Methylation-Mediated Silencing of *RBP7* Promotes Breast Cancer Progression Through PPAR and PI3K/AKT Pathway

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Abstract

Purpose Retinoid-binding protein (*RBP7*) is a member of the cellular retinol-binding protein (CRBP) family, which is involved in the pathogenesis of breast cancer (BRCA). The study aims to illustrate the prognostic value and the potential regulatory mechanisms of *RBP7* expression in BRCA.

Methods We utilized a series of bioinformatics tools, including HPA, GEPIA, UALCAN, ONCOMINE, Kaplan–Meier plotter, PROGeneV2, TISCH, LinkedOmics, UCSC Xena, MethSurv, SMART APP, bc-GenExMiner4.7, OSbrca, STRING, CARE, SwissDock and R software packages, to investigate the expression, prognostic value and functional regulatory networks of *RBP7* in BRCA.

Results Bioinformatics analysis with the TCGA and CPTAC databases revealed that the mRNA and protein expression levels of RBP7 in normal were higher compared to BRCA tissues. Survival analysis displayed that the lower expression of *RBP7,* the worse the prognosis in ER-positive (ER^+) BRCA patients. Genomic analysis showed that promoter methylation result in transcriptional silencing of *RBP7* in BRCA. Functional enrichment analysis demonstrated that downregulation of *RBP7* expression may exert its biological influence on BRCA through the PPAR pathway and the PI3K/AKT pathway.

Conclusions In summary, we identified *RBP7* as a novel biomarker that is helpful for the prognosis of ER^+ BRCA patients. Promoter methylation of *RBP7* is involved in its gene silencing in BRCA, thus regulating the occurrence and development of ER^+ BRCA through the PPAR and PI3K/AKT pathways.

Introduction

Breast cancer (BRCA) is the most common cancer worldwide, accounting for 30% of female cancers(Siegel et al. 2020). Estrogen receptor-positive (ER^+) BRCA is driven by ER-mediated transcriptional activity, composing the major subtype (approximately 75%) of BRCA (Siersbaek et al. 2018). Although endocrine therapy, including estrogen suppression and direct ER targeting, is widely applied in the treatment of ER^+ BRCA, acquired resistance often occurs and remains a major challenge for the treatment of ER^+ patients (Hanker et al. 2020), thus, novel targets and effective therapeutic strategies for BRCA patients are urgently needed.

Previous studies have confirmed that cellular retinol-binding protein (CRBP) family members are vital for the pathological progression of BRCA. CRBPs belong to the family of fatty acid-binding proteins and are required for vitamin A stability and metabolism (Napoli 2017). Epigenetic silencing of CRBPs is a common event in cancers(Esteller et al. 2002). For example, Kuppumbatti *et al.* reported that CRBPs were underexpressed in 24% of human BRCA (Kuppumbatti et al. 2000). Previous studies demonstrated that CRBPs was involved in the growth inhibition of mammary epithelial cells by restrainin the PI3K/AKT pathway (Kuppumbatti et al. 2001). In breast epithelial cells, CRBP1 dependents on retinoic acid receptor, regulates p85-P110 heterodimer to inhibit PI3K/AKT signalling (Farias et al. 2005a). In addition, CRBP1 downregulation compromises physiological retinoic acid receptor (RAR) activity for a long time, resulting

in loss of cell differentiation and tumour progression (Farias et al. 2005b). Thus, CRBPs may be used to predict the prognosis of BRCA patients.

Retinoid-binding protein 7(*RBP7*), also named CRBP4, belongs to a distinctly different CRBP subfamily, having a relatively different way of retinol binding for this protein. *RBP7* is located on human chromosome 1p36.22, encoding a protein of 134 amino acids in length. The size of the translated exon sequences and intron position of *RBP7* are highly conserved. With a structure similar to other CRBPs, the *RBP7*-encoded protein binds all-trans-retinol with a lower binding affinity than other CRBPs (Folli et al. 2002). *RBP7* has been implicated in several disease processes. For example, the PPARγ-*RBP7*-adiponectin pathway plays a protective role in hypertensive diseases by regulating transcriptional activity (Fang and Sigmund 2020). *RBP7* also plays an important role in adipose tissue during adipogenesis, cold exposure and nutritional treatment (Ahn et al. 2018). Recent studies have demonstrated that *RBP7* is a strong prognostic biomarker for malignant phenotype in colon cancer (Elmasry et al. 2019). However, as a novel member of the CRBP family, the biological significance of *RBP7* in BRCA is still unexplored, and its functional role in BRCA has never been documented.

In this study, we first analysed the differential expression of *RBP7* in BRCA and normal tissues and evaluated the prognostic value of *RBP7* by using data from the TCGA and GEO databases. Next, we determined the localization of *RBP7* expression in BRCA, the functional enrichment of its coexpressed genes and the association between the mRNA expression and DNA methylation of *RBP7*. Then, we performed the association between *RBP7* and multiple molecular subtypes of BRCA and the significant KEGG pathways involved in *RBP7* in ER^+ BRCA. Finally, we screened *RBP7*-targeting drugs from computational analysis of resistance (CARE) databases, which may provide new ideas for the treatment of BRCA.

Materials And Methods

Gene expression and survival analysis

The Human Protein Atlas(Uhlen et al. 2015; Uhlen et al. 2017) (HPA;www.proteinatlas.org) database was used to illustrate *RBP7* mRNA distribution, protein expression and immunohistochemical maps of *RBP7* in normal and BRCA tissues.*RBP7* gene expression levels in pan cancers were identified in Gene Expression Profiling Interactive Analysis(Tang et al. 2017) (GEPIA;gepia2.cancer-pku.cn/#index) and ONCOMINE(Rhodes et al. 2004)(www.oncomine.org). UALCAN(Chandrashekar et al. 2017) (ualcan.path.uab.edu/index.html) and the Gene Expression Omnibus(Edgar et al. 2002) (GEO; https://www.ncbi.nlm.nih.gov/geo/) were used to investigate the different expression of *RBP7* in normal and BRCA tissues. To explore the prognostic role of *RBP7* expression in BRCA, GEPIA ,Kaplan–Meier plotter(Gyorffy et al. 2010) (http://kmplot.com/) and PROGgeneV2(Goswami and Nakshatri 2014) (http://www.compbio.iupui.edu/proggene) were used to determine the prognostic significance. In this study, we analysed the prognosis of *RBP7* in BRCA with detailed hazard ratios (HRs) by setting the expression threshold at the medium or best cut-off and *a* log-rank *p value* less than 0.05.

Tumour immune single-cell hub (TISCH)

TISCH(Sun et al. 2021) (http://tisch.comp-genomics.org) integrates single-cell transcriptome profiles of nearly 2 million cells for 27 cancer types.In this study, we utilized the “multiple-dataset comparison” model to visualize the averaged gene expression distributed in single cells and the “Gene” module to display the heat map of the cell-type averaged expression of *RBP7*.

LinkedOmics

The coexpressed genes of *RBP7* were screened from the TCGA BRCA cohort through the “LinkFinder” module in LinkedOmics(Vasaikar et al. 2018) (www.linkedomics.org/login.php) databases, and the correlative significance was tested by the spearman correlation coefficient. The top 50 positively and negatively correlated genes are presented as heat maps. Gene Ontology biological process (GO_BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed with gene set enrichment analysis (GSEA) in the “LinkInterpreter” module.

