Missense mutations of GPER1 in breast invasive carcinoma: Exploring gene expression, signal transduction and immune cell infiltration with insights from cellular pharmacology

YU ZHANG[1], CHONG DU[1], SHU-QUN ZHANG[1], HUI-XIA YU[2], HAO-LIN MO[2], QI-YUAN YANG[3] and YANG LI[2]

[1]Department of Oncology, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi 710004, P.R. China;
[2]College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi 712100, P.R. China;
[3]Department of Molecular, Cell and Cancer Biology, University of Massachusetts Medical School, Worcester, MA 01605, USA

Received May 24, 2024; Accepted October 15, 2024

DOI: 10.3892/br.2024.1900

Abstract. G protein-coupled estrogen receptor 1 (GPER1) plays a crucial role in the progression of breast cancer and has emerged as a promising therapeutic target. However, while missense mutations in GPER1 have been detected in breast invasive carcinoma (BIC) samples, the resulting molecular, cellular and pharmacological changes remain unclear. The present study categorized BIC samples from The Cancer Genome Atlas database based on mutation information available in the cBioPortal database. Subsequently, survival analysis was conducted and the samples screened for differentially expressed genes (DEGs). Using these DEGs, the present study performed Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses, protein-protein interaction network analysis and hub gene selection. After assessing the prognostic value of hub genes, the immune cell infiltration between mutant and wild-type (WT) groups was analyzed. Finally, a luciferase reporter system was used to assess the cyclic AMP (cAMP) production mediated by GPER1 following treatment with the agonist G-1 for each mutation. The results revealed a significant decrease in progression-free survival and disease-specific survival in the GPER1 mutant group compared with the WT group. Gene expression analysis identified 60 DEGs, all of which were upregulated and significantly enriched in GO terms related to tumor progression, such as organic anion transport, glycosaminoglycan binding and monoatomic ion-gated channel activity. DEGs were also significantly enriched in the PI3K-Akt signaling pathway in KEGG. Hub gene selection and prognostic evaluation identified three genes significantly associated with survival: IL33, STAB2 and CFTR. Immune cell infiltration analysis revealed a significant decrease in CD8 T cell content in the GPER1 mutant group compared with the WT group. Luciferase reporter assays demonstrated that four missense mutations in GPER1 (L129M, E218Q, S235F and A345G) significantly attenuated the induction of cyclic adenosine monophosphate production mediated by its agonist. These findings provided valuable insights for the design of breast cancer drugs targeting GPER1 and for precision medicine initiatives.

Correspondence to: Dr Chong Du, Department of Oncology, The Second Affiliated Hospital of Xi'an Jiaotong University, 157 West 5th Road, Xincheng, Xi'an, Shaanxi 710004, P.R. China
E-mail: duchong@xjtu.edu.cn

Dr Yang Li, College of Animal Science and Technology, Northwest A&F University, 22 Xinong Street, Yangling, Shaanxi 712100, P.R. China
E-mail: yangli2001@nwsuaf.edu.cn

Key words: breast invasive carcinoma, G protein-coupled estrogen receptor 1, missense mutations, differentially expressed genes, signaling

Introduction

Breast invasive carcinoma (BIC) refers to a type of breast cancer where cancer cells invade the surrounding breast tissue. It encompasses various subtypes such as invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC). IDC, the most common type of breast cancer, originates in the milk ducts and invades nearby breast tissue. On the other hand, ILC is characterized by a single-cell infiltration pattern and loss of cell cohesion (1,2). Studying BIC holds significant clinical importance, as breast cancer not only stands as the most commonly diagnosed malignant neoplasm but also ranks as the second leading cause of cancer-related mortality among women worldwide (3). Among breast cancers, BIC accounts for ~80% of cases (4). BIC, particularly IDC, is associated with malignant cells breaching basement membranes. This infiltration into surrounding breast tissues often leads to a poorer prognosis (2). Additionally, BIC can present with unique characteristics such as lymphovascular invasion, nodal metastasis and lymphatic invasion, which can affect disease progression and treatment outcomes (1,5).

G protein-coupled estrogen receptor 1 (GPER1) plays a pivotal role in breast cancers, including BIC. Research conducted by Yang *et al* (6) underscored the involvement of GPER1 activation in estrogen-induced proliferation and endocrine resistance in breast cancer cells. Furthermore,

studies have identified that GPER1 stabilizes MORC2 via the PRKACA-CMA pathway, leading to amplified proliferation of breast cancer cells (6-8). Additionally, GPER1 has been linked to the metastatic behavior of triple-negative breast carcinoma cells, influencing the response to selective ERβ agonists and the sensitivity to tamoxifen (9). While some studies have reported a connection between GPER1 expression and poor postoperative outcomes in non-small cell lung cancer (10,11), its prognostic implications in breast cancer patients remain a topic of debate (12).

GPER1 can signal through $G\alpha_s$, $G\alpha_{i/o}$ and $G\beta\gamma$ subunits, as well as through β-arrestin, to modulate different signaling cascades (13). Through the $G\alpha_s$ subunit, GPER1 can elevate intracellular cyclic AMP (cAMP) levels, while via $G\alpha_{i/o}$, it can activate the extracellular signal-regulated kinase (ERK) pathway and the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway (14,15).

As a G-protein coupled receptor (GPCR), GPER1 is located on the cell membrane and is considered a promising drug target for the treatment of various diseases. However, its multifunctionality has led to controversy within the academic community on whether to agonize or antagonize this target. Wnuk *et al* (16) propose that blocking or antagonizing GPER1 signaling could have a beneficial impact on the treatment of estrogen receptor-positive breast cancer. Conversely, Morelli *et al* (17) discovered that treating Waldenström Macroglobulinemia cell lines with the GPER1-selective agonist G-1 could activate the TP53 pathway, halt the G_2/M cell cycle and induce apoptosis. These findings were also confirmed in mouse models. This research offers valuable insights into using GPER1 as a therapeutic target for addressing other human malignancies.

While GPER1 presents itself as a promising drug target, it is crucial to consider the effect of amino acid mutations on its ligand binding and signal transduction capabilities. Researches have shown that mutations in GPCRs can either weaken or enhance their efficacy as drug targets. For instance, the F282L mutation in the β2-adrenergic receptor has been found to weaken information flow compared with the wild type, leading to alterations in signal transmission pathways (18). In studies on the melanocortin-4 receptor, Tao (19) categorized the effects of GPCR mutations, particularly missense mutations, into five types: Defective protein production, intracellular retention, defective binding, defective signaling and unknown defect. In the case of GPER1, there are only sporadic reports on the physiological effects of its mutations. For example, Pupo *et al* (20) observed that mutations in the N-linked glycosylation site of GPER1 can result in its localization to the nucleus, where it functions as a transcription factor. However, Tutzauer *et al* (21) found that mutations do not affect the expression levels of GPER1. Overall, our understanding of GPER1 mutations remains limited and the cellular and physiological effects of these mutations remain to be elucidated. This limitation presents a challenge in using GPER1 as a target for BIC treatment, particularly in the context of precision medicine, where the same drug may elicit greatly different effects in patients with different genotypes.

To address these knowledge gaps and explore the implications of GPER1 mutations in BIC, the present study used the resources of The Cancer Genome Atlas (TCGA) database to categorize BIC samples based on GPER1 missense mutations and non-mutation status. The present study first screened for differentially expressed genes (DEGs) between the two groups. The identified DEGs were then subjected to gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses and protein-protein interaction (PPI) network analysis. Based on the PPI network, the present study further screened for hub genes and evaluated their prognostic value. Additionally, it analyzed the immune cell infiltration profiles between the mutation and wild-type (WT) groups. Finally, using mutation data from the cBioPortal website, eukaryotic expression vectors were constructed for the mutant and WT GPER1 proteins, and their pharmacological properties were examined in cell-based functional assays.

Materials and methods

Chemicals, reagents and plasmids. The highly selective agonist G-1 of GPER1 (purity ≥98%) was obtained from Tocris (cat. no. 3577; Tocris Bioscience). Dulbecco's modified Eagle's medium (DMEM) and other cell culture reagents were purchased from Thermo Fisher Scientific, Inc. As G-1 is not soluble in water, it was first dissolved in a 10% DMSO solution to establish a stock solution with a concentration of 1 mM. Prior to use, the stock solution was diluted to the working concentration using a serum-free DMEM medium.

The luciferase assay kit was obtained from Beyotime Institute of Biotechnology. Additionally, the fluorescent luciferase reporter plasmid pGL4.29 containing the cAMP response element (CRE) was purchased from Promega Corporation.

The Coding Sequence (CDS) of WT human GPER1 was obtained from NCBI (accession no. NM_001505.3), while the sequences of four missense mutations (L129M, E218Q, S235F and A354G) were sourced from the cBioPortal database (https://www.cbioportal.org; accessed on 22 October 2023). The WT and mutant GPER1 CDS were both synthesized by Beijing Augct DNA-Syn Biotechnology Co., Ltd. and cloned into the pcDNA3.1 (+) vector (Thermo Fisher Scientific, Inc.).

RNA sequencing data. RNA-seq data for BIC were obtained from TCGA database (namely TCGA-BRCA dataset; https://portal.gdc.cancer.gov; accessed on 22 October 2023), which was downloaded through UCSC Xena (https://xena.ucsc.edu; accessed on 22 October 2023).

The genetic mutation information and survival status of these BIC samples were accessed via cBioPortal (https://www.cbioportal.org; accessed on 22 October 2023). Samples from four studies were included in the mutation and survival analysis: TCGA Cell 2015 (818 samples), TCGA Firehose Legacy (1,108 samples), TCGA Nature 2012 (825 samples) and TCGA PanCancer Atlas (1084 samples).

Identification of DEGs. Based on the user's guide, the edgeR package (version 4.0.16; https://bioconductor.org/packages/edgeR/) in R software (version 4.3.2; http://www.R-project.org/) (22) was used to identify DEGs between WT and mutant GPER1 in patients with BIC (23). The threshold for DEGs selection was set at log2 |fold change (FC)| ≥2 and $P<0.05$. The heatmap and volcano plot of DEGs were generated using the pheatmap (version 1.0.12;

https://CRAN.R-project.org/package=pheatmap) and ggplot2 (version 3.5.1; https://CRAN.R-project.org/package=ggplot2) packages in the R programming platform.

Enrichment analyses of DEGs. GO and KEGG analyses were performed using the clusterProfiler package in R to identify the categories and signaling pathways enriched for each gene cluster. To address the issue of a high false discovery rate (FDR) resulting from multiple comparisons, *q* values were calculated. An FDR-adjusted $q<0.25$ and $P<0.05$ were considered statistically significant.

