Research Paper

Low level of PDZ domain containing 1 (PDZK1) predicts poor clinical outcome in patients with clear cell renal cell carcinoma

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ABSTRACT

Clear cell renal cell carcinoma (ccRCC) is the most lethal neoplasm of the urologic system. Clinical therapeutic effect varies greatly between individual ccRCC patients, so there is an urgent need to develop prognostic molecular biomarkers to help clinicians identify patients in need of early aggressive management. In this study, samples from primary ccRCC tumor and their corresponding nontumor adjacent tissues ($n = 18$) were analyzed by quantitative proteomic assay. Proteins downregulated in tumors were studied by GO and KEGG pathways enrichment analyses. Six proteins were found both downregulated and annotated with cell proliferation in ccRCC patients. Of these proteins, PDZK1 and FABP1 were also involved in the lipid metabolism pathway. The downregulation of PDZK1 was further validated in TCGA_KIRC dataset ($n = 532$) and independent set ($n = 202$). PDZK1 could discriminate recurrence, metastasis and prognosis between ccRCC patients. Low level of PDZK1 in both mRNA and protein was associated with reduced overall survival (OS) and disease–free survival (DFS) in two independent sets. In univariate and multivariate analyses, PDZK1 was defined as an independent prognostic factor for both OS and DFS. These findings indicated that low level of PDZK1 could predict poor clinical outcome in patients with ccRCC.

1. Introduction

Renal cell carcinoma (RCC) is the most common and lethal cancer of the adult kidney. Clear cell RCC (ccRCC) accounts for approximately 70% to 80% of all RCC. Patients with ccRCC are normally treated by the standard surgical resection. However, after undergoing a nephrectomy, the outcome of ccRCC patients greatly varies. Organ-confined disease confers the best prognosis, only 3% to 29% of patients passed away after five years of nephrectomy (Frank et al., 2005). For patients with locally advanced tumors, 47% to 80% of them passed away after five years of nephrectomy (Frank et al., 2005). In addition, 10% to 28% of ccRCC patients recurred or distantly metastasized (Levy et al., 1998; Figlin, 1999), leading to their poor outcome. The median survival of recurrent and metastatic ccRCC patients is 21 and 13 months, and the 5-year survival rates are reported as 30.5% and <10%, respectively (Eggener et al., 2006; Minasian et al., 1993). These patients would benefit from a more aggressive treatment strategy and a more active monitoring (NCCN Guidelines®). Hence, there is an urgent need to develop prognostic molecular biomarkers to help clinicians identify patients in need of early aggressive management.

The tumor-node-metastasis (TNM) staging system is viewed as a predominant prognostic factor for ccRCC patients. However, the clinical outcomes of patients with ccRCC may vary considerably even within the same tumor stage, suggesting that further clues other than TNM staging system is needed for more accurate assessment of prognosis. Moreover, the TNM cancer staging systems

predict survival on the basis of anatomic and histological extent of the tumor rather than on molecular changes. The molecular basis of this diversity in clinical behavior is due to large variations of molecular pathogenesis which originates from dysregulation of different gene or protein expression within the same TNM stage (Tsui et al., 2000; Frank et al., 2005; Veeratterapillay et al., 2012). Prognosis stratification by molecular markers, such as expression of specific genes could improve accuracy of outcome prediction (Tamayo et al., 2011). Therefore, new molecular prognostic markers which could precisely stratify patients into different risk categories are clearly warranted.

In the present study, isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomics was used to screen for differentially expressed proteins (DEPs) between tumor and adjacent normal tissues in each of four stages. Gene ontology (GO) cell proliferation annotation and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of these DEPs were analyzed to identify a predictor of patient outcome. PDZ domain containing 1 (PDZK1, also called cystic fibrosis transmembrane conductance regulator associated protein of 70 kDa, CAP70) was downregulated in ccRCC tissues and correlated with lipid metabolism pathway. Further, we validated the prognostic significance of PDZK1 by using multiple approaches and independent sets.

2. Materials and Methods

2.1. Patients and Study Design

We obtained 112 pairs of primary ccRCC and adjacent normal kidney tissues (Table 1) from nephrectomies conducted at the Beijing Friendship Hospital between 2013 and 2014. These tissues were cut into two parts: one part was immediately frozen in liquid nitrogen and stored at −80 °C for further use in paired iTRAQ and western blotting (WB) analyses; the other part was formalin-fixed and paraffin-embedded for use in unpaired IHC analysis. We also obtained 90 pairs of samples (81 of 90 samples had follow-up data from 2 to 83 months with a median of 70 months) collected from 2006 to 2008 (Table 1), formalin-fixed and paraffin-embedded samples for tissue microarray (TMA) construction. TMA was used for paired IHC and overall survival (OS) analyses. The tumor specimens were classified according to the 2010 American Joint Committee on Cancer (AJCC) staging system. Before surgery, none of the patients has received chemotherapy or radiotherapy. All specimens were histologically confirmed by uro-pathologists. Patients with a histological diagnosis other than ccRCC were subsequently excluded. The study was approved by the Research Ethics Boards of both Capital Medical University and Beijing Friendship Hospital. All subjects included in the protocol signed a declaration of informed consent.

Table 1
Summary of clinicopathological features of ccRCC patients.

	Discovery set (18 pairs of ccRCC and adjacent normal tissues for iTRAQ analysis)	Independent validation set (532 ccRCC and 72 adjacent normal tissues in TCGA_KIRC dataset for mRNA level, OS and DFS analyses)	Expanded validation set (38 pairs of ccRCC and adjacent normal tissues for WB analysis)	Expanded validation set (112 ccRCC and 19 adjacent normal tissues for unpaired IHC analysis)	Independent validation set (90 pairs of ccRCC and adjacent normal tissues for TMA construction and paired IHC and OS analyses)
Characteristic					
Age (years mean (range)	63.4(46–76)	60(26–90)	61.7(32–79)	61(28–84)	59(29–82)
Gender (no.) male, female, unknown	18, 0	334, 181, 17	26, 12	68, 44	51, 39
Pathological T stage no. (%)					
T1	9(50%)	259(48.6%)	23(60.53%)	63(56.25%)	63(70%)
T2	7(38.87%)	68(12.8%)	8(21.05%)	26(23.2%)	18(20.1%)
T3	1(5.57%)	177(33.3%)	5(13.16%)	19(16.95%)	5(5.5%)
T4	1(5.57%)	11(2.1%)	2(5.26%)	4(3.6%)	
Unknown		17(3.2%)			4(4.4%)
Pathological N stage no. (%)					
NX		261(49.1%)			
N0	17(94.4%)	237(44.5%)	36(94.74%)	106(94.6%)	87(96.7%)
N1		17(3.2%)	1(2.63%)	3(2.7%)	1(1.1%)
N2				1(0.9%)	
N3	1(5.6%)		1(2.63%)	2(1.8%)	
Unknown		17(3.2%)			2(2.2%)
Pathological M stage no. (%)					
MX		14(2.7%)			
M0	17(94.4%)	421(79.1%)	36(94.74%)	110(98.2%)	88(97.8%)
M1	1(5.6%)	79(14.8%)	2(5.26%)	2(1.8%)	2(2.2%)
Unknown		18(3.4%)			
Pathological grade no. (%)					
G1	2(11.1%)	13(2.5%)	8(21.1%)	18(16.1%)	33(36.7%)
G2	14(77.8%)	229(43.0%)	26(68.4%)	82(73.2%)	42(46.7%)
G3	2(11.1%)	205(38.5%)	2(5.25%)	7(6.2%)	14(15.5%)
G4		77(14.5%)			1(1.1%)
Unknown		8(1.5%)	2(5.25%)	5(4.5%)	
AJCC TNM stage, no. (%)					
I	8(44.4%)	255(47.9%)	23(60.5%)	61(54.5%)	60(66.7%)
II	7(38.9%)	56(10.5%)	8(21.1%)	26(23.2%)	18(20%)
III	1(5.6%)	127(23.9%)	4(10.5%)	19(17.0%)	4(4.4%)
IV	2(11.1%)	81(15.2%)	3(7.9%)	6(5.3%)	2(2.2%)
Unknown		13(2.5%)			6(6.7%)

