DOK1/3/6 Serve as Potential Prognostic Biomarkers and Immunotherapy Targets for Pancreatic Cancer

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Abstract

Background: Downstream of kinase (DOKs), a family of adapter proteins, are frequently depicted as pivotal components of immune regulation complexes involved in the tumor progression in a wide range of cancers. Regrettably, little is known about the expression patterns and exact roles of 7 identified DOKs in pancreatic cancer (PC).

Methods: In this study, we investigated the distinct expression and biological function of DOKs in PC using multiple public databases, including ONCOMINE, GEPIA, cBioPortal, and Kaplan-Meier plotter. The correlations between DOKs and cancer immune infiltrates was investigated via TIMER. In addition, we subsequently verified those in an independent cohort.

Results: The expression levels of DOKs were found to be significantly upregulated in PC, interestingly higher DOK1/3/6 expressions were correlated with shorter overall survival (OS). Moreover, DOK1/2/3/5/6 had a dramatical positive correlation with the immune infiltration of PC and programmed cell death-ligand 1 (PD-L1).

Conclusion: DOK1/3/6 may function as potential prognostic biomarkers and even promising immune checkpoints for PC immunotherapy.

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Pancreatic cancer (PC) is a highly malignant tumor with an unfavorable prognosis, that is expected to become the second leading cause of cancer related fatality in the next decade[1]. Constrained by the lack of efficient diagnostic markers at the early stage, patients with PC are usually diagnosed at advanced stage accompanied by distant metastasis[2]. The application of molecular-targeted agents can improve the clinical outcome of patients suffering from PC. However, how to predict the response of patients to these drugs and the patient selection of optimal treatment remains a big challenge due to their potential to cause serious complications. Accordingly, there is a vital urgency to identify innovative prognostic biomarkers and potential molecular targets for PC.

A prominent desmoplastic stromal reaction is one of hallmarks of PC, which seriously interferes with the efficacy of chemo-therapeutic agents[3]. Tumor-infiltrating immune cells (TIICs) cooperate with other stromal cells to participate in PC progression[4]. Although immunotherapeutic strategies have been proven to be effective against various tumors, to date, among all the immune checkpoints, only the PDL1/PD-1 has been approved by Food and Drug Administration (FDA) as a therapeutic target for cancers. Unfortunately, only a minority of PC patients can benefit from the PDL1/PD1 blockade[5, 6]. Thus, a better understanding of the regulation of immune responses in PC is urgently warranted.

Downstream of kinase (DOKs) family comprise 7 identified members (DOK1 to DOK7). Based on phylogenetic analyses, DOKs are subdivided into 3 subgroups: DOK1/2/3, DOK4/5/6, and DOK7[7]. As essential adapter-family proteins, DOKs play important roles in the formation of multi-molecular

complexes of protein tyrosine kinase (PTK) pathway, and mediate cytokine and immunoreceptor signaling in innate and adaptive immune systems[8–10]. Recent studies have noticed that abnormal expression of DOKs family members is associated with tumorigenesis. The elevated DOK1 in gliomas biopsies and cell lines positively facilitates platelet-derived growth factor (PDGF)-BB-mediated glioma cell motility via BCAR1-Rap1 axis[11]. DOK4/5/7 are over-expressed in acute myelocytic leukemia (AML), which may contribute to molecular classification of AML for precise treatment and prognostic assessment[12]. Nevertheless, the functions of distinct DOKs family members in PC still remains confused.

To evaluate the comprehensive value of DOKs in PC, multiple bioinformatic tools were used to anatomize the correlation between gene expression pattern and PC clinical features, and subsequently the data from bioinformatics analysis were well verified in our own cohort. Furthermore, the regulatory mechanisms of abnormal DOKs and the correlation between DOKs and immunologic microenvironment were also investigated. These results will provide a perspective to the unearth of prognostic biomarkers and novel molecular-targeted candidates for PC therapy.

Results

Aberrant Expression of DOKs in PC

To investigate expression profile of DOKs family in patients with PC, the mRNA expression of DOKs in pan-cancers were initially measured. From ONCOMINE analysis, DOK1/4/5 expression were dramatically uplifted in PC compared to normal tissues (Fig. 1). In Buchholz Pancreas dataset, 1.878-fold up-regulation in DOK4 mRNA expression was found in PC samples (P = 2.63E-6) and Pei Pancreas showed that DOK4 was also elevated in PC tissues with a fold change of 2.444. Significant up-regulation of DOK5 transcription levels, with a fold change of 3.042, was similarly unveiled in Badea Pancreas dataset. Next, the mRNA expression patterns of 7 DOKs were further validated by GEPIA databases. The transcription levels of DOK1/2/3/4/5 were significantly up-regulated in PC tissues compared to normal samples, but no statistical difference in DOK6/7 expression (Fig. 2A). Moreover, DOKs mRNA levels were higher in a panel containing 6 PC cell lines (MiaPaCa2, BxPC3, AsPC1, CFPAC1, HPAF-II, SW1990) than that in a HPDE cell line (Fig. 2B). ROC analysis was conducted to assess the power of DOKs in discrimination, and results demonstrated that DOKs have the promising ability to differentiate PC and non-cancerous tissues (Figure S1).

IHC was also carried out in tumor tissues and their counterparts to assess DOKs protein expression. As shown in Fig. 3, DOKs protein primarily stained in the cell membrane and cytoplasm. Consistent with increase of transcription level, the protein level of DOKs was also elevated in PC, suggesting that DOKs protein was overexpressed in tumor compared to pericarcinomatous tissue.

Prognostic Value of DOKs in PC

In order to explore the critical effects of DOKs in the survival outcomes of PC patients, Kaplan-Meier Plotter analysis, a web-based tool, was utilized. As depicted in Fig. 4A, patients with higher DOKs expression had woeful outcome. The increased mRNA of DOK1 (HR = 0.59, 95% CI: 0.39–0.9, and P = 0.014), DOK3 (HR = 0.59, 95% CI: 0.36–0.98, and P = 0.041), DOK4 (HR = 1.86, 95% CI: 1.08–3.19, and P = 0.023) and DOK6 (HR = 0.56, 95% CI: 0.36–0.88, and P = 0.01) were strongly associated with poor overall survival (OS). However, the transcription levels of DOK2/5/7 were not related with OS. These results indicated that higher expression of DOK1/3/4/6 predictes worse outcome for PC patients. To corroborate these findings, a prognostic evaluation was carried out in our cohort, and results were consistent with the aforementioned conclusion (Fig. 4B). DOK1/3/6 may have the potential to be clinical prognostic survival indicators as well as targeted therapies, which require further developed.

