Transcriptomic Profiling Identifies DCBLD2 as a Diagnostic and Prognostic Biomarker in Pancreatic Ductal Adenocarcinoma

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Research

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Abstract

Background: Accumulating evidence showed that the elevated expression of DCBLD2 is associated with unfavorable prognosis of various cancers. However, the correlation of DCBLD2 expression value with the diagnosis and prognosis of pancreatic ductal adenocarcinoma (PDAC) has not yet been elucidated.

Methods: Univariate Cox regression analysis was used to screen robust survival-related genes. Expression pattern of selected genes was investigated in PDAC tissues and normal tissues from multiple cohorts. Kaplan–Meier (K-M) survival curves, ROC curves and calibration curves were employed to assess prognostic performance. The relationship between DCBLD2 expression and immune cell infiltrates was conducted by CIBERSORT software. Biological processes and KEGG pathway enrichment analyses were adopted to clarify the potential function of DCBLD2 in PDAC.

Results: Univariate analysis, K-M survival curves and calibration curves indicated that DCBLD2 was a robust prognostic factor for PDAC with cross-cohort compatibility. Upregulation of DCBLD2 was observed in dissected PDAC tissues as well as extracellular vesicles from both plasma and serum samples of PDAC patients. Both tissue and extracellular vesicle-encapsulated DCBLD2 expression have significant diagnostic value. Besides, DCBLD2 expression is correlated with infiltrating level of CD8 + T cells and macrophage M2 cells. Functional enrichment revealed that DCBLD2 may be involved in cell motility, angiogenesis, and cancer-associated pathways.

Conclusions: Our study systematically analyzed the potential diagnostic, prognostic and therapeutic value of DCBLD2 in PDAC. All the findings indicated that DCBLD2 may play a considerably oncogenic role in PDAC with diagnostic, prognostic and therapeutic potential. These preliminary results of bioinformatics analyses need to be further validated in more prospective studies.

Background

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest cancers in the world and is featured with relatively late diagnosis and extremely poor prognosis, with a < 9% five-year survival outcome [1]. Curative resection is the only established treatment and it remarkably improves the five-year survival rate to 20–30% [2]. Unfortunately, only a small minority of PDAC patients have surgical indication since over 80% patients are diagnosed with advanced-stage tumors [3]. Accumulating evidences have demonstrated that diagnosis of PDAC at an earlier, resectable stage is likely to result in dramatic improvement of patient outcome [4]. Thus, novel development of diagnostic and prognostic biomarkers with satisfactory sensitivity and specificity is an area of utmost priority for PDAC patients.

The discoidin, CUB and LCCL domain-containing protein 2 (DCBLD2) is a type-I transmembrane protein and has considerable tumor-specific functions [5]. DCBLD2 is upregulated in lung cancer and promotes cell motility [6]. In colorectal cancer, high DCBLD2 expression is associated with poor patient survival, as well as tumorigenesis, invasion and metastasis of cancer cells [7]. In gastric cancer, DCBLD2 is downregulated by epigenetic modification, and it exhibits suppressive role in cancer cell proliferation and

invasion [8]. In the context of PDAC, the elevated expression of DCBLD2 is correlated with poor clinical outcome, indicating DCBLD2 may serve as an appealing biomarker for PDAC diagnosis and prognosis [9, 10]. However, these PDAC studies fail to evaluate the diagnostic and prognostic potential of DCBLD2 from a holistic and multidimensional perspective.

With continuous improvements of high-throughput methods, it is now possible to conduct in-depth and systematical research on genetic landscape of most tumors [11]. In this study, we identified and validated DCBLD2 as a robust diagnostic and prognostic biomarker for PDAC based on the integrating and analyzing of genomic and transcriptomic data. Furthermore, DCBLD2 was found to be associated with several important biological processes of PDAC. In conclusion, DCBLD2 could effectively distinguish PDAC samples from normal samples and predict patient survival, and it has the prospect for clinical application.

Materials And Methods

PDAC cohorts

The ten PDAC cohorts included in this study for survival analyses were the MTAB-6134 cohort (N=288), PACA-AU cohort (N=62), PACA-CA cohort (N=181), TCGA cohort (N=139), and six microarray cohorts, GSE21501 (N = 97), GSE28735 (N = 42), GSE57495 (N = 63), GSE62452 (N = 64), GSE71729 (N = 123) and GSE85916 (N = 79). Eleven cohorts including GSE15471, GSE16515, GSE28735, GSE32676, GSE41368, GSE55643, GSE60979, GSE62165, GSE62452, GSE71729 and GSE71989, which contained both PDAC tissue samples and normal tissue samples, were employed to evaluate the expression of genes. In addition, GSE133684 cohort, which provided expression profiles of extracellular vesicles in human plasma samples from PDAC, chronic pancreatitis (CP) and healthy individuals, was chosen to investigate the potential implication of genes in liquid biopsy. The normalized gene expression data and clinical information of all GSE cohorts were downloaded from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/). Data of MTAB-6134 cohort was downloaded from ArrayExpress database (https://www.ebi.ac.uk/arrayexpress/). All data of PACA-AU and PACA-CA cohorts was obtained from the International Cancer Genome Consortium (ICGC, https://icgc.org/). The TCGA data was obtained from the TCGA hub at UCSC Xena (https://tcga.xenahubs.net). In each cohort, patients whose clinical data was incomplete or whose histopathological type was not PDAC were removed from this study. Patients with a survival time of <1 month were excluded. The baseline characteristics of all PDAC patients with global clinical information are detailed in Additional file 1: Table S1.

In-house serum samples

Serum samples from patients with CP (n=5), PDAC (n=73), and from healthy donors (n=42) were collected at the Department of General Surgery of Ruijin Hospital from March 2018 to December 2018 and frozen at −80°C. None of the patients received preoperative chemotherapy or radiotherapy. Written

informed consent was obtained from all patients. The Ethics Committee of Ruijin Hospital affiliated with Shanghai Jiao Tong University approved the study.

Survival-related genes screening

The univariate Cox regression analysis was conducted to identify the survival-related genes in six independent microarray cohorts (GSE21501, GSE28735, GSE57495, GSE62452, GSE71729 and GSE85916 cohorts). Venn diagram (https://www.omicshare.com/tools/Home/Soft/venn) was used to screen common prognostic genes with $P<0.05$ in all six cohorts. The identified genes were deemed as robust survival-related genes.

Prognostic validation of robust survival-related genes

Patients in each cohort were divided into low- and high-expression groups according to the optimal cut-off value calculated by X-Tile software [12]. Kaplan–Meier (K-M) survival curves were utilized to assess the survival differences between low- and high-expression groups. Calibration plots comparing the predicted and observed clinical outcome were adopted to evaluate the predictive performance. ROC curves were used to compare the efficiency of genes with that of clinical indicators for prognosis prediction.

Estimation of tumor immune infiltrates

We estimated the relative proportions of the 22 subtype immune cells in each sample by the software CIBERSORT. Samples with a $P<0.05$ were included. The CIBERSORT software can use the deconvolution algorithm to estimate the composition of immune infiltrating cells according to gene expression matrix [13]. We further used the Pearson correlation analyses to determine the correlation of gene expression level with the immune infiltrates.