In addition, mRNA data and clinical information for patients in The Cancer Genome Atlas (TCGA_KIRC, Table 1) dataset were used for differential mRNA expression analysis.

2.2. Protein Sample Preparation and iTRAQ-based Proteomics

To identify DEPs between ccRCC and paired adjacent normal tissues, the protein samples from 18 ccRCC patients which were distributed in four AJCC TNM stages were extracted separately. Then according to stage and tissue types, eight groups (four tumor tissue groups I–IV and four paired adjacent normal tissue groups I–IV) were divided. Equal amounts of protein in stage III ($n = 1$) and equally pooled proteins in stage I ($n = 8$), stage II ($n = 7$) and in stage IV ($n = 2$), respectively were analyzed by iTRAQ-based proteomics as previously reported (Uhlen et al., 2015).

Peptides were labeled with the 8-plex iTRAQ kit (AB SCIEX, Darmstadt, Germany) and subjected to a nanospray ionization source followed by tandem mass spectrometry (MS/MS) in Q Exactive™ Plus (Thermo Scientific, Waltham, MA) coupled online to the Ultra Performance Liquid Chromatography (UPLC). For MS scans, the m/z scan range was 350 to 1600. The fixed first mass was set as 100 m/z.

2.3. Database Search

The MS/MS data were processed using the Mascot search engine (v.2.3.0, Matrixscience, London, UK) and searched against SwissProt_human database concatenated with reverse decoy database. Trypsin was specified as the cleavage enzyme allowing up to two missing cleavages. Mass error was set to 10 ppm for precursor ions and 0.02 Da for fragment ions. The false discovery rate (FDR) < 0.01 was considered as statistically significant.

2.4. GO and KEGG Analyses

To explore the functional annotation and involved pathways of genes, the GO and the KEGG analyses were executed by online analysis tools–Database for Annotation, Visualization, and Integrated Discovery (DAVID) The (http://david.abcc.ncifcrf.gov/) and the WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt/option.php), respectively.