Predicted Functions and Pathways of DOKs in PC

We evaluated the mutation frequency of DOKs in PC using cBioPortal. DOKs were altered in 47 (27%) of the 177 patients queried. The percentages of genetic variations in DOKs family ranged from 3–7% (Fig. 5A). Next, the correlation of DOKs with each other was evaluated by analyzing their transcriptional expression. The results displayed a significant positive correlation between DOK1 and DOK2, DOK3 and DOK7, DOK2 and DOK3, DOK5 and DOK2, respectively (Fig. 5B). Further, a network of DOKs and their 20 related genes was constructed by GEPIA 2.0 (Fig. 5C).

To further appreciate the functions and regulatory mechanism of those related genes, GO annotation and KEGG analyses was conducted within clusterProfiler. Enrichments results revealed that biological process of those genes was mainly enriched in GTPase activity, regulation of leukocyte mediated immunity and T cell activation (Fig. 5D). Cellular component was largely focused on phagocytic cup, extrinsic component of cytoplasmic side of plasma membrane and extrinsic component of plasma membrane (Fig. 5E). Molecular function mostly participated in small GTPase binding, Ras GTPase binding and GTPase regulator activity (Fig. 5F). In KEGG analysis, we found that chemokine signaling pathway, Rap1 signaling pathway and B cell receptor signaling pathway were significantly associated with PC (Fig. 5G).

DOKs Correlated with the Immune Microenvironment in PC

The number and activation state of TIICs affect the prognosis of tumor and the response to immune checkpoint blockers. The relationship between DOKs and TIICs was further probed by TIMER analysis. As depicted in Fig. 6A, elevated expression of DOK1-3, DOK5, DOK6 not only have a significant negative correlation with tumor purity, but significantly associated with TIICs (Figure S2). However, there was no close connection between DOK4/7 and infiltrating immune cells, except a weak link of DOK4 with B cells and a weak negative link of DOK7 with macrophages. Moreover, we explored the relationship between DOKs and infiltration of various immune subtypes. The results revealed that DOK1-3/DOK5/DOK6 were closely related to CD8 + T cells, M2 macrophages, DC cells, and T exhaustion (Fig. 6B, Figure S3).

Association among DOKs and Immune-checkpoint Genes

DOK1-3/DOK5/DOK6 were strongly linked to PDL-1 and PD1, two classic immune-checkpoint molecules. To verify these results, the protein expression of PDL-1 was detected by IHC. As expected, the positive rate of PDL-1 (67.2% in PC) was significantly higher in high DOKs expression group. DOK1-3/DOK5/DOK6 were closely associated with PDL-1 (Fig. 7).

Discussion

Tumorigenesis is a result of dysregulation of various signal pathways. DOK family members exist in multiple tumor types via providing a docking platform for the assembly of multi-molecular signal transduction[16]. The roles of DOKs in tumorigenesis are still emerging and controversial. Some researches indicated that DOKs functionally act as anti-oncogenes that positively correlated with cancer patient survival[17, 18]. Conversely, others reported that DOKs perform as oncogenes in tumor progression and motility. *Tong* et al. demonstrated that DOK1 was significantly higher in EBV + gastric cancer tissues compared with EBV- tissues[19]. And higher DOK4 mRNA was found in clear cell renal cell carcinoma biopsies compared with matched normal tissue[20]. Little work, as far as we know, has been done on the effects and mechanisms of DOKs in PC. The present research revealed, in both the transcription and protein levels, that all 7 DOKs family members were over-expressed in PC tissues. Furthermore, DOKs mRNA levels were higher in PC cell lines than that in normal cells. The protein expression was consistent with mRNA expression level, suggesting DOKs may serve as oncogenes promoting PC carcinogenesis and progression. High expression of DOK1/3/6 mRNA forecasted a poor prognosis in PC, but increased of DOK4 expression indicated good prognosis. Notably, DOK4 protein did not show visible relations with survival. Further investigation of DOK4 is needed for validation.

Mounting evidence suggests that DOKs negatively modulate the biological activity of immune cells in the context of physiology in human. The specific subtypes of TIICs in TME may affect tumor cell survival, metastasis and resistance to treatments[21, 22]. Nevertheless, little is known about the impact of these DOKs on tumor immune infiltration, which dictates the functional orientation of DOKs. To determine why DOKs predicted poor survival, we performed enrichment analysis of DOKs and relevant 20 genes in PC which were mainly involved in immunomodulatory. Next, the relationships between DOKs and TIICs were anatomized. Results implied that DOK1/2/3/5/6 were prominently associated with CD8 + T cell, Treg, T exhaustion, macrophages and dendritic cells (DCs) in PC. T cell immune response is the central event in antitumour immunity[23]. Studies have illustrated that TIICs (especially CD8 + T cells) are associated with better outcomes in multiple malignancies, such as breast, lung, melanoma, colorectal, and brain cancers[24, 25]. Although DOK1/2/3/5/6 was strongly associated with CD8 + T cell, high expression of DOKs contributed to poor prognosis. It is feasible that DOKs were mainly related to T cell exhaustion which presented a state of cytotoxic CD8 + T dysfunction in the TME. Moreover, our results also confirmed this hypothesis. Abundant Treg cell infiltration into TME is associated with poor clinical outcomes in PC[26]. Treg cell depletion in combination with conventional chemotherapy has been demonstrated to enhance CD8 + T activation[27]. DCs are took into account the most efficient antigen-presenting cells (APCs) and the only type of cells at stimulating CD8 + T cells[28]. Despite the presence of

DCs in TME and their potential to generate anti-tumor immunity, tumor-infiltrating DCs (TIDCs) often exhibit impaired or dysfunctional functions[29, 30]. DOKs may also negatively regulate TIICs in PC, leading to immune evasion and tumor progression.