RBP7 DNA methylation analysis

Heat maps of *RBP7* in the cohort of BRCA patients were constructed through data mining in TCGA BRCA by using the University of California Santa Cruz (UCSC) Xena(Zweig et al. 2008) (Casper et al. 2018) (http://xena.ucsc.edu/). MethSurv (biit.cs.ut.ee/methsurv/) is used to survival analysis ground on CpG methylation patterns(Modhukur et al. 2018), we verified the methylation levels of probes of *RBP7,* and four of them with high methylation in the promoter were chosen to display the distribution of methylation under different clinical stages. SMART(Li et al. 2019) (Xu et al. 2019) (**http://www.bioinfo-zs.com/smartapp/**) is to further identify the association of the mRNA expression and methylation of *RBP7*.

Bc-GenExMiner online tool and OSbrca

Based on common clinical parameters, we utilized Bc-GenExMiner (v4.7) (Jezequel et al. 2021) (http://bcgenex.ico.unicancer.fr) to analyse the expression data and survival curves of *RBP7* in different molecular subtypes of BRCA, including ER, PR and HER-2 (IHC). The OSbrca (Yan et al. 2019) (http://bioinfo.henu.edu.cn/BRCA/BRCAList.jsp) was utilized to validate prognostic value of *RBP7* in BRCA.

Protein–protein interaction (PPI) network analysis

PPI network analysis of RBP7 was conducted in the STRING (https://string-db.org/) database (Szklarczyk et al. 2011). The regulatory relationships between genes were visualized *via* Cytoscape (ver. 3.4.0). Then, we use the starBase v3.0(Li et al. 2014) (http://starbase.sysu.edu.cn/index.php) to analyse the correlation between *RBP7* and PIK3R3 in BRCA.

Differentially expressed genes (DEGs) analysis

We used the *R* software *Limma*package to screen DEGs by filtering the *p.adj*-value of Student's t-test and the fold change (FC) and dividing the DEGs into two groups with high or low *RBP7* expression. A volcano plot was generated by using the ggplot2 R software package to display the DEGs with statistical significance, i.e., *p*.adjust-value <0.05 and absolute FC value >1. KEGG pathway analysis was performed on those DEGs by using the cluster profiler package, and the pathways with statistical significance (adjusted $p<0.05$) werevisualized by hierarchical clustering of a heat map (Kanehisa et al. 2012).

Computational analysis of resistance (CARE)

A positive CARE score represented a high expression value, which was related to drug response in CARE (Jiang et al. 2018) (http://care.dfci.harvard.edu/), and vice versa.In this study, we utilized 3 databases, i.e., Cancer Cell Line Encyclopedia (CCLE), Cancer Therapeutics Response Portal (CGP), and Genomics of Drug Sensitivity in Cancer (CTRP), to analyse the drugs targeting *RBP7*.

SwissDock

The PDF file of the *RBP7* protein was downloaded from the RCSB Protein Data Bank (PDB) database, and the ligand and water molecules were then removed by using PyMOL software. The mol2 file of the nilotinib small molecule was downloaded from the PubChem database and converted using Open Babel software. Finally, we uploaded the two files to the SwissDock (Grosdidier et al.
2011) (http://www.swissdock.ch) page for docking.

Results

Gene expression profiles of *RBP7* in normal and cancer tissues

We utilized the HPA database to analyse the mRNA and protein expression profiles of *RBP7* in human normal tissues and found that *RBP7* expression varied significantly in different human tissues with relatively high expression in breast and adipose tissues (Figure 1A). Consistently, the protein expression of *RBP7* was highly expressed in the breast and adipose tissues (Figure 1B). To assess the variation in *RBP7* expression in pan-cancer, we performed analysis with the GEPIA online server from the TCGA and GTEx projects. Compared to the matched normal tissues, *RBP7* was highly expressed in adrenocortical carcinoma (ACC), kidney renal clear cell carcinoma (KICH) and liver hepatocellular carcinoma (LIHC). In contrast, *RBP7* expression was relatively lower in urothelial bladder carcinoma (BLCA), breast invasive carcinoma (BRCA), cervical squamous cell carcinoma and so on (Figure 1C).

To further verify the significance of RBP7 expression in cancers, the fluxionary expression of *RBP7* in tumour and normal tissues was analysed by using ONCOMINE. We found that *RBP7* was overexpressed

in liver cancer and lymphoma, but decreased expression of *RBP7* was found in brain and CNS cancer, BRCA, oesophageal cancer, head and neck cancer, leukaemia, and ovarian cancer (Figure S1A).

Prognostic value of *RBP7* expression in BRCA

To investigate the role of *RBP7* expression in BRCA, the UALCAN database was utilized to analyse the expression of *RBP7* in 114 normal tissues and 1097 primary BRCA tissues. The TCGA database results revealed that *RBP7* transcript levels were significantly reduced in patients with BRCA (Figure 2A), which was further validated by the GSE37751 dataset from the GEO database (Figure S1B). Then, we discovered that the protein expression of RBP7 was higher in normal tissues through CPTAC database (Figure 2B), which was consistent with the result of *RBP7* expression in the TCGA database.

We subsequently utilized the transcriptomic sequencing data in the GEPIA database to assess the prognostic value of *RBP7* in BRCA and found that a high level of expression of *RBP7* was favourable to the prognosis of BRCA (Figure 2C). Survival analysis was performed with the GSE20685 (Figure 2D) and GSE42568 (Figure 2E) datasets from the GEO database, which showed that Shorter OS was observed in patients with lower expression of *RBP7*. Furthermore, we used FROGgeneV2 to confirm the association between *RBP7* expression and survival rates of BRCA patients, and the results are consistent with the above (Figure 2F). These results reveal that there is a significant association between *RBP7* expression and BRCA prognosis and that *RBP7*has a protective effect on BRCA prognosis.

RBP7 expression in different cells from BRCA tissues

It demonstrated that *RBP7* exhibits cell type-specific expression in endothelial and epithelial cells in TISCH database (Figure 3A and 3B). A heat map of the gene module analysis depicts the expression of *RBP7* in different cell types in BRCA datasets in which endothelial and epithelial cells are characterized by high expression of *RBP7* (Figure S1C). Then, we utilized the immunohistochemistry (IHC) data detected by the HPA-034749 antibody from the HPA database to determine the protein expression of *RBP7* in BRCA and adjacent/healthy tissues. The results showed that adipocytes were highly stained in normal breast tissues, while glandular and myoepithelial cells were mildly stained, mainly in the nucleus (Figure 3C). In the BRCA samples, the expression level of *RBP7* in tumour cells was ranked as weak, moderate and strong, which was scored by the staining intensity in the pathological IHC (Figure 3D-3F). Interestingly, as a nuclear receptor, *RBP7* was found to be mainly localized in the nucleus, indicating the vital role of *RBP7* in the regulation of gene expression in epithelial cells of BRCA tissues.

RBP7 coexpression networks in BRCA

To in-depth knowledge the biological meaning of RBP7 in BRCA, *RBP7* coexpression genes were analysed by the functional module of LinkedOmics. The top 50 significant genes that were positively and negatively correlated with *RBP7* were selected as heat maps (Figure 4A and 4B) in which *RBP7* displayed a strong positive related with the expression of FAM107A, GPIHBP1 and FXYD1. Remarkably, the top 50 negatively coexpressed genes had a high probability of being high-risk markers in BRCA, of which 15

genes had significantly high HRs (*p value* < 0.05). In contrast, there were no genes with high HRs (*p*< 0.05) in the top 50 positively coexpressed genes. These results further confirmed that *RBP7* performs a protective role in the progression of BRCA (Figure 4C).

