Clinical and biological significance of estrogen receptor-positive/progesterone receptor-negative in invasive breast cancer: bioinformatic analysis

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Abstract

Improved prognostication and management of Invasive Breast Cancer (IBC) requires more precise knowledge of the molecular pathways that lead to the development and progression of cancer. Thus, we aimed to identify potential candidate genes in Estrogen Receptor-positive (ER+) and Progesterone Receptor-negative (PR-) IBC to clarify the molecular mechanisms underlying this subtype of breast cancer. A retrospective invasive breast cancer cohort was utilized in this study. An integrated bioinformatic approach was developed to assess the associations between clinical outcomes and data obtained from the Cancer Genome Atlas (TCGA). Differentially Expressed Genes (DEGs) were identified using MultiExperiment Viewer. Multiple online tools were employed to conduct functional enrichment analysis. STRING was used to create protein-protein interaction networks for the identified DEGs. The Breast Cancer Gene Miner online database was employed to analyze the associations of key hub genes with tumor features and clinical outcomes. Overall, 33 and 88 genes were found to be upregulated and downregulated, respectively, in ER+PR- IBC. The upregulated genes are mainly associated with cell proliferation, cell division, mitosis, cell-cell adhesions, and autophagy; the downregulated genes are implicated in lymphocyte migration and negative regulation of immune system processes. Analysis of the protein-protein interaction networks and gene-gene co-occurrence identified the upregulated hub genes *PIP4K2C, CDH1*, and *CLTC* are closely related to key nodes. High *PIP4K2C, CDH1*, and *CLTC* expression were associated with more aggressive tumor features ($p<0.05$) and high *PIP4K2C* and *CDH1* expression were associated with poorer disease-free survival ($p<0.05$). Overall, the study highlights key molecular biomarkers and mechanisms that could be targeted to improve the treatment of ER+PR- IBC.

Introduction

Invasive Breast Cancer (IBC) is a group of structurally complex tumors that exhibit heterogeneity in morphology, gene mutations, protein and metabolic profiles, and tumor microenvironment components.[1,2] IBC is traditionally classified into several molecular subtypes based on the expression of the biomarkers Estrogen Receptor (ER), Progesterone Receptor (PR), and Human Epidermal growth factor Receptor 2 (HER2). These classifications are predominantly used to determine the clinical treatment of patients with IBC.[3-5]

ER and PR are routinely evaluated in pathology laboratories by Immunohistochemistry (IHC). Clinical decision-making regarding treatment for patients with IBC mainly depends on the ER status of the tumor.[6] Approximately two thirds of primary IBC are ER positive (ER+). Endocrine therapy is the standard systemic treatment for ER+ IBC;[7,8] patients with ER+ IBC achieve superior outcomes after endocrine therapy compared to patients with other molecular subtypes of IBC.[9] However, approximately 30–50% of patients with ER+ IBC develop progression and recurrence,[7] and distant metastasis occurs in about 30–40% of those patients.[8] ER is a nuclear transcription factor that plays a key role in cell division and is regulated by a variety of corepressor proteins.[10] ER controls the transcription of various proteins, including PR. Expression of PR (*i.e.*, PR+ status) may reflect the presence of a functional ER pathway in IBC. Co-expression of ER and PR also indicates a functional, intact estrogen response pathway.[11,12] However, some breast tumors only express one of these hormone receptors, and approximately 7-16% of tumors are ER+/PR-.[13] Some research studies indicate that ER+ PR− breast tumors exhibit aggressive clinical characteristics and respond poorly to endocrine therapy compared to ER+PR+ IBC.[14,15] Thus, the biology and prognostic value of PR expression must be better defined to provide further insight into the clinical utility of PR status in IBC. The prognosis of patients with ER+PR- tumors remains uncertain due to the inability to predict relapse and resistance to therapy.

An explanation of the downregulation and absence of PR expression in some ER+ IBC cases is needed. The nonfunctional ER in certain tumors fails to activate the *PR* gene.[16,17]. The PR promoter methylation may silence *PR* gene transcription. Additionally, crosstalk between *ER* and growth factor signaling pathways may reduce PR protein expression.[15,18] However, the molecular mechanisms that promote tumor progression in PR–IBC are still poorly characterized. Differential Gene Expression (DGE) analysis can provide useful insights to improve our understanding of IBC and potentially lead to the discovery of the molecular distinction of underling genomic regulation in different subtypes of IBC.[7]

PR expression assessment is one of the main prognostic biomarkers in clinical practice, however the prognostic and biological value of *PR* expression in IBC is not fully understood. The aims of this study were to i) investigate the differentially expressed genes associated with the ER+PR- subtype of IBC (compared to other subtypes of IBC), ii) identify the key pathways and biological function of the potential DGE related to ER+ PR- IBC, and iii) identify the prognostic significance of candidate regulators of the ER+ PR- IBC subtype.