PPI and hub gene screening. To obtain the PPI network and hub genes associated with GPER1 in BIC, the DEGs were entered into the search tool for the retrieval of interacting genes (STRING) database (https://cn.string-db.org/) for analysis with a confidence score >0.4. Subsequently, the network generated by STRING was imported into Cytoscape 3.7.2 (24) for visualization. The cytoHubba plugin was used to filter key protein modules and after calculation with 12 algorithms, the top 10 genes with the highest scores were selected as hub genes.

Analysis of immune cell infiltration. CIBERSORT, a deconvolution algorithm, was used to assess the proportions of 22 immune lymphocyte in various BIC tumor samples (25). The results obtained were further used to compare the levels of immune cell infiltration between WT and mutant GPER1 patients. The number of permutations was set to 1,000, where $P<0.05$ serves as the criterion for successful computation in each sample.

Cell culture. The 293T cells were obtained from Beyotime Biotechnology (cat. no. C6008, Shanghai, China) and cultured in DMEM containing 10% fetal bovine serum, in a humidified atmosphere with 5% CO_2 at 37˚C. Upon purchase, all cells underwent mycoplasma testing and the results were negative. Cell line authentication was conducted using short tandem repeat (STR) analysis, which confirmed that the purchased cell line was 293T.

The cells were plated in 6-well plates and cultured for 24 h prior to assays. The Countess 3 cell counter (Invitrogen; Thermo Fisher Scientific, Inc.) was used for cell counting.

Detection of intracellular cAMP levels using a luciferase reporter. The effect of WT and mutant GPER1 on intracellular cAMP accumulation was assessed using a luciferase reporter assay that had been previously established (26). Specifically, 500 ng pcDNA3.1 (+) vectors carrying the WT GPER1 and four mutant variants (designated as pGPER1-WT, pGPER1-L129M, pGPER1-E218Q, pGPER1-S235F and pGPER1-A354G) were respectively mixed with 1,000 ng pGL4.29 vector (Promega Corporation) and 300 ng pEGFP-N1 (serving as an internal control for transfection efficiency and normalization in luciferase assays) and then transfected into 293T cells using polyethyleneimine transfection reagent (Shanghai Fushen Biotechnology Co., Ltd.).

Following transfection, the cells were cultured in the original medium for 24 h at 37˚C, then transferred to 48-well plates and allowed to grow for an additional 24 h to reach a density of $2x10^5$ cells per well. Subsequently, the agonist G-1 (Tocris Bioscience), diluted in serum-free DMEM medium, was added to the cells in the 48-well plates and incubated for 6 h.

After treatment, the cells were lysed and a substrate was added to induce luminescence using a Firefly Luciferase Reporter Gene Assay Kit (cat. no. RG006) obtained from Beyotime Biotechnology. The luminescence signal was detected using a Tecan M200 microplate reader (Tecan Group, Ltd.) to determine the relative luciferase activity in each well. According to a previous study, enhanced green fluorescent protein (EGFP) was used as an internal control to normalize the bioluminescent signal of the luciferase reporter pGL4.29 (27). The fluorescence of EGFP was detected under excitation/emission wavelengths of 488/507 nm. Firefly luciferase values were divided by the EGFP fluorescence values to assess normalized reporter gene activity. Each agonist concentration was tested in triplicate independent experiments and the data were presented as mean ± standard error of the mean.

Statistical analyses. The statistical analyses were performed using R software (version 4.3.2; http://www.R-project.org/) (22) and GraphPad Prism 7.0 (Dotmatics). The comparison of gene expression between GPER1 wild-type and mutant patients with BIC was conducted using the Mann-Whitney U test. The differences in immune cell proportion between the GPER1 wild-type and mutant groups were evaluated using the Mann-Whitney U test. Kaplan-Meier method with log-rank test was employed for survival analysis. The luciferase activities after agonist treatment in cells were converted into fold change relative to the control group (DMEM serum-free medium) and fitted to a dose-response curve using a nonlinear regression analysis. For the half-maximal effective concentration (EC_{50}) and maximum response concentration (R_{max}) of the wild-type and mutant groups, the Kruskal-Wallis test was performed with Dunn's post hoc analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Survival analyses and GPER1 expression. The cBioPortal online survival analysis tool used data from four studies sourced from TCGA, encompassing a total of 3,835 samples from 3,827 patients. Among these, 29 patients lacked mutation data, 3,743 patients had no mutations in GPER1 and 55 patients had mutations in GPER1. The findings suggested that, although no noteworthy disparities were observed in overall survival (OS) and disease-free survival (DFS) between the GPER1 WT and mutant cohorts, there were notable variances in progression-free survival (PFS) and disease-specific survival (DSS) outcomes across the two groups. The mutant group showed significantly reduced PFS and DSS compared with the non-mutant group ($P<0.05$; Fig. 1B-E).

As for the mRNA expression levels of GPER1 in the WT and mutant groups, RNAseq samples obtained from the UCSC Xena were analyzed. Out of the 1,126 samples downloaded, normal adjacent samples and those with missing clinical data and duplicates were excluded, resulting in a final dataset of 1,098 samples. Among these, 5 samples had missense mutations according to cBioPortal. The results showed no significant difference in GPER1 mRNA expression between the WT and mutant groups (Fig. 1A).

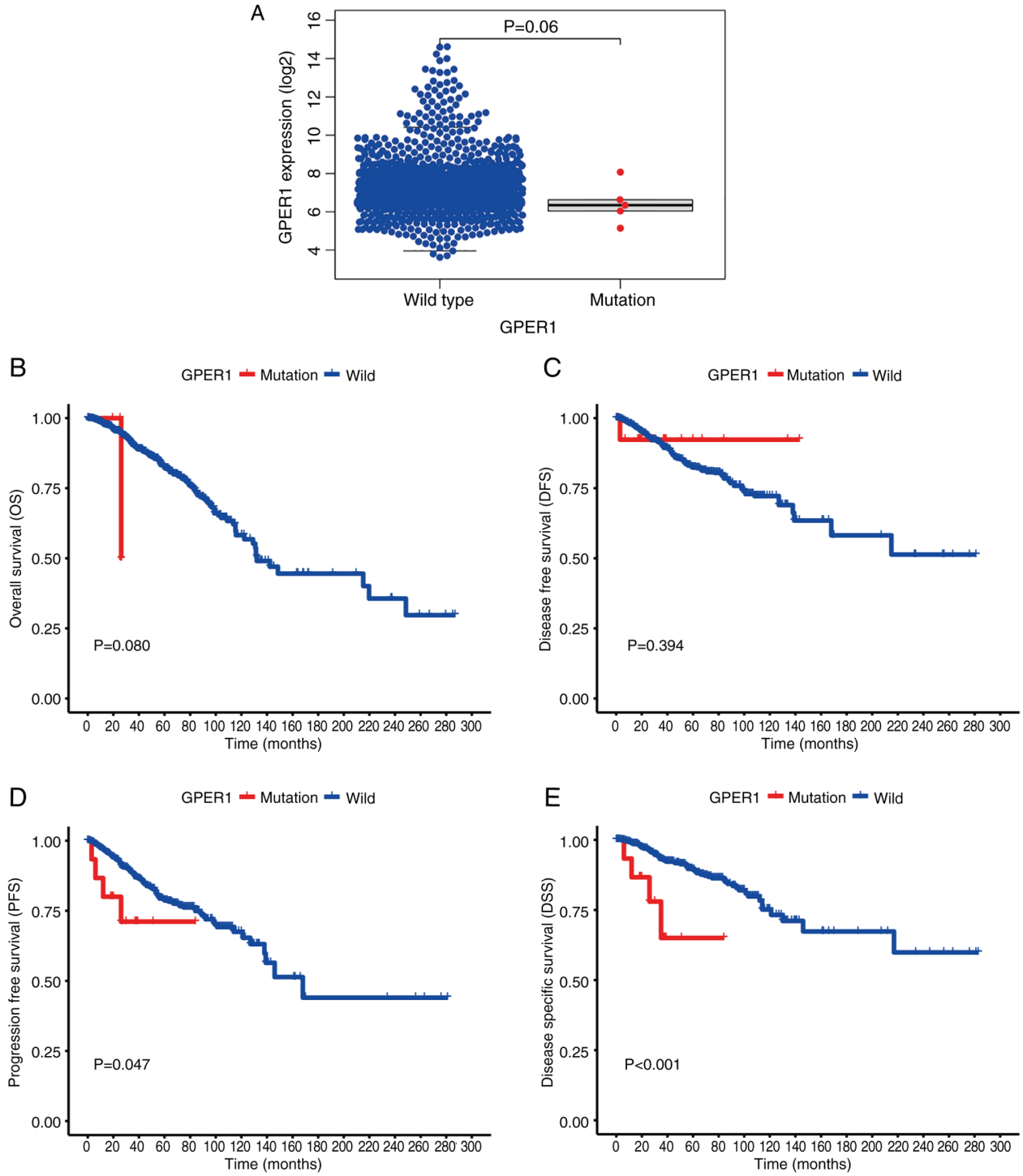

Figure 1. Comparison of mRNA expression and survival between WT and mutated GPER1 in patients with BIC. (A) mRNA expression of GPER1 in tissues of patients with BIC with WT and mutated GPER1. The analyzed samples were obtained from a filtered set of 1,098 samples downloaded from UCSC Xena. (B-E) Kaplan-Meier survival analysis of patients with GPER1 mutations. The analyzed samples were sourced from four studies on cBioPortal: TCGA Cell 2015 (818 samples), TCGA Firehose Legacy (1,108 samples), TCGA Nature 2012 (825 samples) and TCGA PanCancer Atlas (1,084 samples). WT, wild type; GPER1, G protein-coupled estrogen receptor 1; BIC, breast invasive carcinoma; TCGA, The Cancer Genome Atlas.

Identification of DEGs. The identification of DEGs was also conducted using the 1,098 samples obtained from the UCSC Xena. The analysis, performed with the edgeR package in R, identified 60 DEGs meeting the criteria of $\log_2$ |fold change (FC)| ≥2 and P<0.05, all of which exhibited upregulation (Table SI). The volcano plot and heatmap illustrating the DEGs are presented in Fig. 2.

