Pan-cancer Analysis Combining with Experiments Indicates the Sensitivity of Renal Carcinogenesis to DLGAP5 Expression

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Abstract

Background: Discs large-associated protein 5 (DLGAP5), a kinetochore fibers-binding protein, has been found to function as an oncoprotein in many cancers. However, its expression patterns in normal and cancer tissues across pan-cancer, as well as the cell lines, are far from clear.

Methods: Data from genotype-tissue expression (GTEx) and The Cancer Genome Atlas (TCGA) was used to analyze the DLGAP5 expression in normal tissues and cancer cell lines, respectively. The analysis of DLGAP5 expression in cancer tissues and adjacent tissues was based on data from a combined TCGA and GTEx. The associations between the expression, prognosis and cancer immune infiltrates in pan-cancer were also investigated based on TCGA and Tumor Immune Estimation Resource (TIMER), respectively. Furthermore, the analysis results of ccRCC was verified using cell lines via RNAi, western blotting, and the cytological analysis.

Results: The low expression levels of DLGAP5 were observed in 31 types of common human tissues, including kidney tissue. However, its expression displayed upregulation in all the 21 tested cancer cell lines, of which kidney cancer cell lines showed a minimal upregulation. As predicted, the significant overexpression of DLGAP5 occurred in at least 26 types of common cancer tissues compared with the adjacent normal tissues. Surprisingly, in three types of kidney cancer (KICH, KIRC/ccRCC, KIRP), DLGAP5 exhibited a statistically significant, but minor, overexpression among 26 types of tested cancers. Furthermore, the survival probability of some tested cancers, including kidney cancer, were significantly related to the upregulated expression of DLGAP5. In addition, among 33 types of tested cancers, KIRC/ccRCC, LGG and LIHC showed a significant positive correlation between DLGAP5 expression and immune infiltration levels. DLGAP5 expression level was also significantly positive correlated with clinical TNM stage of ccRCC patients. Regarding ccRCC tissues and the cell lines, upregulation expression of DLGAP5 was also detected. Its knockdown inhibited the cells viability and proliferation, and compromised the cells migration and invasion.

Conclusions: DLGAP5 overexpression occurred in common human cancers, including the kidney cancers. Notably, ccRCC, seemed to be particularly sensitive to the expression. DLGAP5, therefore, may be as a robust independent prognostic biomarker in ccRCC diagnosis.

Background

Kidney cancer or renal cell carcinoma (RCC) is one of the top ten most commonly diagnosed cancers worldwide [1]. Clear cell renal cell carcinoma (ccRCC) is the predominant subtype of RCC, accounting for about 75% of all cases and the majority of cancer-related deaths [2]. It is well established that each of the seven known kidney cancer genes, including hypoxia sensing gene VHL, is involved in responses to metabolic stress or nutrient fluctuations [3], making RCC a metabolic disease, even a model of the Warburg effect in cancers [4]. Despite several currently treatment agents that target cellular pathways stemmed from genomic analyses, the therapeutic responses, however, need further evaluation [5].
Therefore, the multi-omic analysis of ccRCC were carried out by two multidisciplinary research groups [4, 6]. These studies have identified discriminating features of ccRCC, and highlighted the value of underlying molecular mechanisms, including metabolic shift, genomic instability, as well as microenvironment cell signatures. These results are absolutely essential to stratify ccRCC patients and to prospectively explore personalized clinical care. Nevertheless, ccRCC is a common group of chemotherapy-resistant cancers [4], indicating that there is an urgent need to better understand the different roles of specific target molecules under various cellular contexts. To this end, it is necessary to explore the novel functions of potential therapeutic target molecules in the various carcinomas.