Functional enrichment Analysis

To shed light on the biological function, co-expressed genes ($P<0.05$) were screened by Pearson correlation analysis in MTAB-6134 cohort and TCGA cohort respectively. Top 1000 positively correlated genes were subjected to biological process and The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis on DAVID online website [14].

Isolation of extracellular vesicles and extracellular vesicular RNA

For each case, 1.2 mL of serum was used, and an exoRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) was used to extract extracellular vesicles following the manufacturer's instructions. Extracellular vesicles were eluted with 100 μL phosphate-buffered saline (PBS), half of them was used for characterization and the rest for RNA isolation. Morphology of extracellular vesicles was observed by transmission electron microscopy (TEM, JEOL, Japan) on a JEOL-1230 instrument. The density and size distribution of the extracellular vesicles were measured by nanoparticle tracking analysis (NTA) using a ZetaView PMX 110 (Particle Metrix, Germany). extracellular vesicular RNA was extracted using QIAzol (Qiagen, Hilden, Germany).

Quantitative real-time polymerase chain reaction (qRT-PCR)

The extracellular vesicular RNAs of total 120 samples (Ruijin cohort) were reverse-transcribed using an Evo M-MLV RT Kit (Accurate Biology, China). Real-time PCR was conducted with an ABI 7900 instrument using ChamQ SYBR qPCR Master Mix (Vazyme, Nanjing, China). Quantitation was performed in triplicate and expression was computed using the $2^{-\Delta\Delta CT}$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference. The primer sequences for amplified mRNAs are as follows: DCBLD2-Forward: 5'-GCTCCAACTCCTCCTCCTTCTCC-3'; DCBLD2-Reverse: 5'-GTGTCCACATCCATCACCTTGCTG-3'; GPADH-Forward: 5'- GCACCGTCAAGGCTGAGAAC-3'; GAPDH-Reverse: 5'- TGGTGAAGACGCCAGTGGA-3'.

Statistical analysis

The statistical analysis and graphical work were carried out in the R environment (version 3.5.2). Cox regression analyses and K-M survival curves were plotted by the 'survival' package. The ROC curves for diagnosis were derived from the 'pROC' package while the ROC curves for prognosis were generated from the 'survivalROC' package. Boxplots were depicted using the 'ggpubr' package. Calibration curves were produced by the "rms" package. Correlation curves were plotted by the "ggstatsplot" package. The enriched pathways and biological processes were illustrated by the "ggplot2" package. A two-sided log-rank $P<0.05$ was considered signifcant.

Results

DCBLD2 was associated with unfavorable survival in PDAC

Fig. 1 shows the research workflow of this study. The univariate Cox regression analysis screened hundreds of survival-related genes in each cohort, while only DCBLD2 was identified as a robust prognostic gene by Venn diagram. In all available PDAC cohorts with clinical data, DCBLD2 was significantly associated with overall survival (OS) of patients (GSE21501: HR = 1.21, 95% CI = 1.06–1.39, P = 0.0065; GSE28735: HR = 1.98, 95% CI = 1.24–3.14, P = 0.004; GSE57495: HR = 1.53, 95% CI = 1.16–2.01, P = 0.0029; GSE62452: HR = 1.68, 95% CI = 1.24–2.29, P = 0.0009; GSE71729: HR = 1.28, 95% CI =

1.00–1.64, P = 0.0457; GSE85916: HR = 1.89, 95% CI = 1.43–2.51, P < 0.0001; MTAB-6134: HR = 1.39, 95% CI = 1.19–1.56, P < 0.0001; PACA-AU: HR = 1.52, 95% CI = 1.13–2.05, P = 0.0056; PACA-CA: HR = 1.14, 95% CI = 1.03–1.25, P = 0.0098; TCGA: HR = 1.24, 95% CI = 1.03–1.49, P = 0.0199).

DCBLD2 is upregulated in PDAC with diagnostic potential

We first investigated the expression pattern of DCBLD2 in PDAC patients. As illustrated in Fig. 2a, DCBLD2 was remarkably overexpressed in PDAC tissues compared with normal tissues in seven GEO datasets. According to the expression data from Gene Expression Profiling Interactive Analysis (GEPIA), DCBLD2 was significantly elevated in PDAC tissues (Fig. 2b). Fig. 2c-f demonstrated that DCBLD2 expression was also increased in PDAC tissues compared with paired normal tissues. Furthermore, we explored the diagnostic potential of DCBLD2 in four GEO cohorts (GSE32676, GSE60979, GSE62165 and GSE71729). The area under the curve (AUC) value of DCBLD2 was 0.800, 0.874, 0.984 and 0.818, respectively (Fig. 2g-j), which was no less than that of an established diagnostic marker, CA19-9, whose AUC value was approximately 0.84 [15]. In addition, the expression of DCBLD2 was remarkably increased in patients with high grade (P<0.05), suggesting that DCBLD2 was related to high tumor malignancy (Additional file 2: Fig. S1).

Prognostic performance of DCBLD2

We next assessed the prognostic efficiency of DCBLD2 in ten independent PDAC cohorts. K-M survival curves illustrated that DCBLD2 could precisely capture the survival differences between low- and high-expression patients (Fig. 3). The calibration curves revealed that the clinical outcomes predicted by DCBLD2 were in good accordance with the actual observations (Fig. 4).

We further compared the robustness of DCBLD2 with clinical indicators, including histological grade, N stage and T stage, in MTAB-6134, PACA-AU and TCGA cohorts. The AUC value of DCBLD2 was 0.708, 0.753 and 0.690, respectively, which is greater than that of clinical factors in all three cohorts (Fig. 5). This finding suggested that DCBLD2 outperformed traditional indicators in predicting PDAC survival. Moreover, patients in the high-expression group had a significantly decreased disease-free survival (DFS) compared with low-expression group in MTAB-6134 and TCGA cohorts, indicating that DCBLD2 may serve as a prognostic indicator of DFS (Additional file 2: Fig. S2).

Relationship between immune cell infiltration and DCBLD2 expression

We subsequently determined the relationship between DCBLD2 expression and immune cell infiltration in PDAC samples from MTAB-6134 and TCGA cohorts by the software CIBERSORT. The abundance of macrophage M0 and M2 was positively related to DCBLD2 expression, while CD8 + T cells had negatively

correlation with DCBLD2 expression in MTAB-6134 cohort (P<0.05, Fig. 6a-c). Similar trends were observed in TCGA cohort (P<0.05, Fig. 6d-f).

Biological function and pathway of DCBLD2

In order to clarify the function mechanism of DCBLD2, we performed biological process and KEGG pathway enrichment analyses on top 1000 positively co-expressed genes of DCBLD2. For biological process, DCBLD2 was found to be primarily involved in angiogenesis, cell adhesion, cell motility and cell migration in both cohorts (Fig. 7a-b). For pathway enrichment, DCBLD2 was mainly associated with PI3K-AKT signaling pathway, Hippo signaling pathway, Rap1 signaling pathway and pancreatic cancer in both cohorts (Fig. 7c-d).