GO and KEGG enrichment analyses. Using the clusterProfiler package in R software, GO analysis of the 60 DEGs revealed significant enrichment patterns. Specifically, in terms of Biological Process (BP), the DEGs were primarily enriched in organic anion transport. For Molecular Function (MF), the DEGs showed enrichment in glycosaminoglycan binding, monoatomic ion gated channel activity and gated channel

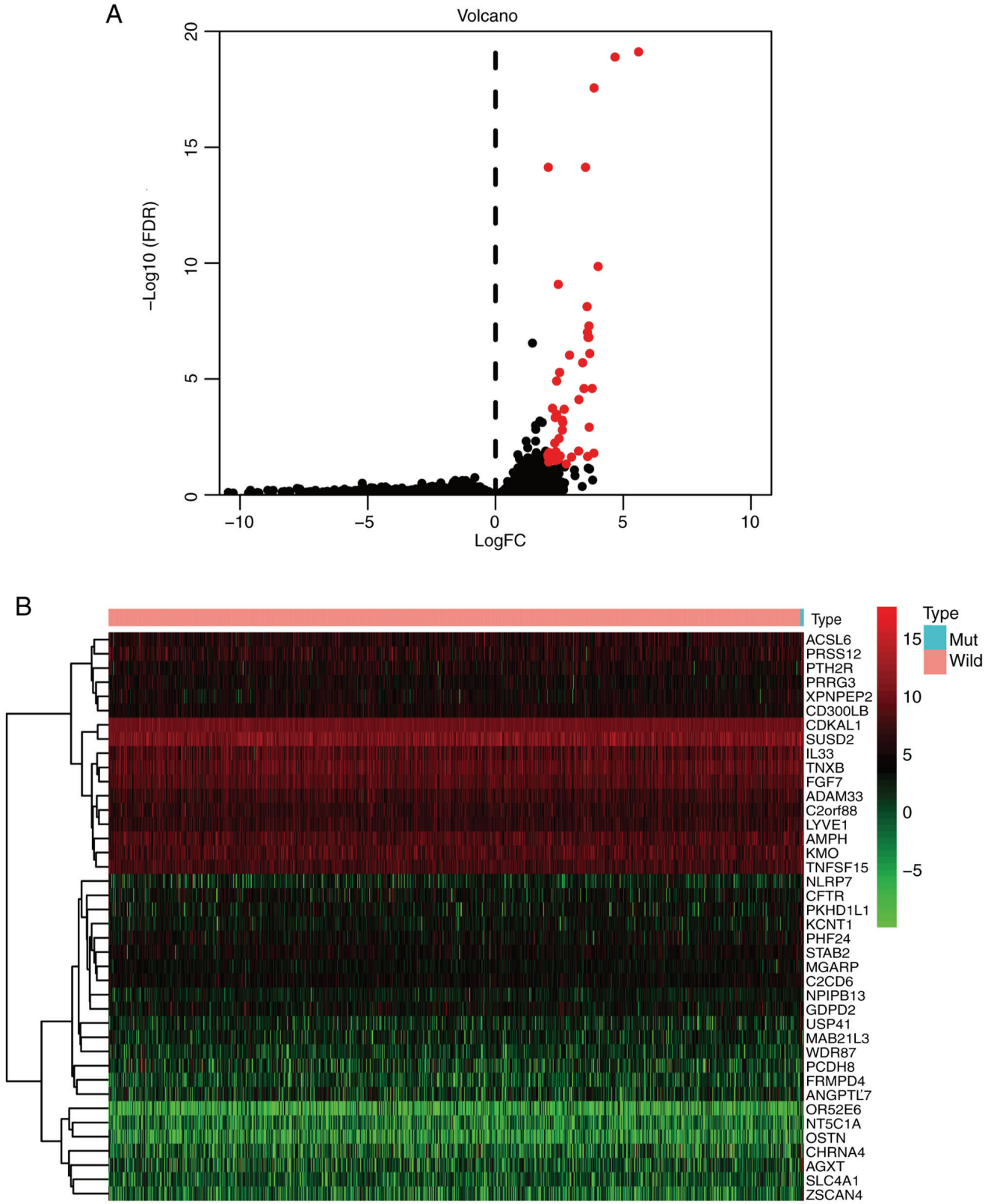

Figure 2. Volcano plot and heatmap of differentially expressed genes. (A) The volcano plot shows the results of gene expression analysis on BIC tumor samples in the TCGA database, categorized by GPER1 missense mutation. Red dots indicate upregulated genes, while black dots represent non-differentially expressed genes. (B) The heatmap displays 20 upregulated and 20 downregulated genes, with red indicating upregulation and green indicating downregulation. TCGA, The Cancer Genome Atlas; GPER1, G protein-coupled estrogen receptor 1.

activity. Regarding Cellular Component (CC), the DEGs were predominantly enriched in the external side of the plasma membrane (Fig. 3).

The KEGG analysis of the DEGs using the clusterProfiler package revealed significant enrichment in the PI3K-Akt signaling pathway, with subsequent enrichment observed in

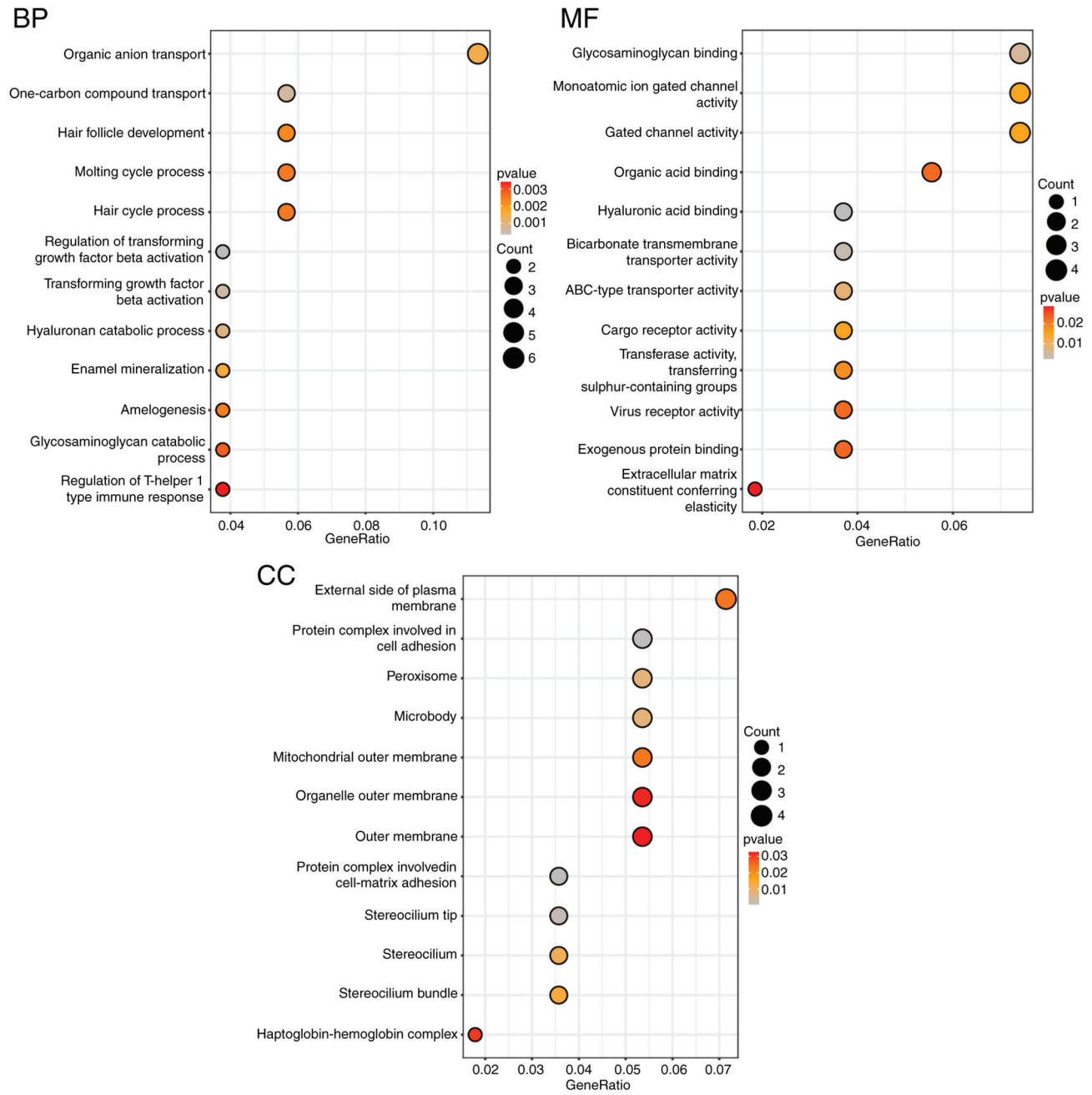

Figure 3. GO enrichment of differentially expressed genes. GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function.

ABC transporters, Peroxisome, ECM-receptor interaction and Bile secretion pathways (Fig. 4).

PPI network and hub genes. The protein-protein interaction network of DEGs obtained using the STRING online tool is shown in Fig. 5A. This network was further analyzed using the cytoHubba plugin in Cytoscape software with 12 algorithms to calculate the top 10 hub genes. Each algorithm yielded the same result, identifying IL33 and LYVE1 as the highest-scoring genes, followed by CFTR and STAB2 and finally FGF7, HBD, SLC4A1, TNFSF15, ABCB5, ITGB6 (Fig. 5B; Table SII).

Prognostic values of the hub genes. Kaplan-Meier survival analysis, employing the log-rank test, demonstrated that out of the 10 hub genes examined, only IL33 and STAB2 displayed significant survival disparities between the high and low expression cohorts (P<0.05). Additionally, CFTR, another hub gene, exhibited a nearly significant difference in expression between the two groups (P=0.059; Fig. 6).

GPER1 mutation-related immune cell infiltration. The analysis of tumor immune cell infiltration based on the CIBERSORT algorithm revealed that the proportions of various immune cells in each sample (Fig. 7A) and found that the content of CD8 T cells in the GPER1 mutation group was significantly lower than that in the WT group (P<0.05; Fig. 7C). Furthermore, the correlation matrix of immune cells showed that the content of memory resting CD4 T cells and CD8 T cells was negatively correlated with M0 macrophages, while naive B cells and Plasma cells exhibited a positive correlation.