Materials and Methods

Databases

The gene expression RNA sequenced dataset for IBC and non-cancerous breast tissues in the Cancer Genome Atlas (TCGA) cohort were retrieved from the TCGA Firehose data portal (https://gdac.broadinstitute.org/).[19] The TCGA project was launched in 2006. Multiple studies have used this online database.[20-22] This dataset was constructed using a specific gene expression measurement platform, Agilent Human 1A Oligo UNC custom Microarrays. Data was retrieved for a total of 875 breast tissue samples, including normal tissue samples and samples from different subtypes of IBC. The available TCGA dataset comprised 100 normal breast tissue samples and 732 IBC samples; samples of unknown subtype (n = 43, 5%) were excluded. The IBC dataset contained 563 (64%) ER+ tumors, which were divided into two categories according to PR expression. ER and PR status were considered positive if ≥ 1% of cells were positive in immunohistochemical staining.[23] The reads per kilobase of transcript per million mapping reads (RPKM) values of the reads after log2 transformation were extracted from the dataset. Z-transformation (z-scores) was applied to normalize the gene expression dataset.[24] For Further validation, the association between the candidate hub genes, and IBC subtypes, tumor features and patient's outcome were analyzed using BC gene miner online tool (BC gene expression database: http://bcgenex.ico.unicancer.fr/). The IBC gene miner is an online tool that includes 15,428 IBC cases.

Analysis of differentially expressed genes

T-tests (Welch approximation) were performed with standard Bonferroni correction to identify Differentially Expressed Genes (DEGs) in each subtype of IBC compared to the normal breast samples. Differential mRNA expression was defined as a fold-change ≤ -2 or ≥ 2 and p value < 0.01 between groups. This analysis was performed using MultiExperiment Viewer (MeV) software version 4.9.[25] The differentially expressed mRNA genes in the BC subtypes (ER+PR+, ER+PR-, ER- PR+, and ER-PR- subtypes) were plotted using Venn diagrams.

Functional enrichment analysis of DEGs

Gene ontology and functional assessments were performed using the Protein Analysis Through Evolutionary Relationships (PANTHER) database (http://www.pantherdb.org/). PANTHER is a web-based system that integrates gene function, ontology, pathways, and statistical analysis tools to allow scientists to evaluate large-scale genome-wide experimental data.[26] Functional enrichment analysis of DEGs was performed using the Metascape tool (https://metascape.org/), an integrated program for functional enrichment, interactome analysis, and gene annotation analysis.[27] The differentially expressed genes in the ER+ PR- subtype were considered for this study. In addition, the PANNZER (Protein ANNotation with Z-score) tool was employed for more detailed functional annotation of the DEGs.[28]

Protein- protein interaction network and prognostics significant analysis

The STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins; https://string-db.org/) was used to predict the Protein-Protein Interaction (PPI) networks and co-occurrence of the DEGs in the ER+PR- subtype of IBC. The STRING, a comprehensive biological database, was employed to analyze the PPI networks based on experimentally studied and predicted protein associations and known functional interactions between proteins.[29,30] Furthermore, the Network Analysis tool (https://www.networkanalyst.ca/)[31] was used to map PPI networks using the STRING interactome module, with a confidence score cutoff > 0.70. The study was carried out at Shaqra University, KSA, in 2023.

Results

Identification of DEGs in different subtypes of IBC

The TCGA cohort contained 563 (64%) cases of ER+ IBC, of which 478 (55%) were ER+/PR+, 85 (10%) were ER+/PR-, 169 (19%) were ER-, 12 (1%) were ER-/PR+, and 157 (18%) were ER-/PR-. Compared to normal breast tissues, 3,855 DEGs were identified in ER+/PR- IBC, 4332 in ER+/PR+ IBC, 348 in ER-/PR+ IBC, and 4,866 in ER-/PR- IBC. Venn diagrams (Figure 1; A and B) indi-

cated that 311 DEGs were common to all four subtypes of IBC, including 106 upregulated and 205 downregulated DEGs. The ER+ subtypes (PR+ and PR-) of IBC shared 759 common genes while 121 DEGs. Overall, 33 and 88 upregulated and downregulated DEGs were identified in the ER+PR- subtype, respectively, and 704 and 6 of these DEGs were common to ER+PR+ and ER-PR+ IBC, respectively (Figure 1). The upregulated and downregulated DEGs in the ER+ PR- subtype are listed in Table 1.