Epidemiological evidence suggested that tumor-associated macrophages (TAMs) were often associated with poor outcomes in different forms of cancer (e.g. pancreatic and breast cancer)[31, 32]. TAMs were the most common TIICs in PC, which can be divided into M1 and M2 subtypes according to their state. M1 cells secrete pro-inflammatory factors to activate the inflammatory response and anti-tumor effects. TGF-β, IL-6 and other immunosuppressive factors secreted by M2 cells induce the dysregulation of T cell receptor signaling pathway, thereby exerting a tumor-promoting effect[33]. Interestingly, in animal models of PC, M2 cells were the major source of PD-L1 in TME and negatively regulated CD8 + T cells[34]. Our results confirmed that DOK1/2/3/5/6 emerged a significant correlation with M2, however, it was not associated with M1. DOKs may promote PC development and metastasis by promoting macrophages polarization from M1 to M2 phenotype. However, causality between DOKs and TAMs cannot be inferred in this study and the specific mechanism needs to be further explored.

Numerous studies have revealed that bright prospects of immune checkpoint blockers (ICBs), such as CTLA-4 and PDL-1 blockade, to promote T-cell responses by preventing T-cell exhaustion, have bright prospects. However, only part of patients with PC can benefit from ICBs clinically[35]. ICBs exert a crucial part in the activation and infiltration of cytotoxic CD8 + T cells after tumor antigen recognition. The classical TLA-4 and PD-1/PDL-1 axis modulate different inhibitory pathways and have non-overlapping mechanisms. Clinical trials have shown that more than 80% of patients with advanced melanoma have a better outcome after ICBs combination therapy[36]. Therefore, it is worth studying the immunoserotyping and regulation mechanisms of PC. In our research, DOK1/2/3/5/6 were co-expressed with CD247 and PDCD1, and high DOK1/2/3/5/6 expression in tumor accompanied with a high positive rate of PDL-1 protein. DOK1/2/3/5/6 may have the potential to be a new immune checkpoint molecule similar to PDL-1, but its role requires further research in future.

Conclusions

To summarize briefly, findings gained in the present study for the first time corroborate that increased DOK1/3/6 in PC predicted poor prognosis, and may mechanically act as a negative regulator of TIICs. The importance of DOK1/3/6 in PC prognosis, and as potential immune checkpoints for immunotherapy with PC has been highlighted in current work, which has improved our understanding of DOKs' functions in PC. However, as a retrospective analysis still has limitations. Given a lack of effective evaluation of DOK in PC prognosis and treatment, a multi-center study with a large sample size is required. Moreover, biases need to be further verified by prospective experiments.

Materials And Methods

Oncomine Analysis

Transcriptional levels of DOKs in distinct cancers were confirmed by excavating the ONCOMINE database (http://www.oncomine.org), the largest database of cancers integrated microarray data from published literatures. To compare the differential expression of DOK1-7 between tumors and normal tissues, Students t test was conducted. The cut-off criteria were restricted as follows: $P < 0.05$, a 2-fold change and top 10% gene rank.

Gene Expression Profiling Interactive Analysis (GEPIA)

GEPIA (http://gepia.cancer-pku.cn/detail.php) is a comprehensive platform for assessing mRNA expression data on the basis of RNA sequencing profiles from The Cancer Genome Atlas (TCGA) and Genotype Tissue Expression (GTEx) databases. The DOKs expression in PC were also tested by GEPIA database that contains 179 PC tissues and 171 normal pancreatic tissues.

Cell Culture and Quantitative Real-time PCR (qRT-PCR)

All PC cell lines (MiaPaCa2, BxPC3, AsPC1, CFPAC1, HPAF-II, SW1990) and a non-malignant pancreatic cell lines (HPDE) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). These cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). All cells cultured in a humidified atmosphere of 5% CO_2 at 37°C. Total RNA was isolated by Trizol method and was subjected to reverse transcription (RT) with Evo M-ML RT Kit with gDNA Clean for qPCR (AG11711, Accurate Biotechnology, Hunan, China). We conducted qRT-PCR using SYBR Green Pro Taq HS in an ABI 7500 Fast PCR device with Biosystems. All operations were performed according to the operation manual. Primers were listed in Table S1. $2^{-\Delta\Delta CT}$ method was used to show the relative mRNA expression.

Human Tissue Specimens

Commercial tissue microarrays (TMAs) of PC (HPanA120Su02) were obtained from Shanghai Outdo Biotech (Shanghai, China). The ethical permit number was YB M-05-02. The TMAs are comprised of paraffin-embedded tumor samples (n = 66) and peritumor samples (n = 54) from patients who underwent surgery and confirmed by pathological examination that were collected from 2009 to 2012. The group included 38 men and 33 women with a mean age of 65.3 years (range 38–90 years). Complete clinical information and follow-up data were summarized in Table S2.

Immunohistochemistry (IHC)

We performed IHC experiment to validated DOKs protein expression. Primary antibodies against DOK1 (ab227147, abcam, Cambridge, USA), DOK2 (D262993, sangon, ShangHai, China), DOK3 (D120564, sangon), DOK4 (D220562, sangon), DOK5 (D220561, sangon), DOK6 (D162867, sangon) and DOK7 (D262868, sangon) were added to detect the expression of DOKs, respectively. IHC experiment protocol was depicted as previously[13]. The results were appraised by two experienced pathologists who were blinded to the clinical outcomes. DOKs expression was scored by the staining intensity and percentage of

positive cells[14]. Finally, we defined score = 2 as cut-off criterion to subdivided patients into low and high expression groups. PD-L1 positive was defined as positive cells > 1%. Additionally, clinical significance of DOKs was further explored based on clinical information of 66 patients.

The Kaplan-Meier Plotter

The prognosis value of DOKs in PC was appraised using the Kaplan-Meier plotter (www.kmplot.com), a user-friendly database contained gene expression profiles and follow-up data, which is widely applied in tumor prognosis analyses. On the basis of median values of specific gene expression as the cut-off criteria in this tool, patients were divided into 2 groups: the high expression group and the low expression group. The results were presented in Kaplan-Meier survival plots with HRs, 95% CIs, P-values and number-at-risk cases[15].

cBioPortal and GEPIA 2.0

The online software cBioPortal (www.cbioportal.org) is based on other authoritative databases and subjected to scheduled update. In this study, Mutation maps polifts of DOKs were generated using cBioPortal. A network of DOKs and their 20 related genes was further constructed by GEPIA 2.0 (www.gepia2.cancer-pku.cn/).