GO term annotation of biological processes showed that *RBP7* coexpressed genes mainly participate in the adrenergic signalling pathway, excretion, endothelium development, regulation of transporter activity, cell communication by electrical coupling and G protein-coupled receptor signalling pathway, with inhibition of the biological processes including double-strand break repair, cargo loading into vesicle, DNA conformation change, ncRNA transcription and protein localization to chromosome (Figure 4D). KEGG pathway analysis showed that there was an enrichment in the regulation of lipolysis in adipocytes, PPAR signalling pathway, ovarian steroidogenesis, and drug metabolism (Figure 4E), indicating a widespread impact of *RBP7* on the global transcriptome.

RBP7 methylation in BRCA

We displayed hierarchical clustering analysis of *RBP7* mRNA expression related to DNA methylation by using the UCSC Cancer Genomics Browser. The results showed that *RBP7* methylation mainly occurred in primary BRCA, and hypermethylation at the promoter region downregulated the mRNA expression of *RBP7* (Figure 5A), indicating a potential correlation between transcription level and methylation on the promoter of *RBP7*.

Next, we performed methylation analysis with the Methsurv database and found that 4 methylation probes, namely, cg20413202, cg03406535, cg10796749 and cg14202757, in the promoter of *RBP7* were highly methylated in BRCA (Figure 5B). According to this standard, we found that the β values of 3 of the 4 detected methylation probes, i.e., cg20413202 (β value=0.947), cg10796749 (β value=0.798) and cg14202757 (β value=0.694), were higher than 0.6, indicating that almost complete methylation occurred on the promoter of *RBP7* (Figure S2A-D). Consistently, we found that most of the median β values of different clinical stages were also above 0.6 (Figure S2E-H), suggesting that promoter methylation leads to the possession of RBP7 gene transcription in BRCA.

Subsequently, we used the SMART App to verify the Spearman correlation between transcription level and DNA methylation of the probes of *RBP7* in BRCA. *RBP7* expression was significantly negatively correlated with the methylation probes cg03406535, cg27083689, cg18086187, cg03994053, cg27561954, cg15090005 and cg07224455 (Figure 5C). The aggregation plot for the 12 methylation probes performed that there was a significant negative correlation between *RBP7* transcription levels and DNA methylation (Figure S1D).

Clinicopathological association of *RBP7* and its prognostic value

BRCA is a complex disease with various morphological, clinical and molecular features. Molecular subtypes and optimal treatments for BRCA are usually based on immunohistochemical markers such as

ER, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). We checked the relevance of *RBP7* expression and different clinicopathological features by using the web-based tool bc-GenExMiner. We found that the expression of *RBP7* was highly expressed in ER^+ BRCA patients compared with ER-negative (ER^-) patients (Figure 6A). *RBP7* expression was significantly decreased in PR-negative compared with in PR-positive in BRCA patients (Figure 6B), whereas *RBP7* expression was higher in HER2-negative BRCA patients than in HER2-positive patients (Figure 6C). To further determine the correlation of *RBP7* expression and hormone receptors (HRs), we utilized DNA microarray data to perform correlation analysis of *RBP7* expression with different combinations of ER and PR expression statuses, i.e., ER^+/PR^+, ER^+/PR^-, ER^-/PR^+ and ER^-/PR^-. The results showed that there were remarkable differences in *RBP7* mRNA expression in some hormone receptor combinations, i.e., ER^+/PR^+ *vs.* ER^-/PR^-, $p<0.01$ and ER^+/PR^+ *vs.* ER^+/PR^-, $p<0.01$ (Figure 6D).

Furthermore, we performed survival analysis of BRCA patients with different ER/PRcombinations. Downregulated *RBP7* expression was only significantly associated with poor prognosis in ER^+/PR^+ and ER^+/PR^- patients but not in ER^-/PR^+ and ER^-/PR^- BRCA patients (Figure 6E-6L). OSbrca was used to verify the prognostic value of RBP7 in these 4 groups with the TCGA database. Consistently, we discovered that down-regulation of *RBP7* expression was significantly correlated with shorter OS in ER^+/PR^+ and ER^+/PR^- patients (Figure S3).

Potential regulatory mechanisms and target drugs of *RBP7* in BRCA

To further explore the PPI network of *RBP7* in BRCA, we used STRING to identify genes that may interact with *RBP7*. The interactions between *RBP7* and proteins encoded by the functional genes, including *SLC45A1*, *SLC25A33*, *UBE4B*, *TMEM201*, *NMNAT1*, *SOCS7*, *SOCS4*, *GPR157*, *PIK3R3* and *POLR2G*, are shown in Figure 7A. Interestingly, in this interacting network, PIK3R3, a regulatory subunit of phosphatidylinositol 3-kinase (PI3K), was an important factor in BRCA. Therefore, we further analysed the correlation between *RBP7* and PIK3R3 in BRCA and found that the expression levels of *RBP7* and PIK3R3 were negatively correlated (R = -0.231, p = 6.79e-15) (Figure 7B). Then, we acquired ER^+ BRCA data from the TCGA database, which were quarterly ranked according to the expression level of *RBP7*. The high and low *RBP7* expression groups were defined as the first and fourth quarters of ER^+ BRCA data, respectively, and the differential genes ($|\log_2(FC)|>1$) between these two groups were analysed by using the Limma software package for display as a volcano plot (Figure 7C).

KEGG pathway analysis was conducted to investigate the functional implications of the DEGs, and it was found that several tumour-related pathways, such as the PPAR signalling pathway, regulation of lipolysis in adipocytes and tyrosine metabolism, were significantly enriched (Figure 7D). In addition, the PI3K-AKT signalling pathway, which is important in ER^+ BRCA, was also enriched (Figure 7D).

Furthermore, we used the CARE database to analyse the association of the molecular alteration of *RBP7* with drug efficacy and found that *RBP7* was negatively associated with drug efficacy in the CCLE, CGP

and CTRP databases (Figure 7E). Interestingly, we found that only one drug (nilotinib) was common among the resistant drugs from these 3 databases (Figure 7F). Finally, we visualized the binding site of *RBP7* with nilotinib by SwissDock (Figure 7G).

Discussion

RBP7 is a member of the CRBP family and participates in cell response mediated by retinoic acid (Choder 2004). Previous studies demonstrated that the retinol signalling pathway might be relevant to BRCA progression. However, the prognostic value of *RBP7*, a new member of the CRBP family, in BRCA is still unclear. In this study, we utilized various databases to explore the expression, prognosis, cellular localization, coexpression network, DNA methylation and function of *RBP7* in BRCA.

Gene expression analysis displayed that *RBP7* is widely expressed in various normal tissues, including thyroid, testis, breast and adipose tissues. Notably, in BRCA tissues, *RBP7* is mainly expressed in epithelial cells with nuclear localization. Importantly, we found that both the mRNA and protein expression levels of *RBP7* were significantly reduced in BRCA tissues. Survival analysis with 4 different databases showed that high expression of RBP7 is a favourable prognostic factor in BRCA patients. To further illuminate the role of *RBP7* in the progression of BRCA, we analysed the expression and prognosis of *RBP7* in different molecular subtypes of BRCA. *RBP7* mRNA expression in ER^+ patients was higher than that in ER^- patients, and higher expression of *RBP7* was associated with better OS and DFS in ER^+ BRCA patients, revealing that down-regulation of *RBP7* may promote carcinogenesis.