Expression of DCBLD2 in extracellular vesicles from human plasma samples

Early diagnosis of PDAC remains challengeable, and extracellular vesicles have emerged as attractive diagnostic biomarkers for early detection of PDAC. Since DCBLD2 was upregulated in PDAC tissues, we wondered whether DCBLD2 was also highly expressed in extracellular vesicles from plasma samples of PDAC patients. As Fig. 8a illustrated, expression of DCBLD2 in extracellular vesicles from plasma samples of PDAC patients was higher than that of normal donors (P=0.0029) or CP patients (P<0.0001). In addition, DCBLD2 in extracellular vesicles could serve as a moderate diagnostic biomarker for PDAC as the AUC value was 0.627 (Fig. 8b).

Validation of DCBLD2 expression in extracellular vesicles from human serum samples

Extracellular vesicles can be isolated from both plasma and serum of whole blood, and we had proved that DCBLD2 in extracellular vesicles from plasma samples had diagnostic value based on the public data. We next analyzed our own data to evaluate the diagnostic value of DCBLD2 in extracellular vesicles from serum samples. The results of NTA analysis and TEM demonstrated typical characteristics of isolated extracellular vesicles (Fig. 9a-b). Fig. 9c showed that the expression of DCBLD2 in extracellular vesicles from serum samples was markedly elevated in PDAC patients compared with normal donors (P<0.0001) or CP patients (P=0.0018). Similarly, DCBLD2 in extracellular vesicles from serum samples also bore moderate diagnostic value for PDAC as the AUC value was 0. 756 (Fig. 9d).

Discussion

For PDAC patients, early detection is laudable and beneficial for long-term survival, but it is challenging. New strategies and better biomarkers are urgently needed to help identify early, potentially curable PDAC

[17]. In the current study, we identified a robust diagnostic and prognostic biomarker, DCBLD2, with cross-platform compatibility through bioinformatic analyses. The proposed gene exhibited satisfactory predictive performance and could be detected in extracellular vesicles from both human plasma and human serum samples. Mechanistically, DCBLD2 was correlated with immune infiltrates and was involved in regulation of cell motility and several essential oncogenic pathways.

DCBLD2 is a neuropilin-like transmembrane scaffolding receptor and participates in regulation of receptor tyrosine kinase (RTK) signaling pathway [18–20]. Elevated expression of DCBLD2 was significantly associated with decreased OS time in various cancers including PDAC [9], colorectal cancer [21], hypopharyngeal squamous cell carcinoma [22] and melanoma [23]. Although previous studies have reported that DCBLD2 is upregulated in PDAC and indicates unfavorable OS, the study was limited to a small sample. In this study, we screened and integrated all freely available PDAC cohorts with survival data, and investigated the prognostic potential in these cohorts. The results showed that DCBLD2 was a stable biomarker in ten independent cohorts, indicating the possibility of clinical application to populations with different nationalities and races. What's more, ROC analyses showed that DCBLD2 expression outperformed clinical indicators in predicting patient survival, which further strengthened the clinical relevance of DCBLD2.

In addition to prognostic potential, we also attempted to characterize the diagnostic potential of DCBLD2 in tissue samples, plasma samples and serum samples. DCBLD2 expression can be a useful diagnostic biomarker to evaluate invasive properties of myxofibrosarcoma [24], but its diagnostic value in PDAC remains unclear. We profiled DCBLD2 expression in several cohorts containing either matched or unmatched PDAC tissues and adjacent normal tissues. Increased DCBLD2 expression in PDAC tissues compared with normal tissues revealed an oncogenic role of DCBLD2. The diagnostic ability of DCBLD2 in tissues was satisfactory, as the AUC value was close to or no less than CA19-9, an established diagnostic biomarker for PDAC [25]. Extracellular vesicles contain proteins, lipids and RNA from donor cells and can be an attractive source of diagnostic biomarkers for human cancers [26]. More and more researches have focused on the application of extracellular vesicular protein markers in the diagnosis of human cancers [27, 28]. Based on the RNA-seq data from GSE133684 dataset, we found that DCBLD2 existed in extracellular vesicles from plasma samples and could serve as a moderate marker in the early diagnosis of PDAC. We also experimentally verified the diagnostic value of DCBLD2 in extracellular vesicles from serum samples. These findings highlighted the clinical implication of DCBLD2 in liquid biopsy.

To preliminarily elucidate the mechanism underlying DCBLD2-resulted poor prognosis, we investigated the immune infiltrates and biological processes associated with DCBLD2 expression. We observed that the DCBLD2 expression was negatively correlated with the CD8 + T cells infiltration while DCBLD2 expression was positively correlated with the infiltrating level of M2 macrophage cells. Above findings further confirmed the oncogenic role of DCBLD2. Biological function analysis demonstrated that co-expressed genes with DCBLD2 were mainly involved in cell motility and multiple classic oncogenic

pathways including PI3K-AKT and Hippo signaling pathways. Therefore, this study provided the reference for clarifying the potential biological role of DCBLD2 in tumor immunology and PDAC progression.

However, this study, after all, is a retrospective study and has several limitations. First, the clinical application of DCBLD2 in PDAC management should be tested and validated in more prospective studies. Second, more in vivo and in vitro experiments are needed to verify the abovementioned bioinformatic findings, especially the biological function of DCBLD2 in PDAC tumorigenesis. Finally, we fail to adequately assess the relationship between DCBLD2 expression and clinical factors due to the lack of significant data. With the development of follow-up research, we hope to supplement them in future studies.

In conclusion, we integrated and analyzed genomic data and clinical data of multiple PDAC cohorts to demonstrated that expression value of DCBLD2 was a reliable diagnostic and prognostic factor and was significantly associated with immune and oncogenic signaling pathways in PDAC. DCBLD2 might facilitate tumor progression and bore strong diagnostic, prognostic and therapeutic value in PDAC.

Abbreviations

AUC: area under the curve; CP: chronic pancreatitis; DCBLD2: The discoidin, CUB and LCCL domain-containing protein 2; DFS: disease-free survival; GEO: Gene Expression Omnibus; GEPIA: Gene Expression Profiling Interactive Analysis; KEGG: The Kyoto Encyclopedia of Genes and Genomes; K-M: Kaplan-Meier; OS: overall survival; PDAC: pancreatic ductal adenocarcinoma; ROC: receiver operating characteristic; RTK: receptor tyrosine kinase; QRT-PCR: Quantitative real-time polymerase chain reaction

Declarations

Acknowledgements

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Author's Contributions

ZF and CP were involved in conception and design of the study and wrote the manuscript. KL, JL, and YF participated in data analysis, discussion, and language editing. MS collected the serum samples. YW reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available in the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/ geo/), ArrayExpress (https://www.ebi.ac.uk/arrayexpress/), International Cancer Genome Consortium (https://icgc.org/), and The Cancer Genome atlas (https ://cancergenome.nih.gov/) databases. R code is available upon reasonable request.