Functional enrichment analysis of DEGs in the ER+PR- IBC subtypes

The biological functions of the 33 upregulated and 88 downregulated DEGs identified in the ER+PR- IBC subtype were assessed using the PANTHER and PANNZER tools. PANTHER revealed that the upregulated and downregulated DEGs are mainly associated with cellular process, biological regulation, metabolic process, signaling, and response to stimulus (Figure 1; C and D). Detailed functional assessments of the upregulated and downregulated DEGs in terms of their biological processes, molecular functions, and cellular components based on PANNZER are listed in *Supplementary Tables 1 and 2*. Furthermore, functional enrichment analysis using Metascape revealed the upregulated DEGs in the ER+PR- IBC subtypes are functionally associated with genes that are upregulated in basal-like BC. Similarly, we observed that the E12_Q6 gene set was also functionally enriched among the upregulated DEGs in the ER+PR- IBC subtype (Figure 1E). As shown in Figure 1F, functional enrichment analysis of the downregulated DEGs identified in ER+PR- IBC indicate that these genes are mainly involved in the negatively regulated immune system processes and lymphocyte migration.

Table 1. List of up- and down-regulated differentially expressed genes in the BR+PR- invasive breast cancer subtype.

Regulation	Differentially expressed genes (gene symbol)
Upregulated	*NXPH1*, *CKMT1B*, *CDH1*, *RAP1GAP*, *SIX1*, *INHA*, *PRR5-ARHGAP8*, *KIAA0319*, *AIFM3*, *SLC12A5*, *GDPD2*, *LOC100144603*, *IGSF8*, *ESRP2*, *MRPS23*, *LOC389493*, *NCDN*, *FHDC1*, *BEYLA*, *DARS2*, *CLTC*, *PTRH2*, *SRD5A3*, *ADIPOR1*, *COL24A1*, *MAP7*, *C1orf9*, *PIP4K2C*, *ORMDL2*, *TMC6*, *TRIM62*, *ARID3A*, *ELL3*
Downregulated	*C5orf56*, *ARHGAP28*, *ARHGAP21*, *SHMT1*, *IL27RA*, *AKR1B15*, *NNMT*, *TRIM61*, *LRRC34*, *FAS*, *HCG27*, *OAF*, *KIAA1409*, *PKN3*, *NHEDC2*, *PGCP*, *ADPRH*, *ARSJ*, *CUTC*, *PHLDA1*, *C21orf49*, *AHR*, *NR1H3*, *C14orf149*, *VNN1*, *TRIM22*, *SDC2*, *C10orf25*, *SLCO3A1*, *SHBG*, *EPM2A*, *LONRF1*, *CRIP3*, *GPR45*, *AK3*, *LOC284688*, *COL27A1*, *JAZF1*, *ARHGEF33*, *DCBLD2*, *RASA3*, *OTOP1*, *FAM65B*, *IRAK2*, *SLFN12*, *CD59*, *PRF1*, *GVIN1*, *C20orf197*, *P2RX1*, *CLYBL*, *VSIG4*, *ST3GAL3*, *PAM*, *SCARA3*, *CENPV*, *MBNL1*, *TNFRSF1B*, *EID3*, *TGFB2*, *GIMAP4*, *C4orf19*, *ATL1*, *ELAC1*, *DYRK3*, *CAND2*, *ZFP36L1*, *BXO32*, *KLRK1*, *LOC728989*, *CD69*, *APOL6*, *RGS9*, *LIPN*, *FCRL6*, *CASP1*, *DPEP3*, *TNFRSF11B*, *GBP2*, *ASB2*, *BHLHE22*, *OR52N2*, *PLCE1*, *NOG*, *FKBP5*, *FAM46B*, *KLK1*, *KRT23*