2.5. The Western Blotting

Western blotting (WB) was performed as previously described (Zheng et al., 2010a, 2010b). Anti-PDZK1 and anti-β-actin antibodies were purchased from BD Biosciences (Cat# 612660, RRID: AB_399904, San Jose, CA) and Sigma–Aldrich (Cat# A5441, RRID: AB_476744, St. Louis, MO), respectively. HRP-conjugated secondary antibody was purchased from Amersham Biosciences (Cat# NA931 RRID: AB_772210, Little Chalfont, UK). The blots were quantified using NIH Image 1.62 program. The protein level was normalized with β-actin.
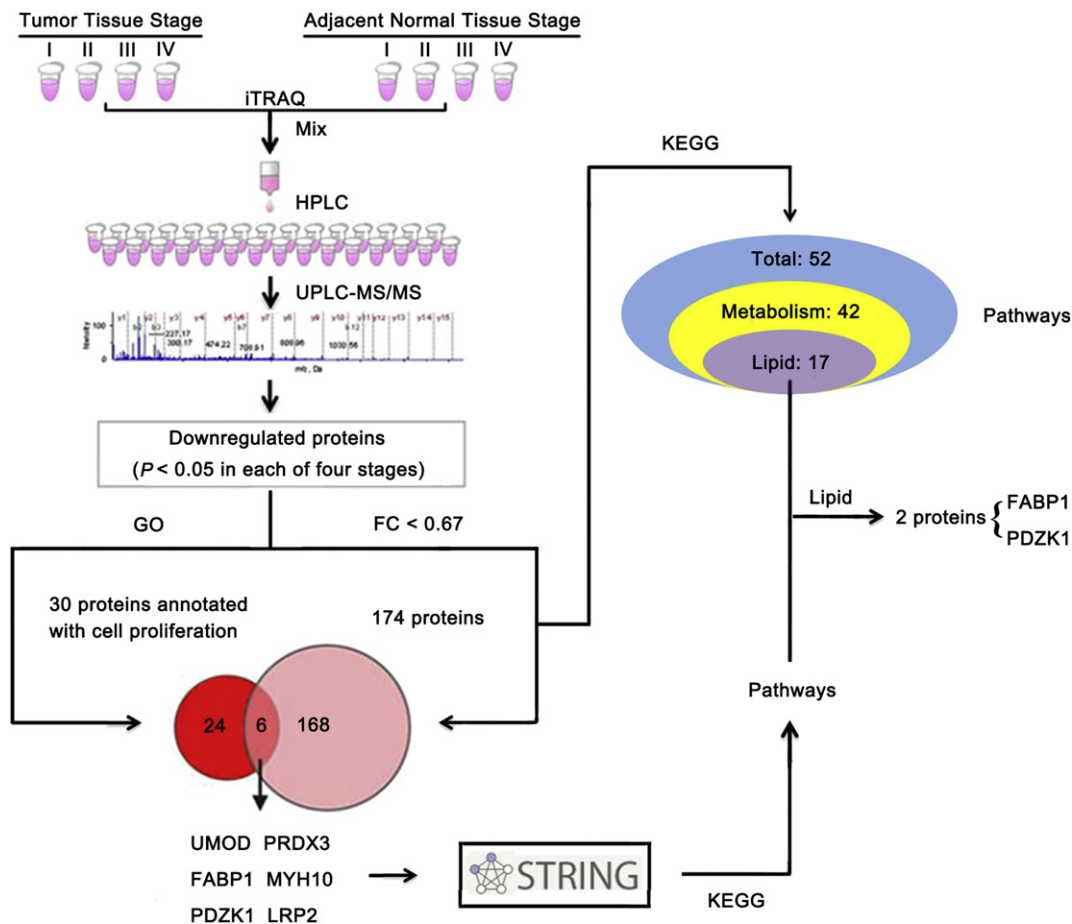
Fig. 1 PDZK1 and FABP1, which are involved in both cell proliferation and lipid metabolism pathways, are downregulated in ccRCC tissues. The proteins of paired ccRCC and its adjacent normal tissues from 18 ccRCC patients were extracted respectively. Protein lysates from the same TNM stage (stage I, $n = 8$; stage II, $n = 7$; stage III, $n = 1$; stage IV, $n = 2$) were combined as a group, respectively (Tumor Tissue Stage I, II, III, IV; Adjacent Normal Tissue Stage I, II, III, IV) in equal amounts and analyzed by iTRAQ quantitative proteomics. The downregulated proteins were performed with the GO and KEGG pathway analysis. Results revealed that 174 proteins were both downregulated and participated in 52 KEGG pathways, mainly lipid metabolism pathways. Six proteins were both downregulated and annotated with cell proliferation. Two proteins annotated with cell proliferation (FABP1 and PDZK1) participated in lipid metabolism pathways.
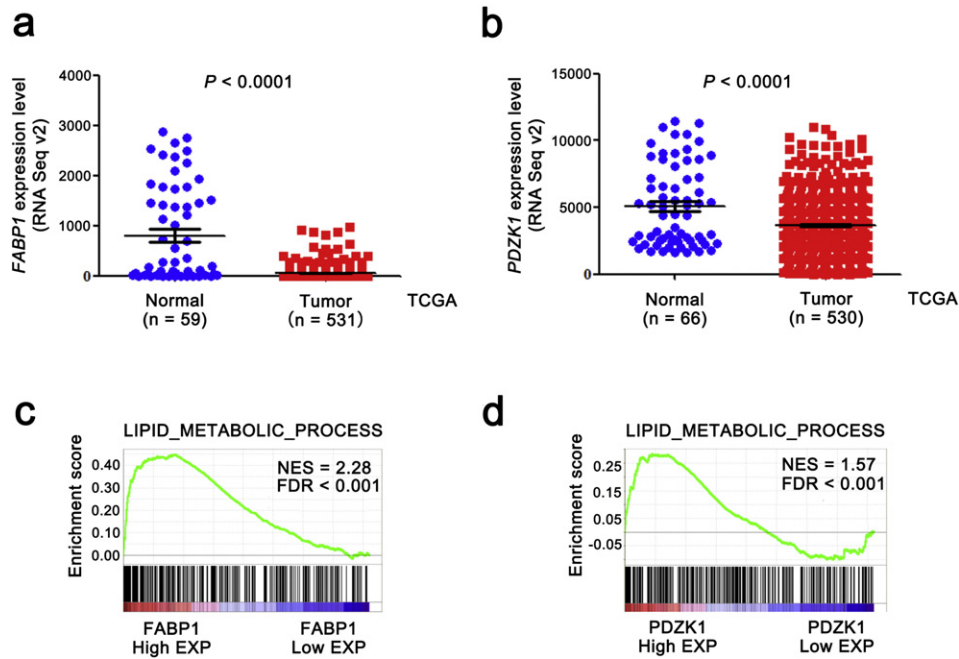
Fig. 2 The levels of both PDZK1 and FABP1 mRNA are downregulated in ccRCC tissues and correlated with lipid metabolism in ccRCC. (a,b) The mRNA levels of both *FABP1* and *PDZK1* in ccRCC by RNA sequencing were obtained from the TCGA_KIRC dataset. Seventy-two normal tissues and 532 ccRCC tissues were sequenced. Outlier values (>mean ± 3 SD) were removed. (c,d) The correlation between lipid metabolism process gene set and mRNA level of *FABP1* or *PDZK1* was analyzed by GSEA assay. FDR < 0.001 was considered as statistically significant.

2.6. Immunohistochemistry

Immunohistochemistry (IHC) was performed as previously reported (Ma et al., 2015). Sections were incubated at the optimal conditions with rabbit monoclonal anti-PDZK1 antibody (GeneTex, CA, Cat# GTX114628 RRID: AB_10620236, 1:1000). Image–Pro plus 6.0 (MediaCybernetics Inc., SilverSpring, MD) was used to analyze optical densitometry.

2.7. Bioinformatics Analysis

mRNA (RNA Seq v2) levels of genes in ccRCC patients, normal kidney tissues and clinical information about recurrence, metastasis, OS, disease–free survival (DFS) of patients in TCGA_KIRC dataset were obtained from https://www.synapse.org and http://www.cbioportal.org/public-portal, respectively. The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (http://string-db.org/) was used to screen for interacting protein with a combined score of C0.4.

The gene set enrichment analysis (GSEA) was performed as previously described (Peng et al., 2016) to assess whether genes from pre-defined gene-sets are enriched among the highest- (or lowest-) ranked genes by calculating a pathway Enrichment Score (ES).

2.8. Statistical Analysis

The results of the iTRAQ, WB and IHC of paired samples were analyzed by paired sample *t*-test. The results of RNA seq v2 and IHC of un-paired samples were analyzed by independent sample *t*-test. Receiver operator characteristic (ROC) curve and area under the curve (AUC) analyses were applied to detect the optimal cutoff point that yielded the highest total accuracy with respect to discriminating different clinical classifications [good (≥5 years, living) and poor (≤2 years, die) prognosis]. The log-rank test for the generated Kaplan–Meier (KM) curve was conducted to evaluate the association between the expression level of PDZK1 and the survival rate. Univariate and multivariate Cox proportional hazard regression analyses were used to estimate the

prognostic significance of PDZK1 in ccRCC. Statistical significance was set at two-tailed *P* values < 0.05. All statistical analyses were performed using SPSS Statistics 19.0 (IBM SPSS, Chicago, IL).

3. Results

3.1. Proteomic Analysis Identifies 212 Differentially Expressed Proteins in ccRCC

To assess DEPs between ccRCC and normal tissues, a total of 3816 proteins were identified by using LC-MS/MS. Of these proteins, 2985 proteins were quantitatively analyzed. Thirty-eight upregulated proteins (>1.5 fold over normal, $P < 0.05$) and 174 downregulated proteins (<0.67 fold over normal, $P < 0.05$) were found in each of four stages in tumor tissues (Table S1).

3.2. Six Proteins are both Downregulated and Related to Cell Proliferation in ccRCC

To search the proteins correlated with cell proliferation in ccRCC, GO annotation was used to analyze the downregulated proteins, and six proteins were identified as follows: UMOD, FABP1, PDZK1, PRDX3, MYH10 and LRP2 (Table S2).