Ethics approval and consent to participate

The Ethics Committee of Ruijin Hospital affiliated with Shanghai Jiao Tong University approved the study. Informed consent was obtained from all individual participants included in the study.

Consent for publication

Consent to publish has been obtained from all authors.

Competing interests

The authors declare that they have no competing interests.

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Figures

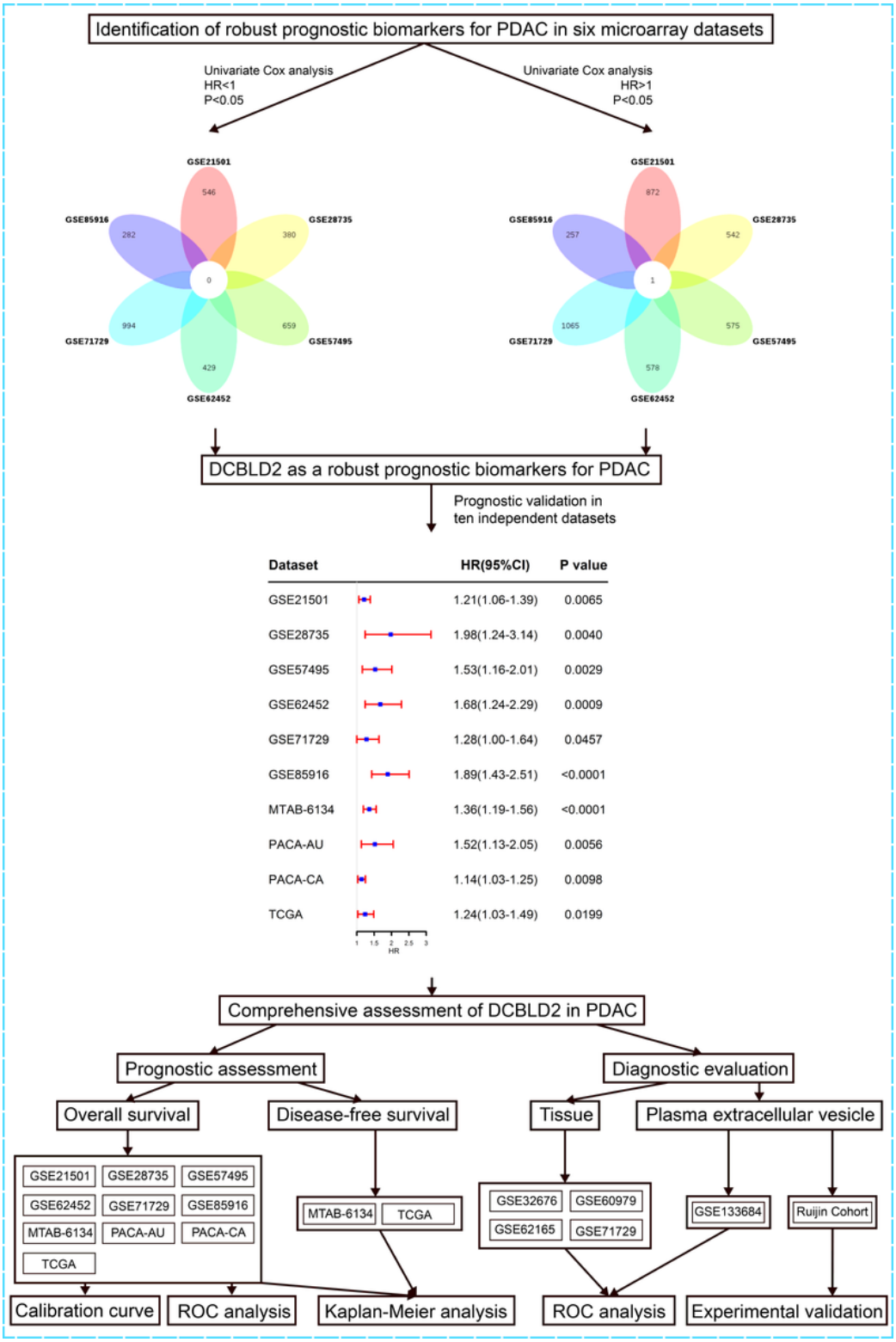

Figure 1

Overall study design and data analyses of this study.