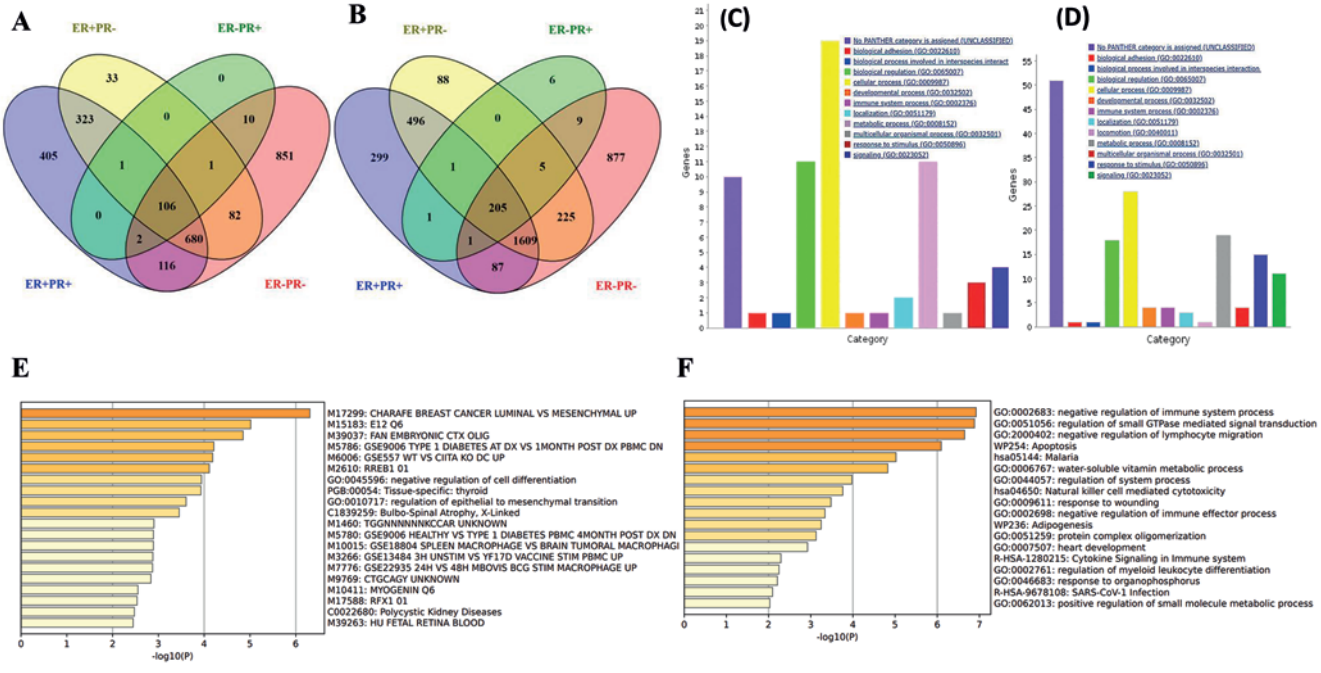

Figure 1. Venn diagrams show (A) upregulated and (B) downregulated genes in all IBC subtypes; 33 genes were uniquely upregulated, and 88 were uniquely downregulated in the ER+PR- IBC subtype. Functional enrichment analysis predicted that (C) upregulated and (D) downregulated ER+PR- subtype genes are primarily involved in cellular processes, biological regulation, metabolic processes, signaling, and response to stimulus. ER+PR- gene enrichment. (E) downregulated, (F) upregulated. Upregulated: associated with genes upregulated in basal-like, E12_Q6. Downregulated: associated with genes involved in immunity, migration, GTPase, apoptosis.

