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Clinical studies

Reduced expression of ferroportin1 and ceruloplasmin predicts poor prognosis in adrenocortical carcinoma

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ABSTRACT

Introduction: Iron metabolism is tightly controlled in human cells. Dysregulation of iron metabolism-related genes has been characterized as a promising prognostic biomarker in cancers. However, the expression patterns and prognostic roles of iron metabolism-related genes remain unknown in adrenocortical carcinoma (ACC).
Objectives: The primary objective of this study was to explore the expression patterns and prognostic roles of iron metabolism-related genes in ACC using publicly available datasets.
Methods: In the present study, we compared the expression patterns of 36 iron metabolism-related genes between ACC tumors (n = 77) and normal adrenal tissues (n = 128) based on The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) data. The associations between clinical variables (including survival rate and pathological stage) and expression levels of iron mentalism-related genes were further explored. All the bioinformatics analyses were performed using the GEPIA or the Metascape tool.
Results: Twelve iron metabolism-related genes were differentially expressed between ACC tumors and normal controls. Among them, reduced expression levels of ferroportin1 (FPN1) and ceruloplasmin (CP) were significantly correlated with poor survival of ACC patients. Specially, the expression levels of FPN1 were negatively correlated with the pathological stages of ACC. A pan-cancer analysis characterized the reduced expression of FPN1 and CP as an ACC-specific signature among 33 types of cancers. Functional enrichment analysis suggested that both FPN1 and CP might be implicated in several immune processes.
Conclusion: Reduced expression of FPN1 and CP was identified as a potential signature for poor prognosis of ACC in this study. Mechanisms underlying the prognostic value of FPN1 or CP in ACC deserve further experimental investigation.

Abbreviations: Tf, Transferrin; STEAP3, Six-transmembrane epithelial antigen of the prostate-3; HJV, Hemojuvelin; DMT1, Divalent metal transporter 1; Dcytb, Duodenal cytochrome b; SCARA5, Scavenger receptor class A member 5; ZIP, Zrt/IRT-like protein; FLVCR, Feline leukemic virus, sub-group C receptor; HRG-1, Heme-responsive gene-1; NTBI, Non transferrin bound iron; HO, Heme oxygenase; LIP, Labile iron pool; Mfrn, Mitoferrin; ABCB7, ATP-binding cassette B7; IRP, Iron regulatory protein; FPN1, Ferroportin1; CP, Ceruloplasmin; HEPH, Hephaestin; ALAS1, 5-aminolevulinic acid synthase 1; FtMt, Ferritin mitochondrial; FtH(L), Ferritin heavy (light) chain; PCBP, Poly(rC) binding protein; NCOA4, Nuclear receptor coactivator 4; FBXL5, F-box and leucine rich repeat protein 5; FAM96A, Family with sequence similarity 96 member A; TPM, Transcripts per million; ACC, Adrenocortical carcinoma; BLCA, Bladder urothelial carcinoma; BRCA, Breast invasive carcinoma; CESC, Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, Cholangio carcinoma; COAD, Colon adenocarcinoma; DLBC, Lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, Esophageal carcinoma; GBM, Glioblastoma multiforme; HNSC, Head and neck squamous cell carcinoma; KICH, Kidney chromophobe; KIRC, Kidney renal clear cell carcinoma; KIRP, Kidney renal papillary cell carcinoma; LAML, Acute myeloid leukemia; LGG, Brain lower grade glioma; LIHC, Liver hepatocellular carcinoma; LUAD, Lung adenocarcinoma; LUSC, Lung squamous cell carcinoma; MESO, Mesothelioma; OV, Ovarian serous cystadenocarcinoma; PAAD, Pancreatic adenocarcinoma; PCPG, Pheochromocytoma and paraganglioma; PRAD, Prostate adenocarcinoma; READ, Rectum adenocarcinoma; SARC, Sarcoma; SKCM, Skin cutaneous melanoma; STAD, Stomach adenocarcinoma; TGCT, Testicular germ cell tumors; THCA, Thyroid carcinoma; THYM, Thymoma; UCEC, Uterine corpus endometrial carcinoma; UCS, Uterine carcinosarcoma; UVM, Uveal melanoma