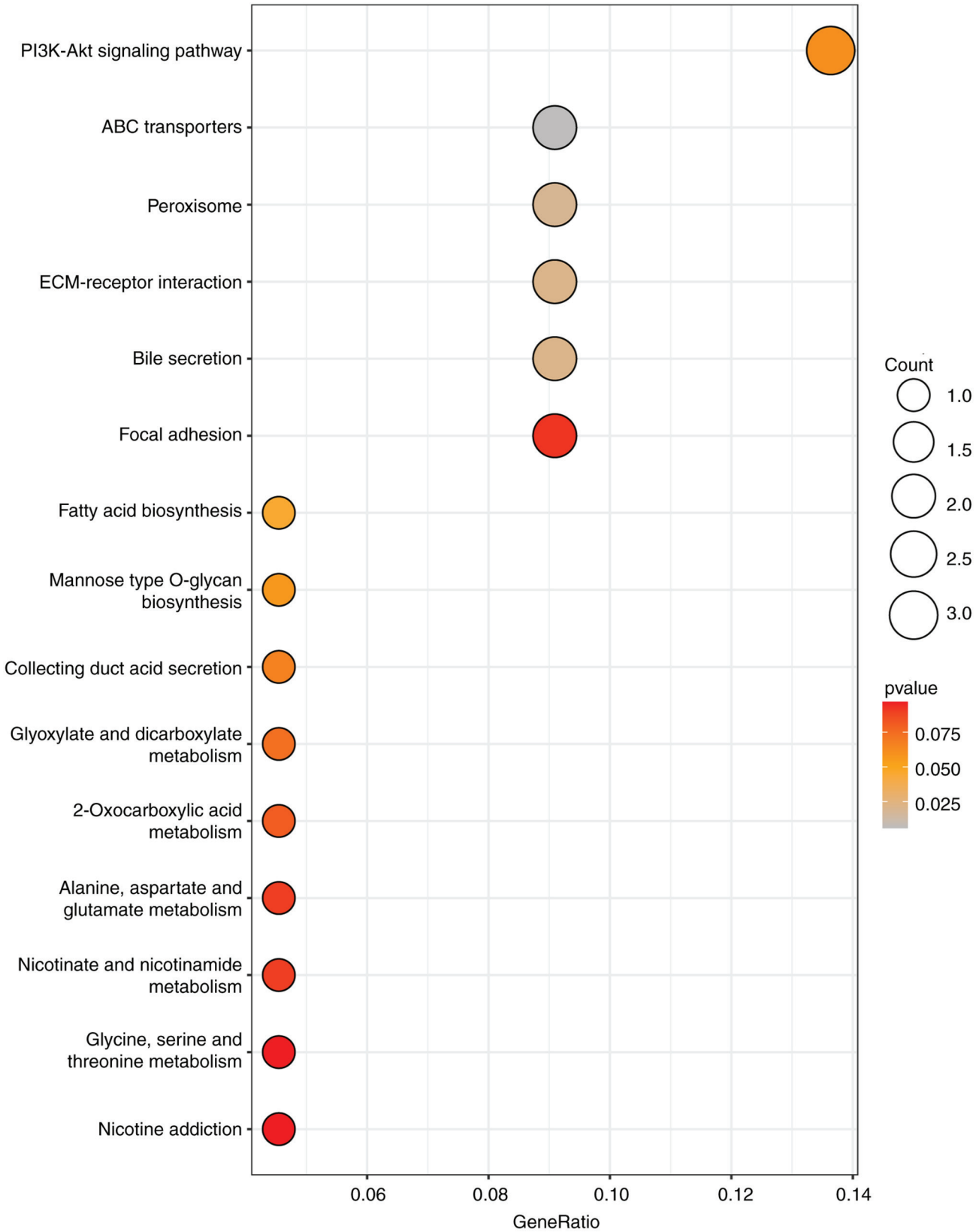

Figure 4. Kyoto Encyclopedia of Genes and Genomes enrichment analysis of differentially expressed genes.

Similarly, gamma and delta T cells and M1 macrophages also showed a certain degree of positive correlation (Fig. 7B).

Signaling properties of the GPER1 mutants. As a cell membrane surface receptor, GPER1 is capable of binding with the agonist G-1, activating intracellular adenylyl cyclase and facilitating the production of the canonical second messenger cAMP (14). To investigate the effect of missense mutations on the signal transduction of GPER1, a luciferase reporter system was used with CRE in cells transfected with WT and

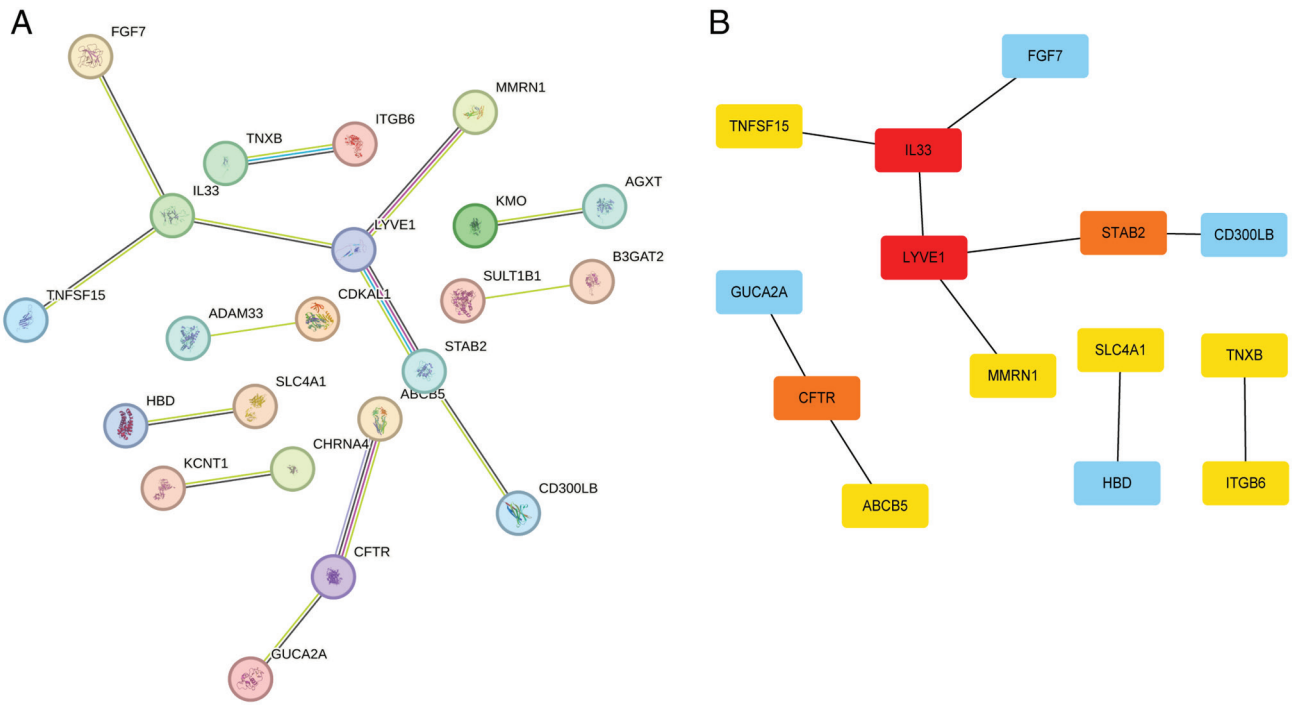

Figure 5. The PPI network and hub genes. (A) PPI network generated by STRING database. (B) The hub gene network constructed by Cytoscape. Red signifies the hub gene with the highest score (score=3), orange denotes the hub gene with the second-highest score (score=2), whereas blue and yellow represent hub genes with the lowest scores (score=1). PPI, protein-protein interaction; STRING, search tool for the retrieval of interacting genes.

mutant GPER1 vectors for analysis. The findings revealed that in comparison with the WT receptor, the response to the agonist G-1 were significantly attenuated in all the four missense mutations, as evidenced by a notable increase in their EC50 values (P<0.05) or undetectable responses (L129M and A345G) (Fig. 8; Table I). In terms of the response intensity, three mutants (E218Q, S235F and A345G) displayed a reduction in maximal response (R_{max}<100%), while one mutant (L129M) exhibited an enhancement in maximal response (R_{max}>100%; Table I).

Discussion

Previous studies have demonstrated that GPER1 can influence the proliferation, metastasis and drug sensitivity of breast cancer cells (6,9); However, whether mutations in this receptor, especially missense mutations, will cause a series of cellular and pharmacological changes similar to those observed in other GPCRs and thereby affect the regulation of tumor-related signaling pathways, remains to be elucidated. The present study analyzed the BIC samples from TCGA database, stratifying them into GPER1 missense mutation and WT groups. It compared the survival differences between the two groups, identified DEGs and subjected the DEGs to GO, KEGG and PPI network analyses. From the PPI network, 10 hub genes were identified and their prognostic value assessed. Additionally, the present study compared the immune cell infiltration profiles between the mutation and WT groups. Using cell-based pharmacological approaches, it also examined the impact of the GPER1 mutations on the receptor's regulation of intracellular cAMP accumulation in response to agonist stimulation.

Analysis of GPER1 gene expression in both the WT and mutant groups revealed that the difference between the two groups nearly reached statistical significance (P=0.06). This non-significant result, although consistent with the findings of Tutzauer *et al* (21), may be attributed to the limited sample size of mutants, warranting further sample collection for validation. Regarding survival analysis, while OS and DFS did not show significant differences between the two groups, PFS and DSS were significantly lower in the mutant group compared with the WT group. According to medical statistics, DSS is generally considered more accurate than OS in assessing treatment impact on specific diseases (28). Therefore, it can be inferred that mutations in GPER1 reduce the treatment efficacy for patients with BIC, resulting in poorer prognosis. Considering the limited number of mutant individuals included in the study (n=55) and the various types of mutations, this conclusion requires further support from additional case reports and larger cohort studies.

Using the criteria of log2 |fold change (FC)| ≥2 and P<0.05 in the edgeR package of R software, 60 DEGs were identified between the GPER1 mutant and WT groups. To assess whether the small number of DEGs identified was due to overly stringent filtering criteria, the present study further relaxed the thresholds to log2 |fold change (FC)| ≥1 and P<0.05. Despite this adjustment, only 80 DEGs were identified. The scarcity of DEGs hints at the possibility that GPER1 mutations in BIC may primarily influence a select few cellular functions and signaling pathways. An intriguing observation is that all identified DEGs were upregulated. This expression pattern contrasts with findings from other tumor-related DEG screenings (29-31), suggesting that GPER1 mutations may elicit a distinct gene expression profile in BIC.