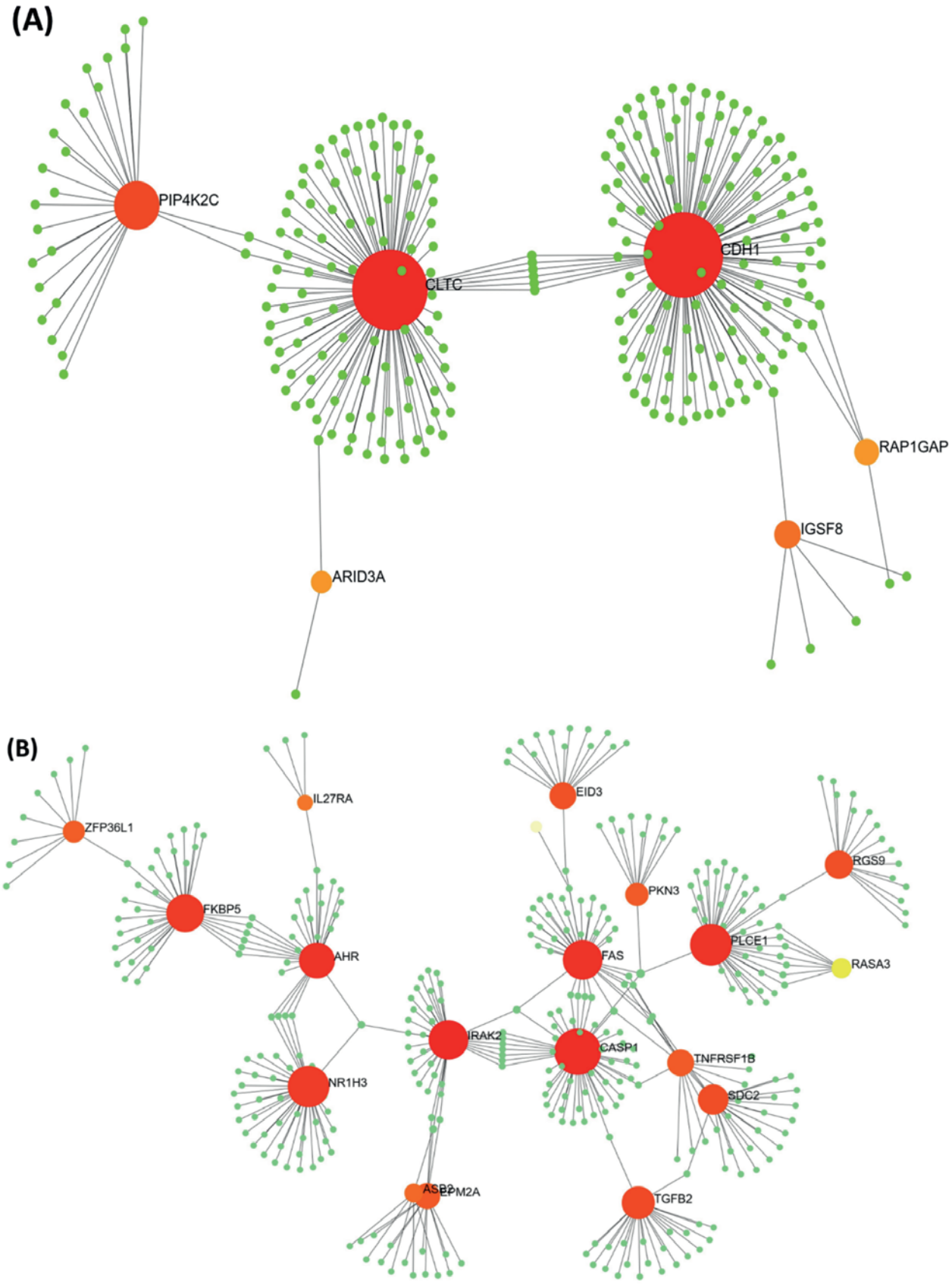

Figure 2. Protein-protein interaction networks of ER+PR- subtype genes: (A) upregulated, highlighting hub genes *PIP4K2C*, *CDH1*, and CLTC; (B) downregulated, showing high connectivity for seed genes *PLCE1*, *CASP1*, *NR1H3*, and *IRAK2*.

In addition, the DEGs downregulated in the ER+PR- IBC subtype also altered the regulation of small GTPase-mediated signal transduction and apoptosis (Figure 1F). More detailed information on the functional enrichment of the DEGs in ER+PR- IBC is shown in Figure 1; E and F.

Protein-protein interaction network analysis

Network analysis online tool STRING was used to explore PPI and establish the interactions between the 33 upregulated DEGs and 88 downregulated DEGs identified in the ER+PR- subtype of IBC. The network topology and module analyses for standard functions were assembled based on the set of interactions that comprise co-regulated nodes and nodes. This network and module analysis identified three main active hotspots for the upregulated DEGs and more than fifteen hotspots for the downregulated DEGs. The upregulated hub genes *PIP4K2C, CDH1*, and *CLTC* are densely connected to nodes (Figure 2A). The network topology and module analyses indicated a higher number of network modules with fewer degrees of nodes for the downregulated DEGs in ER+PR- IBC compared to the upregulated DEGs. As shown in Figure 2B, more than 15 of the significant hub genes with a score > 7 were downregulated DEGs. The module seed genes *PLCE1*, *CASP1, NR1H3,* and *IRAK2* exhibit high connectivity, while the other DEGs have fewer nodes.

Verification of the expression patterns and the clinical significance of hub genes

Further validation, the association between the candidate hub genes that were obtained from PPIs analysis, and IBC subtypes, tumor features and patient's outcome were analyzed using BC gene miner online tool (bc-GenExMiner). The results confirmed that *PIP4K2C, CDH1*, and *CLTC* were highly expressed in ER+PR- subtype compared with other IBC subtypes ($p<0.05$) (*Supplementary Figure 1*). High expression level of *PIP4K2C, CDH1,* and *CLTC* tumors were associated with more aggressive features such as lymph node positive, group 3 Nottingham prognostic index, and histological type (all $p<0.05$), as shown in *Supplementary Materials, Figures 2-4*.

PIP4K2C was associated with short disease-free survival ($p=0.039$, Figure 1A). *CDH1* was associated with poor overall BC survival ($p=0.0287$), short disease-free survival ($p=0.003$), and distant metastasis free survival ($p=0.003$) as shown in Figure 3 (B, C and D, respectively). According to the human protein atlas (The Human Protein Atlas), the protein expression for all hub genes is expressed in the BC. *PIP4K2C* and *CLTC* protein expressions were mainly localized in the cytoplasm of the tumor cells, while *CDH1* was mainly localized in the membrane and cytoplasm of the tumor cells, with the staining intensities varying from moderate to high (Figures 4, A, B, C).