DLGAP5 (disc large (drosophila) homolog-associated protein 5), also known as DLG7, DAP-5 or HURP, originally discovered as a cell cycle regulator in hepatocellular carcinoma (HCC) and involved in the control of M phase progression by modulation of the function of kinetochore-microtubule (K-MT) [7]. HURP is a ran-importin β-regulated protein, forming a protein complex with other spindle assembly-related proteins including Aurora A, and essential for stabilization of the mitotic kinetochore fiber (k-fiber) [8, 9]. It has been found that HURP can also bind Kif18A, which is an important microtubule plus-end depolymerase, and modulate its K-MT plus-end localization. Both HURP and Kif18A localized to the plus end of K-MTs in a comet-like gradient pattern [10]. HURP is highly dynamic, the enrichment in its phosphorylated form at the kinetochore and in its unphosphorylated form at the centrosomes, and trafficking between centrosomes and kinetochores driven by Aurora A-dependent phosphorylation and PP1/PP2A-associated dephosphorylation [11]. Recently, Dudka and his colleagues found that centrosomes also control HURP accumulation on k-bers by setting k-ber length, which it accumulates on k-fibers inversely proportionally to half-spindle length [12]. HURP is periodically expressed during the cell cycle and peaks at G2/M, CDK1/cyclin B phosphorylation results in ubiquitination of HURP by SCF and targets it for proteosomal degradation [13].

Although it remains to be fully elucidated what the exact functional contributions of HURP in the mitotic spindle assembly are, the strong evidence mentioned above provides important insights into the role of HURP in the control of cell cycle. Importantly, as expected, the upregulated of HURP in many common cancers is detected, including HCC [8, 14], bladder cancer [15], prostate cancer [16, 17], breast cancer [18, 19], lung cancer [20, 21], colorectal cancer [22], as well as endometrial carcinoma [23], but not in normal tissues, indicating that misregulation of its expression plays roles in carcinogenesis.

Given that DLGAP5 is an essential component in the assembly of the spindle during cell division, firstly, we want to know whether it is overexpressed in all the most common cancers, including ccRCC, in which the role of DLGAP5 has hardly been considered since the discovery as a regulator of cell division. Second, apart from promoting cancer cells proliferation, what other functions does it have? Indeed, based on bioinformatics analysis, it has been found that DLGAP5 involved in the occurrence, development and prognosis of malignant tumors [18, 24], and may serve as potential molecular biomarkers for a set of carcinomas, including glioblastoma [24], prostate cancer [16, 17, 25]. Interestingly, DLGAP5 protein has been found to be one of top ten oxygen-sensitive proteins in prostate cancer [16, 26], and is proposed as an independent prognostic biomarker in this tumor [16]. By using the published date, it has been found
that several putative HIF binding sites located at promoter region of \textit{DLGAP5}, most of which are highly methylated region [16, 25]. In addition, the inhibitory effect of \textit{DLGAP5} knockout on prostate cancer is related to androgen receptor [17]. In Hep3B cells overexpressing HBx, \textit{DLGAP5}/HURP upregulated promotes p53 ubiquitination and degradation by the proteasome, and preventing p53-mediated apoptosis during cancer progression [14]. These results indicate that the functions of \textit{DLGAP5} in cancers may not just involved in spindle assembly during the cell cycle. Uncovering its other functions are thus of great significance for cancer targeted treatments.

In the present study, using the bioinformatics approach, the comprehensive pan-cancer analysis for \textit{DLGAP5} was carried out, especially focusing on the relationship between its upregulated, prognosis, immune infiltration in most common cancers. we found that it is upregulated in at least 26 most common cancers. Surprisingly, among the evaluated cancers, ccRCC tissues seems to be particularly sensitive to this gene expression.

**Materials And Methods**

**\textit{DLGAP5} expression in normal tissues, cell lines and pan-cancer**

The basic expression profiles of \textit{DLGAP5} in a diverse set of normal human tissues were first examined based on the Genotype-Tissue Expression (GTEx) database (https://commonfund.nih.gov/GTEx/data). For cancer cell lines, the gene expression was analyzed according to the tissue source via Cancer Cell Line Encyclopedia (CCLE) database (https://portals.broadinstitute.org/ccle). The levels of \textit{DLGAP5} mRNA across the pan-cancer, however, were analyzed using data obtained from The Cancer Genome Atlas (TCGA) (https://xena.ucsc.edu). Considering the small number of normal samples in TCGA, we combined the normal tissue data from GTEx database with the TCGA tumor tissue data to investigate the differential expression of \textit{DLGAP5} in 33 cancerous tissues and adjacent tissues.