3.3. Downregulated Proteins in ccRCC are Mainly Involved in the Lipid Metabolism Pathway

To investigate the relevant pathways of the downregulated proteins, we performed a KEGG pathway enrichment analysis and found that these proteins participated in 52 KEGG pathways, including metabolic processes (42 pathways, 80.8%) and sodium reabsorption, *etc.* (10 pathways). Of metabolic processes, 17 pathways (32.7%) were related with lipid metabolism (Fig. 1). Results of GSEA study further verified that lipid metabolism pathway was dysregulated in ccRCC tissues (data not shown), which is consistent with the previous reports (Wettersten et al., 2015).

3.4. Cell Proliferation Associated Protein FABP1 and PDZK1 Are Involved in Lipid Metabolism Pathway

To explore whether the six proteins annotated with cell proliferation (UMOD, FABP1, PDZK1, PRDX3, MYH10 and LRP2) also participated in these metabolic pathways, especially lipid metabolism pathway, we first investigated their binding partners by using STRING database as most proteins usually exerted functions coordinately with their binding partners (Zheng et al., 2010b). A number of possible interacting proteins were identified. As shown in Table S3, UMOD, FABP1, PDZK1, PRDX3, MYH10 and LRP2 interacted with 10, 12, 16, 12, 14 and 13 proteins, respectively. Further KEGG pathway enrichment analysis demonstrated that UMOD, PRDX3, MYH10, LRP2 and their respective partners were not involved in metabolic pathways. FABP1 and its binding partners participated in two lipid metabolism pathways, PDZK1 and its binding partners participated in four metabolism-related pathways, with three of which involved in lipid metabolism (Fig. 1, Table S4). Thus, we further studied the clinical significance of FABP1 and PDZK1 in ccRCC.

3.5. mRNA Levels of FABP1 and PDZK1 Are Downregulated and Correlate With Lipid Metabolism Pathway

To confirm the results of our proteomics studies, we further analyzed the mRNA levels of FABP1 and PDZK1 from TCGA_KIRC dataset, and found that mRNA levels of both FABP1 and PDZK1 were significantly decreased in tumor tissues compared with normal tissues (Fig. 2a,b, $P < 0.0001$).

Intracellular storage of lipid in the neoplastic cells of ccRCC leads to its distinctive pale and glassy cytoplasm (Sundelin et al., 2012). To confirm whether the FABP1 and PDZK1 were involved in lipid metabolism, GSEA was performed. The results showed that the mRNA levels of both FABP1 and PDZK1 positively correlated with the lipid metabolism process in renal tissues (FDR < 0.001, Fig. 2c,d).

3.6. Low Level of PDZK1 mRNA Predicts Poor Prognosis of ccRCC Patients

We further analyzed the correlation of the mRNA levels of FABP1 and PDZK1 with TNM stage in ccRCC patients. The change of FABP1 mRNA level had no correlation with the T stages, and failed to distinguish between N0 and N1 (without and with lymph node metastasis, data not shown), non-distant metastasis and metastasis, non-recurrence and recurrence (Fig. S1a). These results suggest that FABP1 is not a potential prognostic biomarker as least in ccRCC.