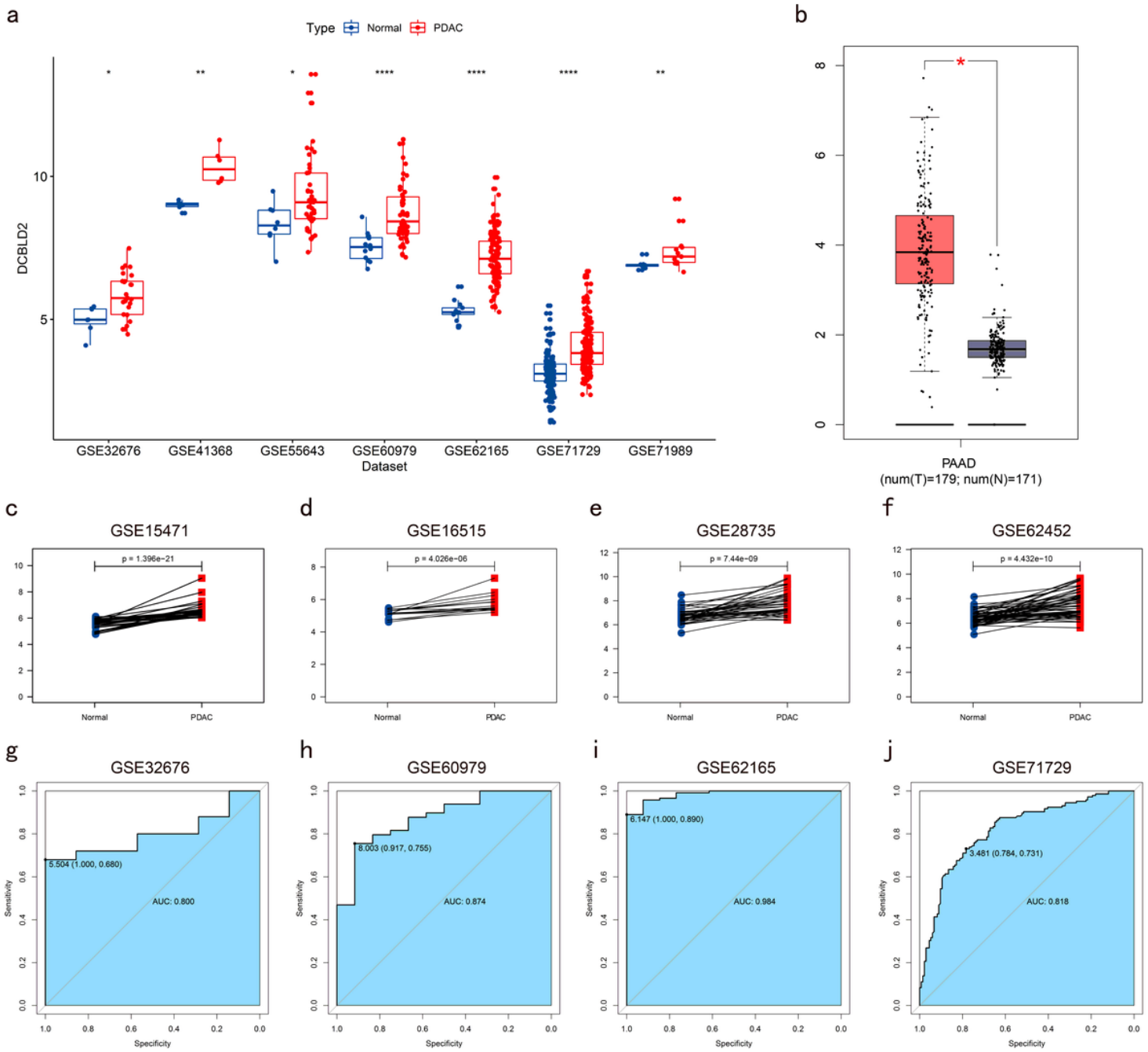

Figure 2

Expression of DCBLD2 in PDAC tissues and its diagnostic value. (a) The expression of DCBLD2 in unpaired PDAC tissues and normal tissues in seven independent PDAC cohorts. (b) Expression profile of DCBLD2 based on the GEPIA database. (c-f) Expression pattern of DCBLD2 in PDAC tissues and matched adjacent normal tissues in four independent PDAC cohorts. (g-j) ROC curves illustrated the value of DCBLD2 in the diagnosis of PDAC in four independent PDAC cohorts. The statistical significance of differential expression was assessed by Wilcoxon test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

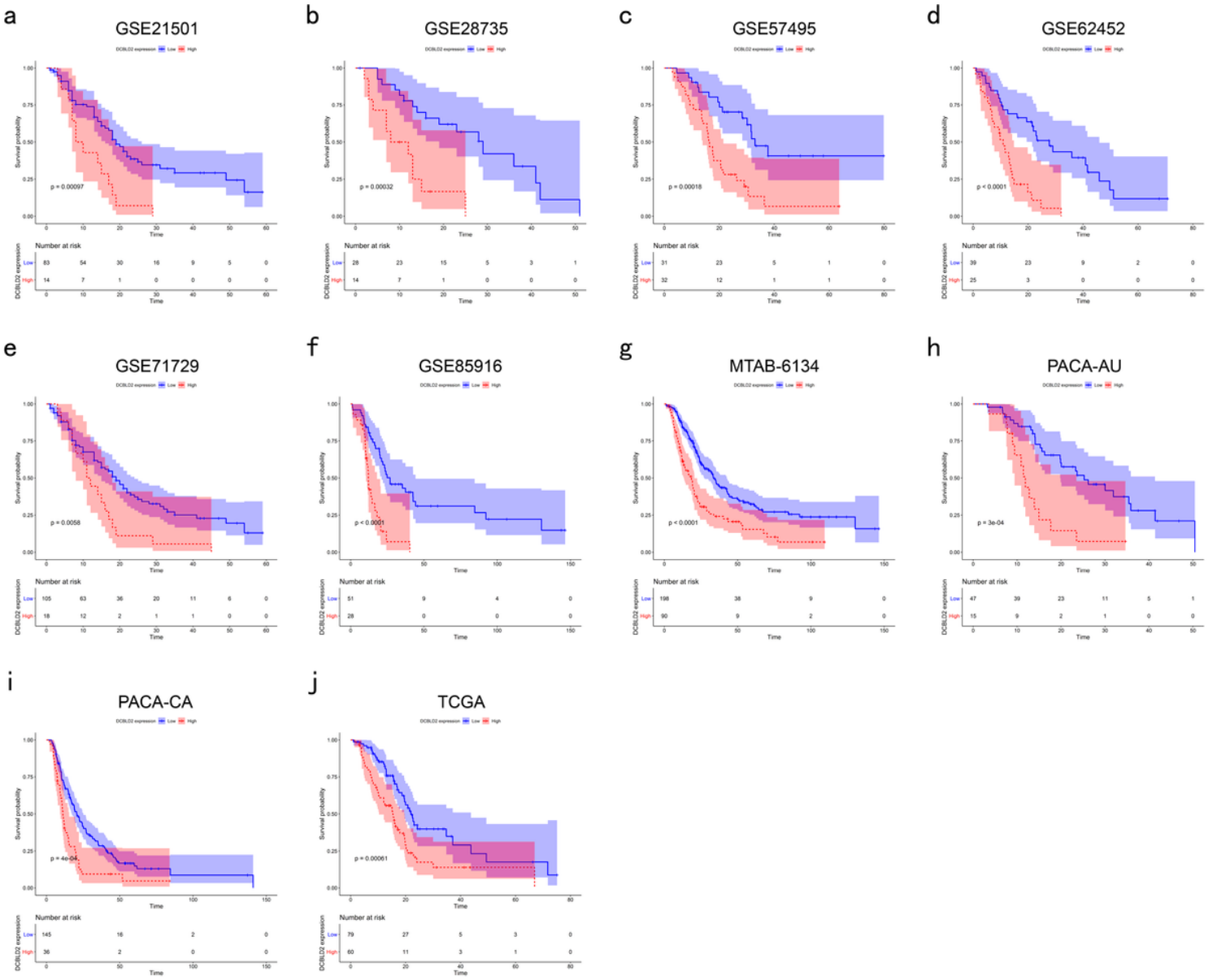

Figure 3

Prognostic validation of DCBLD2 in PDAC. (a-j). K–M curves estimated the OS difference between low- and high-expression groups in ten independent PDAC cohorts.