**The pan-cancer analysis for \textit{DLGAP5} prognostic values**

The correlation between \textit{DLGAP5} expression and survival in various types of cancers was analyzed using the data from TCGA. Based on the expression levels \textit{DLGAP5}, samples were divided into two groups: the high expression and the low expression group. Kaplan-Meier survival analysis was used to compare the differences in cancer types. Firstly, the expression patterns and patient overall survival (OS) were assessed to identify any correlations using a single-factor survival analysis in 33 types of cancer from TCGA. Secondly, considering the possibility of non-tumor death during follow-up, the analysis for relationship between \textit{DLGAP5} expression and prognosis DSS (disease-specific survival) of 33 types of tumor was carried out in TCGA database. Furthermore, the relationship between the expression and prognosis PFI (disease-free interval) was also analyzed in TCGA 33 tumors.
**DLGAP5 expression and its relationship with immune infiltration in pan-cancer**

Tumor Immune Estimation Resource (TIMER) is a comprehensive resource for systematic analysis of immune infiltrates across diverse cancer types (https://cistrome.shinyapps.io/timer/) [27]. TIMER applies a deconvolution previously published statistical method [28] to infer the abundance of tumor-infiltrating immune cells (TIICs) from gene expression profiles. We analyzed DLGAP5 expression in different types of cancer and its relationship with the abundance of immune infiltrates, including B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells, via gene modules. The abundance of cells was scored and analyzed by Spearman’s correlation, and the gene expression level was displayed with (log2 TMP+1). Firstly, we downloaded the score data of six immune infiltrating cells in 33 cancers from the TIMER database. Then, immune score, matrix score, and ESTIMATE score for each tumor sample were analyzed using the R Software Package Estimate.

**DLGAP5 expression and its relationship with clinical progression of ccRCC**

Based on the above analysis of prognosis and immune infiltration in pan-cancer, we further focused on the effects of DLGAP5 expression on the clinical progression of ccRCC. Data of ccRCC (KIRC) cases and normal controls were downloaded from the University of California Santa Cruz Xena website (https://xena.ucsc.edu). The tumor samples, thus, were matched to TNM stage and G stage [29] in order to obtain data on DLGAP5 expression and clinical progression, their relationships were analyzed.

**Immunohistochemistry**

The clinical cancer tissues and adjacent tissues of ccRCC were obtained from the Affiliated Hospital of Yan’an University. Sections were deparaffinized in xylene and rehydrated in a descending ethanol series at room temperature. Deparaffinized sections were blocked with 10% goat serum working solution and incubated with 50µl endogenous peroxidase inhibitor (both from OriGene Technologies, Inc.), according to the manufacturer’s protocol, both at room temperature for 30 min. Antigen retrieval and blocking was subsequently performed. Tissue sections were incubated with primary antibody directed against DLGAP5 (working dilutions: 1:50, #12038-1-AP, Proteintech, China) overnight in a moist chamber at 4°C, washed in PBS, incubated with a biotinylated goat anti-rabbit antibody for 1 hour at room temperature and stained with 3,3-diaminobenzidine tetrahydrochloride (DAB). Finally, the sections were counterstained with Mayer’s hematoxylin, dehydrated and mounted. A negative control was prepared by replacing the primary antibody with normal rabbit IgG. The slides were independently reviewed under light microscopy by two pathologists. Positive staining was analyzed by measuring the gray pixels using Image-pro Plus (version 6.0; Media Cybernetics, Inc.).
Cell culture and RNA transfection