Previous studies have also confirmed the role of *RBP7* in BRCA. For example, Kinyamu et al. used genome-wide transcriptional profiling to demonstrate that *RBP7* is positively regulated by estradiol (E2) in BRCA cells (Kinyamu et al. 2008); Calvo et al. reported that blockers of estrogen receptors inhibited the expression of estradiol-modulated genes, including *RBP7*, in the mouse mammary gland (Calvo et al. 2012). It is reasonable to propose that upregulation of *RBP7* by E2 leads to a good prognosis in ER^+ BRCA. Previous studies demonstrated that the proliferation of BRCA cells is regulated by signalling pathways involving nuclear steroid thyroxine receptors (Schneider et al. 1999), especially RARs, which show growth inhibitory activity against BRCA cells both *in vitro* and *in vivo* (Peng et al. 2004).Interestingly, RAR and ER share a common coactivator, estradiol. Pemrick et al. proved that both RAR and ER have high affinity for β-estradiol by constructing chimeric RARs containing the ligand-binding domain of ER (Pemrick et al. 1998). Furthermore, Fonja et al. found that the intracellular crosstalk of RAR, ER and Her-2 may acts as a growth inhibition signal in BRCA cells (Afonja et al. 2004). In the retinoid pathway, RAR activation is associated with CRBP1-mediated retinol storage(Farias et al. 2005b) (Bushue and Wan 2010). *RBP7*, another member of the CRBP family, may also engage in crosstalk with RAR; thus, it may be in mechanism regulation via RAR/ER in BRCA. Based on the results above, we conclude that *RBP7* may be a tumour suppressor gene and that *RBP7* expression had maintained a low level with the emergence and development of BRCA, leading to a poor prognosis.

Bioinformatics analysis with UCSC Xena showed that there was a significant negative correlation between *RBP7* mRNA expression and promoter methylation. The different DNA methylation patterns between cell subpopulations drive the phenotypic changes in BRCA, which is significant for epigenetic heterogeneity within tumours (Almendro and Fuster 2011).It is reported that the promoter methylation as well as intragenic and intergenic regions is involved in the modulation of tumour development and invasion (Rauscher et al. 2015).The methylation of CRBPs was reported to be associated with tumour development. For example, the methylation of CRBPs squint towards increase in prevalence in foci with worse pathological changes in the esophageal mucosa of patients with esophageal squamous cell carcinoma (ESCC) in the high-risk population (Roth et al. 2006). The methylation profile of CRBPs in bladder cancers is also correlated with the clinicopathological features of poor prognosis (Brait et al. 2008). DNA hypermethylation brings about epigenetic silencing of CRBPs in human and mouse BRCA (Arapshian et al. 2004). For example, CRBP1 gene silencing was found in 60% of G2 and 66.7% of G3 carcinoma cells due to CRBP1 promoter methylation (Doldo et al. 2014). DNA methylation can occur in the whole genome, including the promoter, gene body, 3'-untranslated region (UTR) and intergenic regions, while the promoter can be further divided into TSS200, TSS1500, 5'-UTR and the 1st exon. DNA methylation in gene promoters generally has a negative regulatory effect on gene expression (Shenker and Flanagan 2012). We utilized the MethSurv database to identify the methylation sites of *RBP7* in BRCA and found 4 probes, namely, cg20413202, cg03406535, cg10796749 and cg14202757, located in TSS1500-N_Shore with high methylation. Analysis using SMART APP web tools showed that promoter methylation was negatively correlated with *RBP7* mRNA expression. In summary, our results demonstrate that the promoter methylation of *RBP7* result in its transcriptional silencing, which may be a reasonable explanation for the gene downregulation of *RBP7* in BRCA.

With the concept that downregulation of *RBP7* leads to short OS of ER^+ BRCA patients, we further performed KEGG pathway analysis in ER^+ BRCA and found that *RBP7* exerts its biological function through crosstalk with the PPAR and PI3K/AKT signalling pathways. PPARs represent a nuclear receptor superfamily that includes PPARα, PPARβ/δ and PPARγ. It was reported that the activation of PPARγ inhibits the cell growth of different cancers, such as colon cancer, gastric cancer and liposarcoma (Mirza et al. 2019). For example, after cleavage by caspase-1 at Asp64, PPARγ translocates to mitochondria, leading to attenuation of medium-chain acyl-CoA dehydrogenase (MCAD) activity and inhibition of fatty acid oxidation, which brings about the accumulation of lipid droplets and differentiation of tumour-associated macrophages (TAMs), thus resulting in an ultimate suppression of tumour growth (Niu et al. 2017). Another study by Mueller et al. reported that PPARγ is highly expressed in human primary and metastatic BRCA, and ligand activation of this receptor in BRCA cells causes extensive lipid accumulation, which results in a reduction in the growth rate and clonogenic capacity of tumour cells(Mueller et al. 1998) (Apostoli et al. 2015). Intriguingly, application of the PPARγ agonist rosiglitazone in combination with the MEK inhibitor trametinib can terminally differentiate BRCA cells that have undergone epithelial-mesenchymal transition (EMT) into adipocytes (Ishay-Ronen et al. 2019).

As a PPARγ target gene, *RBP7* is also an upstream regulator of some other PPARγ target genes in the endothelium. PPARγ and *RBP7* control the oxidative state of blood vessels by forming a transcriptional regulatory circuit (or hub) in the endothelium. Loss of *RBP7* impairs this regulatory circuit, resulting in oxidative stress and dysfunction in endothelial cells (Hu et al. 2017). Cancer cells have to endure oxidative stress throughout tumourigenesis, including during initiation, matrix detachment, transmission in the circulation, and relapse after therapy (Hayes et al. 2020). In addition, endothelial injury is closely related to tumourigenesis and accompanies malignant cancer cells in almost every stage of the metastatic process(Blazejczyk et al. 2015). Thus, impairment of the regulatory circuit between *RBP7* and PPARγ increases the opportunity to promote the occurrence of cancer.

PI3K/AKT is the most frequently activated signalling pathway that promotes tumour growth (Miller et al. 2011b) and progression of BRCA (Saal et al. 2007). Bonofiglio *et al.* revealed that the ERα and PPARγ pathways have an opposite effect on the regulation of the PI3K/AKT signal transduction cascade (Bonofiglio et al. 2005). The nuclear receptor ERα has been shown to be involved in the pathophysiological process of BRCA. Membrane-anchored ERα can activate various cytoplasmic kinases, including components of the PI3K/AKT pathway, through rapid nongenomic actions (Khatpe et al. 2021). There are two signalling pathways involved in the activation of the PI3K/AKT pathway in ER^+ BRCA cells. The estrogen-dependent pathway activates the PI3K/AKT signalling process by directly binding to the p85α regulatory subunit of PI3K, thus enhancing the transcriptional activity of targeted genes in BRCA cells (Simoncini et al. 2000). For the estrogen-independent pathway, the interaction of EGFR with growth factor can directly induce ERα transcriptional activity through the PI3K/AKT signalling pathway(Miller et al. 2011a). Consequently, the downstream signalling of both pathways is activated, leading to the proliferation and survival of tumour cells. In contrast, PPARγ can inhibit the PI3K/AKT pathway by upregulating PTEN transcription in BRCA cells (Bonofiglio et al. 2005). As an antagonist of the PI3K/AKT pathway, PTEN plays a key role in preventing tumourigenesis (Huang et al. 2012). Based on our results and previous studies, we hypothesize that *RBP7* may regulate PTEN by targeting PPARγ, thereby suppressing the activation of the PI3K/AKT pathway. The PPI network from bioinformatics analysis revealed that *RBP7* may directly interact with PIK3R3, resulting in activation of the PI3K/AKT pathway (Figure 8).