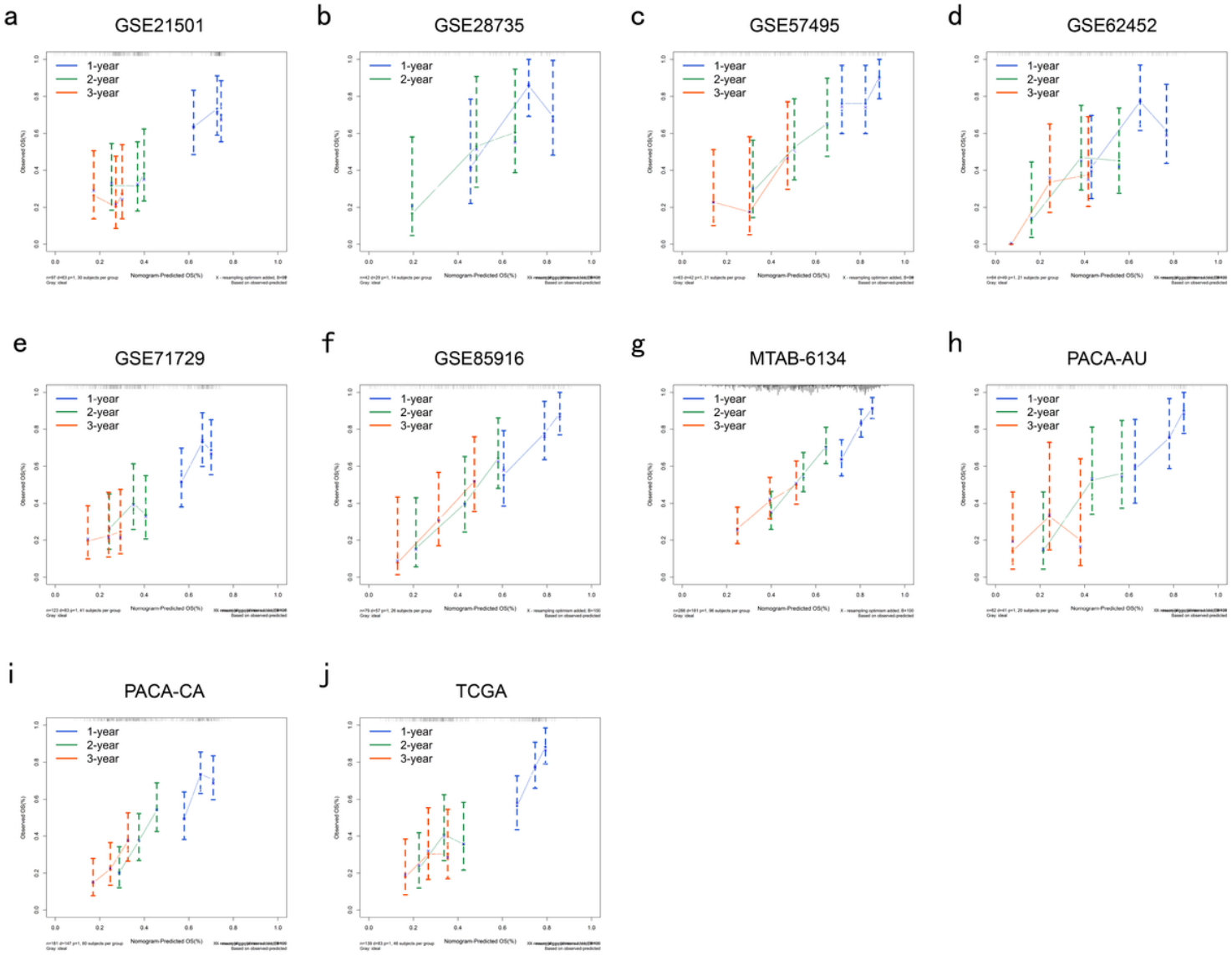

Figure 4

Prognostic performance of DCBLD2 in PDAC. (a-j). Calibration curves for DCBLD2 in ten independent PDAC cohorts.

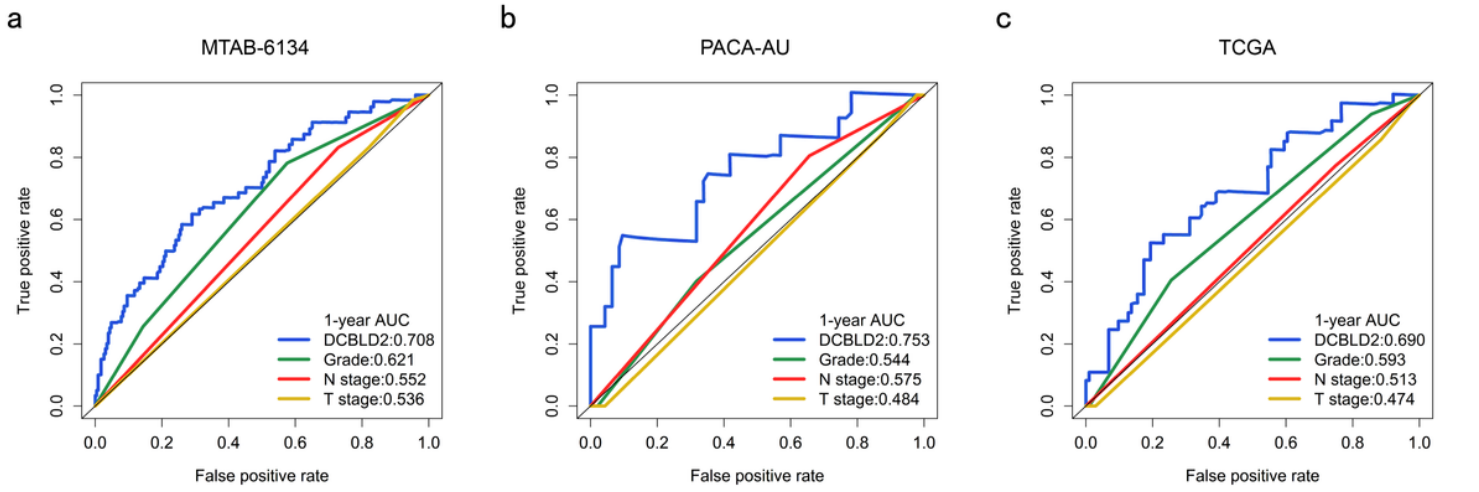

Figure 5

Comparison of predictive accuracy of DCBLD2 and clinical parameters. (a-c) ROC curves compared the predictive abilities of DCBLD2 and clinical parameters for OS in the MTAB-6134, PACA-AU and TCGA cohorts, respectively.