Enrichment Analysis

In this study, Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed on DOKs and their 20 related genes using clusterProfiler in R (17). The assigned GO terms were mainly classified into 3 categories, including biological process (BP), cellular component (CC), and molecular function (MF), which predicted the function of DOKs and their 20 related genes. The P value < 0.05, adjusted by Benjamini-Hochberg method was used.

TIMER Analysis

Tumor infiltrating lymphocytes are associated with tumor genesis and outcome. TIMER, a reliable database for online analysis (https://cistrome.shinyapps.io/timer/), integrates high-throughput sequencing data of 32 diverse cancers in TCGA to calculate the abundance of TIICs in tumor tissues according to gene expression profile. We used the TIMER to assess the correlation of DOKs with 6 different typical tumor-associated immune cells. Heatmap showed the purity-corrected partial Spearman's correlation and statistical significance. In view of the specific genetic markers expressed by immune cells in each state, we then calculated the association between DOKs and each TIICs based on the genetic markers set, as described in a previous study (Assessment of the expression of the immune checkpoint molecules PD-1, CTLA4, TIM-3 and LAG-3 across different cancers in relation to treatment response, tumor-infiltrating immune cells and survival).

Statistical Analysis

Receiver operating characteristic (ROC) analysis using pROC package generated a score to differentiate PC and non-cancerous tissues. The prognosis value of DOKs protein was analyzed by Kaplan-Meier

analysis using survminer R package (https://CRAN.R-project.org/package=survminer). All hypothetical tests were two-tailed and P values < 0.05 were considered statistically significant.

Abbreviations

Downstream of kinase (DOKs), pancreatic cancer (PC), Overall survival (OS), Programmed cell death-ligand 1 (PD-L1), Tumor-infiltrating immune cells (TIICs), Food and Drug Administration (FDA), Protein tyrosine kinase (PTK), Platelet-derived growth factor (PDGF), Acute myelocytic leukemia (AML), Gene Expression Profiling Interactive Analysis (GEPIA), Immunohistochemistry(IHC), The Cancer Genome Atlas (TCGA), Genotype Tissue Expression (GTEx), Commercial tissue microarrays (TMAs), Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), biological process (BP), Cellular component (CC), Molecular function (MF), Receiver operating characteristic (ROC), Dendritic cells (DCs) , Antigen-presenting cells (APCs), Tumor-infiltrating DCs (TIDCs), Tumor-associated macrophages (TAMs), Immune checkpoint blockers (ICBs).

Declarations

Ethical Approval and Consent to participate

The project was approved by the Ethics Committee of First Affiliated hospital of Dalian Medical University. This study was ratified by the Ethics Committee of Shanghai Outdo Biotech Company. All participants offered written informed consent before surgery. The study conforms to the provisions of the Declaration of Helsinki. The data sets analyzed in this study are available on the public databases.

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

No potential conflicts of interest were disclosed.

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Authors' contributions

DW and LJ contributed to the design of the study and drafting and revising of the manuscript; QZ, CP and FY analyzed the data as well as drew the figures; HX edited the manuscript. DS reviewed the manuscript.

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Figures

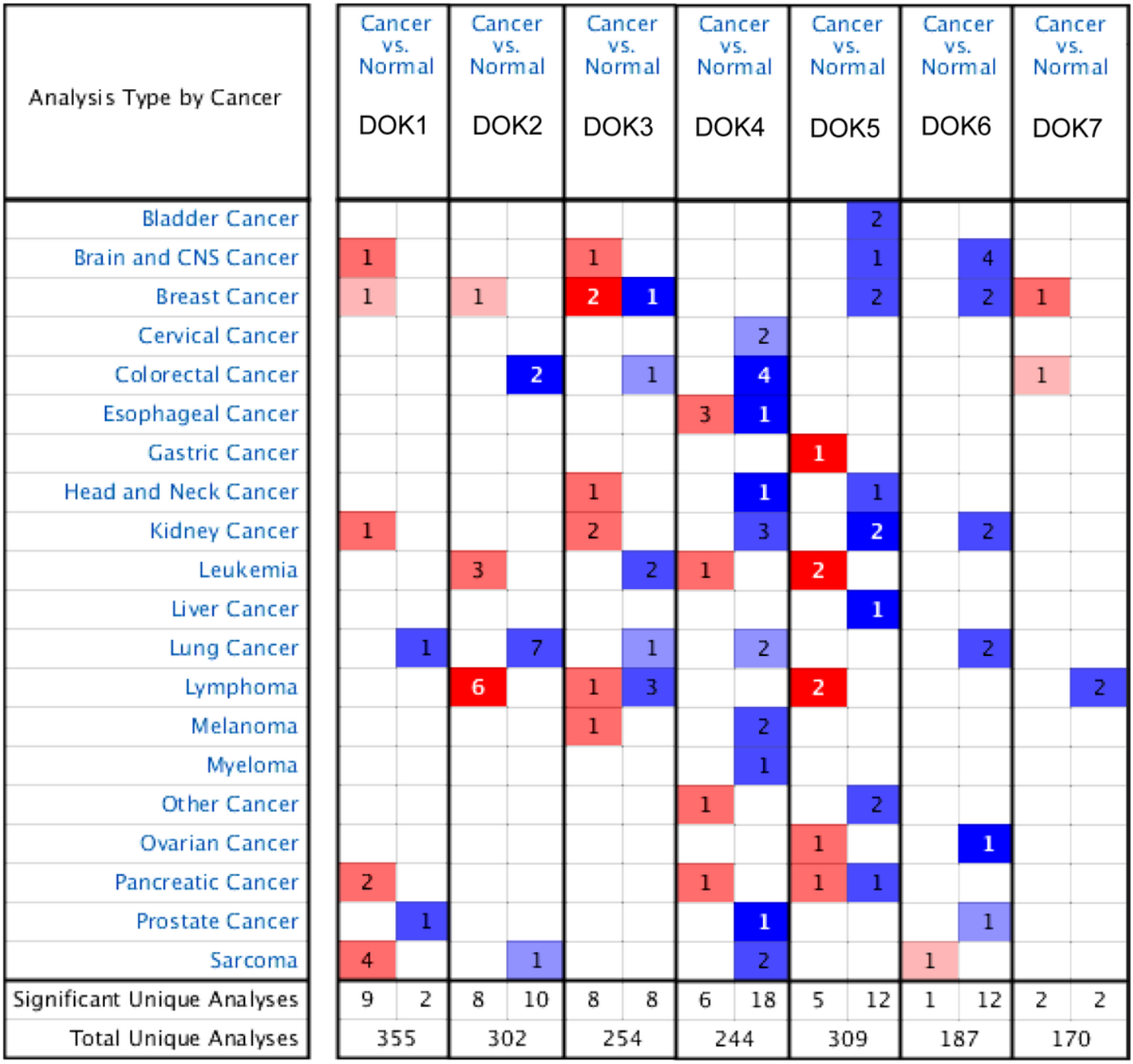

Figure 1

Transcriptional expression of DOKs in different types of tumors. The number of datasets in ONCOMINE database with significant differences in the expression of DOKs genes. Red represents high expression and blue represents low expression.