Conclusions

In conclusion, this study provides the first evidence that RBP7 downregulation in BRCA is associated with promoter methylation. Furthermore, we found that RBP7 has prognostic value for ER^+ BRCA. Deep bioinformatics analysis of *RBP7*-related pathways reveals some vital information for the regulatory mechanism in ER^+ BRCA. However, these results need to be further validated by both in vitro and in vivo experiments. This study is helpful in providing novel approaches for clinical diagnosis and treatment.

Declarations

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Author contributions HL, QH, YH and YJ conceived and designed research. HL and QH analysed data. HL, JW and ZZ interpreted results. HL, QH and JW prepared figures. HL drafted manuscript. HHL, YH and YJ edited and revised manuscript. HL, JW, QH, ZZ, HHL, YH and YJ approved the final version of the manuscript.

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Data availability The original contributions presented in the study are included in the article/Supplementary material, and further inquiries can be directed to the corresponding authors.

Compliance with ethical standards

Conflict of interest No conflicts of interest, financial or otherwise, are declared by the authors.

Ethics approval All data of this study were public and required no ethical approval by an institutional review board or ethics committee.

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Figures

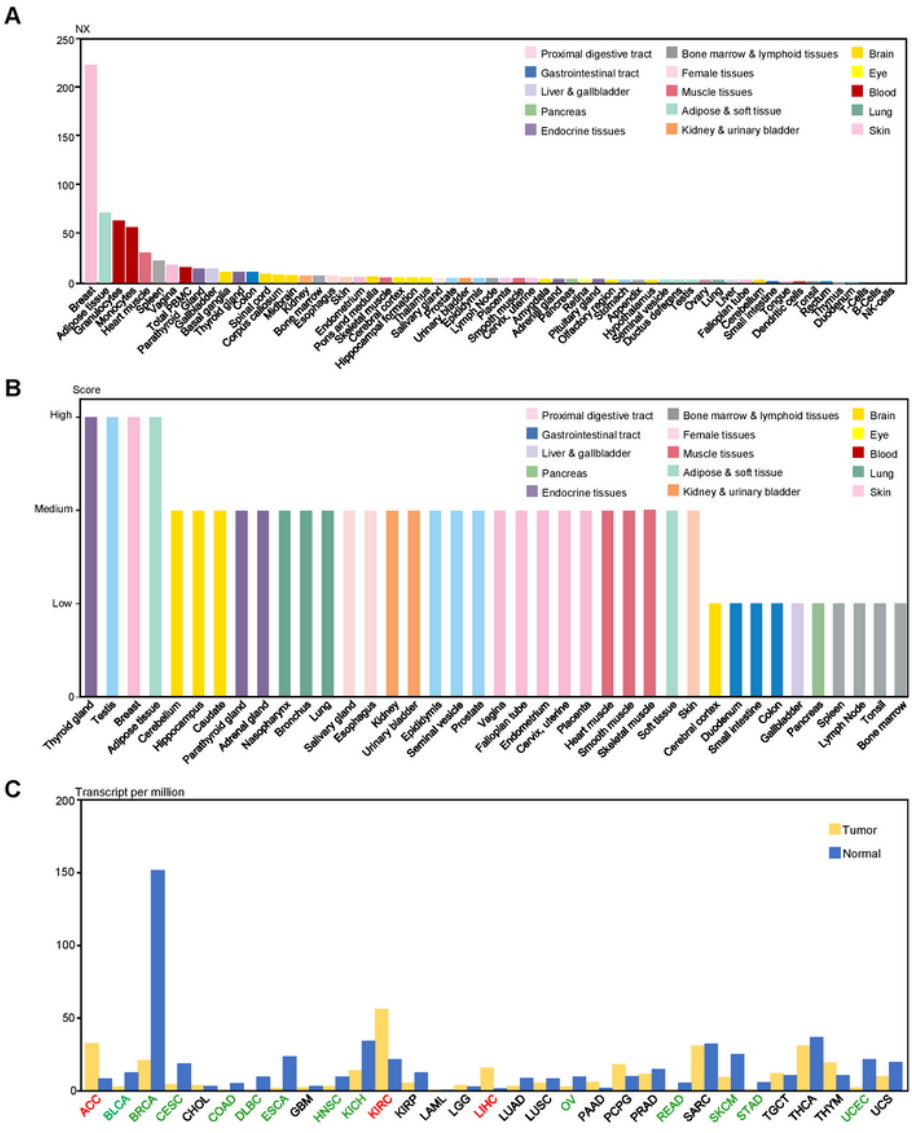

Figure 1.

Figure 1

The expression of RBP7 in different human tissues and pan cancers. (A) RBP7 expression profiles in normal human tissues. NX, normalized expression. (B) The protein expression of RBP7 in normal human tissues. (C) Pan cancer analysis of RBP7 expression from the GEPIA database. The red and green letters

represent increased or decreased gene expression of RBP7 in tumours in comparison with normal tissues, respectively.

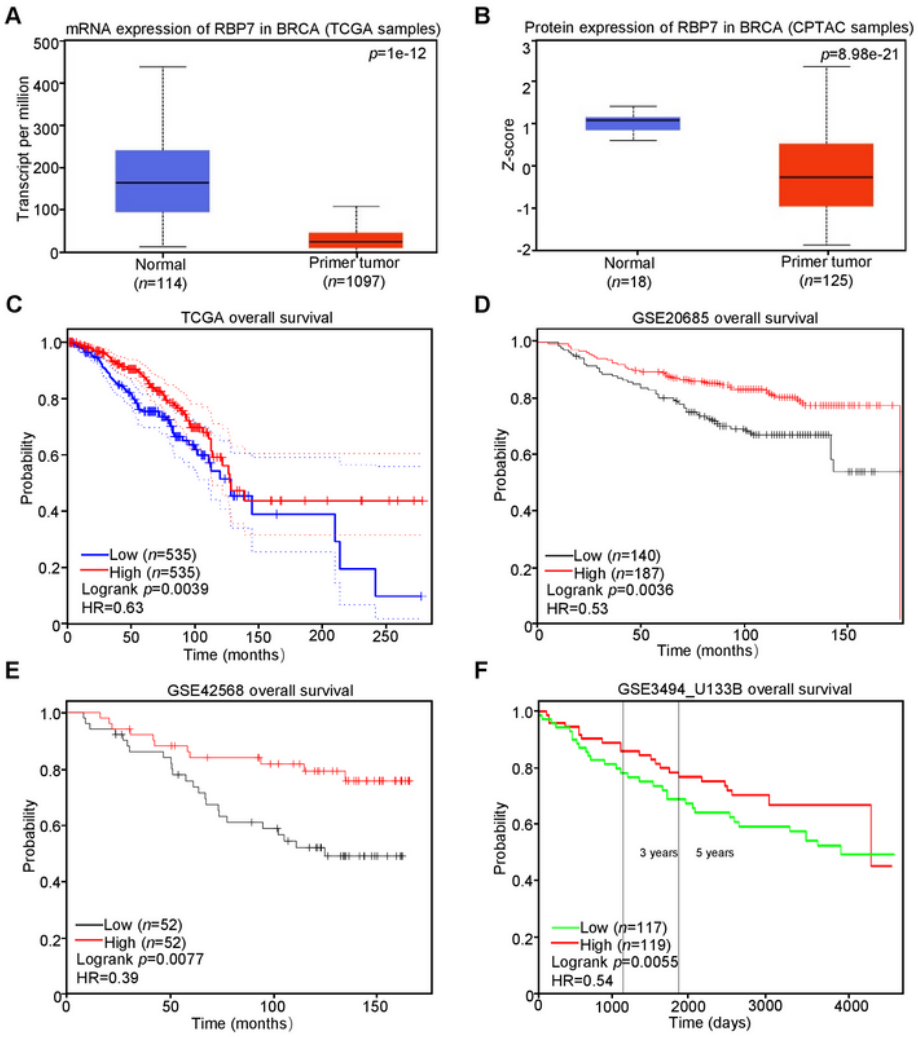

Figure 2.