The ccRCC cell lines, (786-O and CAKI-1) and a human tubular epithelial cell line (HK-2) were obtained from the Cell Repository of Shanghai Institute of Life Sciences, Chinese Academy of Sciences (Shanghai, China). 786-O cell were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Biological Industries) and 1% penicillin/streptomycin (PS). CAKI-1 cells were cultured in McCoy’s 5a Modified Medium (Thermo Fisher Scientific, Inc.) supplemented with 15% FBS and 1% PS. All cell lines were cultured in an incubator with 5% CO$_2$ at 37˚C, until they reached 60-80% confluence. Small interfering (si)RNA duplexes targeting human DLGAP5 were synthesized and purified by Shanghai Gene-Pharma Co., Ltd. Non-specific siRNA sequences, purchased from Shanghai Gene-Pharma Co., Ltd., were used as a negative control. A total of two siRNAs were used, and the sequences were as follows: DLGAP5 siRNA-1; sense, 5'-GUG CCA UAU UUC AGA AAU ATT-3' and, antisense 5'-UAA UUC UGA AAA AUG GCA CTT-3'; and DLGAP5 siRNA-2; sense, 5'-CGA GGU AUA AAG GAG ACU ATT-3' and antisense, 5'-UAG UCU CCC UUA UAC CUC GTT-3'. The non-specific siRNA sequence was as follows: sense, 5'-UUC UCC GAA CGU GUC ACG UTT-3' and antisense, 5'-ACG UGA CAC GUU CGG AGA ATT-3'. A total of 20µM of siRNA was used, and 4µl was added to each well of the six-well plate. Transfection of siRNAs was performed using jetPRIME reagent (Polyplus-transfection SA), according to the manufacturer's protocol.

Quantification of DLGAP5 expression by RT-qPCR

Total RNA was extracted from transfected ccRCC cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol and expression levels were quantified using a Nano Drop spectrophotometer (Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using the PrimeScript™ RT Reagent kit (GenStar Bio, Inc.), according to the manufacturer's protocol. qPCR was subsequently performed using the iQ5 Optical real-time PCR system (Bio-Rad Laboratories, Inc.) with SYBR Green Ex Taq™ II (GenStar Bio, Inc.). The following primer sequences were used for the qPCR: DLGAP5; Forward, 5'-TCC GAC CTG GTC CAA GAC AA-3' and reverse, 5'-GAC GTG GGC ATT ACA GGC T-3' and GAPDH; Forward, 5'-TGA AGG TCG GAG TCA ACG GAT T-3' and reverse, 5'-CTT GGA AGA TGG TGA TGG GAT T-3'. The following thermocycling conditions were used for the qPCR: Initial denaturation at 95°C for 10 min; 40 cycles of 95°C for 15 sec, annealing at 60°C for 15 sec and extension at 72°C for 30 sec. DLGAP5 mRNA levels were quantified using the $2^{\Delta\Delta C_q}$ method [30] and normalized to the internal reference gene GAPDH.

Western blot analysis

Total proteins were extracted from ccRCC cells at 48h post-transfection using RIPA buffer supplemented with protease inhibitors and phosphatase inhibitors (Invitrogen; Thermo Fisher Scientific, Inc.). Protein concentrations were determined using a BCA protein assay kit (Takara Bio, Inc.) and 20µg protein/lane was separated via SDS-PAGE on a 10% polyacrylamide gel and transferred to PVDF membranes for
blotting. The separated proteins were subsequently transferred onto a methanol-activated polyvinylidene membrane (EMD Millipore) and blocked with 5% non-fat milk in Tris-buffered saline (pH 7.4) containing 0.1% Tween, for 1h at room temperature. The membranes were incubated with primary antibodies against DLGAP5 (working dilutions: 1:500, #12038-1-AP, Proteintech, China), overnight at 4˚C. Following the primary incubation, membranes were incubated with horseradish peroxidase-labeled secondary antibodies for 1h at room temperature. Protein bands were visualized by enhanced chemiluminescence (FD™FD bio-Dura Ecl Hangzhou China). The optical density of the image was analyzed using ImageJ software (version 1.4.3.67; National Institutes of Health) and protein levels were normalized to β-actin (1:5,000, cat. no. ab822, Abcam).