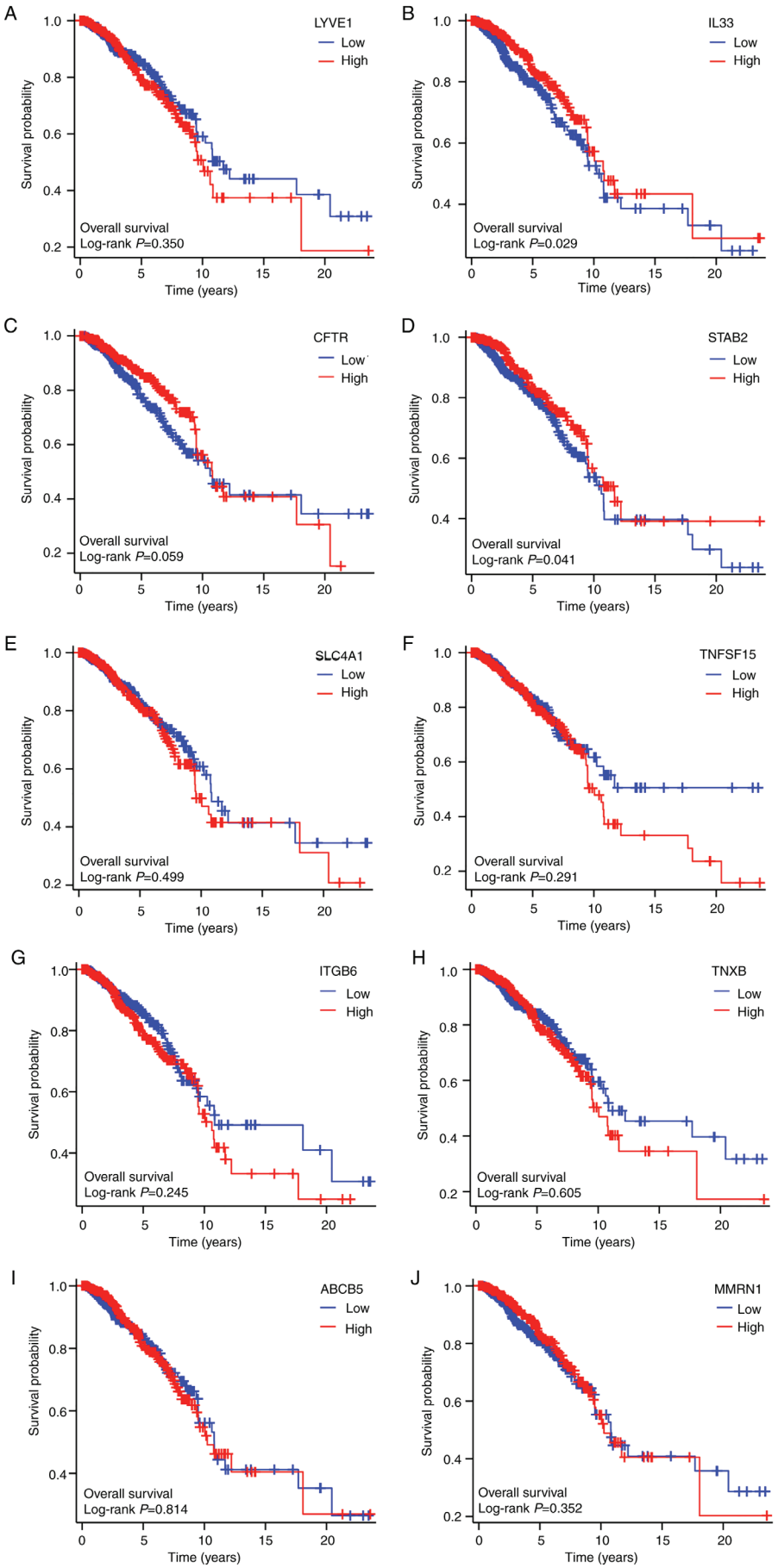

Figure 6. Prognostic value of 10 hub genes. Kaplan-Meier log-rank survival curves of the 10 selected hub genes grouped by expression levels in patients with BIC. (A) LYVE1, (B) IL33, (C) CFTR, (D) STAB2, (E) SLC4A1, (F) TNFSF15, (G) ITGB6, (H) TNXB, (I) ABCB5 and (J) MMRN1. BIC, breast invasive carcinoma.

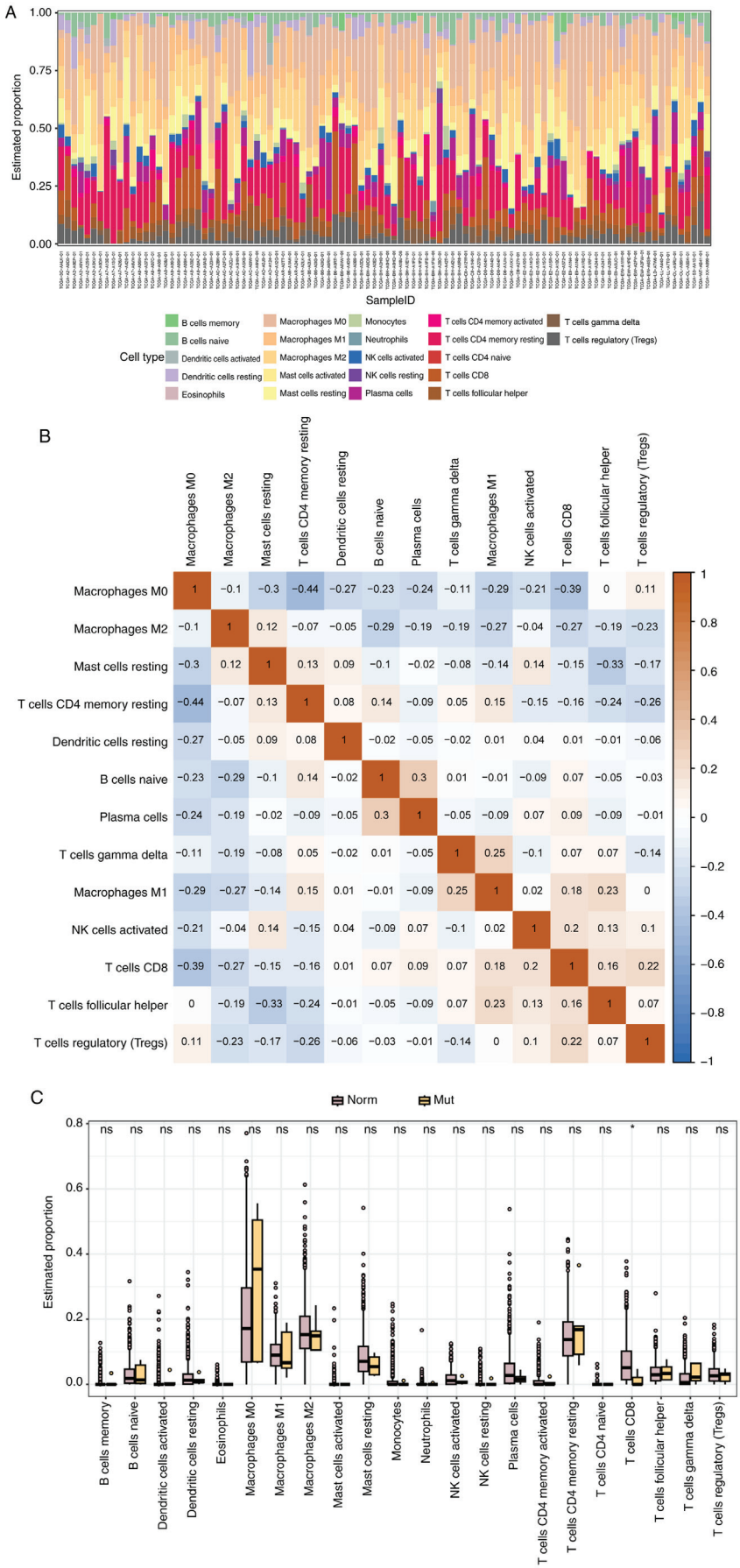

Figure 7. Immune cell infiltration in breast invasive carcinoma samples grouped by GPER1 mutation status. (A) Estimated proportions of 22 immune cell types in each sample. (B) Correlation matrix of immune cells. Brown indicates positive correlation, blue indicates negative correlation. (C) Differences in the proportions of 22 immune cell types between samples with and without GPER1 mutations. Wilcoxon rank-sum test was used, P<0.05. GPER1, G protein-coupled estrogen receptor 1; norm, wild type; mut, mutant; ns, not significant.

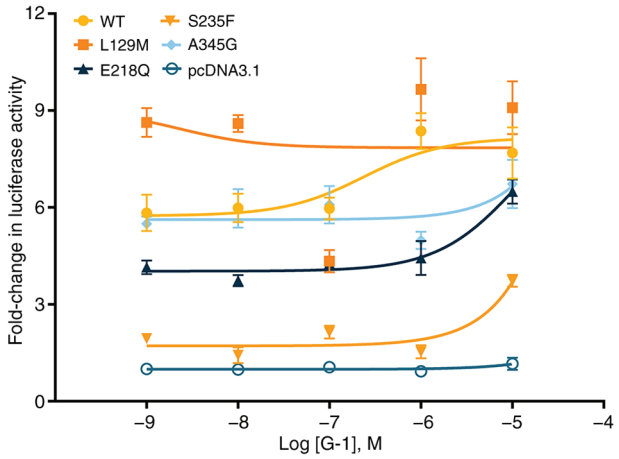

Figure 8. Signaling properties of WT and mutant GPER1s. WT and mutant GPER1 vectors were transiently transfected into human embryonic kidney 297T cells and the pGL4-CRE luciferase reporter system (pGL4.29 vector) monitored the effects of agonist G-1. Results are presented as fold change of agonist-treated groups relative to DMEM serum-free medium-treated group. Empty vector pcDNA 3.1 served as a negative control. Each data point represents mean ± standard error of the mean of 3 replicates. WT, wild-type; GPER1, G protein-coupled estrogen receptor 1.

Table I. Agonist-stimulated cAMP response of WT and mutant GPER1s.

WT and mutant GPER1s	G-1 stimulated cAMP response	
	EC_{50} (μM)	R_{max} (% WT)
WT	0.20±0.08	100
L129M	ND[b]	115.40±6.60
E218Q	157.12[a]±45.98	77.60±2.60
S235F	10.66±2.70	44.60±1.30
A345G	ND[b]	80.40±5.10

Values are expressed as the mean ± standard error of the mean of three independent experiments. [a]P<0.05 vs. wild type GPER1, Kruskal-Wallis test followed by Dunn's post hoc analysis. [b]ND, could not be determined. GPER1, G protein-coupled estrogen receptor 1; WT, wild type; EC_{50}, concentration of agonist that causes 50% maximal cAMP response; cAMP, cyclic AMP; R_{max}, maximal response.

