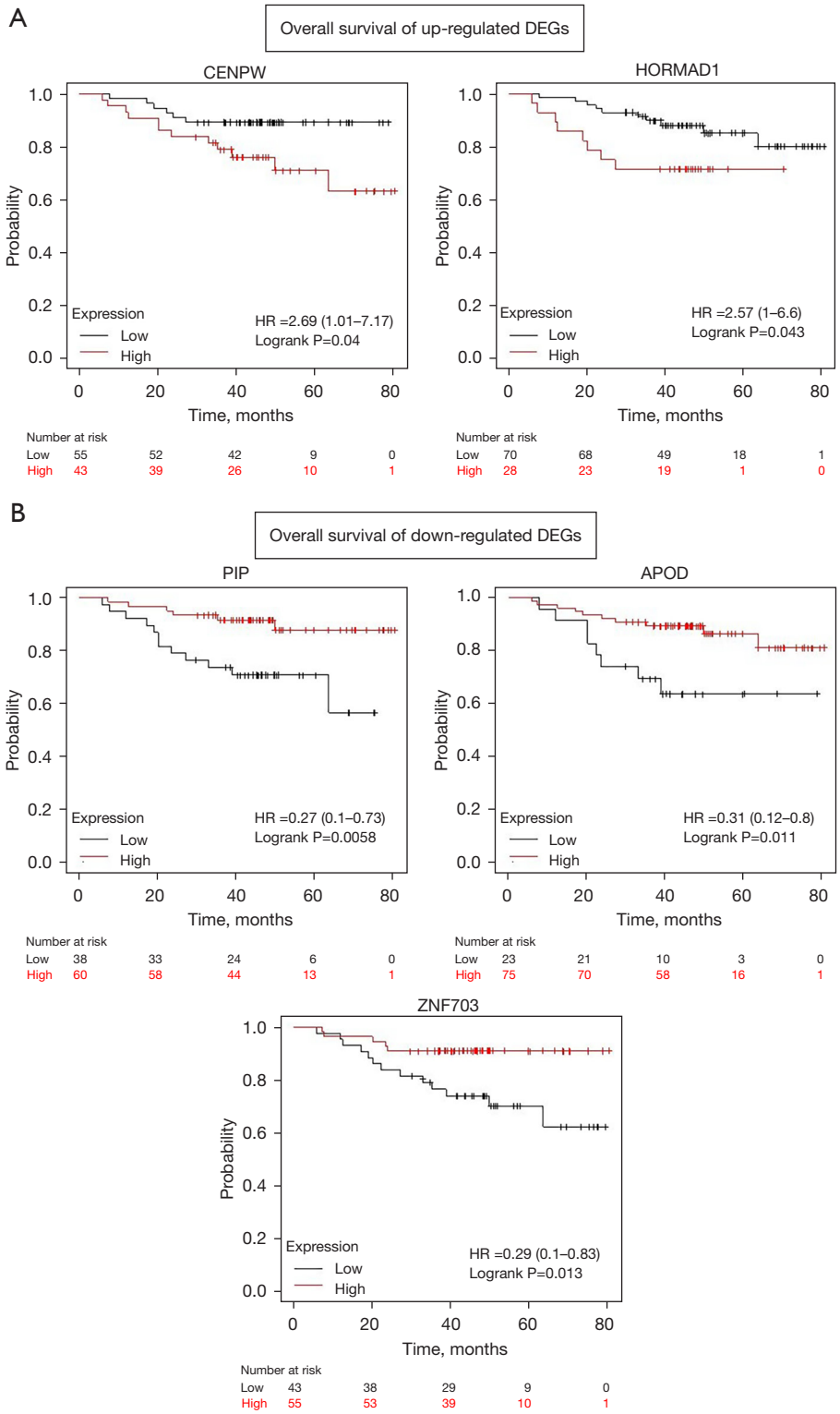

Figure 6 OS analysis of key genes in TNBC patients using Kaplan-Meier plotter. High expression levels of *CENPW* and *HORMAD1* (A) and low expression levels of *APOD*, *PIP*, and *ZNF703* (B) were significantly associated with unfavorable prognosis of TNBC patients. Log-rank P<0.05 was calculated and considered to indicate a statistically significant difference. DEGs, differentially expressed genes; OS, overall survival; TNBC, triple-negative breast cancer.