The PDZK1 mRNA level was decreased as T stage progressed and negatively correlated with the size and weight of tumors (Fig. S1b). It also exhibited differences between the patients with non-metastasis and metastasis (Fig. 3a), indicating that PDZK1 may be a potential
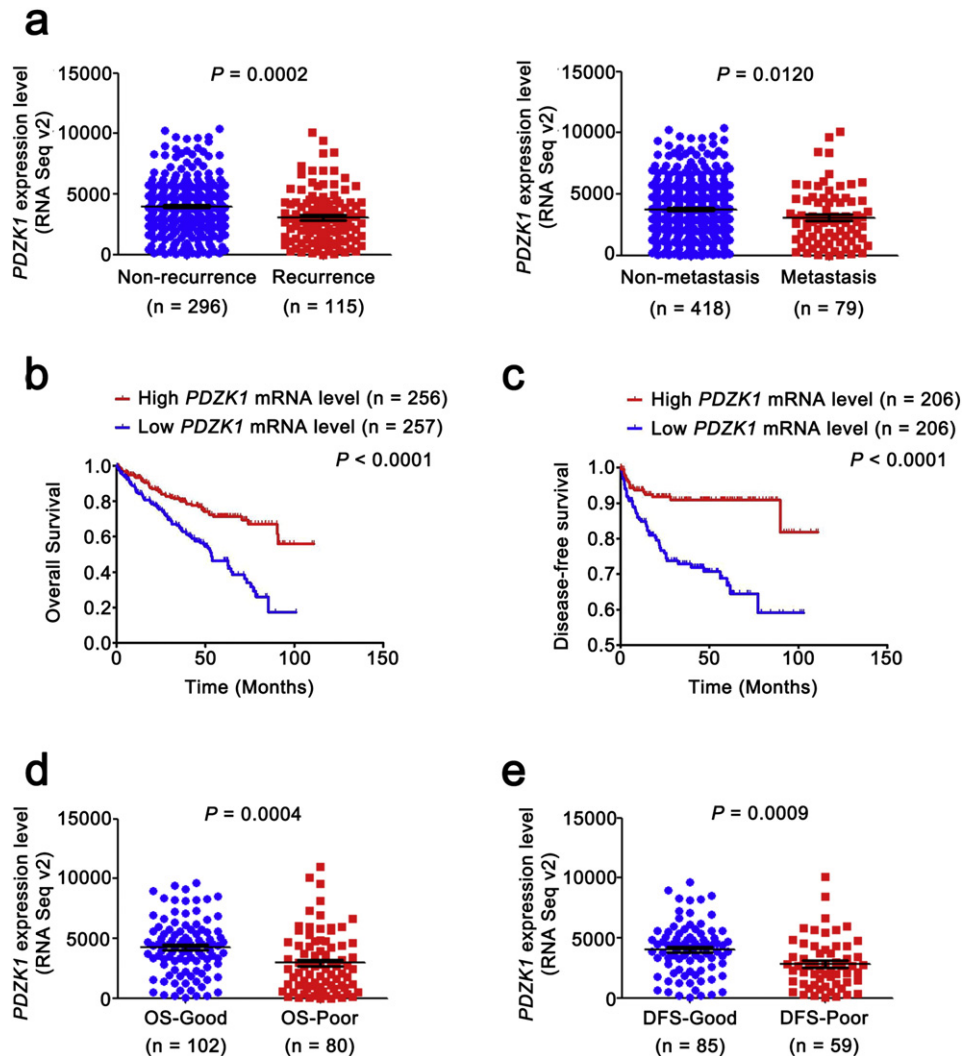
Fig. 3. Low level of PDZK1 mRNA predicts poor prognosis of ccRCC patients. (a) The mRNA level of PDZK1 between ccRCC patients with and without recurrence, metastasis was compared by independent sample t-test. (b, c) Patients were divided into high and low groups by median value of PDZK1 mRNA level. Kaplan-Meier (KM) curves for OS and DFS of patients were performed. (d, e) The mRNA level of PDZK1 between ccRCC patients with good and poor prognosis for OS and DFS was compared by independent sample t-test.
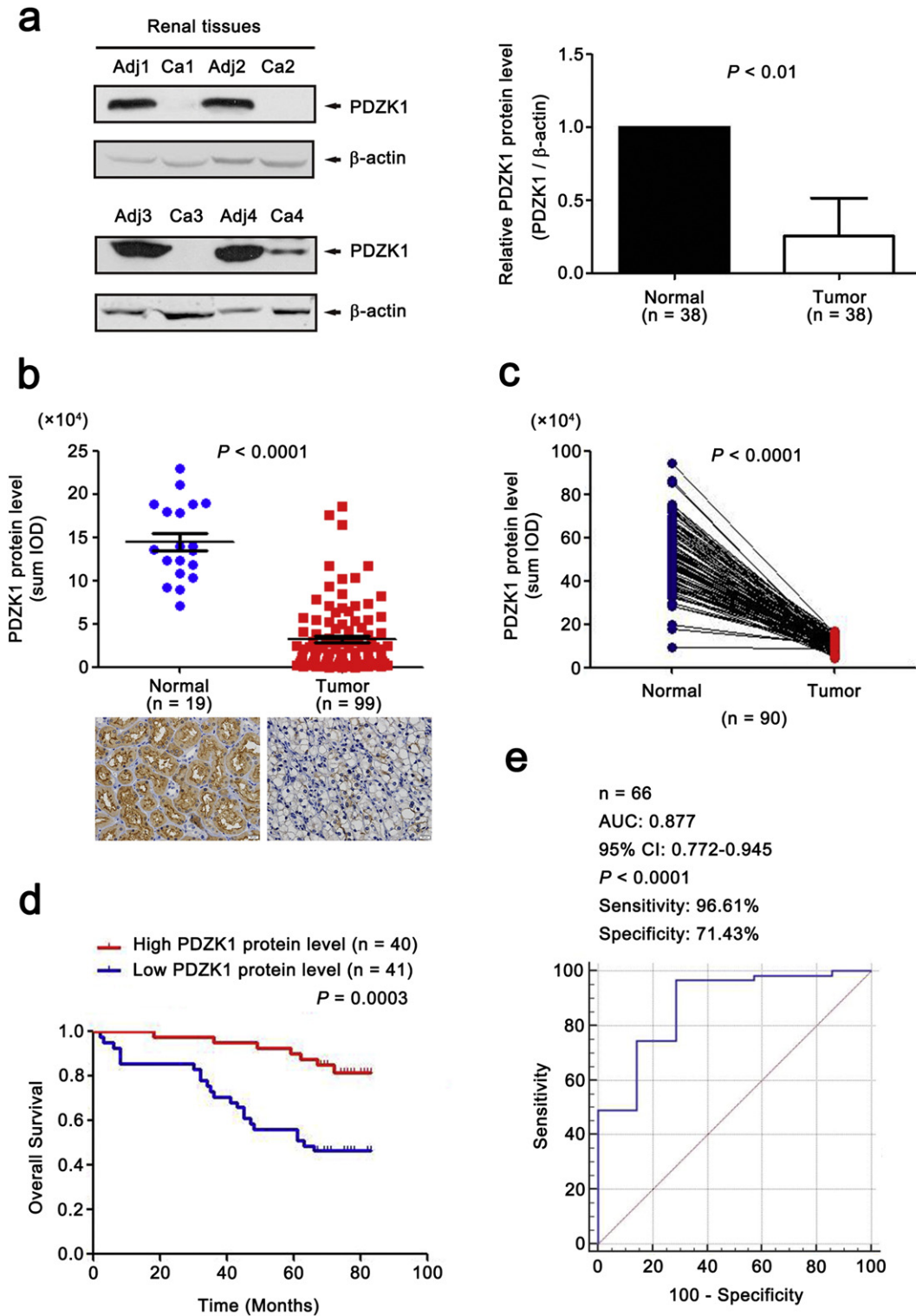
Fig. 4 Low level of PDZK1 protein predicts poor clinical outcome in patients with ccRCC. (a) The protein level of PDZK1 in paired biopsies of tumor (Ca) and normal (Adj) tissues from 38 ccRCC patients were analyzed by WB analysis. Representative blots of PDZK1 protein level in normal and ccRCC tissues were shown. β-actin was used as a loading control. (b) IHC was performed with anti-PDZK1 antibody in 19 normal tissues and 112 tumor tissues. Outlier values (>Mean ± 3 SD) were removed. Representative IHC images (20 × magnification, all panels) were shown. (c) The protein level of PDZK1 in 90 paired ccRCC samples was detected with IHC. (d) Patients were divided into high and low groups by median value of PDZK1 protein level. KM survival curve for OS was performed. (e) ROC curve for PDZK1 protein level in classifying ccRCC patients with good and poor prognosis for OS. The area under the receiver operating characteristic curve (AUC) was 0.877 (95% CI, 0.772–0.945).

prognostic biomarker for ccRCC. To investigate the prognostic significance of *PDZK1*, we compared *PDZK1* mRNA level between patients with and without recurrence. *PDZK1* mRNA expression was in significantly lower level with recurrent ccRCC (Fig. 3a).

To further evaluate the association of *PDZK1* mRNA expression level with the survival time of ccRCC patients, KM survival curves were plotted. Patients were divided into 'low' and 'high' groups based on the median values of *PDZK1* RNA seq quantification results. Patients with

low *PDZK1* mRNA level had shorter OS and DFS time (Fig. 3b, c, $P < 0.0001$), even for patients in early stage of ccRCC (Fig. S2a, $P < 0.05$). Moreover, the mRNA level of *PDZK1* was able to classify ccRCC patients with a good or poor prognosis (Fig. 3d, e, $P < 0.001$).

3.7. Low Level of PDZK1 Protein in ccRCC Tissues is Validated by WB and IHC

To further confirm our results, both WB and IHC assays were used on an expanded cohort of patients ($n = 38$ and 112, respectively). IHC was also performed on an independent set of 90 paired tissues. All results showed that PDZK1 protein level from ccRCC tissues was significantly lower than adjacent normal tissues (Fig. 4a,b,c, $P < 0.01$), and was gradually decreased with the increase of AJCC stage (data not shown).

Table 2
Univariate and multivariate analyses of *PDZK1* mRNA level and patient survival.