Cell viability assay

The effect of DLGAP5 knock down on the viability of 786-O and CAKI-1 cells were measured using the cell counting kit-8 (CCK-8) assay (Fudebio-tech, Hangzhou, China). Cells were plated into 96-well plates at a seeding density 3×10^3 cells per 100 µl culture media per well. Transfections were performed the following day. The culture medium was changed with fresh medium and cells were cultured for 24, 48, and 72 hours. Then, 10µL of CCK-8 solution was added to the cells for an additional 1.5h at 37°C. The absorbance of samples was measured at 450 nm using a high-throughput universal microplate reader (BMG Labtech GmbH).

Cell proliferation monitoring

786-O and CAKI-1 cells were seeded in 96-well plates at a density of 1.5×10^3 cells per 100 µl culture media per well. Transfections were performed the following day. The culture medium was changed with fresh medium. Cell proliferation was monitored using a Cytation™5 Cell Imaging Multi-Mode Reader (Biotek, Winooski, VT, USA) at the time points of 12, 24, 36, and 48h. Suspension cells were counted separately using a Cytation™5 Cell Imaging Multi-Mode Reader with corresponding Gen5 Image software version 3.08.

Colony forming assay

786-O and CAKI-1 cells were plated into 12-well plates at a seeding density of 5x10^4 cells/well and transfection was performed the following day. At 24h post-transfection, cells were reseeded into 6-well plates at a seeding density of 1,000 cells/well in triplicate and incubated at 37°C with 5% CO₂ for 7-10 days until they reached 80% confluence. Cells were then fixed with 4% paraformaldehyde and stained with 0.1% crystal violet (Sigma Aldrich; Merck KGaA) for 30 min at room temperature. Photos were captured and colonies were counted using the Quantity One® software (version 4.3.1; Bio-Rad Laboratories, Inc.).
Wound-healing assay

786-O and CAKI-1 cells were grown to 80%-90% confluence in 6-well plates and then transfected with siRNA-1 and siRNA-2 to knock down DLGAP5 expression. A wound was made by dragging a plastic pipette tip across the cell culture surface 24 hours after transfection. The cells were then incubated at 37°C, and phase contrast images of the wound were recorded at 0, 12, 24 and 36 hours. Digital images were obtained using a photomicroscope at x100 magnification (Nikon Corporation). Three separate experiments were performed, and cells transfected with siRNA-NC served as the control.

Cell migration and invasion assays

The migration and invasion assays were conducted in a 24-well Transwell chamber (Millicell®) with uncoated membranes or membranes coated with Matrigel (BD Biosciences, USA). Briefly, 200μL of cell suspensions (786-O (1.5x10⁴) and CAKI-1 (2x10⁴) cells) prepared in serum-free medium was loaded in the upper well of the chamber, while medium containing 10% FBS was placed in the lower wells as a chemoattractant stimulus. The chamber was incubated for 48h, and the non-migratory and non-invasive cells in the upper chamber of the filter were removed with a cotton tip. The invading cells on the bottom surface were fixed with 4% polyformaldehyde for 30min at room temperature and stained with 0.1% crystal violet for 30 min at room temperature. Digital images were obtained using a photomicroscope at x100 magnification (Nikon Corporation). In addition, stained cells on the lower surface of the chamber were dissolved with 33% glacial acetic acid and quantified by measuring the absorbance at 570 nm using a microplate reader (BMG Labtech GmbH).

Statistical analysis.

For analysis of TCGA pan-cancer and GTEx datasets, The Kruskal-Wallis test was used to calculate the significance of difference with a threshold of P < 0.05. Univariate Cox regression analysis was performed with two-sided Wald tests to calculate hazard ratios (HR) and 95% confidence intervals (CIs) for Overall Survival (OS), Disease-Specific Survival (DSS), and Progression-Free Interval (PFI). The threshold was adjusted to a Cox P < 0.05. Kaplan–Meier curves were used to illustrate survival rates. The log rank test was used to compare survival curves. All experiments in vitro were performed in triplicate. Prism Graphpad version 7.0 software was used for statistical analysis. The unpaired Student's t-test and one-way ANOVA analysis followed by Dunnett's post-hoc test was performed for multiple comparison between the groups. A P-value <0.05 was considered statistically significant. All data were presented as the mean ± standard deviation (SD).