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1. Introduction

Iron is the most abundant trace element in the human body [1]. Systemic and cellular iron metabolism is tightly regulated, and numerous proteins are dedicated to the uptake, utilization, storage and export of iron [2]. Most dietary iron is in the ferric form, it needs to be reduced before it can be absorbed. After reduced by duodenal cytochrome b (Dcytb), the dietary iron enters enterocyte *via* divalent metal transporter 1 (DMT1) and is exported into the circulation *via* ferroportin 1 (FPN1) [3]. Absorbed iron binds to plasma transferrin (Tf) and is distributed to tissues throughout the body [4]. Almost all nucleated cells are able to use Tf-bound iron. Diferric Tf binds to its receptor (TFR1) on cell membrane, and the complex is internalized in endosomes. Iron freed from Tf is reduced by the ferrireductase STEAP3 (six-transmembrane epithelial antigen of the prostate-3) in the endosomes, and then released into the cytoplasm *via* DMT1 [5,6]. The hemochromatosis protein HFE binds to TfR1 in competition with Tf, and thus reduces cellular iron uptake [7]. Some cells can also take up non transferrin-bound iron (NTBI), which enters cells *via* DMT1, Zrt/IRT-like protein 8 (ZIP8), or ZIP14, after being reduced to its ferrous form by Dcytb [8]. Heme oxygenases are the rate-limiting enzymes in the degradation of heme, releasing iron, biliverdin, and carbon monoxide, and play an important role in the acquisition of heme-iron [9]. Additionally, several other genes mediate iron uptake in a cell-specific manner, including the scavenger receptor class A member 5 (SCARA5), the feline leukemic virus, sub-group C receptor 2 (FLVCR2), CD163, CD91, and heme-responsive gene-1 (HRG1) [8,10–14]. Once iron enters into the cytosolic labile iron pool (LIP), it is utilized by intracellular iron proteins, stored in ferritin, or transported into mitochondrial *via* mitoferrin (Mfrn), or the ATP-binding cassette B7 (ABCB7) [15,16]. Intracellular iron homeostasis is mainly balanced by iron regulatory proteins (IRPs) [17]. Mechanisms underlying cellular iron export remain not fully elucidated. FPN1 is the only known iron exporter in human cells and plays a critical role in regulating cellular iron homeostasis [18]. As exported iron must be oxidized to its ferric form, FPN1 functions in concert with the ferroxidases hephaestin (HEPH) and ceruloplasmin (CP) [19,20]. Previous studies have also indicated that hepcidin, a major regulator of body iron metabolism, inhibits iron efflux by binds to FPN1, and triggers the internalization and degradation of FPN1 [21].

Cancer cells often require a relatively high amount of intracellular iron to maintain rapid proliferation [22]. Expression pattern of iron metabolism-related genes varies in different types of cancer [8]. The expression signatures of iron metabolism-related genes have been identified as potential prognosis predictors or therapeutic targets in multiple types of cancer [8,23]. Adrenocortical carcinoma (ACC) is a rare endocrine malignancy with poor prognosis and few therapeutic options [24]. Factors and mechanisms affecting the survival of ACC patients remain elusive. Therefore, there is a pressing need to explore novel prognosis predictors and therapeutic targets for ACC.

The association between adrenal glands and iron homeostasis has been reported for over half a century [25]. Subcutaneous injections of excess iron can cause marked damage in the adrenal cortex of guinea-pigs, indicating the potential role of iron homeostasis in adrenal gland diseases [26]. The expression patterns of iron metabolism-related genes in ACC remain unknown due to the limited availability of ACC samples. Nowadays, the convenient access to The Cancer Genome Atlas (TCGA) database allows large-scale global gene expression profiling of indicated genes in rare cancers, including ACC [27]. In the present study, we explored the expression patterns of 36 iron metabolism-related genes in ACC tumors and evaluated the prognostic value of 12 differentially expressed genes for ACC patients.

2. Methods

2.1. Differential expression analysis

GEPIA (http://gepia.cancer-pku.cn/) is a web server for cancer and normal gene expression profiling and interactive analyses based on TCGA and the GTEx projects [28]. Relative mRNA expression levels of 36 iron metabolism-related genes (including hepcidin, CP, HEPH, Tf, TfR1, TfR2, HFE, HJV, STEAP3, DMT1, Dcytb, ZIP14, ZIP8, SCARA5, FLVCR2, HRG-1, CD91, CD163, HO-1, Mfrn1, Mfrn2, frataxin, ABCB7, ALAS1, FLVCR1, FtMt, FtH, FtL, PCBP1, PCBP2, NCOA4, FPN, IRP1, IRP2, FBXL5, and FAM96A) in ACC tumors (n = 77) were compared with their normal counterparts (n = 128). The expression data are first $\log_2$ (TPM + 1) transformed for differential analysis and the $\log_2$FC is defined as median (Tumor) – median (Normal). An adjusted *P*-value (adj. *P*) < 0.01 and $|\log_2 FC| > 1$ were set as the cut-off criteria.

2.2. Survival analysis

Patients with ACC were classified into two subgroups with the median value of the expression level of indicated gene as a cutoff. Overall survival and disease-free survival were calculated using the Kaplan–Meier method, and survival curves were compared using log-rank tests. The log-rank *P* value < 0.05 was selected as a significance threshold. The hazards ratio hazard ratios (HRs) with their 95% CIs were calculated based on Cox PH model.

2.3. Correlation analysis

The Spearman rank analysis was performed to evaluate the correlation between the expression levels of indicated genes. We used the non-log scale for calculation and the log-scale axis for visualization with the GEPIA tool, respectively. A *P* value < 0.05 was considered statistically significant.

2.4. Pan-cancer gene expression analysis

TCGA has profiled more than 10,000 samples derived from 33 types of cancer, including ACC, BLCA, BRCA, CESC, CHOL, COAD, DLBC, ESCA, GBM, HNSC, KICH, KIRC, KIRP, LAML, LGG, LIHC, LUAD, LUSC, MESO, OV, PAAD, PCPG, PRAD, READ, SARC, SKCM, STAD, TGCT, THCA, THYM, UCEC, UCS, and UVM (For full names of these cancer types, see Abbreviations). The pan-cancer gene expression analysis based on TCGA and GTEx data was performed using the GEPIA tool. The expression data are first $\log_2$ (TPM + 1) transformed for differential analysis and the $\log_2$FC is defined as median (Tumor) – median (Normal). An adjusted *P*-value (adj. *P*) < 0.01 and $|\log_2 FC| > 1$ were set as the cut-off criteria.