GO enrichment analysis revealed that the DEGs were predominantly enriched in organic anion transport, glycosaminoglycan binding, monoatomic ion-gated channel activity, gated channel activity and plasma membrane. Organic anion transport plays a pivotal role in tumor cell proliferation and metastasis (32,33), influencing the efficacy of anti-tumor drugs by modulating their intracellular concentrations, thereby affecting their cytotoxic effects on cancer cells (34-36). Glycosaminoglycan binding affects crucial cellular functions such as adhesion, migration and signaling (37). Dysregulation of glycosaminoglycan-binding proteins has been linked to increased invasiveness and aggressiveness of tumors (37). Monoatomic ion-gated channel activity is associated with the regulation of ion flow across cell membranes, essential for maintaining cellular homeostasis and involved in processes such as cell signaling, proliferation and apoptosis (38,39). In tumors, dysregulation of ion channels can contribute to abnormal cell growth, survival and migration, influencing the progression and behavior of cancer cells (38,39). The plasma membrane plays a critical role in cell-cell interactions, nutrient uptake and signal transduction, with alterations in its properties affecting cell adhesion, migration and response to extracellular signals, thereby impacting cancer development and progression (39). In summary, the terms associated with the DEGs identified through GO enrichment analysis are all related to tumor progression and metastasis, potentially influencing the effectiveness of anti-tumor drugs. This finding is consistent with the lower PFS and DSS observed in the GPER1 mutant group in the present study.

The KEGG analysis of these DEGs revealed enrichment for the PI3K-Akt, ABC transporters, Peroxisome, ECM-receptor interaction and bile secretion pathway. This discovery is in line with numerous studies that have identified this pathway in differential gene expression analyses comparing tumor and non-tumor samples (40-42) Activation of the PI3K-Akt pathway is known to promote oncogenes, suppress tumor suppressor genes and contribute to the development of cancer characteristics such as uncontrolled cell growth, evasion of apoptosis, angiogenesis, tissue invasion and metastasis (43,44). For instance, in cervical cancer, the PI3K-Akt pathway plays a role in modulating the expression of HCCR, a downstream component that influences disease progression (45). Overall, activation of the PI3K-Akt signaling pathway is frequently linked to poor prognosis in cancer (46-48). Similar to the PI3K-Akt pathway, the ABC transporter pathway is associated with poor prognosis in cancer patients (49,50). A study identified transporters such as ABCA1, ABCB1 and ABCG2 as being involved in the efflux of chemotherapeutic agents, thereby contributing to the multidrug resistance seen in aggressive breast cancer subtypes (51). Furthermore, some other researches indicate that high expression levels of these transporters are correlated with increased tumor invasiveness (52,53). The peroxisome signaling pathway, particularly through peroxisome proliferator-activated receptors (PPARs), plays a significant role in the development and progression of breast cancer. PPARs are ligand-dependent nuclear receptor transcription factors that regulate genes involved in critical cellular processes such as proliferation, differentiation and metabolism (54). Dysregulation of PPARs has been linked to breast cancer. Additionally, peroxisomes contribute to carcinogenesis by producing reactive oxygen species (ROS), which can lead to DNA damage and initiate tumor development (55,56). Components of the extracellular matrix (ECM), such as fibronectin and collagen, significantly influence breast cancer cell migration and epithelial-mesenchymal transition (EMT), a process whereby epithelial cells acquire mesenchymal traits, enhancing their invasive capabilities (57,58). ECM-receptor interaction signaling pathways have been reported to be closely associated with breast cancer metastasis (59,60). Unlike the previous four signaling pathways, bile acids, although found to accumulate in human breast tumors, have been shown to inhibit tumor growth and improve patient survival (61). The enrichment of DEGs in the bile secretion pathways may be related to the body's mechanisms for counteracting tumor

development. Overall, the KEGG analysis revealed that DEGs were enriched in signaling pathways, with four pathways related to tumor progression and drug resistance and one pathway associated with anti-tumor activity. These findings are generally consistent with the results obtained from the GO analysis and can help explain the lower PFS and DSS observed in the GPER1 mutation group.

Through PPI network analysis and hub gene screening, the present study identified 10 hub genes, among which IL33 and STAB2 showed significant associations with the survival of BIC patients, while CFTR demonstrated near-significant relevance (P=0.059). Of these three genes, only CFTR was found to function as a hub gene in other cancers and diseases, such as colorectal cancer (62), chromophobe renal cell carcinoma (63) and pulmonary arterial hypertension (64), whereas IL33 and STAB2 have not been previously identified as hub genes in other types of cancer. For the other two genes, IL33 has been shown to promote cancer stemness, tumor growth and metastasis by recruiting macrophages into the tumor microenvironment (65,66), while STAB2, a hyaluronic acid receptor, has also been associated with cancer metastasis (67-69). Furthermore, the IL33-STAB2 axis has been demonstrated to modulate immune responses in gastrointestinal cancers (70,71). In summary, IL33, STAB2 and CFTR are all linked to tumor progression, but only CFTR has been identified as a hub gene in some other cancer types, suggesting that IL33 and STAB2 may play distinct roles in GPER1 mutation-mediated tumor progression.

Based on the analysis of immune cell infiltration using the CIBERSORT algorithm, it was observed that the proportion of CD8 T cells in the GPER1 mutation group was notably lower compared with the WT group. CD8 T cells are essential for recognizing and eliminating cancer cells and their diminished presence may lead to immune evasion and tumor progression (72-74). Furthermore, research has indicated that augmenting the infiltration of CD8 T cells, either through interventions such as vitamin D therapy or immune checkpoint blockade, can bolster anti-tumor immunity and improve treatment outcomes for cancer patients (75-77). Consequently, the reduced levels of CD8 T cells in the GPER1 mutation group may contribute to the poorer prognosis observed in this cohort.

Mutations in GPCRs can lead to diverse pharmacological effects that significantly affect drug efficacy and patient responses. These genetic alterations can modify receptor pharmacology by influencing cell surface expression, receptor interactions, basal activity and GPCR-G protein coupling. Such changes can result in varied disease phenotypes or alter drug response (78). For instance, mutations in the adhesion G protein-coupled receptor latrophilin 1 can impair receptor trafficking, contributing to conditions such as obesity (79). Consequently, understanding the molecular consequences of pathogenic mutations in GPCRs is crucial for elucidating disease mechanisms and developing effective therapeutic strategies. To assess the influence of mutations on the response of GPER1 to agonists, the present study employed a luciferase reporter system with a CRE element to quantify cellular cAMP levels downstream of GPER1 activation by the potent selective agonist G-1. The results revealed that all four mutations, namely L129M, E218Q, S235F and A345G, attenuated cellular cAMP levels to varying extents following G-1 stimulation. While the cAMP signaling pathway demonstrates multifunctionality and can exert either tumor-suppressive or tumor-promoting effects across different types of tumor and cellular contexts (80), activation of cAMP signaling by $G\alpha_s$ in the GPCR system has been implicated as a tumor suppressor in neoplasms derived from ectodermal cells, including neural and epidermal stem/progenitor cells (81). Given that GPER1 activates cAMP via coupling to $G\alpha_s$, the attenuation of cAMP signaling due to mutations may contribute to the unfavorable prognosis observed in patients with BIC with GPER1 mutations. Furthermore, the diminished ligand activation capacity of mutated GPER1 also partially accounts for the reduced PFS observed in patients with BIC with GPER1 mutations.

It is worth noting that, while previous studies have grouped tumors based on mutations and non-mutations and screened for DEGs for subsequent bioinformatics analysis (82-84), the present study has, for the first time, to the best of the authors' knowledge, integrated cell pharmacological experiments to detect the potential effect of mutations on key GPCR downstream signaling pathways in tumors. These intriguing findings provide important insights for precision medicine targeting GPCRs. However, the present study has some limitations, such as not grouping according to the mutation types of GPCRs. Considering the diverse pharmacological effects of GPCR mutations (19), further subgrouping and studying the specific effects of different GPCR mutations on tumor cell biology have become one of the next research priorities.

In summary, the present study explored the molecular and pharmacological effects of missense mutations in GPER1 using the BIC sample information from TCGA data. It found that missense mutations in GPER1 led to adverse prognostic outcomes and reduced treatment effectiveness. Differential gene expression analysis revealed that GPER1 mutations caused upregulation of a subset of genes, which enriched in signaling pathways, such as PI3K-Akt, implicated in tumor progression. Similarly, hub genes selected through PPI network screening, including IL33, STAB2 and CFTR, were all associated with tumor progression. Immune infiltration analysis also demonstrated a reduction in anti-tumor CD8 T cell content with GPER1 mutations. Pharmacological analysis revealed that mutations diminish GPER1's ability to induce cAMP production upon agonist stimulation. These findings provided insights into the design of anti-tumor drugs targeting GPER1 and personalized medicine approaches.

Acknowledgements

The authors appreciate the support of Animal Genetics and Breeding Laboratory, College of Animal Science and Technology, Northwest A&F University (Shaanxi, China).