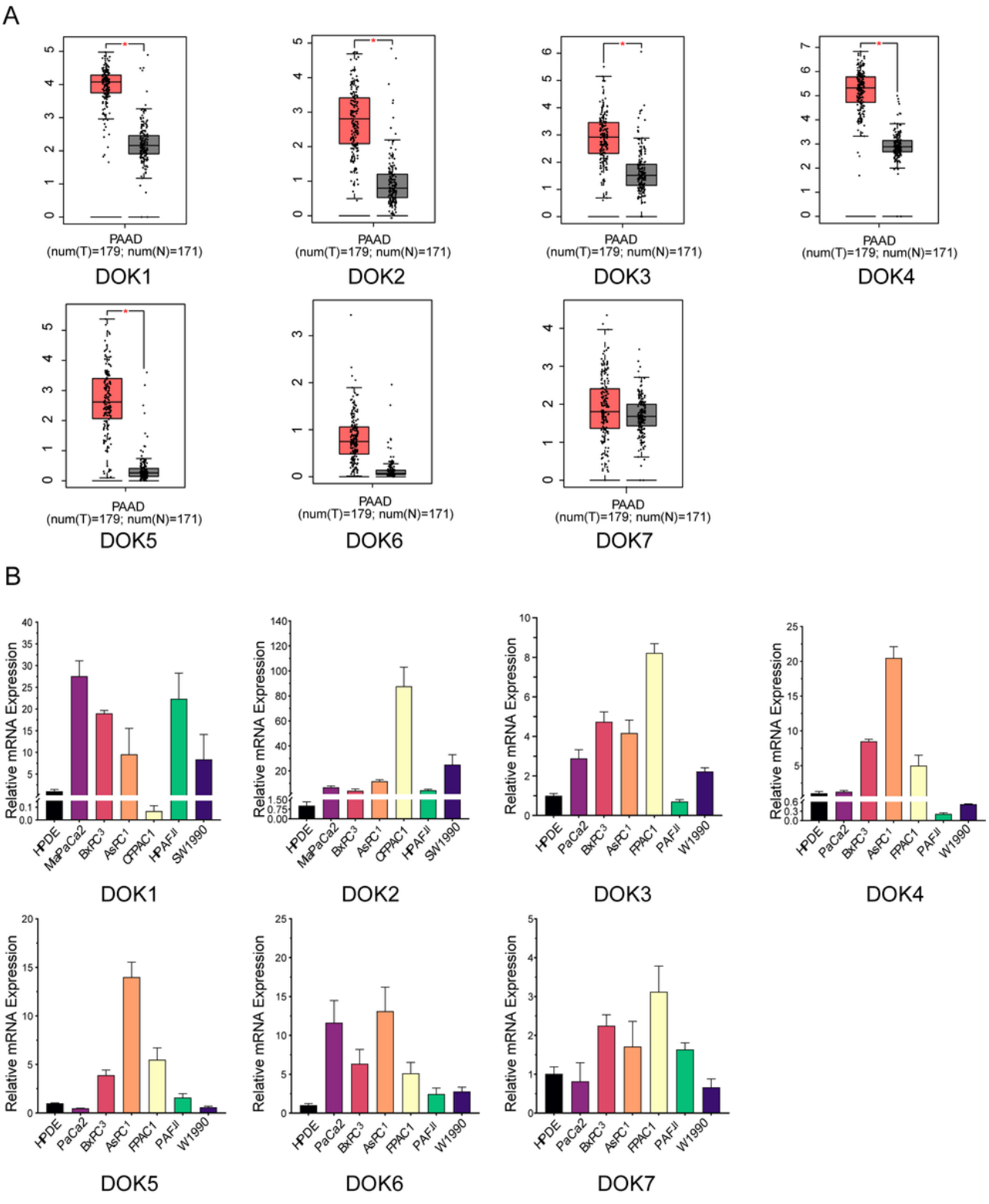

Figure 2

Higher DOKs mRNA expression in PC. A. The mRNA expressions of DOKs were over-expressed in primary PC tissues compared to normal samples based on GEPIA databases. B. RT-qPCR analysis of DOKs mRNA in non-malignant pancreatic cell line and PC cell lines. Compared with HPDE, *P< 0.05.