2.5. Functional and pathway enrichment analysis

The co-expression analysis was performed using the GEPIA tool, and 200 similarly expressed genes of the target gene were subjected to the functional and pathway enrichment analysis. The functional and pathway enrichment analysis was performed using the Metascape tool (www.metascape.org) with the following ontology sources: GO Biological Processes, KEGG pathway, Reactome Gene Sets, Canonical Pathways and CORUM [29]. All genes in the human genome were used as the enrichment background. Terms with an enrichment factor > 1.5, a minimum count of 3, and a *P* value < 0.01 were collected and grouped into clusters based on their membership similarities.

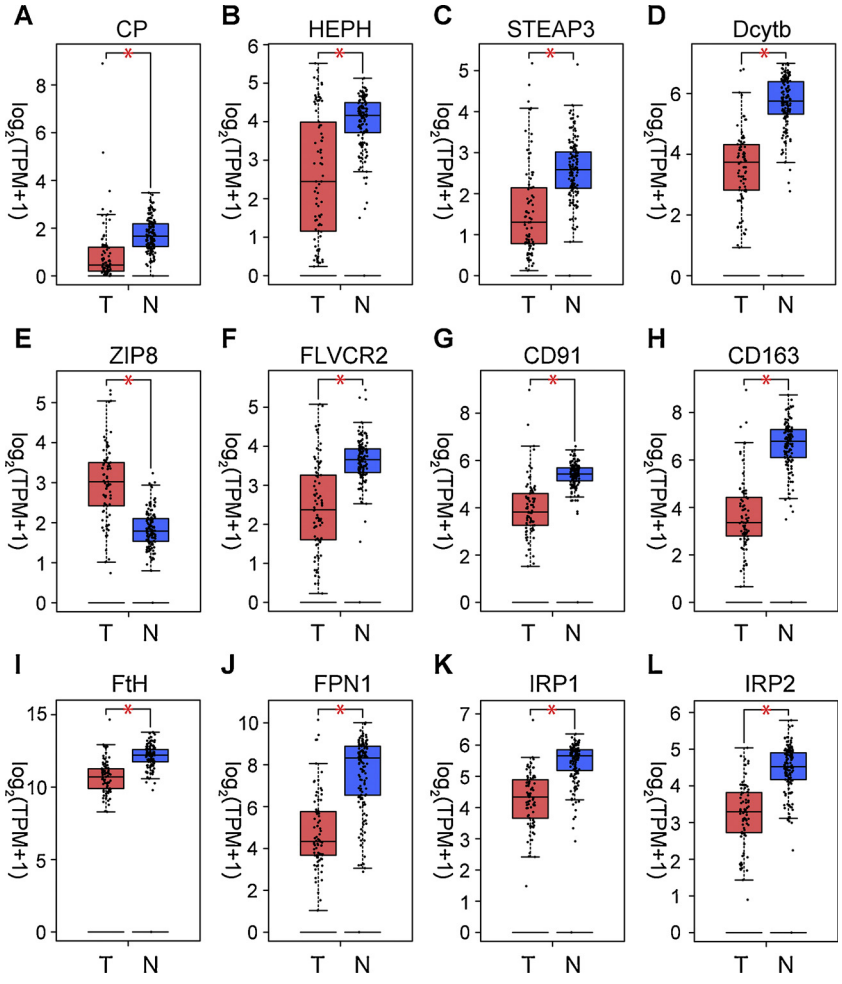

Fig. 1. Differentially expressed genes related to iron metabolism between ACC tumors and normal controls. RNA sequencing data from 77 ACC tumors in TCGA and 128 normal adrenal tissues in GTEx were analyzed, and 12 differentially expressed iron-metabolism genes were identified, including CP (A), HEPH (B), STEAP3 (C), Dcytb (D), ZIP8 (E), FLVCR2 (F), CD91 (G), CD163 (H), FtH (I), FPN1 (J), IRP1 (K) and IRP2 (L). The expression data were $\log_2$ (TPM + 1) transformed for differential analysis, and the $\log_2$FC was defined as median (Tumor) – median (Normal). *Genes with higher $|\log_2 FC|$ values than 1 and lower *P* values than 0.01 are considered differentially expressed. TPM means transcripts per million. FC means fold change.