Variable	Univariate analysis			Multivariate analysis[c]		
	HR[a]	95% CI[b]	P	HR	95% CI	P
Overall survival ($n = 499$)						
Age (years)						
≤50 ($n = 108$)	0.468	0.293–0.748	0.001	0.578	0.358–0.934	0.025
>50 ($n = 391$)						
Gender						
Female ($n = 175$)	1.028	0.747–1.414	0.866			
Male ($n = 324$)						
T stage						
T1 or T2 ($n = 316$)	0.286	0.209–0.393	0.000	0.564	0.386–0.825	0.003
T3 or T4 ($n = 183$)						
N stage						
N0 or NX ($n = 483$)	0.341	0.179–0.647	0.001	0.569	0.293–1.107	0.097
N1 ($n = 16$)						
M stage						
M0 or MX ($n = 421$)	0.217	0.158–0.299	0.000	0.426	(0.292–0.621	0.000
M1 ($n = 78$)						
G grade						
G1 or G2 ($n = 223$)	0.360	0.252–0.514	0.000	0.566	0.385–0.831	0.004
G3 or G4 ($n = 276$)						
PDZK1						
High ($n = 250$)	0.415	0.299–0.577	0.000	0.577	0.410–0.811	0.002
Low ($n = 249$)						
Disease–free survival ($n = 399$)						
Age (years)						
≤50 ($n = 99$)	0.664	0.371–1.190	0.169			
>50 ($n = 300$)						
Gender						
Female ($n = 133$)	0.911	0.556–1.494	0.713			
Male ($n = 266$)						
T stage						
T1 or T2 ($n = 262$)	0.150	0.090–0.252	0.000	0.394	0.218–0.712	0.002
T3 or T4 ($n = 137$)						
N stage						
N0 or NX ($n = 385$)	0.156	0.074–0.331	0.000	0.376	0.175–0.811	0.013
N1 ($n = 14$)						
M stage						
M0 or MX ($n = 347$)	0.087	0.055–0.140	0.000	0.181	0.106–0.307	0.000
M1 ($n = 52$)						
G grade						
G1 or G2 ($n = 188$)	0.197	0.106–0.365	0.000	0.354	0.185–0.675	0.002
G3 or G4 ($n = 211$)						
PDZK1						
High ($n = 200$)	0.298	0.175–0.508	0.000	0.425	0.246–0.735	0.002
Low ($n = 199$)						

[a] Hazard ratio, estimated from Cox proportional hazard regression model.
[b] Confidence interval of the estimated HR.
[c] Multivariate models were adjusted for T, N, M classification, age and gender.

Table 3
Univariate and multivariate analyses of PDZK1 protein level and patient OS.

Variable	Univariate analysis			Multivariate analysis[c]		
	HR[a]	95% CI[b]	P	HR	95% CI	P
Overall survival ($n = 77$)						
Age (years)						
≤60 ($n = 40$)	0.467	0.215–1.012	0.054			
>60 ($n = 37$)						
Gender						
Female ($n = 31$)	1.107	0.524–2.340	0.791			
Male ($n = 46$)						
Grade						
G1 or G2 ($n = 67$)	0.157	0.070–0.351	0.000	0.547	0.202–1.477	0.234
G3 or G4 ($n = 10$)						
AJCC stage						
I or II ($n = 55$)	0.214	0.101–0.454	0.000	0.330	0.135–0.806	0.015
III or IV ($n = 22$)						
PDZK1						
High ($n = 47$)	0.236	0.108–0.513	0.000	0.287	0.125–0.657	0.003
Low ($n = 30$)						

[a] Hazard ratio, estimated from Cox proportional hazard regression model.
[b] Confidence interval of the estimated HR.
[c] Multivariate models were adjusted for T, N, M classification, age and gender.

### 3.8. Low PDZK1 Protein Level Predicts Poor Prognosis of ccRCC Patients

To investigate the clinical relevance of PDZK1 protein level with prognosis in ccRCC patients, we further evaluated the OS time of ccRCC patients from the follow-up data via KM survival analysis. The patients were divided into 'low' and 'high' groups according to the median values of PDZK1 intensity/area. Low PDZK1 protein level was correlated with shorter OS time of ccRCC patients (Fig. 4d, $P < 0.001$). Moreover, PDZK1 protein level of ccRCC patients discriminated between good or poor OS prognosis with the AUC of 0.877 (Fig. 4e). Interestingly, the similar results were observed in the early stage of ccRCC patients (Figs. S2b,2c, $P < 0.05$, AUC 0.857). Consistent with the results of mRNA, the PDZK1 protein level was gradually decreased with the increase of the T stage (Fig. S1b).

3.9. PDZK1 is an Independent Prognostic Marker for ccRCC

The association between PDZK1 mRNA (Table 2), protein level (Table 3) and OS or DFS in the cohort of ccRCC patients was studied using univariate and multivariate analyses. Specimens were grouped into low and high PDZK1 expression categories according to the median value. Univariate analysis indicated that the patients with low PDZK1 level exhibited a shorter OS and DFS [PDZK1 mRNA OS: hazard ratio (HR) 0.415, $P < 0.0001$ and DFS HR 0.298, $P < 0.0001$; PDZK1 protein OS HR 0.236, $P < 0.0001$]. When controlling for other variables in the multivariate analysis, low PDZK1 mRNA and protein level retained its clinical significance as a marker of shorter survival (mRNA OS: HR 0.577, $P = 0.002$ and DFS HR 0.425, $P = 0.002$; protein OS HR 0.287, $P = 0.003$). Taken together, these data suggest that low PDZK1 expression level is an independent predictor of poor prognosis for ccRCC patients.