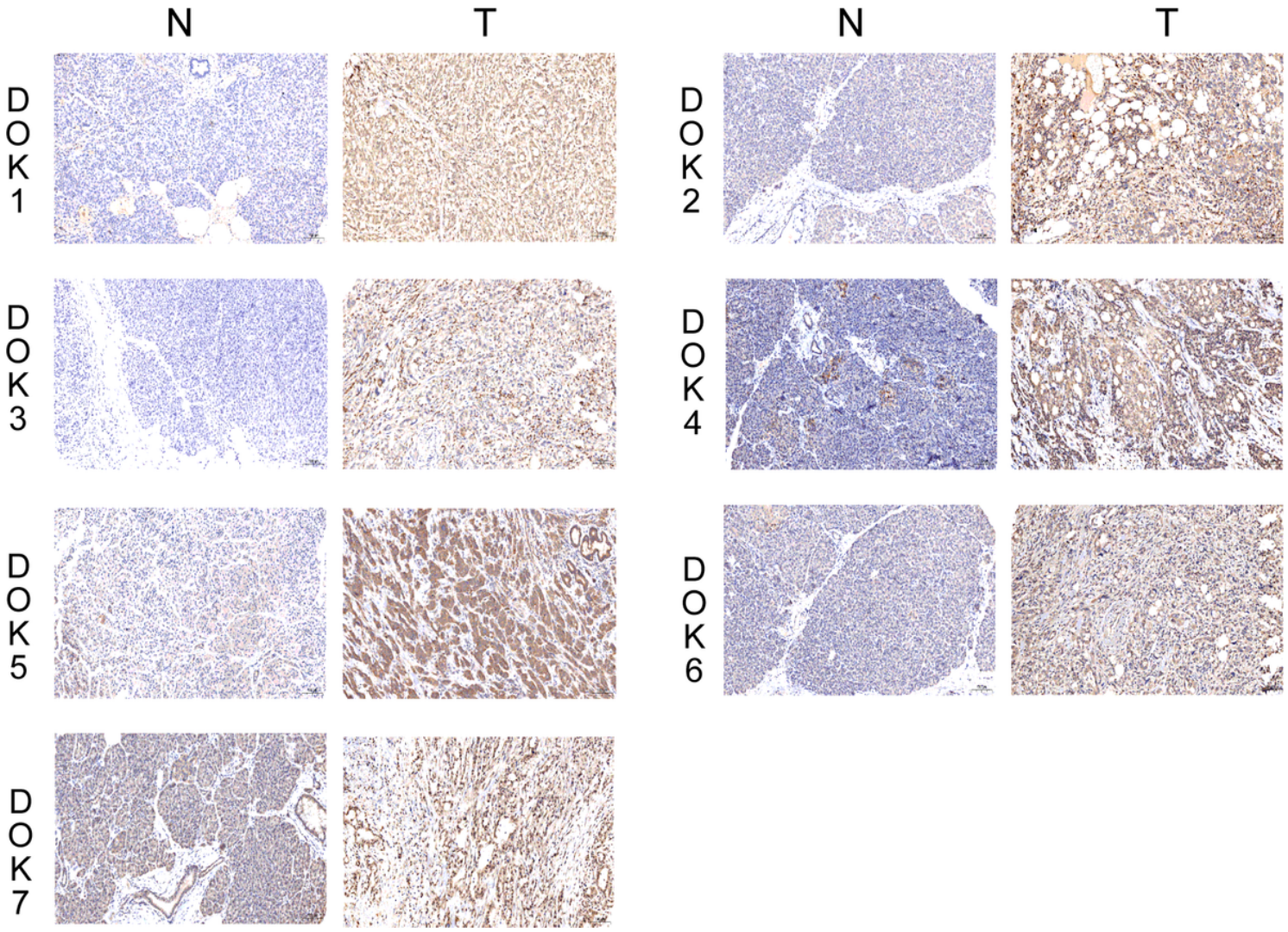

Figure 3

Higher DOKs protein expression in PC. The quantitative analysis of DOKs protein in tumor tissue and non-tumor tissues by IHC experiment. N, non-tumor tissues; T, tumor tissue.

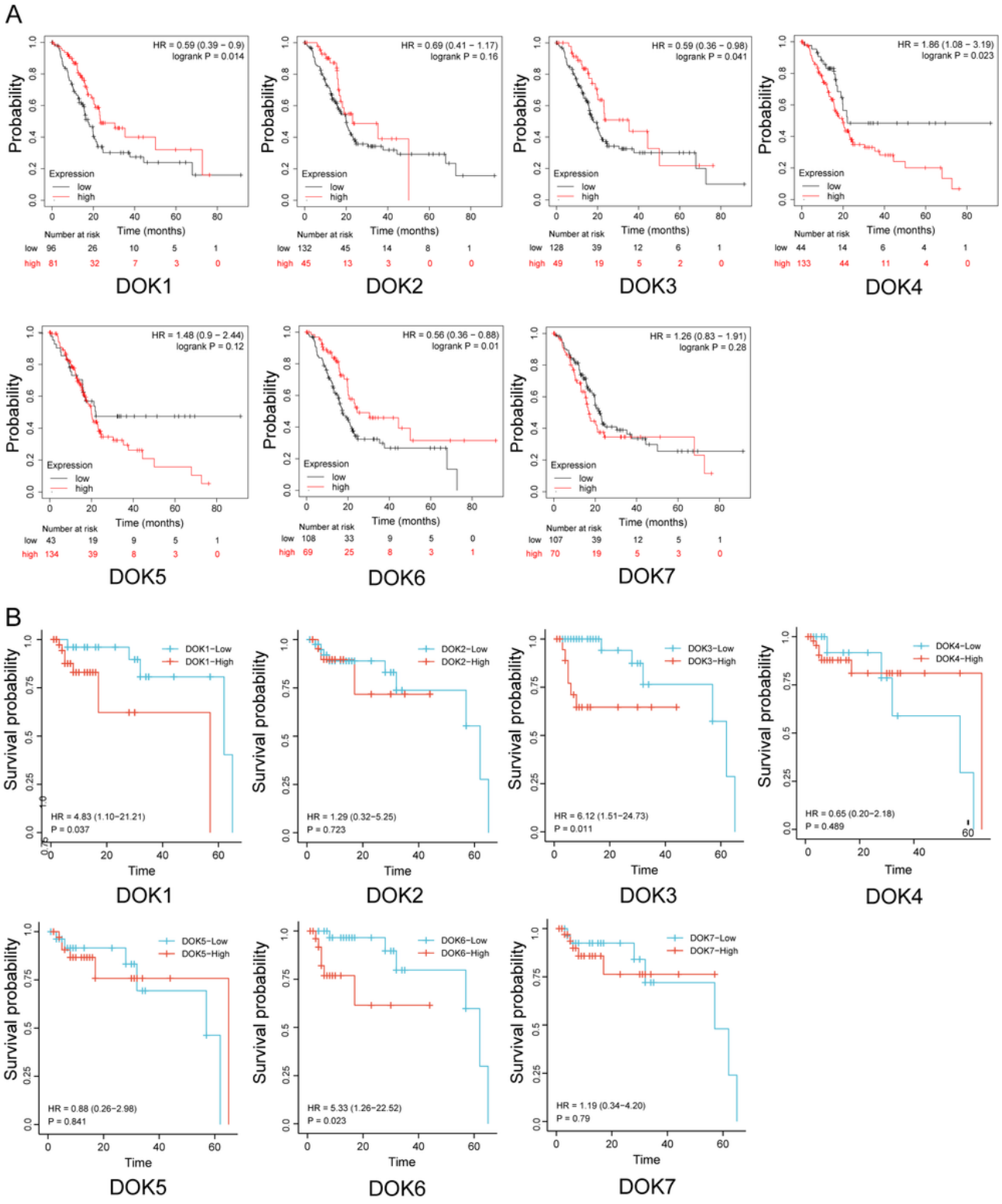

Figure 4

Prognostic value of DOKs in patients with PC.