Funding

The present study was supported by the National Natural Science Foundation of China (grant nos. 82174164, 81901886 and 31502180), Shaanxi Administration of Traditional Chinese Medicine (grant no. 2021-ZZ-JC019), the Key Research and Development Program of Shaanxi (grant nos. 2022SF-411 and 2023-YBSF-565) and the Fundamental Research Funds

for the Central Universities (grant no. xzy012020040), the Natural Science Foundation of Shaanxi Province (grant no. 2023-JC-YB-155).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YL conceived the study and conducted the overall review and revision of the manuscript. CD and SQZ secured the research funding and supervised the progress of the study. HY and HLM performed the cellular pharmacology experiments. YZ conducted the differential analysis of breast cancer data from the TCGA database and drafted the initial manuscript. QYY carried out the immune cell infiltration analysis. HY and HLM confirm the authenticity of all the raw data. All authors read and approved the final manuscript. HY and HM confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Ide Y, Horii R, Osako T, Ogura K, Yoshida R, Iwase T and Akiyama F: Clinicopathological significance of invasive micropapillary carcinoma component in invasive breast carcinoma. Pathol Int 61: 731-736, 2011.
2. Lwin ZM, Guo C, Salim A, Yip GW, Chew FT, Nan J, Thike AA, Tan PH and Bay BH: Clinicopathological significance of calreticulin in breast invasive ductal carcinoma. Mod Pathol 23: 1559-1566, 2010.
3. Siegel RL, Giaquinto AN and Jemal A: Cancer statistics, 2024. CA Cancer J Clin 74: 12-49, 2024.
4. Mei L, Wang K and Gu Y: Improved Fuzzy C-means clustering algorithm-based dynamic contrast-enhanced magnetic resonance imaging features in the diagnosis of invasive breast carcinoma before and after menopause. Comput Math Methods Med 2022: 2917844, 2022.
5. Rakha EA, Martin S, Lee AHS, Morgan D, Pharoah PD, Hodi Z, Macmillan D and Ellis IO: The prognostic significance of lymphovascular invasion in invasive breast carcinoma. Cancer 118: 3670-3680, 2012.
6. Yang F, Xie HY, Yang LF, Zhang L, Zhang FL, Liu HY, Li DQ and Shao ZM: Stabilization of MORC2 by estrogen and antiestrogens through GPER1-PRKACA-CMA pathway contributes to estrogen-induced proliferation and endocrine resistance of breast cancer cells. Autophagy 16: 1061-1076, 2020.
7. Saroha HS, Kumar Guddeti R, Jacob JP, Kumar Pulukuri K, Karyala P and Pakala SB: MORC2/β-catenin signaling axis promotes proliferation and migration of breast cancer cells. Med Oncol 39: 135, 2022.
8. Thomas L, Chutani N, R K, Nair AS, Yellapu NK, Karyala P and Pakala SB: Microrchidia 2/histone deacetylase 1 complex regulates E-cadherin gene expression and function. Biochem J 480: 1675-1691, 2023.
9. Schmitz V, Bauerschmitz G, Gallwas J and Gründker C: Suppression of G Protein-coupled estrogen Receptor 1 (GPER1) Enhances the Anti-invasive efficacy of selective ERβ Agonists. Anticancer Res 42: 5187-5194, 2022.
10. Li ZH, Liu C, Liu QH, Wang J, Wang Y, Wang YF, Deng SJ and Li DB: Cytoplasmic expression of G protein-coupled estrogen receptor 1 correlates with poor postoperative prognosis in non-small cell lung cancer. J Thorac Dis 14: 1466-1477, 2022.
11. Shen Y, Li C, Zhou L and Huang JA: G protein-coupled oestrogen receptor promotes cell growth of non-small cell lung cancer cells via YAP1/QKI/circNOTCH1/m6A methylated NOTCH1 signalling. J Cell Mol Med 25: 284-296, 2021.
12. Yang F and Shao ZM: Double-edged role of G protein-coupled estrogen receptor 1 in breast cancer prognosis: An analysis of 167 breast cancer samples and online data sets. Onco Targets Ther 9: 6407-6415, 2016.
13. Srivastava DP and Evans PD: G-protein oestrogen receptor 1: Trials and tribulations of a membrane oestrogen receptor. J Neuroendocrinol 25: 1219-1230, 2013.
14. Evans NJ, Bayliss AL, Reale V and Evans PD: Characterisation of signalling by the Endogenous GPER1 (GPR30) Receptor in an Embryonic Mouse Hippocampal Cell Line (mHippoE-18). PLoS One 11: e0152138, 2016.
15. Yu M, Xu L, Lei B, Sun S and Yang Y: Tetrachlorobisphenol A and bisphenol AF induced cell migration by activating PI3K/Akt signaling pathway via G protein-coupled estrogen receptor 1 in SK-BR-3 cells. Environ Toxicol 38: 126-135, 2023.
16. Wnuk A, Przepiórska K, Pietrzak BA and Kajta M: Emerging evidence on membrane estrogen receptors as novel therapeutic targets for central nervous system pathologies. Int J Mol Sci 24: 4043, 2023.
17. Morelli E, Hunter ZR, Fulciniti M, Gullà A, Perrotta ID, Zuccalà V, Federico C, Juli G, Manzoni M, Ronchetti D, *et al*: Therapeutic activation of G protein-coupled estrogen receptor 1 in Waldenström Macroglobulinemia. Exp Hematol Oncol 11: 54, 2022.
18. Gao N, Liang T, Yuan Y, Xiao X, Zhao Y, Guo Y, Li M and Pu X: Exploring the mechanism of F282L mutation-caused constitutive activity of GPCR by a computational study. Phys Chem Chem Phys 18: 29412-29422, 2016.
19. Tao YX: Molecular mechanisms of the neural melanocortin receptor dysfunction in severe early onset obesity. Mol Cell Endocrinol 239: 1-14, 2005.
20. Pupo M, Bodmer A, Berto M, Maggiolini M, Dietrich PY and Picard D: A genetic polymorphism repurposes the G-protein coupled and membrane-associated estrogen receptor GPER to a transcription factor-like molecule promoting paracrine signaling between stroma and breast carcinoma cells. Oncotarget 8: 46728-46744, 2017.
21. Tutzauer J, Sjöström M, Bendahl PO, Rydén L, Fernö M, Fredrik Leeb-Lundberg LM and Alkner S: Plasma membrane expression of G protein-coupled estrogen receptor (GPER)/G protein-coupled receptor 30 (GPR30) is associated with worse outcome in metachronous contralateral breast cancer. PLoS One 15: e0231786, 2020.
22. R Core Team: R Core Team 2023 R: A language and environment for statistical computing. R foundation for statistical computing. https://www.R-project.org/. R Foundation for Statistical Computing, 2023.
23. Robinson MD, McCarthy DJ and Smyth GK: edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26: 139-140, 2009.
24. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B and Ideker T: Cytoscape: A software Environment for integrated models of biomolecular interaction networks. Genome Res 13: 2498-2504, 2003.
25. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, Hoang CD, Diehn M and Alizadeh AA: Robust enumeration of cell subsets from tissue expression profiles. Nat Methods 12: 453-457, 2015.
26. Wu L, Yu H, Mo H, Lan X, Pan C, Wang L, Zhao H, Zhou J and Li Y: Functional characterization of melanocortin-3 receptor in a hibernating cavefish onychostoma macrolepis. Animals 12: 38, 2021.
27. Vesuna F, Winnard P and Raman V: Enhanced green fluorescent protein as an alternative control reporter to Renilla luciferase. Anal Biochem 342: 345-347, 2005.
28. Han J, Xiao N, Yang W, Luo S, Zhao J, Qiang Y, Chaudhary S and Zhao J: MS-ResNet: Disease-specific survival prediction using longitudinal CT images and clinical data. Int J Comput Assist Radiol Surg 17: 1049-1057, 2022.