Figure 2

The mRNA and protein expression of RBP7 in BRCA and the overall survival analysis with RBP7 mRNA expression. (A) Boxplot of the mRNA expression of RBP7 in normal and BRCA tissues. (B) Boxplot of the

protein expression of RBP7 in normal and BRCA tissues. (C-F) OS analysis with RBP7 expression in BRCA patients from different databases.

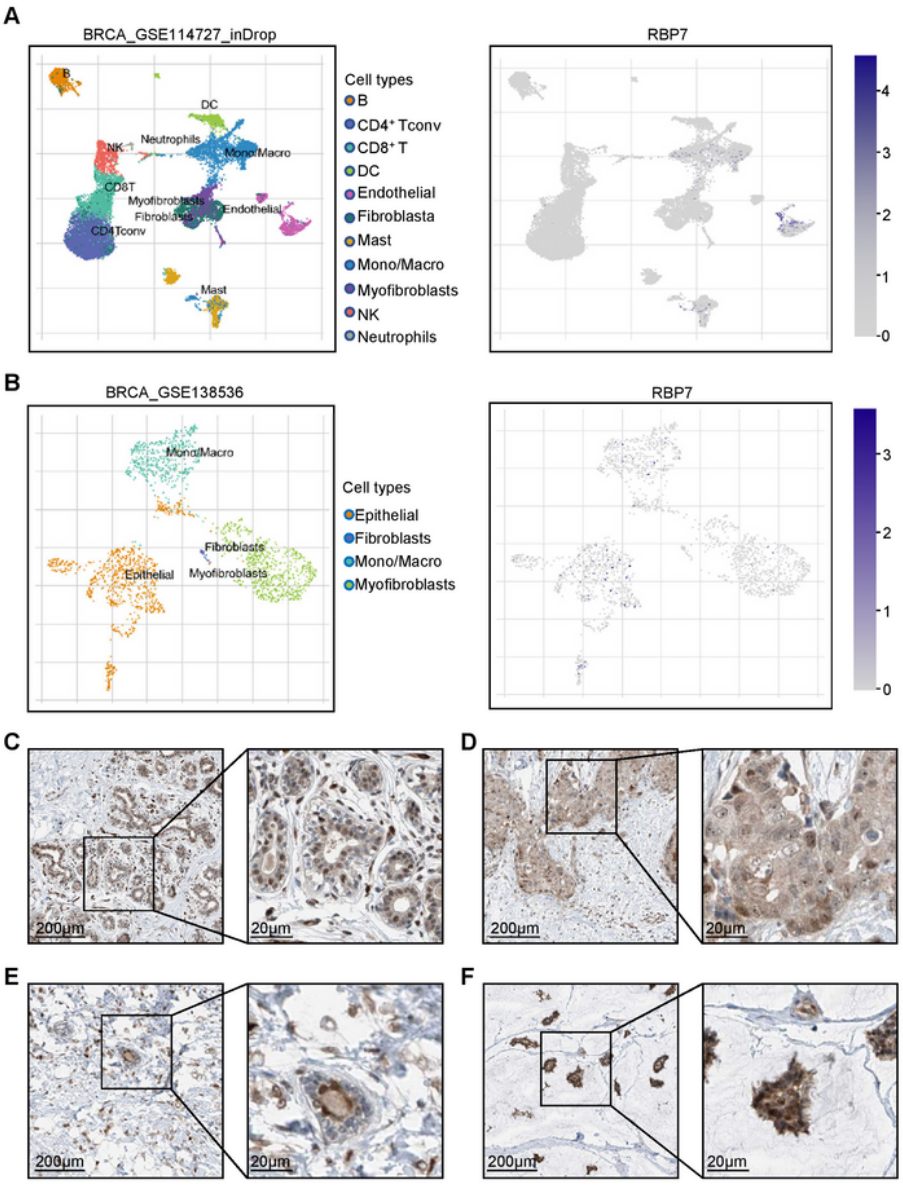

Figure 3.

Figure 3

The cellular and subcellular localization of RBP7 in BRCA. (A-B) The cellular localization of RBP7 mRNA in single BRCA cells. The expression level is coloured by marker intensity. (C-F) Representative immunohistochemical staining of normal tissues (C) and BRCA with weak (D), moderate (E) or strong (F)

RBP7 expression. Scale bars with lengths of 200 μm or 20 μm are displayed in the left and right panels, respectively.

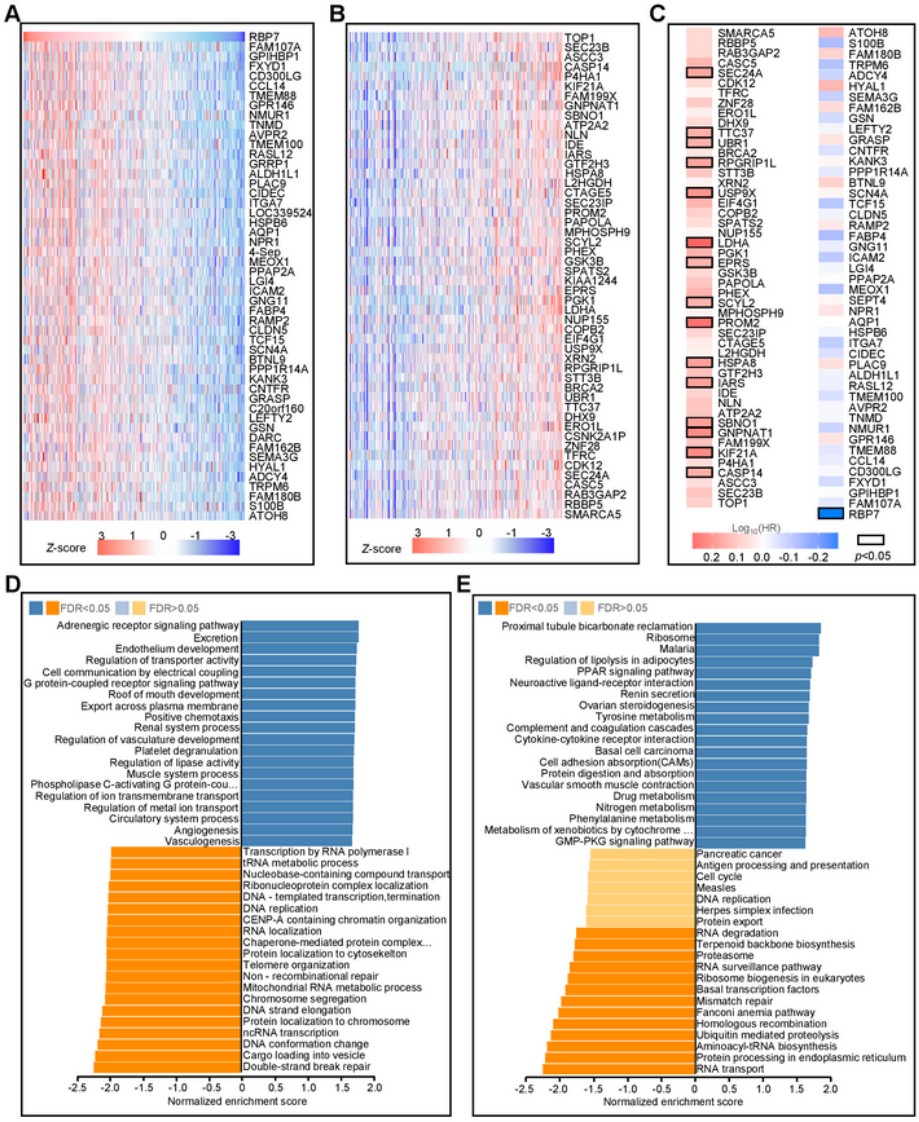

Figure 4.