Result
DLGAP5 expression in normal human tissues and tumor cell lines

The patterns of DLGAP5 expression across the diversity of normal human tissues can provide a baseline for comparison to cancer tissues. Therefore, we first examined the basic expression profiles in 31 human tissues from GTEx database. As shown in (Fig. 1a), the low expression levels were observed in most differentiated human tissues, including kidney tissue, except for the high expressions in bone marrow and testis, which usually undergo vigorous cell division. It tallies with a vital role of DLGAP5 in spindle assembly and mitotic progression. Furthermore, we also analyzed the expression in a various tumor cell lines based on CCLE data sets. As expected, it is overexpressed in all 21 tumor cell lines tested, of which kidney tumor cells showed a relatively low expression of DLGAP5 compared with other cell lines (Fig. 1b).

Figure 1 DLGAP5 expression in normal human tissues and tumor cell lines (a) GTEx database analysis of the expression in normal human tissues (K-W test, P = 0). (b) CCLE database analysis of the expression in cancer cell lines (K-W test, P = 3e-17)

Upregulated patterns of DLGAP5 in pan-cancer

As described above, extensive studies have demonstrated that DLGAP5 is overexpressed in many cancers. The pan-cancer gene expression patterns were therefore performed to examine the differential expression of DLGAP5 across TCGA cancer types. The results were shown that the upregulated levels were observed in all tissues of cancer types compared with the corresponding adjacent tissues (Fig. 2a). Interestingly, the degree of upregulated in three types of kidney cancer (KICH, KIRC/ccRCC, KIRP) were not very high at P < 0.001 level, which exhibited a similar expression manner to that of kidney tumor cells (Fig. 1b). Considering the small number of normal samples in TCGA, and to extend this comparison, we combined the normal tissue data from GTEx database and TCGA tumor tissue data to analyze the expression of 33 tumors, and found also the upregulated of DLGAP5 in all tumors tested (*P < 0.05, **P < 0.01, ***P < 0.001), of which kidney cancers, including ccRCC, again showed the relatively low expressions (Fig. 2b).

Figure 2 DLGAP5 expression in Pan-cancer (a) The expression of DLGAP5 in different human tumors within TCGA. (b) DLGAP5 expression in 33 human tumors was analyzed by integrating normal tissue data from GTEx database and TCGA tumor tissue. (TCGA sample data and GTEx normal sample data of 33 tumors, Additional file 1: Table 1) (blue: normal; red: tumor; log2(TPM + 1)*P < 0.05, **P < 0.01, ***P < 0.001).

Prognostic analysis of DLGAP5 in pan-cancer

To investigate the potential prognostic value of DLGAP5 upregulated in ccRCC and other cancers, data of the gene expression profiles from TCGA 33 tumor samples was analyzed for the relationship between expression and prognosis (overall survival, OS) using univariate analysis, the survival forest map.
comparing 33 tumors is presented in (Fig. 3a), and part of K-M survival plots are presented in (Additional file 2: Figure S1). Among these, about half of patients with upregulated show a worse prognosis than those with low expression (P < 0.05), and KIRC/ccRCC, KIRP, as well as LGG show a worse prognosis at a very significant level (P < 1 × 10\(^{-10}\)). Considering that there may be factors other than tumor death during the follow-up period, a comparison of the disease-specific survival (DSS) and the progression-free interval (PFI) was also presented in Fig. 3b and 3c. (part of K-M survival plots as shown in Additional file 3: Figure S2 and Additional file 4: Figure S3), respectively. The similar patterns of prognostic values, however, were also observed in those contexts, showing that KIRC/ccRCC, KIRP, as well as LGG exhibit a worse prognosis at a very significant level (P < 1 × 10\(^{-8}\)).

Figure 3 The survival forest map for prognostic analysis of DLGAP5 expression in pan-cancer. (a)HR(95% CI) for OS. (b)HR(95% CI) for DSS. (c)HR(95% CI) for PFI (Using a single-factor survival analysis, DSS, disease-specific survival; OS, overall survival; PFI, progression-free interval; *P < 0.05, **P < 0.01, ***P < 0.001).