Discussion

Invasive BC is a heterogeneous disease and can be classified into subgroups of biologically distinct entities with specific biological features and pathological characteristics.[32] The ER is one of the main prognostic and predictive factors in IBC.[33] BC cells that express the ER and/or PR typically require estrogen and/or progesterone to proliferate and survive.[32] IBC is classified as ER+ or ER-. ER+ tumors account for 70% of cases of IBC, while ER- tumors represent the other 30% of cases of IBC.[34] The ER+ tumors can be further categorized into two subgroups based on PR expression: i) ER+PR+ and ii) ER+PR-.[35] The role of PR expression in carcinogenesis of ER+ IBC subtype remains to be elucidated. A study reported that 12.2% of ER+ tumors are ER+PR- subtype and these subtype tumors exhibit a more aggressive phenotype than ER+PR+ IBC.[36] Moreover, higher PR expression in IBC is associated with a better prognosis.[32] An analysis of 666,852 cases of hormone receptor-positive IBC using the Surveillance, Epidemiology, and End Results (SEER) reported that ER+PR- tumors were less responsive to Selective ER Modulators (SERMs), such as tamoxifen.[36] Although several studies reported that loss of PR expression is implicated in disease progression and poorer response to therapy in IBC, patients with ER+ IBC frequently receive hormone therapy, regardless of PR status.[37] Therefore, understanding the biological and molecular characteristics of the ER+PR- subtype may provide new insights to enable more precise diagnosis and prognostic assessment for patients with IBC. In the current study, a comprehensive analysis of the TCGA database was conducted to identify genes associated with ER+PR- IBC subtype. Overall, 64% of the TCGA cohort employed in the current study had ER+ IBC, with ER+ PR+ being the most common type; ER+PR- tumors accounted for about 10% of the cohort, in agreement with previous studies.[32,36] We detected a significant number (n=13,401) of DEGs in the ER+/PR-, ER+/PR+, ER-/PR+, and ER-/PR- subtypes of IBC when compared to normal breast tissues. These differentially expressed genes are associated with a large variety of cellular processes, including transcription of mRNAs, signal transduction, cellular adhesion, cell cycle, transport, and metabolism that are altered in IBC compared to the normal luminal epithelium. In agreement with our findings, a previous analysis of the expression profiles contributing to malignant transformation of the luminal epithelium identified 14,245 unique transcripts in IBC compared to 10,249 individual transcripts in normal luminal epithelial cells.[38] Furthermore, we identified a total of 121 significant DGEs in ER+PR- tumors compared to the other subtypes of IBC, which indicates ER+PR- tumors exhibit distinct molecular profiles that could result in different outcomes.[39] We identified that the DEGs in ER+PR- IBC are involved in several basic biological functions, such as the control of cell division, metabolism, and responses to external stimuli, which further suggests that the pathways affected by these DEGs are involved in tumor progression and may affect the outcome of patients with ER+PR- IBC. Previous studies suggested that the growth factor signaling pathways that interact with *ER* cause downregulation of PR protein levels in IBC.[15,16,18] Functional enrichment analysis suggested that the DEGs in ER+PR- IBC exert similar functionality as genes that are implicated in basal-like BC. Basal-like BC has more genetic complexity and is distinct from other IBC subtypes.[40] Basal-like BC are more aggressive, and patients have shorter metastasis-free survival compared to patients with the luminal subtype of IBC.[41] Some upregulated DEGs in ER+PR- IBC are also functionally similar to the E12_Q6 pathway, which contains several transcription factors. About 20% of known oncogenes are transcription factors. Cancer cells require the constitutive expression of a variety of transcription factors to sustain the various biological processes that support their cellular transformation, growth, and survival.[42]

The PPI analysis suggested that upregulated differentially expressed genes *PIP4K2C, CDH1* and *CLTC* are densely connected network modules, which are likely to involve together in promoting the growth and development of cancer cells. For further validation, the Breast Cancer Gene Expression Miner database showed that the *PIP4K2C, CDH1* and *CLTC* are highly expressed in ER+PR- subtype compared to other subtypes.