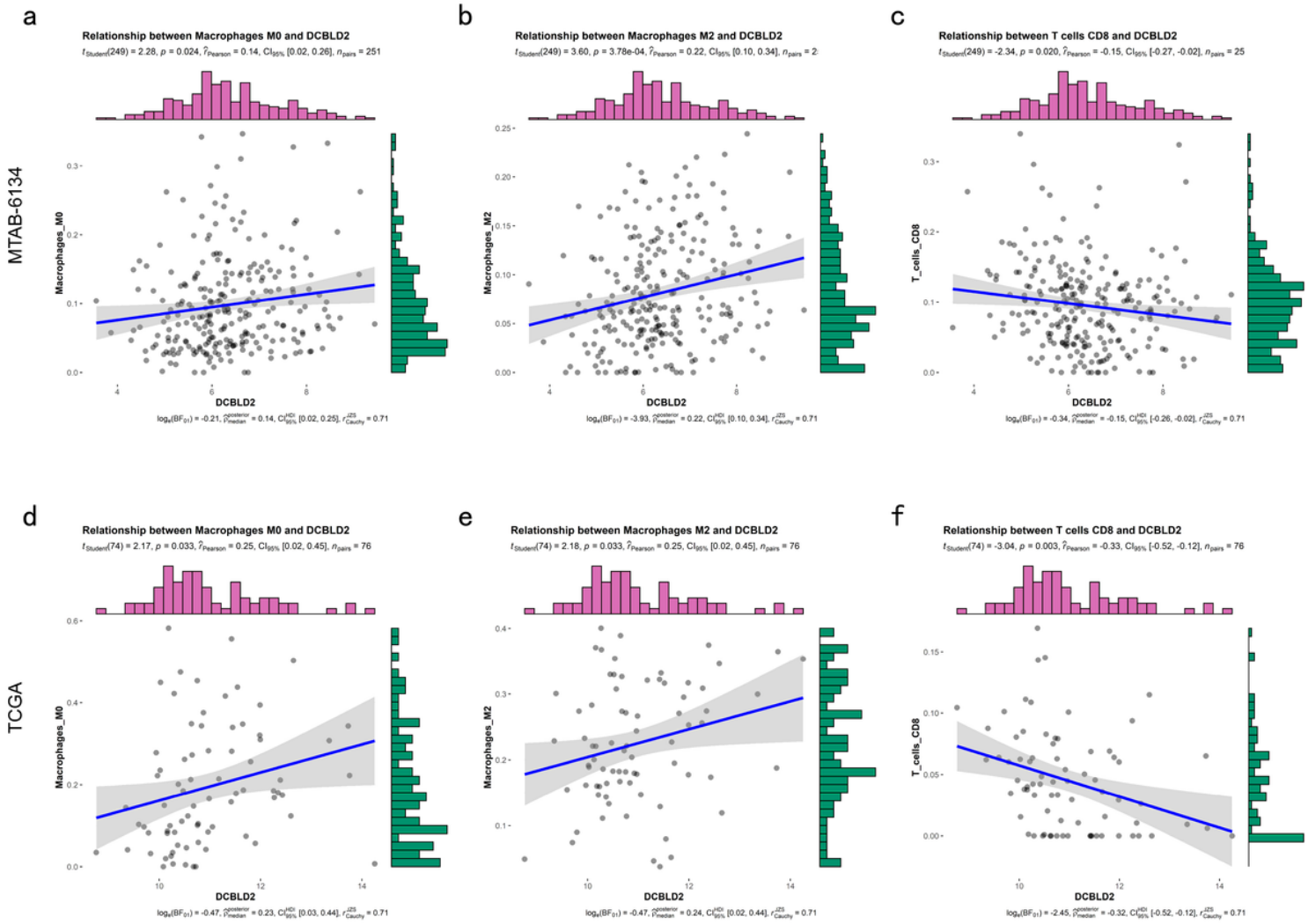

Figure 6

Correlation analysis between DCBLD2 expression and immune infiltrates. (a-c) The correlation between DCBLD2 expression and the abundance of macrophage M0, macrophage M2 and CD8+ T cell in MTAB-6134 cohort, respectively. (d-f) The correlation between DCBLD2 expression and the infiltration of macrophage M0, macrophage M2 and CD8+ T cell in TCGA cohort, respectively.

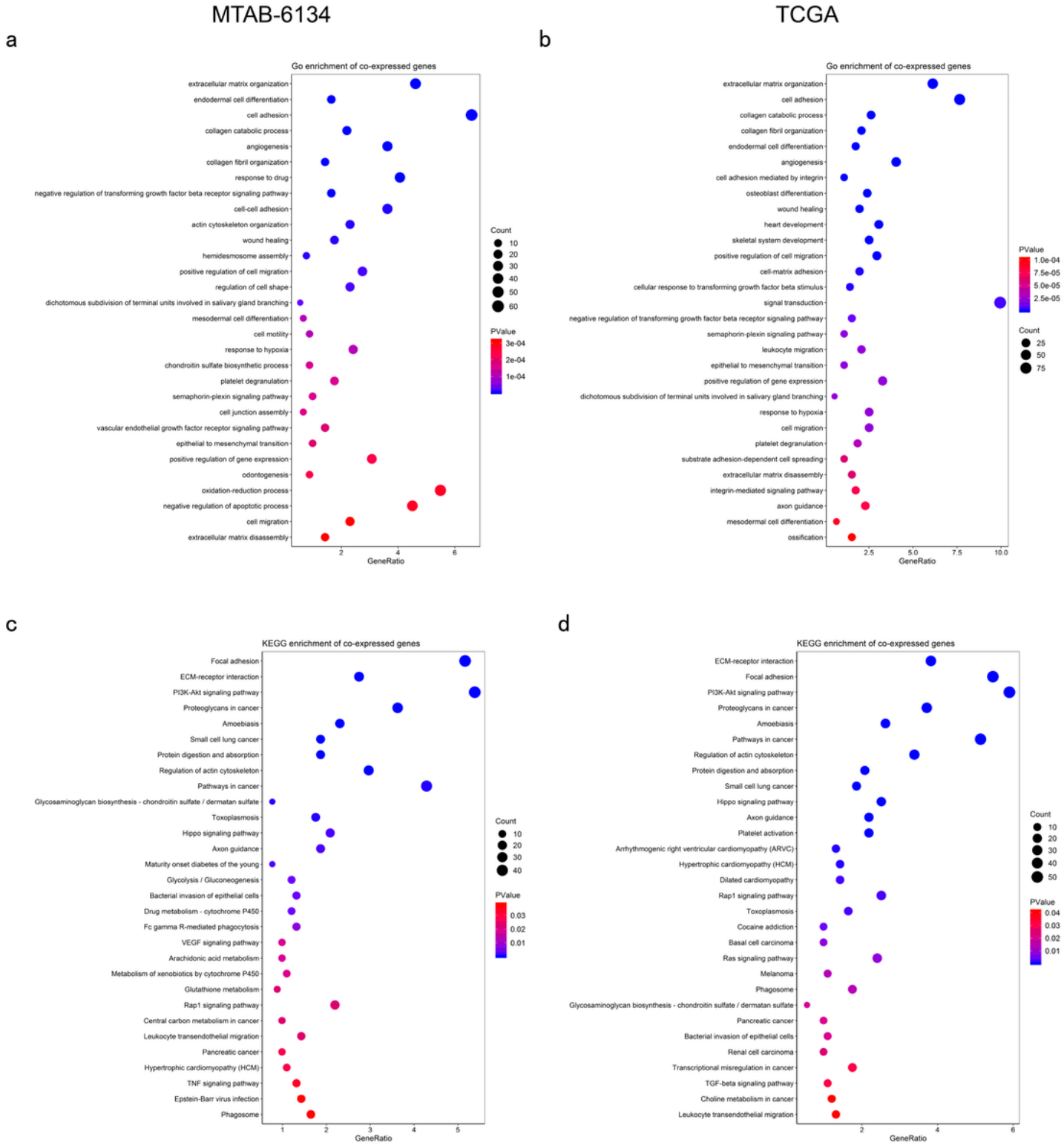

Figure 7

Biological function of DCBLD2. (a-b) Biological process analysis of top 1000 positively co-expressed genes of DCBLD2. (c-d) KEGG pathway enrichment analysis of top 1000 positively co-expressed genes of DCBLD2.