Figure 4

GO and KEGG pathway enrichment analysis of the coexpressed genes of RBP7 in BRCA. (A-B) Heat maps of the top 50 genes that are positively (A) or negatively (B) correlated with the expression of RBP7 in BRCA. (C) Survival maps of the top 50 positive or negative coexpressed genes of RBP7 in BRCA. (D)

GO_BP enrichment analysis of the coexpressed genes of RBP7 in BRCA. (E) KEGG pathway analysis of the coexpressed genes of RBP7 in BRCA.

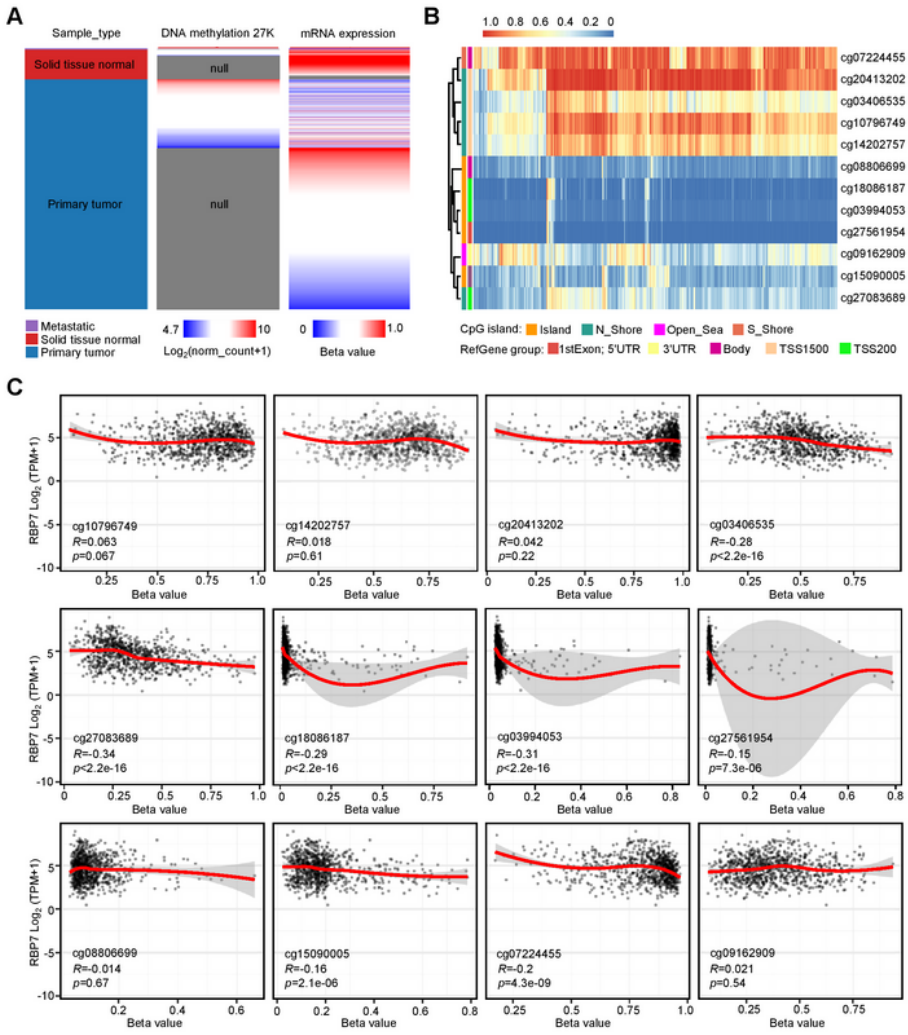

Figure 5.

Figure 5

The methylation analysis of RBP7 in BRCA. (A) Heat maps for the mRNA expression and DNA methylation of RBP7 in the TCGA database. (B) Visualization of methylation on different methylation

probes or gene regions of RBP7. (C) Analysis of the correlation between methylation on different methylation probes and mRNA expression of RBP7 in BRCA.

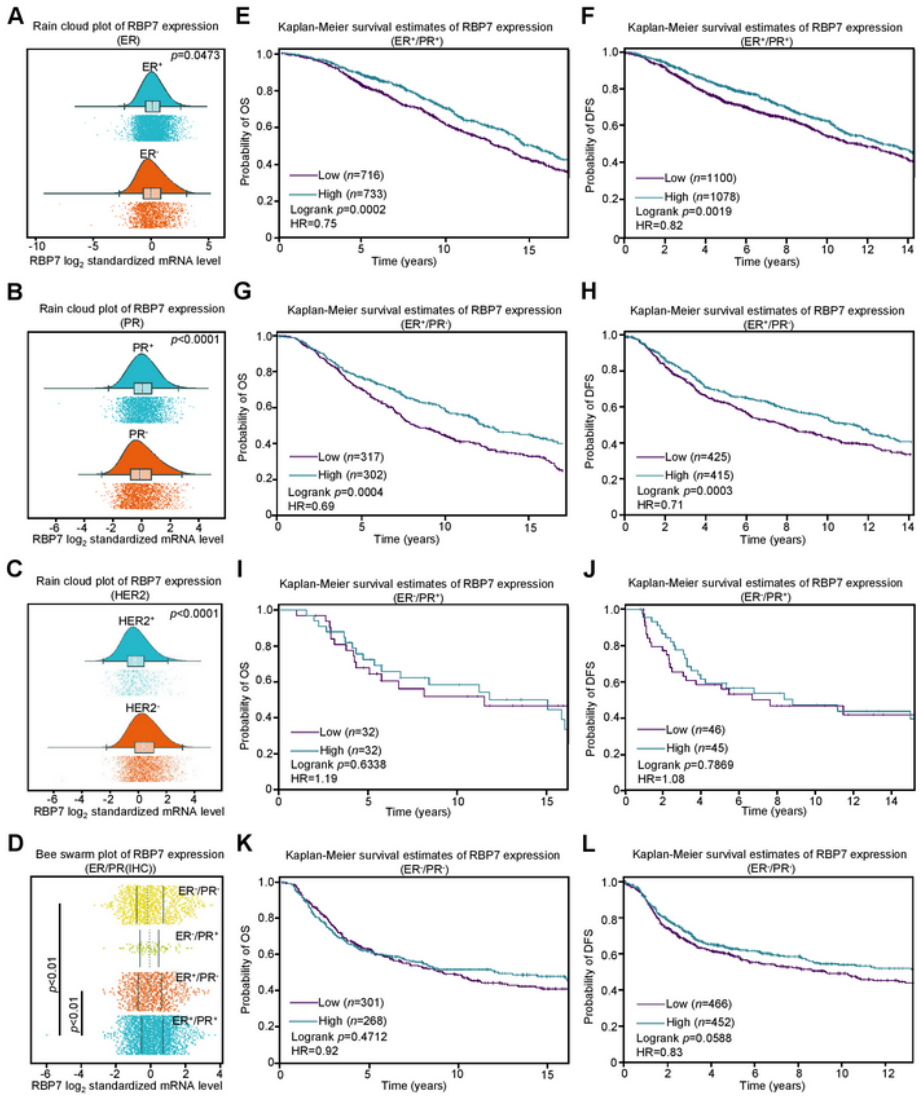

Figure 6.