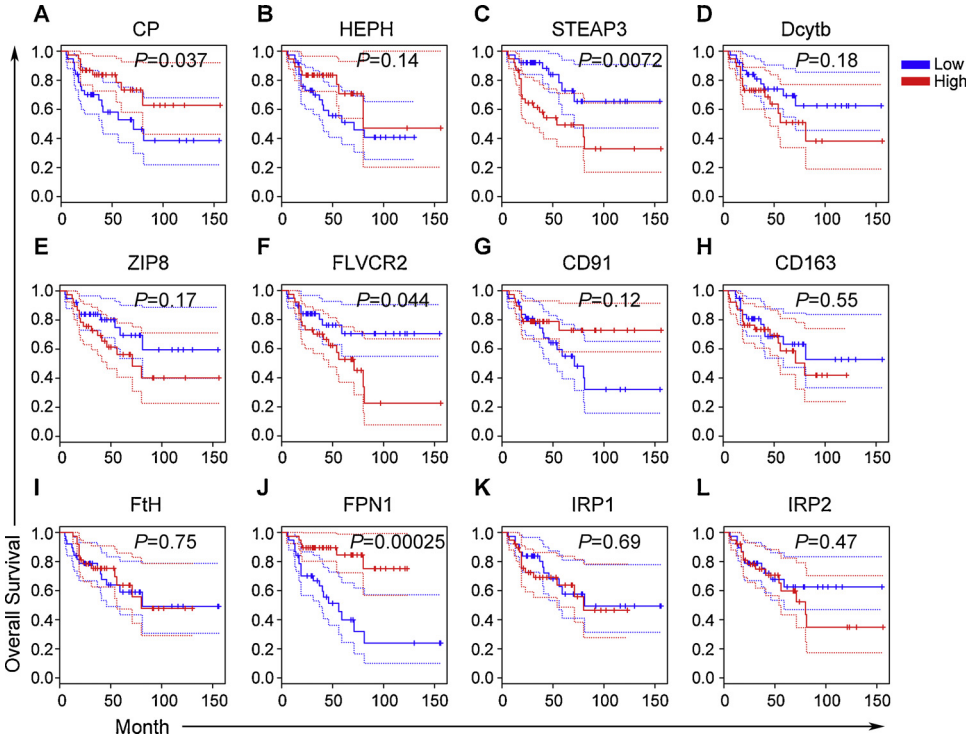

Fig. 2. Association between the expression levels of 12 differentially expressed genes (related to iron metabolism) and the overall survival in patients with ACC. The patients with ACC (n = 77) were classified into two subgroups based on the expression level of indicated genes, including CP (A), HEPH (B), STEAP3 (C), Dcytb (D), ZIP8 (E), FLVCR2 (F), CD91 (G), CD163 (H), FtH (I), FPN1 (J), IRP1 (K) and IRP2 (L). The overall survival probabilities in subgroups were determined with Kaplan-Meier survival analysis and compared using the log-rank test. The median TPM was selected as the cut-off level for subgrouping (Red line: high expression group; Blue line: low expression group). The hazards ratio was calculated based on Cox PH model, and 95% Confidence Interval was added as dotted line. All data were extracted from TCGA using the GEPIA tool. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

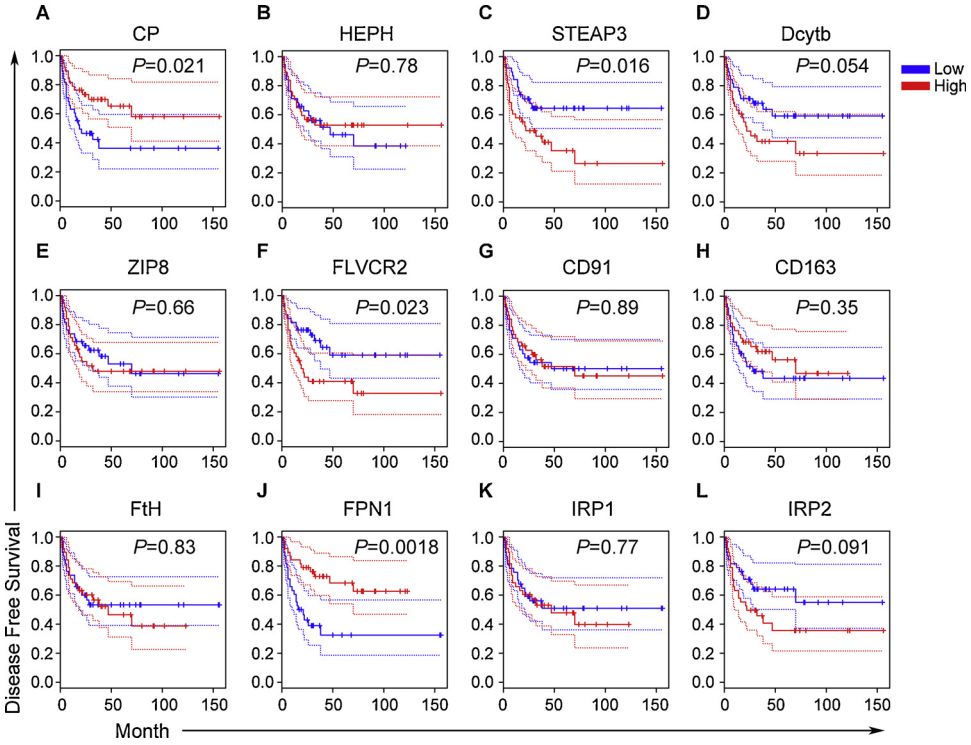

Fig. 3. Association between the expression levels of 12 differentially expressed genes (related to iron metabolism) and the disease-free survival in patients with ACC. The patients with ACC (n = 77) were divided into two subgroups based on the expression level of indicated genes, including CP (A), HEPH (B), STEAP3 (C), Dcytb (D), ZIP8 (E), FLVCR2 (F), CD91 (G), CD163 (H), FtH (I), FPN1 (J), IRP1 (K) and IRP2 (L). The disease-free survival probabilities in subgroups were determined with Kaplan-Meier survival analysis and compared using the log-rank test. The median TPM was selected as the cut-off level for subgrouping (Red line: high expression group; Blue line: low expression group). The hazards ratio was calculated based on Cox PH model, and 95% Confidence Interval was added as dotted line. All data were extracted from TCGA using the GEPIA tool. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