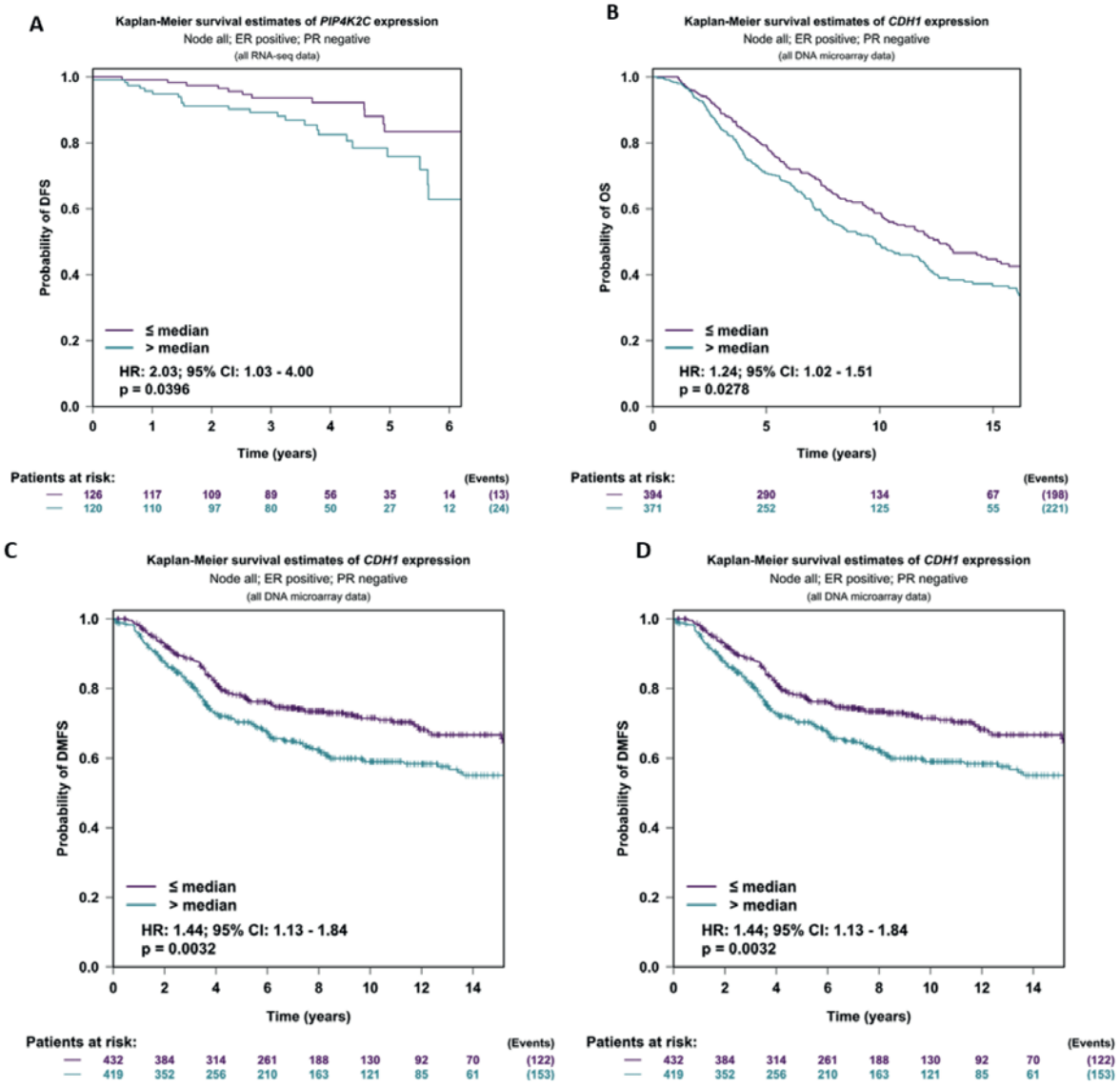

Figure 3. Kaplan-Meier survival curves in ER+PR- IBC. (A) *PIP4K2C* expression and disease-free survival; (high *PIP4K2C* associated with shorter disease-free survival (DFS). (B) Overall survival (OS), (C) distant metastasis-free survival (DMFS), (D) DFS stratified by *CDH1* expression (low *CDH1* associated with poor OS, short DFS, and DMFS).