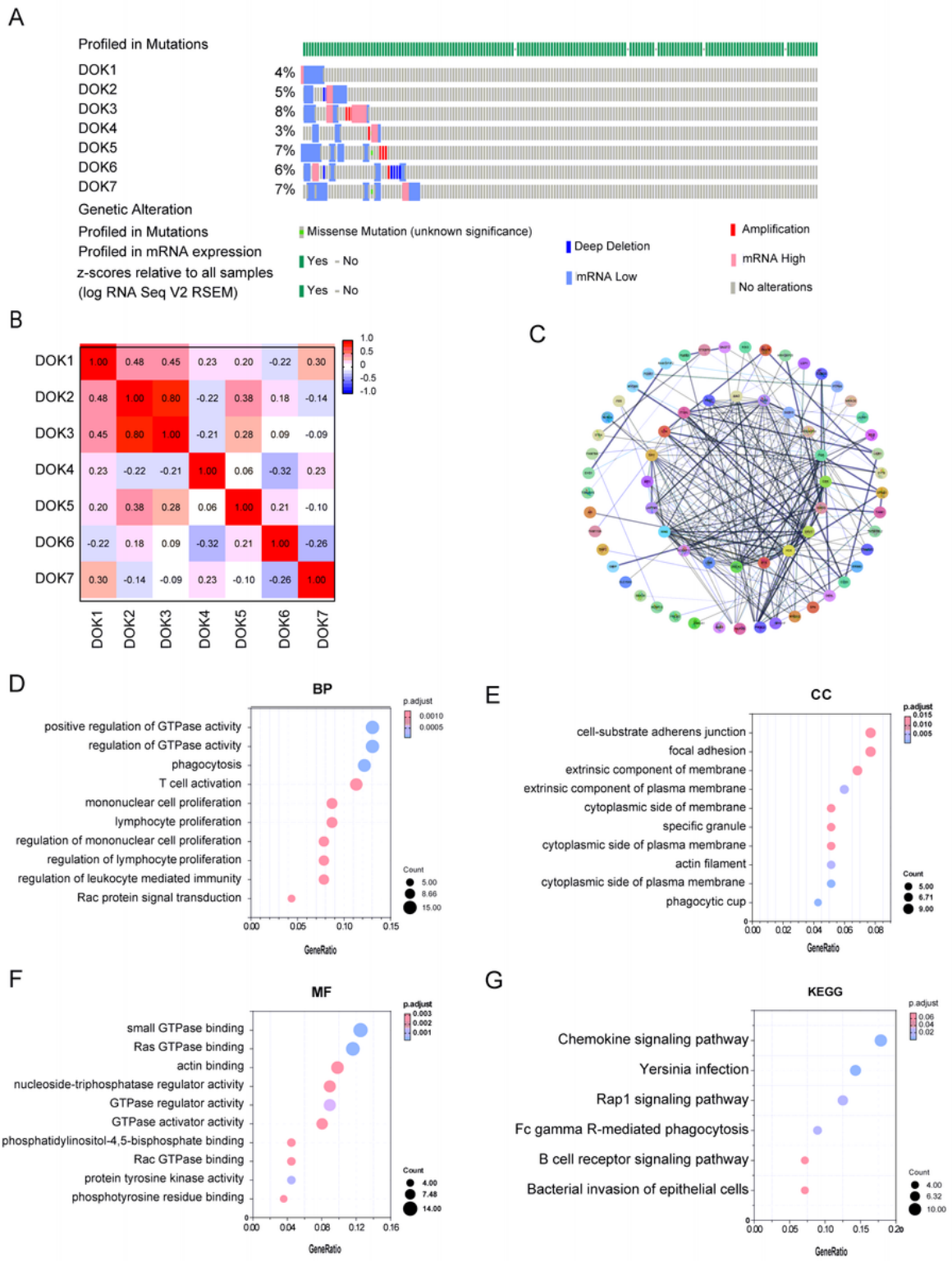

Figure 5

DOKs mutation and enrichment analysis of DOKs and related 20 genes in PC. A. DOKs gene expression and mutation analysis in PC. B. The correction between different DOKs in PC. C. A network of DOKs and their 20 related genes. D-F. GO enrichment in cellular component terms (D), biological process terms (E) and molecular function terms (F). G. KEGG enriched terms.