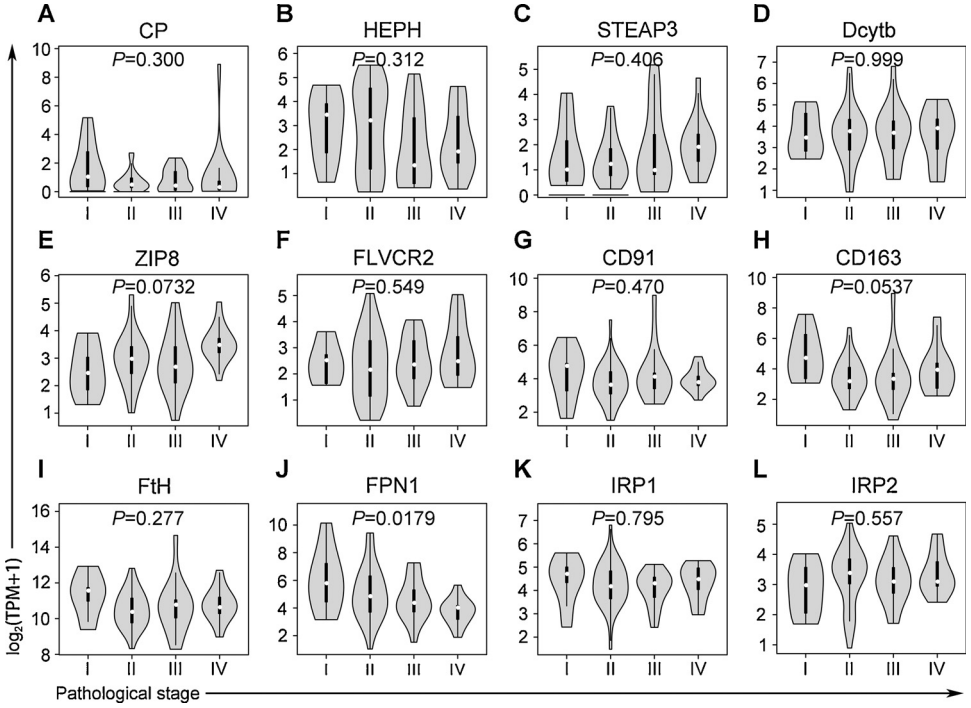

Fig. 4. Association between 12 differentially expressed genes (related to iron metabolism) and the pathological stages of ACC. The patients with ACC (n = 77) were divided into four subgroups based on pathological stages (from I to IV), the expression levels of CP (A), HEPH (B), STEAP3 (C), Dcytb (D), ZIP8 (E), FLVCR2 (F), CD91 (G), CD163 (H), FtH (I), FPN1 (J), IRP1 (K) and IRP2 (L) in different subgroups were compared using a one-way ANOVA respectively. All data were extracted from TCGA and $\log_2$ (TPM + 1) transformed for differential analysis using the GEPIA tool.

3. Results

3.1. Reduced expression of CP, HEPH, STEAP3, Dcytb, FLVCR2, CD91, CD163, FtH, FPN1, IRP1 and IRP2, but increased expression of ZIP8 was identified in ACC tumors

We first compared the expression levels of 36 iron metabolism-related genes between ACC tumors (n = 77) and normal adrenal gland tissues (n = 128), including 17 genes for iron uptake (hepcidin, Tf, TfR1, TfR2, HFE, HJV, STEAP3, DMT1, Dcytb, ZIP14, ZIP8, SCARA5, FLVCR2, HRG-1, CD91, CD163, and HO-1), 12 genes for iron utilization and storage (Mfrn1, Mfrn2, frataxin, ABCB7, ALAS1, FLVCR1, FtMt, FtH, FtL, PCBP1, PCBP2, and NCOA4), 4 genes for intracellular iron balance (IRP1, IRP2, FBXL5, and FAM96A), 2 genes encoding ferroxidases (CP and HEPH) and one iron exporter gene FPN1. We found that the expression levels of CP, HEPH, STEAP3, Dcytb, FLVCR2, CD91, CD163, FtH, FPN1, IRP1 and IRP2 were significantly decreased while the expression level of ZIP8 was significantly increased in ACC tumors compared to normal controls (Fig. 1).

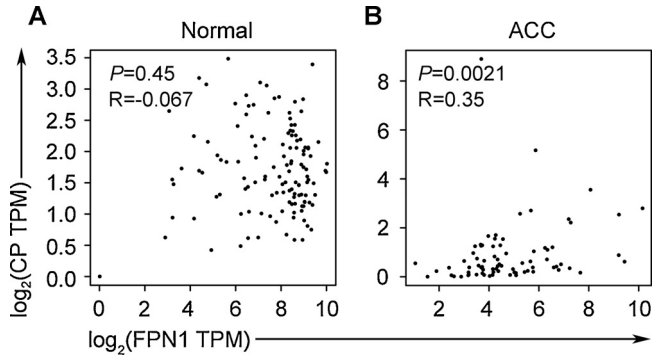

Fig. 5. The correlation analysis on the expression levels of FPN1 and CP in ACC tumors or normal adrenal tissues. Correlation between FPN1 and CP expression levels in normal adrenal glands (A) or ACC tumors (B) was analyzed by Spearman's rank correlation test. All data were extracted from TCGA (n = 77) or GTEx (n = 128) using the GEPIA tool. We used the non-log scale for calculation and use the log-scale axis for visualization. TPM means transcripts per million.