**Association of DLGAP5 expression with immune infiltration in pan-cancer**

Tumor-infiltrating lymphocytes (TILs) are the independent predictors of overall survival rate and sentinel lymph node status [31, 32]. Therefore, we investigated whether DLGAP5 expression was correlated with immune infiltration levels in different types of cancer. Six main types of immune cells, including B cell, CD4 T cell, CD8 T cell, Dendritic cell, Macrophage, and Neutrophil, were selected, of which the abundance of cells was scored and analyzed by Spearman’s correlation. Among 33 types of cancers analyzed, three types, namely KIRC/ccRCC, LGG and LIHC showed a significant positive correlation between the gene expression and immune infiltration levels, and an extremely significant level was observed in KIRC/ccRCC from R = 0.288, P = 1.99 × 10\(^{-11}\) for CD4 T cell to R = 0.513, P = 5.04 × 10\(^{-37}\) for Dendritic cell. These results indicate that DLGAP5 plays a key role in immune infiltration in ccRCC, especially for Dendritic cell (Fig. 4a).

Furthermore, the tumor microenvironment (TME), including immune cells and stromal cells as the major non-tumour constituents of tumour samples, plays an important role in the occurrence and development of tumors, and has important reference values for tumor diagnosis and prognosis evaluation [33]. In addition, immune cells not only infiltrate the tumour cell region but have also been demonstrated to associate with stromal cells, in a cancer-type-specific manner [34]. We therefore assessed the correlations of DLGAP5 expression with immune and stromal scores, as well as combined stromal and immune scores (estimate score) in 33 TCGA tumor types from the Tumor Immune Estimation Resource (TIMER). As shown in Fig. 4b, both KIRC/ccRCC and THCA, showing positive correlation of the expression with three scores, and both STAD and LUSC, showing negative correlation of the expression with three scores, were found to exhibit the very significant differences (R = 0.356, P = 1.97 × 10\(^{-17}\) for KIRC, R = 0.465, P = 0
for THCA, $R = -0.355$, $P = 1.96 \times 10^{-12}$ for STAD, and $R = -0.293$, $P = 2.64 \times 10^{-11}$ for LUSC;) among tumor types evaluated.

Figure 4 Association of DLGAP5 expression with immune infiltration in Pan-cancer. (a) Scores of six immune-infiltrating cells of 33 cancers were downloaded from TIMER database, and the correlation between DLGAP5 expression and scores of these immune cells was analyzed respectively. The expressions in three cancers (KIRC, LGG, and LIHC) were significantly associated with the Scores. (b) The Stromal Scores, Immune Scores, and ESTIMATE Scores for THCA, KIRC, STAC, and HUSC, with showing a significant correlation between the gene expression and Scores.

**Upregulated DLGAP5 expression associated with clinical stage of ccRCC in TCGA dataset**

To further investigate the effects of DLGAP5 expression on the clinical progression of ccRCC, publicly available CRC gene expression RNAseq datasets from TCGA database were analyzed for the relationship between its expression levels and clinical pathological characteristics. As shown in Fig. 5a-e, compared with the control group, DLGAP5 expression level in tumorous tissues was significantly ($P < 0.05$) positive correlated with clinical TNM stage and Fuhrman grade of patients with ccRCC. The highest level was found in the tissues of patients with stage T4, N1, M1 or G4, the most progressive clinicopathological stage. Thus, as shown in K-M curves (Fig. 5f-h), upregulation of DLGAP5 expression resulted in the low survival probability of ccRCC patients.