and *HORMAD1* was associated with poor OS of TNBC patients. *CENPW* is identified as a centromeric component and required for chromosome segregation during cell division (22). Its overexpression has been reported in various human cancers. The expression of *CENPW* was significantly decreased in the ER–PR–HER2+ BC subgroup, but was significantly increased in the ER–PR–HER2– BC subgroup (23). In our study, we found that *CENPW* was associated with worse OS of TNBC patients. These genes are involved in cell division, which is a crucial factor supporting cancer growth. Thus, these genes and signaling pathways might be potential prognostic biomarkers for TNBC. Through the gene expression analysis, previous studies revealed that *HORMAD1* has a function as an oncogene in TNBC (24,25). *HORMAD1* is also known as cancer testis antigen 46 and might specifically reflect the poor prognosis of TNBC. Chen *et al.* reported that high *HORMAD1* in TNBC samples has a bad prognosis (24). Due to the limited therapeutic options, chemotherapy is still mainstay of TNBC treatment. *HORMAD1* has been reported to play a role in chemotherapeutic drug resistance. *HORMAD1* silencing increased the sensitivity of TNBC cells to docetaxel (25).

The low expression of *PIP*, *APOD*, and *ZNF703* was related to worse OS in TNBC patients. In this study, we found the low expression of *PIP* and *APOD* in TNBC subtype compared to other BC. Our results are consistent with previous studies that *APOD* and *PIP* were shown to be downregulated in TNBC (26-29). *PIP* plays multiple roles in biology, including fertility, immuno-regulation, and tumor progression (30). However, the expression of *PIP* in BC still controversy in the difference of its expression between normal and BC tissues. *ZNF703* is important oncogene in BC and its expression was significantly higher in estrogen receptor (ER)-positive especially, luminal B than ER-negative cancer (31).

In our study, the high expression of *CENPW* was significantly increased in BC patients with a *TP53* mutation. *TP53* is a tumor suppressor gene that is mutated in approximately 80% of the TNBCs (7,28). BC patients with *TP53* mutations showed shorter recurrence-free survival, progression free survival, and OS (32-35). Analysis of DEGs in *TP53* mutant BCs relative to wild type *TP53* BCs showed that *CENPW*, which is cell cycle regulators, was significantly overexpressed in mutant *TP53* BCs. The poor prognosis of TNBC seems to depend on the multi-layered interaction between tumor cells, tumor stroma, and the tumor immune microenvironment. Previous studies revealed that the level of tumor-infiltrating lymphocytes (TILs) was positively associated with the good prognosis of TNBC (36-38). Thus, the integration of our key gene expression with the measurement of TILs may help to develop as a predictor of prognosis in TNBC.

In conclusion, in this study, we identified key genes and related pathways through bioinformatic analysis of DEGs in TNBC patients. Our study found that both up- and downregulated overlapping DEGs were correlated with tumor progression and poor prognosis. Some were associated with worse OS. Thus, these genes may be potential candidates for developing essential prognostic markers for TNBC patients. However, the main limitations of this study is the lack of experimental validation. *In vitro* and *in vivo* studies are needed to further elucidate the functions of these genes, specifically in relation to TNBC tumorigenesis and prognosis. Second, the TNBC sample size of KM Plotter is small, thus further research with larger sample sizes is still required to confirm our findings.

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-662/rc

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-662/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study has been approved by Human Research Ethics Committee (HREC), Faculty of Medicine, Prince of Songkla University, Thailand (No. REC.65-180-4-2).