3.10. External Comparison of Biomarkers

For external validation of PDZK1 as a prognostic biomarker in ccRCC, we compared it with five previously reported biomarkers. Fascin (FSCN2) (Jin et al., 2006), insulin-like growth factor mRNA binding protein 3 (IMP3) (Jiang et al., 2006) and carbonic anhydrase IX (CA9) (Zhang et al., 2013), *etc.* were the reported prognostic markers for ccRCC. However, these proteins failed to distinguish patients with good and poor prognosis in TCGA_KIRC dataset (Fig. 5). Although lactate
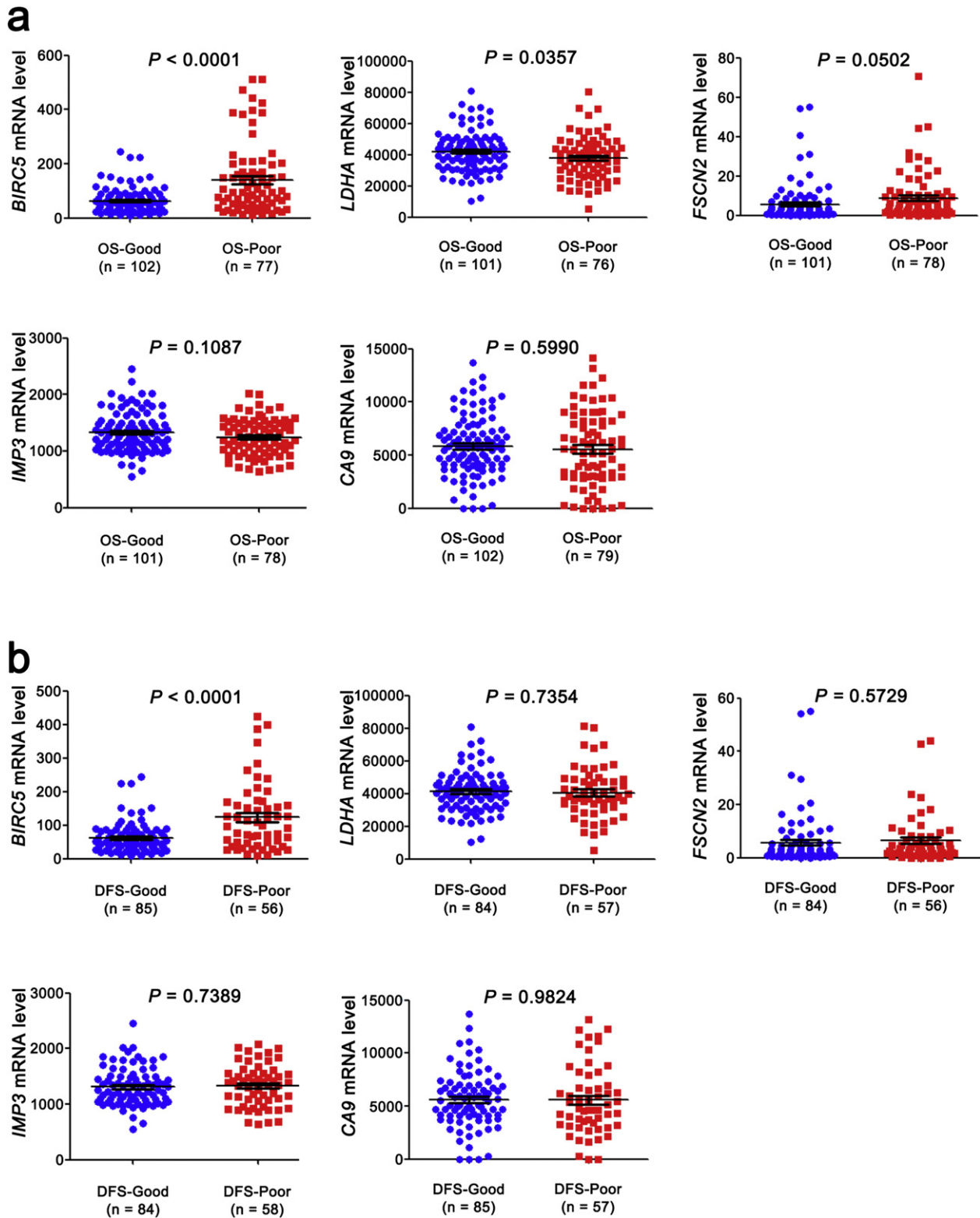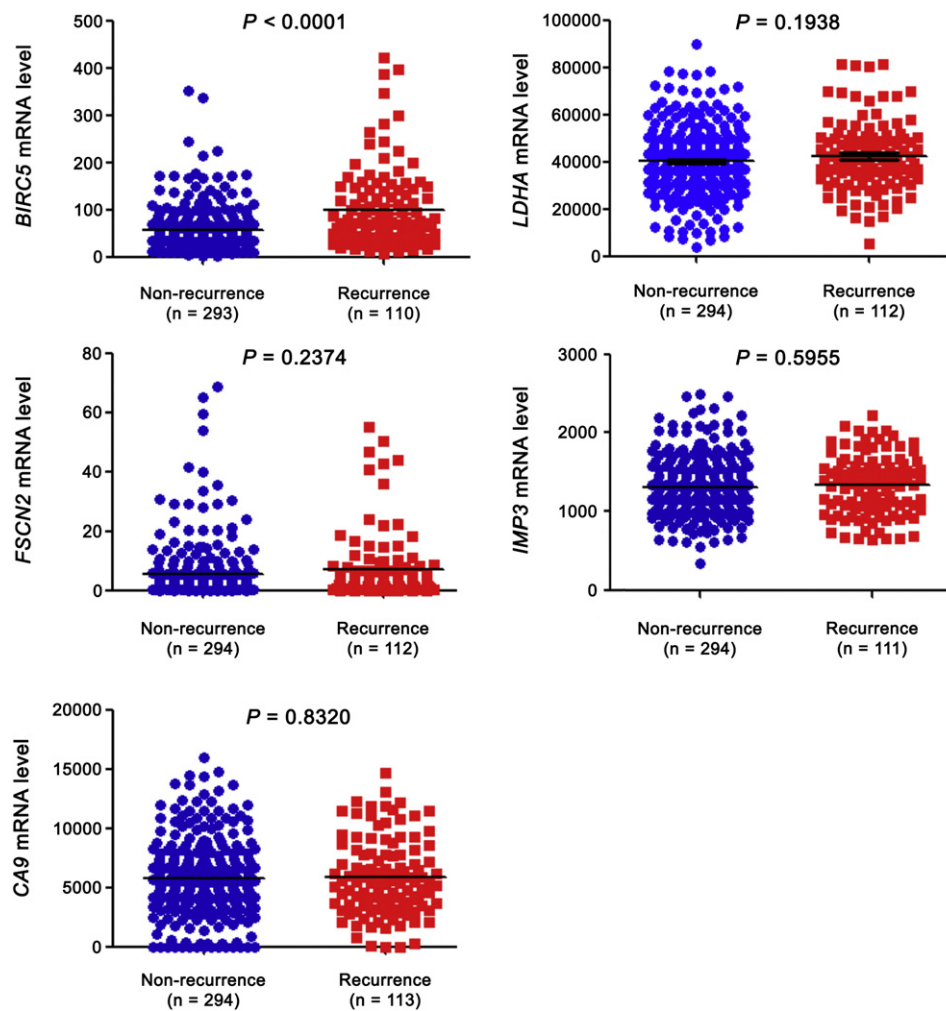
Fig. 5 External comparison of biomarkers-OS and DFS. mRNA levels of five previously reported prognostic markers (*BIRC5*, *LDHA*, *FSCN2*, *IMP3* and *CA9*) were extracted from TCGA_KIRC dataset. mRNA levels between patients with good and poor prognosis were compared by independent sample *t*-test. (a) for OS and (b) for DFS.

dehydrogenase A (LDHA) (Girgis et al., 2014) could distinguish patients with good and poor prognosis for OS, it was not significant in identifying patients with poor prognosis for DFS or with recurrence (Figs. 5, 6). Survivin (BIRC5) (Parker et al., 2006) was able to discriminate between good and poor prognosis patients for both OS and DFS (Fig. 5).