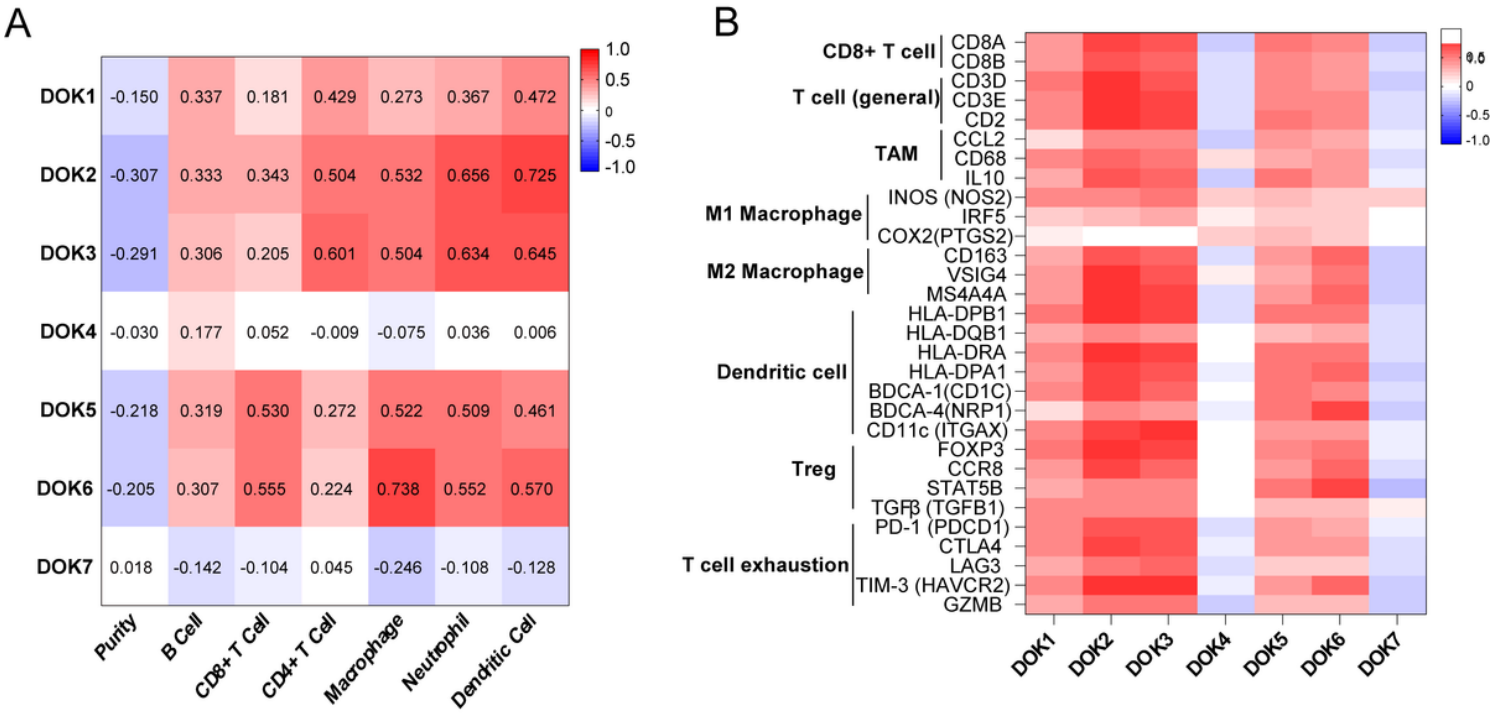

Figure 6

Immune infiltration analysis of DOKs family members by TIMER. A. A heat map showing the correlations between DOKs and infiltrating immune cells. B. A heatmap representing the association between DOKs and immune marker sets.

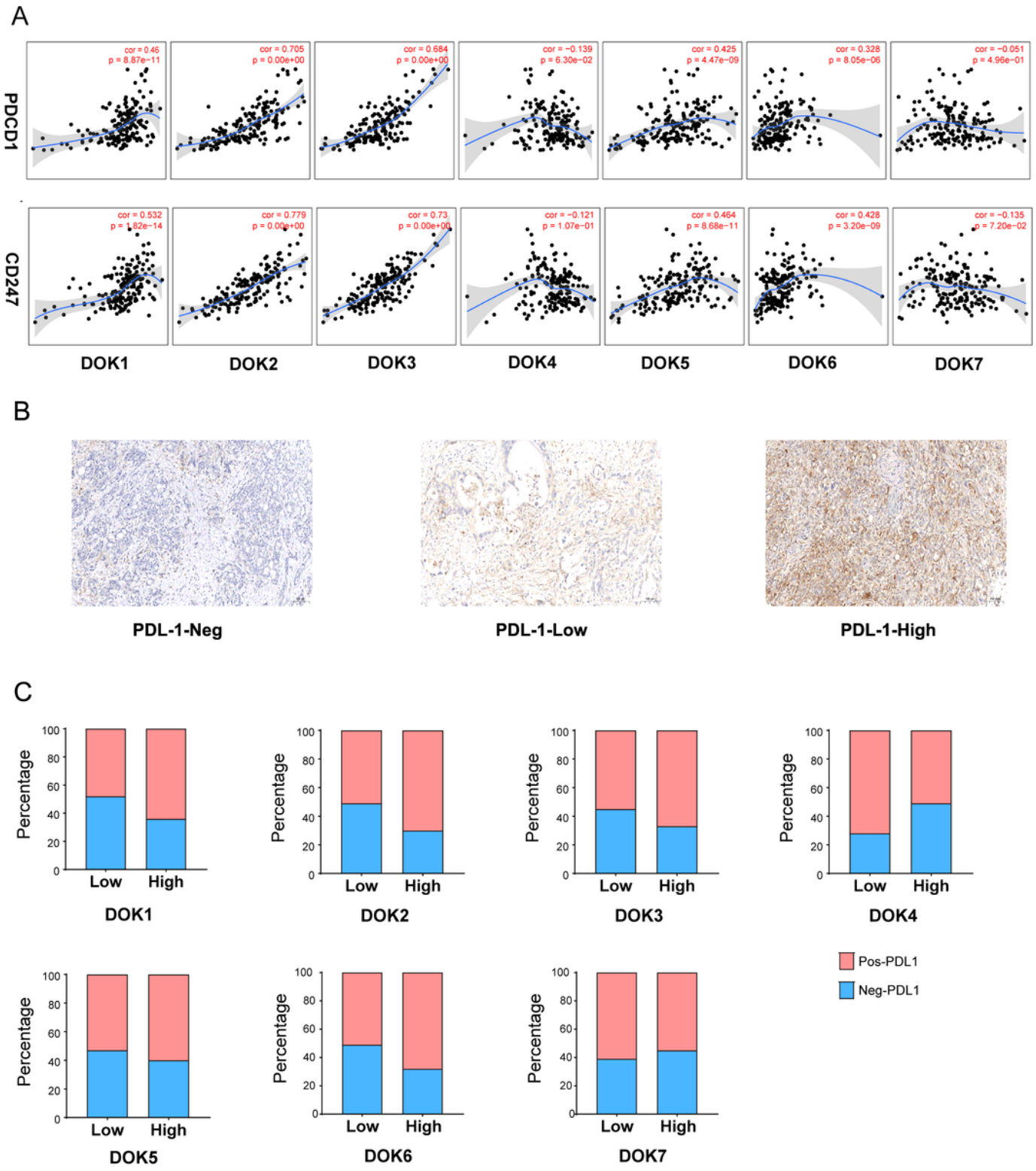

Figure 7

The correlations between DOKs and PDL-1 in PC. A. The correlations between DOKs mRNA and PDL-1 and PD1. B. Representative images of IHC staining of PDL-1 in PC. C. PDL-1 positive rate in DOKs-high group and DOKs-low group.

Supplementary Files

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

- Additionalfile1.pdf
- Additionalfile2.pdf