29. Li Z, Ding B, Xu J, Mao K, Zhang P and Xue Q: Relevance of STK11 mutations regarding immune cell infiltration, drug sensitivity, and cellular processes in lung adenocarcinoma. Front Oncol 10: 580027, 2020.
30. Sun J, Li S, Wang F, Fan C and Wang J: Identification of key pathways and genes in pten mutation prostate cancer by bioinformatics analysis. BMC Med Genet 20: 191, 2019.
31. Fan C, Zhao C, Shugen Li FW and Wang J: Significance of PTEN mutation in cellular process, prognosis, and drug selection in clear cell renal cell carcinoma. Front Oncol 9: 357, 2019.
32. Ono M, Baden A, Okudaira H, Kobayashi M, Kawai K, Oka S and Yoshimura H: Assessment of amino acid/drug transporters for renal transport of [18F]fluciclovine (Anti-[18F]FACBC) in vitro. Int J Mol Sci 17: 1730, 2016.
33. Becchetti A, Munaron L and Arcangeli A: The role of ion channels and transporters in cell proliferation and cancer. Front Physiol 4: 312, 2013.
34. Zhou F, Hong M and You G: Regulation of human organic anion transporter 4 by progesterone and protein kinase C in human placental BeWo cells. Am J Physiol Endocrinol Metab 293: E57-E61, 2007.
35. Xu W, Tanaka K, Sun AQ and You G: Functional role of the C terminus of human organic anion transporter hOAT. J Biol Chem 281: 31178-31183, 2006.
36. You G: Towards an understanding of organic anion transporters: Structure-function relationships. Med Res Rev 24: 762-774, 2004.
37. Baycin-Hizal D, Gottschalk A, Jacobson E, Mai S, Wolozny D, Zhang H, Krag SS and Betenbaugh MJ: Physiologic and pathophysiologic consequences of altered sialylation and glycosylation on Ion channel function. Biochem Biophys Res Commun 453: 243-253, 2014.
38. Zhang G, Yang H, Liang H, Yang J, Shi J, McFarland K, Chen Y and Cui J: A charged residue in S4 regulates coupling among the activation gate, voltage, and Ca2+ sensors in BK channels. J Neurosci 34: 12280-12288, 2014.
39. Thompson AN, Posson DJ, Parsa PV and Nimigean CM: Molecular mechanism of pH sensing in KcsA potassium channels. Proc Natl Acad Sci USA 105: 6900-6905, 2008.
40. Yang R, Zhou Y, Du C and Wu Y: Bioinformatics analysis of differentially expressed genes in tumor and paracancerous tissues of patients with lung adenocarcinoma. J Thorac Dis 12: 7355-7364, 2020.
41. Fan Z, Liu Y, Liu X, Nian W, Huang X, Yang Q, Hou S and Chen F: Phosphorylation of AKT by lysyl oxidase-like 2 activates the PI3K/AKT signaling pathway to promote proliferation, invasion and metastasis in esophageal squamous carcinoma. Clin Transl Oncol 25: 2487-2498, 2023.
42. Chi M, Liu J, Mei C, Shi Y, Liu N, Jiang X, Liu C, Xue N, Hong H, Xie J, *et al*: TEAD4 functions as a prognostic biomarker and triggers EMT via PI3K/AKT pathway in bladder cancer. J Exp Clin Cancer Res 41: 175, 2022.
43. Fadhal E: A comprehensive analysis of the PI3K/AKT pathway: Unveiling key proteins and therapeutic targets for cancer treatment. Cancer Inform 22: 11769351231194273, 2023.
44. Ahmad I, Hoque M, Alam SSM, Zughaibi TA and Tabrez S: Curcumin and plumbagin synergistically target the PI3K/Akt/mTOR pathway: A prospective role in cancer treatment. Int J Mol Sci 24: 6651, 2023.
45. Shi X, Wang J, Lei Y, Cong C, Tan D and Zhou X: Research progress on the PI3K/AKT signaling pathway in gynecological cancer (Review). Mol Med Rep 19: 4529-4535, 2019.
46. Jin T, Li D, Yang T, Liu F, Kong J and Zhou Y: PTPN1 promotes the progression of glioma by activating the MAPK/ERK and PI3K/AKT pathways and is associated with poor patient survival. Oncol Rep 42: 717-725, 2019.
47. Jiang AG, Yu H and Huang JA: Expression and clinical significance of the phosphatidylinositol 3-kinase/protein kinase B signal transduction pathway in non-small cell lung carcinoma. Oncol Lett 8: 601-607, 2014.
48. Li X, Sun H, Hou Y and Jin W: Comprehensive combined proteomics and genomics analysis identifies prognostic related transcription factors in breast cancer and explores the role of dmap1 in breast cancer. J Pers Med 11: 1068, 2021.
49. Andersen V, Vogel LK, Kopp TI, Sæbø M, Nonboe AW, Hamfjord J, Kure EH and Vogel U: High ABCC2 and low ABCG2 gene expression are early events in the colorectal adenoma-carcinoma sequence. PLoS One 10: e0119255, 2015.
50. Yamada A, Ishikawa T, Ota I, Kimura M, Shimizu D, Tanabe M, Chishima T, Sasaki T, Ichikawa Y, Morita S, *et al*: High expression of ATP-binding cassette transporter ABCC11 in breast tumors is associated with aggressive subtypes and low disease-free survival. Breast Cancer Res Treat 137: 773-782, 2013.
51. Sakil HAM, Stantic M, Wolfsberger J, Brage SE, Hansson J and Wilhelm MT: ΔNp73 regulates the expression of the multidrug-resistance genes ABCB1 and ABCB5 in breast cancer and melanoma cells-a short report. Cell Oncol 40: 631-638, 2017.
52. Yuan Y, Xiang Z, Xia Y, Xie J, Jiang X and Lu Z: The role of ATP binding cassette (ABC) transporters in breast cancer: Evaluating prognosis, predicting immunity, and guiding treatment. Channels (Austin) 17: 2273247, 2023.
53. Modi A, Roy D, Sharma S, Vishnoi JR, Pareek P, Elhence P, Sharma P and Purohit P: ABC transporters in breast cancer: Their roles in multidrug resistance and beyond. J Drug Target 30: 927-947, 2022.
54. Zhao B, Xin Z, Ren P and Wu H: The role of PPARs in breast cancer. Cells 12: 130, 2023.
55. Nordgren M and Fransen M: Peroxisomal metabolism and oxidative stress. Biochimie 98: 56-62, 2014.
56. Zhang J, Tripathi DN, Jing J, Alexander A, Kim J, Powell RT, Dere R, Tait-Mulder J, Lee JH, Paull TT, *et al*: ATM functions at the peroxisome to induce pexophagy in response to ROS. Nat Cell Biol 17: 1259-1269, 2015.
57. Brandão-Costa RM, Helal-Neto E, Vieira AM, Barcellos-De-souza P, Morgado-Diaz J and Barja-Fidalgo C: Extracellular matrix derived from high metastatic human breast cancer triggers epithelial-mesenchymal transition in epithelial breast cancer cells through αvβ3 integrin. Int J Mol Sci 21: 2995, 2020.
58. Yu TY, Zhang G, Chai XX, Ren L, Yin DC and Zhang CY: Recent progress on the effect of extracellular matrix on occurrence and progression of breast cancer. Life Sci 332: 122084, 2023.
59. Wu JZ, Yang TJ, Lu P and Ma W: Analysis of signaling pathways in recurrent breast cancer. Genet Mol Res 13: 10097-10104, 2014.
60. Huang Z, Xu E, Ma X, Wang Y, Zhu J, Zhu K, Hu J and Zhang C: Low NT5DC2 expression predicts favorable prognosis and suppresses soft tissue sarcoma progression via ECM-receptor interaction pathway. Transl Oncol 44: 101937, 2024.
61. Tang W, Putluri V, Ambati CR, Dorsey TH, Putluri N and Ambs S: Liver-And Microbiome-derived bile acids accumulate in human breast tumors and inhibit growth and improve patient survival. Clin Cancer Res 25: 5972-5983, 2019.
62. Scott P, Anderson K, Singhania M and Cormier R: Cystic fibrosis, CFTR, and colorectal cancer. Int J Mol Sci 21: 2891, 2020.
63. Wang S, Yu ZH and Chai KQ: Identification of CFTR as a novel key gene in chromophobe renal cell carcinoma through bioinformatics analysis. Oncol Lett 18: 1767-1774, 2019.
64. Wei RQ, Zhang WM, Liang Z, Piao C and Zhu G: Identification of signal pathways and hub genes of pulmonary arterial hypertension by bioinformatic analysis. Can Respir J 2022: 1394088, 2022.
65. Kudo-Saito C, Miyamoto T, Imazeki H, Shoji H, Aoki K and Boku N: IL33 is a key driver of treatment resistance of cancer. Cancer Res 80: 1981-1990, 2021.
66. Fang M, Li Y, Huang K, Qi S, Zhang J, Zgodzinski W, Majewski M, Wallner G, Gozdz S, Macek P, *et al*: IL33 promotes colon cancer cell stemness via JNK activation and macrophage recruitment. Cancer Res 77: 2735-2745, 2017.
67. Twarda-Clapa A, Labuzek B, Krzemien D, Musielak B, Grudnik P, Dubin G and Holak TA: Crystal structure of the FAS1 domain of the hyaluronic acid receptor stabilin-2. Acta Crystallogr D Struct Biol 74: 695-701, 2018.
68. Hirose Y, Saijou E, Sugano Y, Takeshita F, Nishimura S, Nonaka H, Chen YR, Sekine K, Kido T, Nakamura T, *et al*: Inhibition of Stabilin-2 elevates circulating hyaluronic acid levels and prevents tumor metastasis. Proc Natl Acad Sci USA 109: 4263-4268, 2012.
69. Han MW, Lee JC, Park SY, Kim YM, Cho KJ, Kim SW, Lee M, Nam SY, Kim IS and Kim SY: Homotypic interaction of stabilin-2 plays a critical role in lymph node metastasis of tongue cancer. Anticancer Res 36: 6611-6618, 2016.
70. Eissmann MF, Dijkstra C, Jarnicki A, Phesse T, Brunnberg J, Poh AR, Etemadi N, Tsantikos E, Thiem S, Huntington ND, *et al*: IL-33-mediated mast cell activation promotes gastric cancer through macrophage mobilization. Nat Commun 10: 2735, 2019.
71. Eissmann MF, Buchert M and Ernst M: IL33 and mast cells-the key regulators of immune responses in gastrointestinal cancers? Front Immunol 11: 1389, 2020.

72. Yoon HH, Orrock JM, Foster NR, Sargent DJ, Smyrk TC and Sinicrope FA: Prognostic impact of FoxP3+ regulatory T cells in relation to CD8+ T lymphocyte density in human colon carcinomas. PLoS One 7: e42274, 2012.
73. Duhen T, Duhen R, Montler R, Moses J, Moudgil T, de Miranda NF, Goodall CP, Blair TC, Fox BA, McDermott JE, *et al*: Co-expression of CD39 and CD103 identifies tumor-reactive CD8 T cells in human solid tumors. Nat Commun 9: 2724, 2018.
74. Liu Z, Zhou Q, Wang Z, Zhang H, Zeng H, Huang Q, Chen Y, Jiang W, Lin Z, Qu Y, *et al*: Intratumoral TIGIT+CD8+T-cell infiltration determines poor prognosis and immune evasion in patients with muscle-invasive bladder cancer. J Immunother Cancer 8: e000978, 2020.
75. Ma L, Sun L, Zhao K, Dong Z, Huang Z and Meng X: The prognostic value of TCF1+CD8+T in primary small cell carcinoma of the esophagus. Cancer Sci 112: 4968-4976, 2021.
76. Yang B, Deng B, Jiao XD, Qin BD, Lu Y, Zhang W, Guo Y, Chen S, Li D, Li B, *et al*: Low-dose anti-VEGFR2 therapy promotes anti-tumor immunity in lung adenocarcinoma by down-regulating the expression of layilin on tumor-infiltrating CD8+T cells. Cell Oncol (Dordr) 45: 1297-1309, 2022.
77. Karkeni E, Morin SO, Bou Tayeh B, Goubard A, Josselin E, Castellano R, Fauriat C, Guittard G, Olive D and Nunès JA: Vitamin D controls tumor growth and CD8+ T Cell infiltration in breast cancer. Front Immunol 10: 1307, 2019.
78. Wang X, Jespers W, de Waal JJ, Wolff KAN, van Uden L, IJzerman AP, van Westen GJP and Heitman LH: Cancer-related somatic mutations alter adenosine A1 receptor pharmacology-A focus on mutations in the loops and C-terminus. FASEB J 36: e22358, 2022.
79. Dietzsch AN, Al-Hasani H, Altschmied J, Bottermann K, Brendler J, Haendeler J, Horn S, Kaczmarek I, Körner A, Krause K, *et al*: Dysfunction of the adhesion G protein-coupled receptor latrophilin 1 (ADGRL1/LPHN1) increases the risk of obesity. Signal Transduct Target Ther 9: 103, 2024.
80. Zhang H, Kong Q, Wang J, Jiang Y and Hua H: Complex roles of cAMP-PKA-CREB signaling in cancer. Exp Hematol Oncol 9: 32, 2020.
81. Rao R, Salloum R, Xin M and Lu QR: The G protein Gαs acts as a tumor suppressor in sonic hedgehog signaling-driven tumorigenesis. Cell Cycle 15: 1325-1330, 2016.
82. Li Y, Zhou X, Liu J, Yin Y, Yuan X, Yang R, Wang Q, Ji J and He Q: Differentially expressed genes and key molecules of BRCA1/2-mutant breast cancer: Evidence from bioinformatics analyses. PeerJ 8: e8403, 2020.
83. Zhu F, Huang R, Li J, Liao X, Huang Y and Lai Y: Identification of key genes and pathways associated with RUNX1 mutations in acute myeloid leukemia using bioinformatics analysis. Medical Science Monitor 24: 7100-7108, 2018.
84. Chen S, Chen Y, Lu J, Yuan D, He L, Tan H and Xu L: Bioinformatics analysis identifies key genes and pathways in acute myeloid leukemia associated with DNMT3A mutation. Biomed Res Int 2020: 9321630, 2020.