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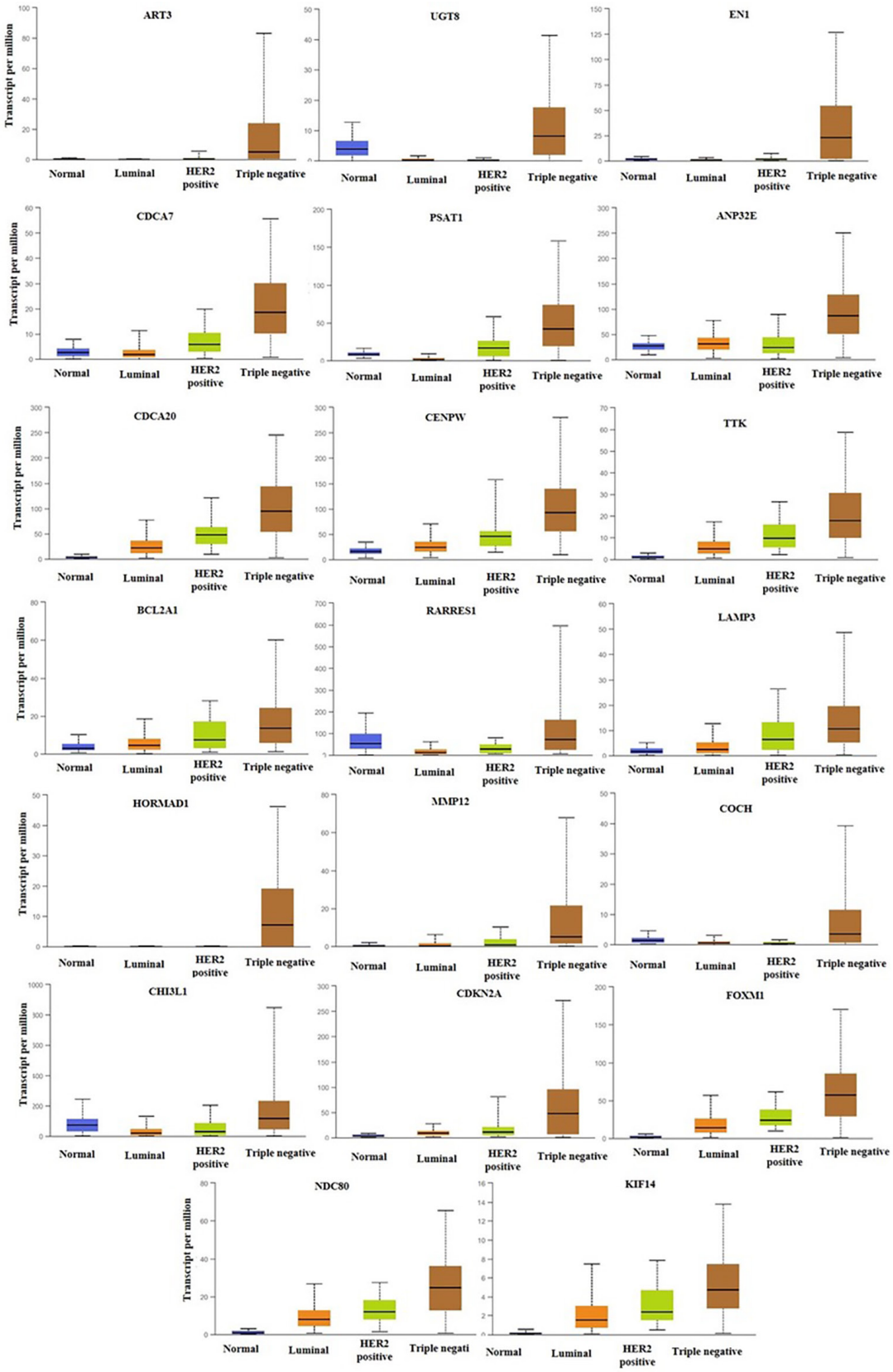

Figure S1 mRNA expression of up-regulated overlapping DEGs. DEGs, differentially expressed genes.