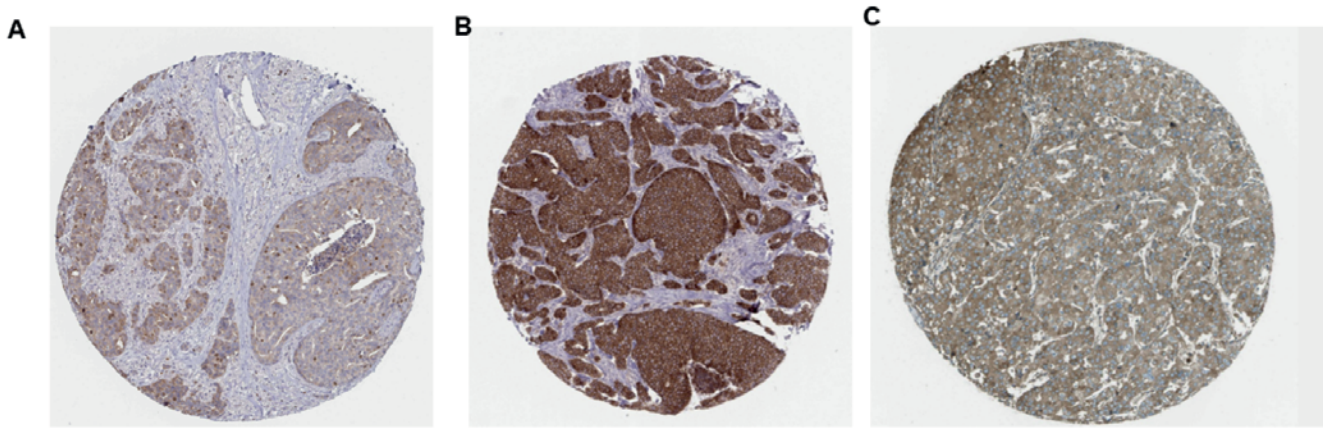

Figure 4. Immunohistochemistry (IHC) of: (A) *PIP4K2C*, (B) *CDH1*, (C) *CLTC*. *PIP4K2C* and *CLTC* showed cytoplasmic localization; *CDH1* showed membrane and cytoplasmic localization. Staining intensity ranged from moderate to high.

Our study finds that high expression of *PIP4K2C* and *CDH1* is significantly associated with poorer outcomes in ER+PR- subtype. Lima *et al.* showed initial evidence of the involvement of *PIP4K2C* in acute myeloid leukemia progression. The study also found a significant correlation between overexpression of *PIP4K2C* and the frequency of *TP53* mutations in higher genetic risk patients, which can be involved in genetic instability.[43] *CDH1* gene acts as a suppressor of invasion, and loss of *CDH1* is associated with Epithelial-to-Mesenchymal Transition (EMT), which can increase the incidence of metastasis in BC.[42,44] We identified that high *CDH1* expression in ER+PR- IBC was associated with a poorer prognosis. High levels of *CDH1* expression may promote strong cell-cell adhesion, which in turn may enhance tumor cell invasion and metastasis by allowing groups of tumor cells to extravasate and migrate more efficiently than individual tumor cells.[45] In addition, high levels of *CDH1* expression may also promote tumor progression by affecting the cell-cell adhesion interactions between tumor cells and cells in the stroma, such as fibroblasts or immune cells.[46] *CLTC* has also been reported to be involved in tumorigenesis.[47] High expression of *CLTC* is an independent prognostic factor for tumor-free survival and overall survival in patients with osteosarcoma.[48] These findings suggest that these genes could be therapeutic targets for the ER+ PR- IBC subtype. ER+ PR- patients constitute approximately 10% to 12% of IBC cases in various studies; these relatively small number of ER+ PR- cases may restrict the generalizability of the findings. In recent years, many studies have underlined the downregulation and lack of PR expression in some cases of ER+ IBC and explained the origin of the ER+ PR- subtype. One theory suggests that ER is non-functional in some breast tumors and thus cannot stimulate transcription of the gene that encodes PR.[16,17] A second potential mechanism is downregulation of PR at the mRNA level in the presence of intact PR promoter activity.[17] Methylation of PR promoter has been reported in 21–40% of ER+PR- tumors and could silence transcription of the gene that encodes PR.[17] Evidence also suggests that crosstalk between ER and other growth factor signaling pathways may downregulate PR protein expression in IBC.[15,18] The results of this study provide a foundation for future research. Larger-scale experimental studies are necessary to validate and expand upon the findings.

Conclusions

The integrated bioinformatics analysis in this study provided new insights into the unique molecular pattern of the ER+PR- subtype of IBC. The identified hub genes contribute to ER+PR- subtype IBC progression and poor patient outcomes, suggesting potential therapeutic targets. The results of this study also showed that PR expression plays an important role in ER+ IBC progression. Further experimental and functional studies are required to confirm these findings.

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Online supplementary material:

Table 1. Functional enrichment analysis of upregulated genes in ER+PR- IBC subtype

Table 2. Functional enrichment analysis of down-regulated genes in ER+PR- IBC subtype

Figure 1. Box plots of PIP4K2C *(A),* CDH1 *(B), and* CLTC *(C) expression levels in IBC, stratified by IBC molecular subtypes.*

Figure 2. Box plots of PIP4K2C *(A),* CDH1 *(B) and* CLTC *(C) expressions stratified by lymph node status in IBC.*

Figure 3. Box plots of PIP4K2C *(A),* CDH1 *(B) and* CLTC *(C) expressions stratified by histological subtype in IBC.*

Figure 4. Box plots of PIP4K2C *(A),* CDH1 *(B) and* CLTC *(C) expressions stratified by Nottingham prognostic index.*