3.2. Expression levels of FPN1 and CP were negatively correlated with the survival of patients with ACC

We then explored the association between the 12 differentially expressed genes and patient survival using the Kaplan-Meier survival analysis. We found that lower expression levels of FPN1 and CP, but higher expression levels of STEAP3 and FLVCR2 predicted poorer overall survival (Fig. 2) and disease free survival (Fig. 3) in patients with ACC. Considering that the expression levels of FPN1, CP, STEAP3 and FLVCR2 were all decreased in ACC tumors, FPN1 or CP might be more appropriate for prognostic prediction than STEAP3 or FLVCR2.

3.3. Decreased expression of FPN1 was correlated with the progression of ACC

In patients with ACC, the prognosis is depending on tumor stage. Once the tumor spreads outside the adrenal gland, 5-year survival drops from 58 to 66% (patients with intra-adrenal ACC) to 0–24% (patients with extra-adrenal ACC) [30]. The associations between 12 differential expressed genes related to iron metabolism and the stages of ACC were explored. We found a significant negative correlation between FPN1 levels and pathological stages of ACC (Fig. 4). These data suggested that FPN1 down-regulation might participate in the progression of ACC.

3.4. Expression levels of FPN1 and CP were positively correlated in ACC tumors, but not in normal controls

We then explored the correlations of FPN1 and CP in ACC tumors and normal controls, respectively. A weak but significant positive correlation between FPN1 and CP expression was observed in ACC tumors but not in normal controls (Fig. 5). These results suggested that the

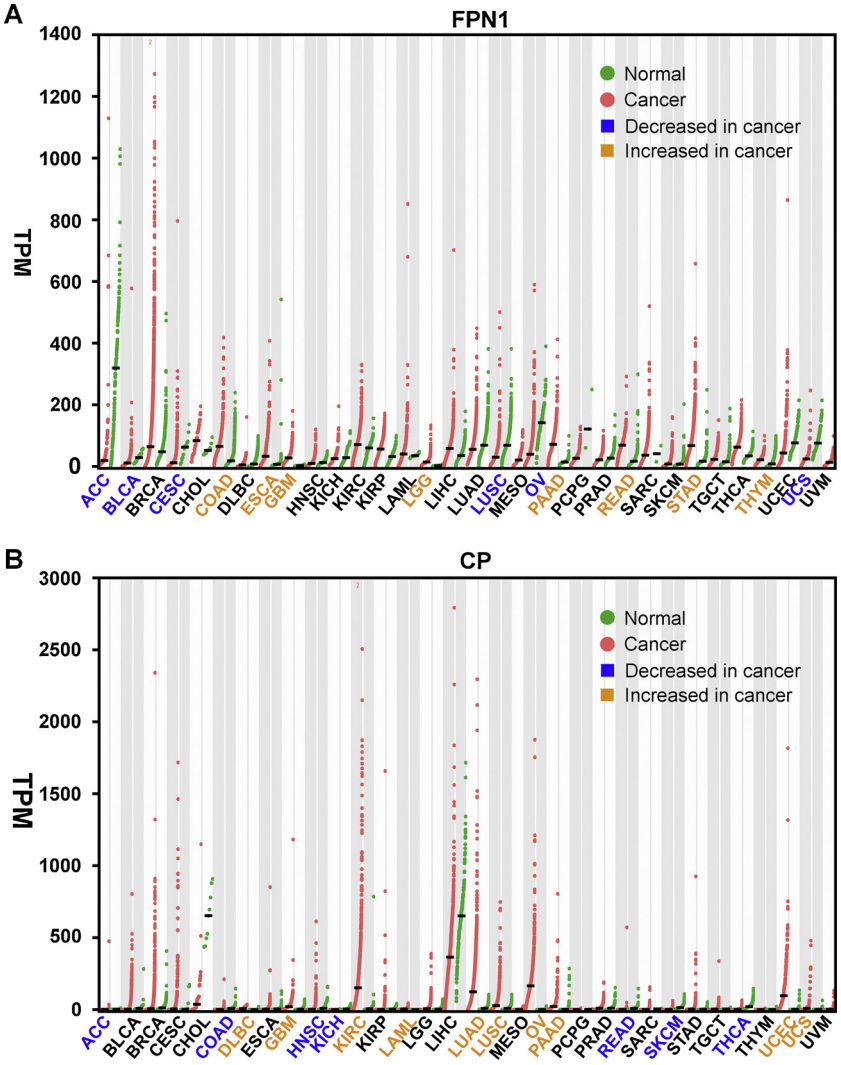

Fig. 6. The pan-cancer analysis of FPN1 and CP expression. The expression patterns of FPN1 (A) and CP (B) in 33 types of tumor tissues and their matched normal controls were shown. All data were extracted from TCGA or GETx, and then $\log_2$ (TPM + 1) transformed for differential analysis. The $\log_2$FC was defined as median (Tumor) – median (Normal). *Genes with higher $|\log_2 FC|$ values than 1 and lower q values than 0.01 are considered differentially expressed. For full name of these cancer types, see Abbreviations. Expression data on normal controls for MESO or UVM are unavailable.