Figure 6

The mRNA expression of RBP7 and the Kaplan–Meier survival curve in different molecular subtypes of BRCA. (A-C) The mRNA expression of RBP7 in different molecular subtypes, including ER (A), PR (B) and HER2 (C), of BRCA. (D) Bee swarm plot of RBP7 expression in BRCA with positive or negative ER or PR

expression. (E-L) Kaplan–Meier curves of BRCA with the DNA microarray results of ER+/PR+ (E,F), ER+/PR- (G,H), ER-/PR+ (I,J) or ER-/PR- (K,L).

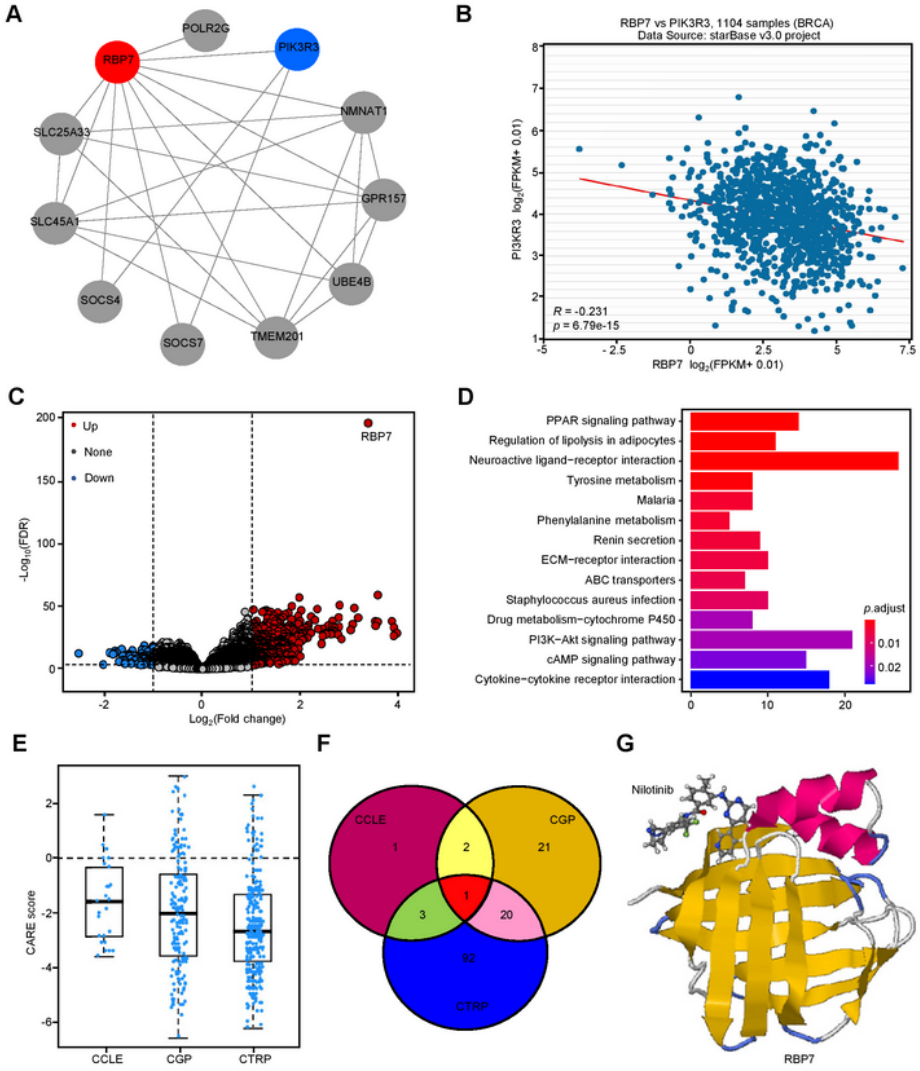

Figure 7.

Figure 7

Analysis of the potential regulatory functions and RBP7-targeting drugs for BRCA. (A) The PPI network for RBP7 and its coexpressed genes by STRING. (B) Correlation analysis of RBP7 and PIK3R3. (C) Volcano plot showing the differentially expressed genes in ER+ BRCA with high or low expression of RBP7. (D)

KEGG pathway analysis of DEGs in ER+ BRCA with high or low expression of RBP7. (E) CARE analysis of the resistance module gene RBP7 in the CCLE, CTRP and CGP databases. (F) Venn diagram showing the overlap of the RBP7-targeting drugs in the 3 databases. (G) Prediction of the binding sites between nilotinib and RBP7.

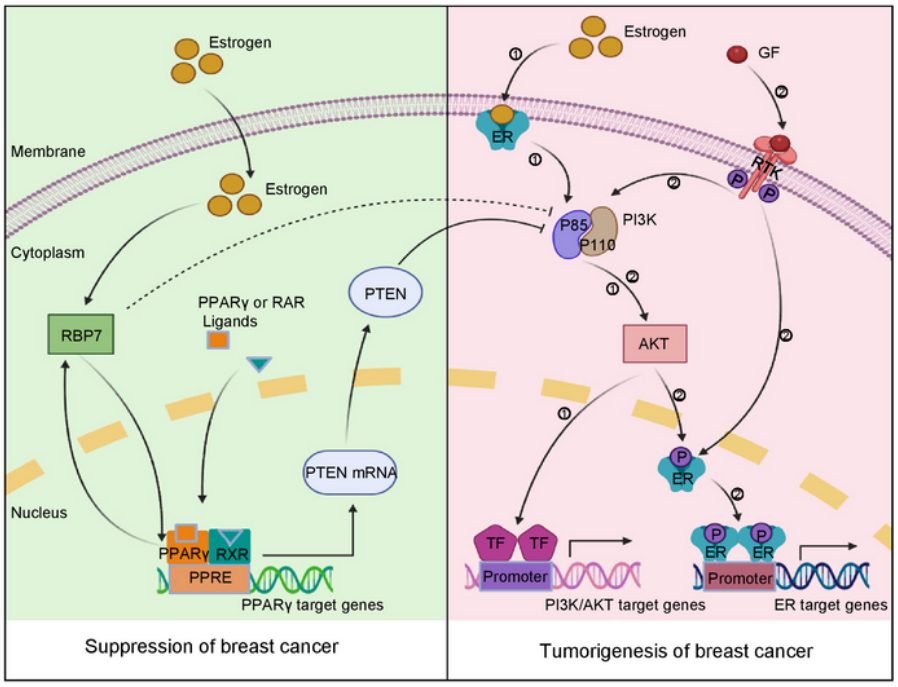

Figure 8.

Figure 8

The hypothetical function of RBP7 in the regulation of the PPAR and PI3K/AKT pathways in ER+ BRCA.

Supplementary Files

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