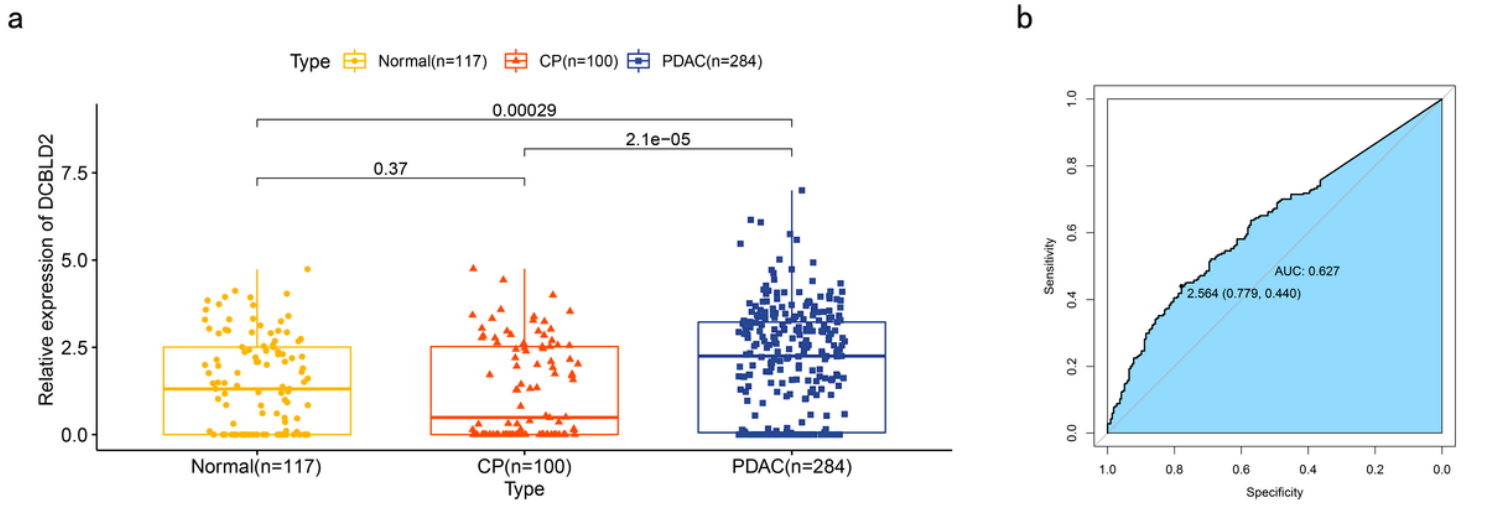

Figure 8

Expression pattern of DCBLD2 in extracellular vesicles from human plasma samples. (a) Boxplots show the distribution of extracellular vesicular DCBLD2 expression in plasma samples from healthy donors, CP patients and PDAC patients. (b) ROC curve showed the diagnostic value of extracellular vesicular DCBLD2 (PDAC vs CP).

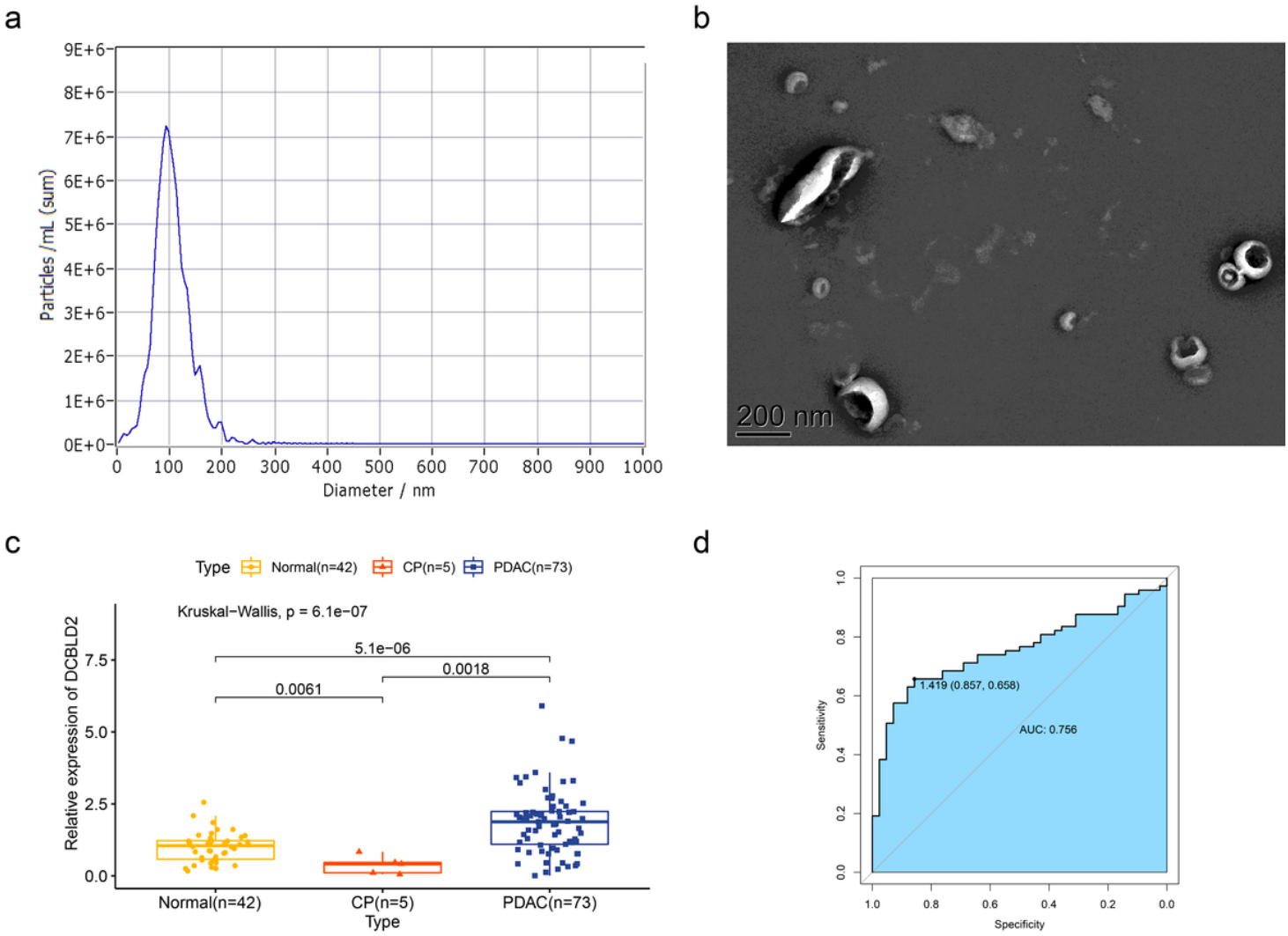

Figure 9

Expression pattern of DCBLD2 in extracellular vesicles from human serum samples. (a) NTA analysis and (b) TEM analysis to assess characteristics of extracellular vesicles. (c) Boxplots show the distribution of extracellular vesicular DCBLD2 expression in serum samples from healthy donors, CP patients and PDAC patients. (d) ROC curve showed the diagnostic value of extracellular vesicular DCBLD2 (PDAC vs CP).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.pdf
- Additionalfile2.pdf