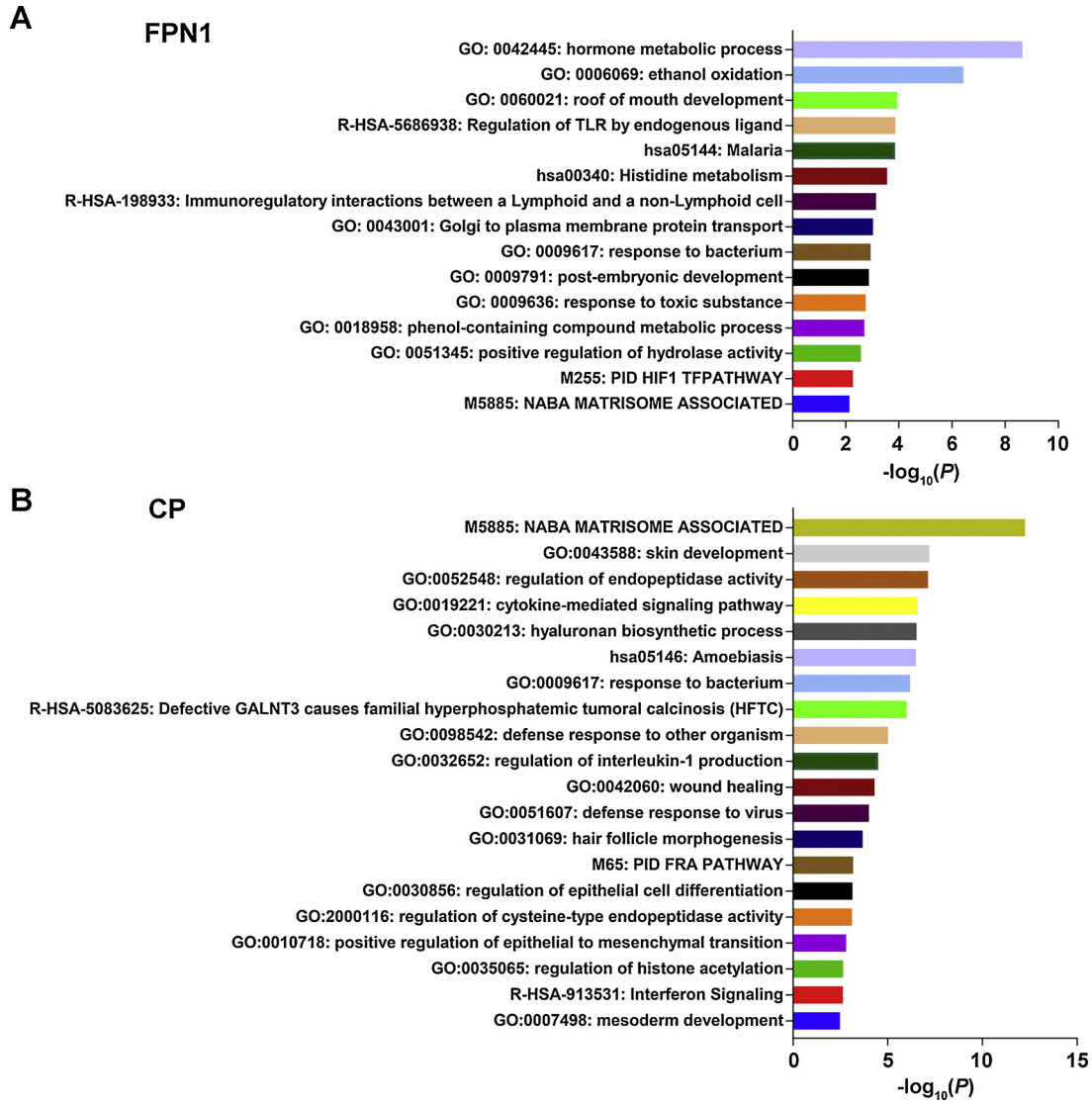

Fig. 7. Functional and pathway enrichment analysis for genes expressed similarly to FPN1 or CP. A list of 200 similarly expressed genes of FPN1 (A) or CP (B) was identified using GEPIA. The enrichment analysis of the gene lists was conducted by the Metascape tool. Terms with an enrichment factor > 1.5, a minimum count of 3, and a *P* value < 0.01 are collected and grouped into clusters based on their membership similarities.

positive correlation of FPN1 and CP expression might be tumor- or even ACC- specific.

3.5. Pan-cancer analysis identified reduced expression of FPN1 and CP as an ACC-specific expression signature

We further explored the expression pattern of FPN1 and CP in another 32 types of cancer. We found that FPN1 was down-regulated in BLCA, CESC, LUSC, OV, and UCS, while CP was down-regulated in COAD, HNSC, KICH, READ, SKCM and THCA, compared to their corresponding normal controls. Moreover, the expression of FPN1 or CP increased in some cancer types. Among these 33 types of cancers, reduced expression of both FPN1 and CP was only found in ACC tumors (Fig. 6). These data indicated that down-regulation of FPN1 and CP might be an ACC-specific expression signature.