3.11. Comparison of DEP Identification Results Between Separate and Pooled Four Stage Samples

By comparison of ccRCC and their corresponding adjacent normal tissues separately in each of four stages, we generated the DEPs,

Fig. 6 External comparison of biomarkers-recurrence. mRNA levels of five previously reported prognostic markers (*BIRC5, LDHA, FSCN2, IMP3* and *CA9*) were extracted from TCGA_KIRC dataset. mRNA levels between patients with and without recurrence were compared by independent sample *t*-test.

which were consistently differentially expressed in each of four stages in this study. Meanwhile, we analyzed virtually pooled four stage samples, *i.e.*, mixtures of biological samples of all four stages ccRCC tissues in one group and mixtures of all corresponding adjacent normal tissues in the other group. Some of DEPs found in virtually pooled groups were not differentially expressed in each of four stages, for example, aldose 1-epimerase was downregulated in pooled ccRCC group compared with pooled normal tissues group (Tumor/Normal: fold change (FC) 0.506, $P < 0.0001$), but not in ccRCC patient of stage IV (Tumor/Normal for stage I: FC 0.433, $P < 0.0001$; stage II: FC 0.424, $P < 0.0001$; stage III: FC 0.638, $P < 0.0001$; stage IV: no fold change, $P = 0.174$). Thus, analysis of consistent DEPs in each stage for separate four-stage samples could identify less DEPs than mixtures of biology samples of all four stages ccRCC tissues ($n = 212$ *vs* 593).

4. Discussion

In this study, PDZK1 was found to be downregulated in ccRCC tissues. The low PDZK1 level was strongly associated with a poor clinical outcome, especially in the early-stage of ccRCC patients.

Cell proliferation plays an important role in cancer development and progression (Feitelson et al., 2015). Proliferation-related proteins were able to predict a poor survival outcome in cancer patients (Yang et al., 2015). Downregulated proteins in ccRCC identified in this study are mainly involved in the lipid metabolism pathway, so we combined both GO cell proliferation annotation and lipid metabolism pathway to

screen for prognostic markers in ccRCC and identified PDZK1 as a promising prognostic marker. PDZK1 could discriminate recurrence, metastasis and prognosis between ccRCC patients (Figs. 3,4). In contrast, ccRCC prognostic markers proposed by other researchers failed to distinguish patients with good and poor prognosis (Fig. 5). The significance of LDHA in identifying patients with poor prognosis for DFS or with recurrence was insufficient. Survivin-BIRC5 was able to discriminate between good and poor prognosis patients for both OS and DFS, but its lower expression level made it not so easy to be detected using conventional method. Interestingly, both LDHA and BIRC5 were involved in lipid metabolism as revealed by KEGG pathway analysis and GSEA. These findings were consistent with previous reports–prognostic markers are usually metabolism pathway-related (Zaravinos et al., 2014), or pathway-derived metabolic products (Arsanious et al., 2009). This result further suggests that lipid metabolism pathway is important in screening prognostic markers for ccRCC. Indeed, we found that the downregulated expression level of *PDZK1* is correlated with dysregulated lipid metabolism in ccRCC and PDZK1 may be a prognostic marker for ccRCC.

In this study, we identified FABP1 and PDZK1 as potential biomarkers by exploring the downregulated proteins involved in cell proliferation and the lipid metabolism pathway. Although the change of *FABP1* mRNA level was not significant in prognosis prediction for ccRCC, it could distinguish ccRCC patients from normal individuals with AUC of 0.820 (95% CI 0.787–0.850). In addition, PDZK1 protein level could also discriminate ccRCC from normal tissues with AUC of

0.944 (95% CI, 0.906–0.982). Thus, the results of this study also verified the important role of cell proliferation and lipid metabolism-related phenotype in identifying biomarkers in ccRCC.

Compared with the strategy of analysis from pooled four stage samples, analysis of DEPs from separate four-stage ccRCC samples identified much less DEPs. Some DEPs found in pooled sample were not identified as differentially expressed in each of four stages, suggesting that not all DEPs found in pooled samples were appropriate as markers. Thus, it is our successful strategy to screen for biomarkers from DEPs which were consistently differentially expressed in each of four stages, and the previously reported markers, such as LDHA and FABP7 (Girgis et al., 2014; Zhou et al., 2015), were also in the list of identified DEPs in this study from the upregulated proteins (Table S1).

PDZK1 was expressed at the apical membrane of the renal proximal tubule cells, from which RCC originates. PDZK1 belong to PDZ proteins (Kocher et al., 1998), which play important roles in cell growth control, tumorigenesis and development (Yao et al., 2012). PDZK1 is also a member of Na^+/H^+ exchanger regulatory factor (NHERF) family. NHERFs were associated with malignant cell transformation (Zheng et al., 2010b; Yao et al., 2012), reminding that PDZK1 was correlated with ccRCC malignancy. PDZK1, as scavenger receptor class B type I (SR-BI)-binding protein, was also closely involved in the regulation of lipid metabolism (Kocher et al., 2008). Metabolism determined the biologically malignant behavior of multiple types of cancer (Ogawa et al., 2015). The disorder of metabolism related proteins, especially the abnormality of lipid metabolism related proteins was correlated with progression of RCC and could predict the prognosis of ccRCC patients (Yu et al., 2013). PDZK1 is most highly expressed in the kidney (Kocher et al., 1998), and significantly downregulated in ccRCC. PDZK1 could discriminate recurrence, metastasis and prognosis between ccRCC patients. Its role in ccRCC remains unknown. Further studies are required to clarify the role of PDZK1 in ccRCC tumorigenesis and progression. However, lines of evidence reported oncogenic activity for PDZK1 in breast cancer (Kim et al., 2013; OLeary et al., 2013). Thus, PDZK1 has been shown to play important and diverse roles in different tissues.

The drawback of this study was the lack of enough specimens with N1 or M1 stage or recurrence in the independent validation set of 81 samples, so we were unable to know if PDZK1 protein level could discriminate patients with lymph node involvement, distant metastasis or recurrence so far. In addition, ignorance of data collection for DFS time led to absence of KM-curve regarding PDZK1 protein level for DFS. These issues need to be further investigated in the future study.

In conclusion, PDZK1 is identified as an independent predictor for prognosis in ccRCC patients. These findings will facilitate patient counseling and individualize the management of patients with ccRCC.

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Conflicts of Interest

The authors declare no conflicts of interest.

Author contributions

Zheng JF, Peng ZQ and He JQ designed the study. Zheng JF and He JQ analysed the data and wrote the report. All authors collected data, interpreted data, and approved the final report.

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