https://dx.doi.org/10.21037/tcr-22-662

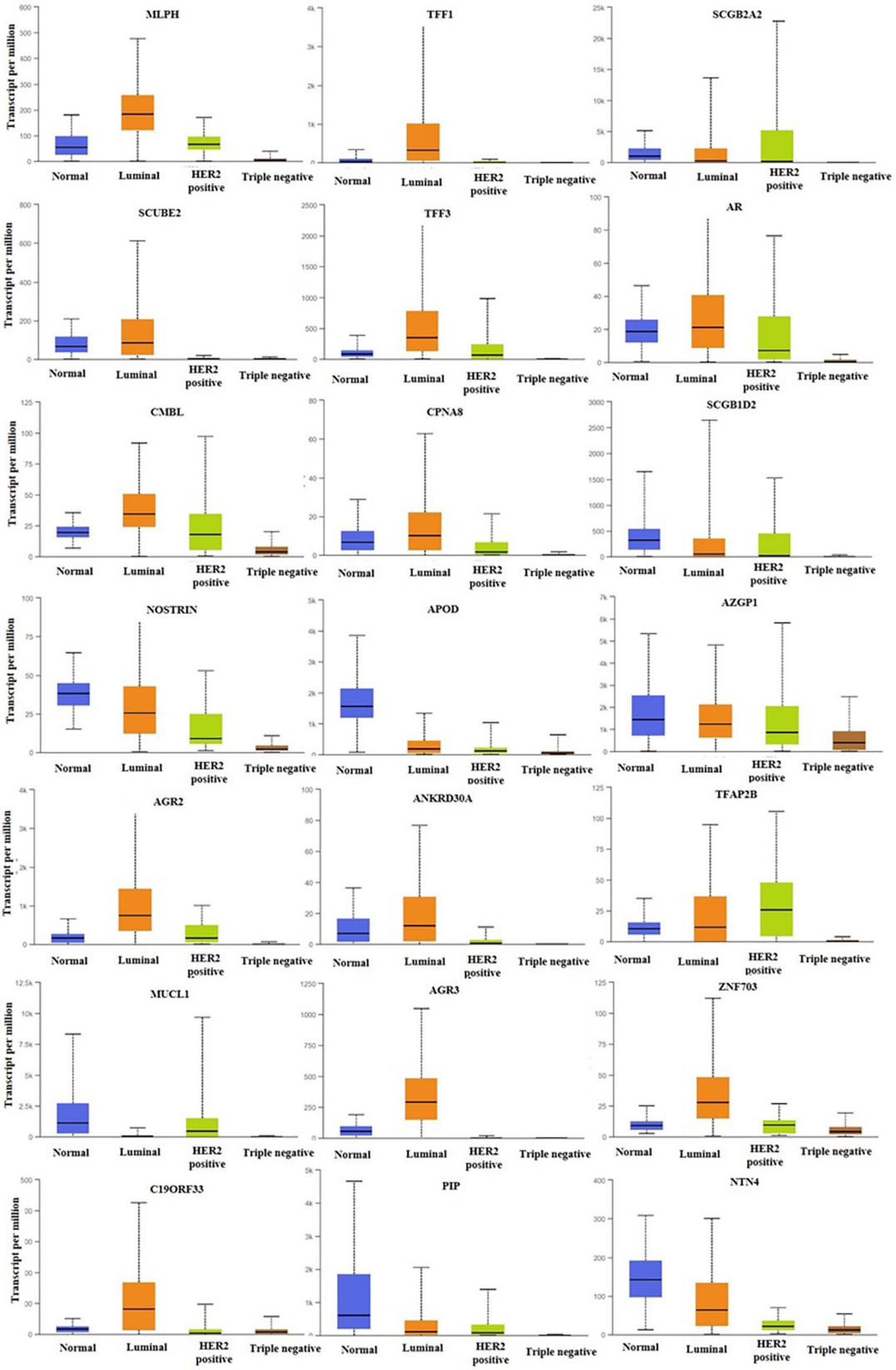

Figure S2 mRNA expression of down-regulated overlapping DEGs. DEGs, differentially expressed genes.

https://dx.doi.org/10.21037/tcr-22-662