3.6. The expression of FPN1 and CP might affect or be affected by the tumor immune microenvironment of ACC

Using the GEPIA tool, we identified 200 similarly expressed genes of FPN1 or CP, respectively. For each given gene list, the functional and pathway enrichment was carried out using the Metascape tool, with the following ontology sources: KEGG Pathway, GO Biological Process, Reactome Gene Sets, Canonical Pathways and CORUM. The involvement of FPN1- and CP-coexpressed genes in immune regulation was demonstrated (Fig. 7). The co-expressed network of FPN1 was involved in TLR signaling, immune-regulatory interactions, and response to bacterium, and the co-expressed network of CP was involved in cytokine-mediated signaling pathway, response to bacterium, regulation of interleukin-1 production, defense response to virus, and interferon signaling. These results indicated that expression of FPN1 and CP might affect or be affected by the tumor immune microenvironment of ACC.

4. Discussion

Different expression signatures of iron metabolism-related genes have been identified to predict prognosis in several cancer types [8,23,31]. However, little is known on the expression pattern and function of iron metabolism-related genes in ACC. In this study, we demonstrated that reduced expression levels of FPN1 and CP were associated with poor prognosis in patients with ACC.

Rapidly proliferating tumor cells often require increased amounts of

iron to maintain abnormally enhanced DNA synthesis [32]. FPN1, the only known iron exporter in mammalian cells, is down-regulated in various types of cancer, including prostate, lung, ovarian and breast cancer [33–38]. Reduced FPN1 may promote proliferation of cancer cells through restricting the efflux of iron. Presently, we demonstrated for the first time that FPN1 expression was significantly decreased in ACC tumors. Pinnix et al. have identified reduced FPN1 expression as an independent predictor of poor prognosis in breast cancer [33]. Consistently, we demonstrated that reduced FPN1 expression was associated with poor prognosis in patients with ACC.

It is generally known that CP is predominantly synthesized by the liver and then released into the circulation under physiological conditions [39]. Increased CP level in serum has been described as a potential prognostic marker for cancers, including bile duct, breast, and colon cancer [40–43]. The transcriptional expression of CP in tumors and its prognostic roles remain unclear. Our findings suggested that reduced CP expression in tumors might predict poor prognosis in patients with ACC. The biological and prognostic roles of CP produced by liver or tumor tissues in ACC deserve further exploration, and it would be useful to know the level of CP transcript expression relative to that of hepatocytes, in normal and cortical tumor cells.

The expression level of FPN1 was weakly but significantly correlated with CP in ACC tumors but not in normal controls, suggesting the existence of a tumor-specific mechanism underlying iron metabolism. We further characterized reduced expression of FPN1 and CP as an ACC-specific signature by a pan-cancer analysis. Additionally, we found that FPN1 expression was continuously and significantly decreased with the increasing stages of ACC. These results suggested the potential of FPN1 and CP, especially FPN1, in the diagnosis and prognosis of ACC.

FPN1-mediated iron export has been characterized as a key component of innate immune responses during infections [44,45]. Zhang et al. have demonstrated that FPN1 deficiency enhances the production of pro-inflammatory cytokines such as TNF-α and IL-6 in mouse macrophages [44]. Conversely, Manfred et al. have found that reduced FPN1 impairs cellular iron homeostasis and attenuates inflammatory immune responses in macrophages during Salmonella infection [45]. These findings suggest that immune regulation involves FPN1. Similarly, CP protein levels in the circulation increase during infections and inflammation [46], as well as in cancer [40–43], suggesting its potential role in tumor immunity regulation. It remains unknown whether and how the CP-FPN1 system of iron export regulates the tumor immune environment. By the functional enrichment analysis, we demonstrated the involvement of FPN1- or CP-similarly expressed genes in several immune processes, suggesting their possible implications on tumor immune microenvironment in ACC. However, it could be that the opposite is true, namely that the tumor or the microenvironment it produces is altering the expression of FPN1 and CP. Several other tumor-associated biological processes or pathways were characterized, including wound healing, epithelial cell differentiation, histone acetylation, hormone metabolic process, histidine metabolism and HIF-1α regulation. These findings may help clarify the mechanisms underlying the prognostic role of FPN1 or CP in ACC. The link between iron metabolism and tumor immune microenvironment deserves further experimental exploration.

Another 10 differential expressed genes related to iron metabolism were identified in ACC tumors, including HEPH, STEAP3, Dcytb, ZIP8, FLVCR2, CD91, CD163, FtH, IRP1 and IRP2. The expression of ferritin, an iron-storage molecule, is increased in esophageal adenocarcinoma and glioblastoma but decreased in breast cancer [47–50]. The expression of IRP1 is increased in breast cancer, and the expression of IRP2 is up-regulated in breast, colorectal, and lung cancer [51–53]. Differently, reduced expression of FtH, IRP1 and IRP2 in ACC tumors was demonstrated in the present study. As a CP homologue, low HEPH expression correlates with poor survival of breast cancer [54]. Consistently, we found reduced HEPH expression in ACC tumors compared to normal controls. However, no association between the expression of FtH, IRP1, IRP2 or HEPH and survival of patients with ACC was found.

In summary, we identified the reduced expression of FPN1 and CP as a potential signature for poor prognosis of ACC patients based on the TCGA database. Mechanisms underlying the prognostic value of FPN1 and CP in ACC deserve further experimental exploration.

Declarations of interest

None.

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