Figure 5 The association of DLGAP5 expression with clinical stage in TCGA dataset. (a) topography (T1, T2, T3 and T4), (b) lymph node metastasis (N0, N1), (c) distant metastasis (M0 and M1), (d) G stages (I, II, III and IV) and (e) pathological stage (I, II, III and IV) of patients with ccRCC. (f-h) Kaplan-Meier survival curves of patients with ccRCC based on DLGAP5 expression levels (f: DSS; g: OS; h: PFI). (DSS, disease-specific survival; OS, overall survival; PFI, progression-free interval; *$P < 0.05$. TCGA, The Cancer Genome Atlas; DLGAP5, DLG Associated Protein 5; ccRCC, clear cell renal cell carcinoma)

**DLGAP5 expressions in ccRCC tissues and cell lines**

Based on the above pan-cancer analysis, we further focused on the DLGAP5 expression in ccRCC tissues and the cell lines, Fig. 6a and b show the results of TCGA and GTEx samples, indicating that DLGAP5 expression was upregulated in cancer tissues, but not in adjacent normal tissues. The results from the western blotting released that the upregulated expression of DLGAP5 in the ccRCC cell lines 786-O and CAKI-1 was also occurred compared with the normal renal epithelial cell line HK-2 (both $P < 0.05$; Fig. 6c). By using immunohistochemistry, three pairs of representative results were presented in (Fig. 6d), positive staining of DLGAP5 protein measured by the gray pixels was higher in ccRCC tissue than normal, indicating that DLGAP5 protein expression was upregulated in ccRCC tissues compared with adjacent normal tissue.
Figure 6 Up-regulated expression of DLGAP5 in ccRCC. (a) Data from TCGA database demonstrated a significant upregulation of DLGAP5 expression in ccRCC tumor samples (n = 531) compared with normal tubular tissue (n = 72). (b) Data from GTEx database and TCGA tumor tissue demonstrated a significant upregulation of DLGAP5 expression in ccRCC tumor samples (n = 531) compared with normal tubular tissue (n = 98). (c) Western blotting results revealed that DLGAP5 protein levels were higher in the ccRCC cell lines 786-O and CAKI-1 compared with the normal renal epithelial cells HK-2. (d) Representative images of DLGAP5 immunohistochemistry in paired tumor and normal tissue samples. (*P < 0.05; **P < 0.01 vs. control. DLGAP5, DLG Associated Protein 5; ccRCC, clear cell renal cell carcinoma; TCGA, The Cancer Genome Atlas.)

DLGAP5 Knockdown inhibits cell viability and proliferation in ccRCC cells.

To evaluate the contribution of DLGAP5 to ccRCC tumorigenesis, we designed two DLGAP-specific siRNAs (siRNA1, 2) for gene knockdown experiments. To test the efficacy of these siRNAs, they were transiently transfected into 786-O and CAKI-1 cells. siRNA-NC was used as a negative control. We found that siRNA1 and siRNA2 significantly knocked down endogenous DLGAP5 expression compared with siRNA-NC (Fig. 7a). siRNA1 and siRNA2 was then used for cell growth analysis after DLGAP5 knockdown in various ccRCC cell lines, including 786-O and CAKI-1. Our data showed that the transient transfection of siRNA1 and siRNA2 suppressed the growth of the 786-O and CAKI-1 cells compared with siRNA-NC (Fig. 7b-d). These data further support the hypothesis that DLGAP5 promotes ccRCC cell viability and proliferation.

Figure 7 The knockdown of DLGAP5 levels inhibits the proliferation capability. (a) Reverse transcriptionqPCR and (b) western blotting were performed and revealed a significant decrease in the mRNA and protein levels of DLGAP5 in siDLGAP51 and siDLGAP52-transfected human ccRCC, compared with those observed in siNCtransfected cells. The band intensities of DLGAP5 were quantified relative to β-actin and normalized to the siNC sample. (c) CCK8 results showed that DLGAP5 knockdown significantly inhibited the proliferation of 786O and CAKI1 cells. (d) Cell proliferation monitoring 786O and CAKI1 cells inhibited the proliferation. DLGAP5 knockdown significantly decreased the number of (e) 786O and CAKI-1 cell colonies compared with the control group. (*P < 0.05; **P < 0.01; ***P < 0.001; vs. control. DLGAP5, DLG Associated Protein 5; si, small interfering; NC, negative control.)

DLGAP5 knockdown inhibits cell migration and invasion in 786-O and CAKI-1 cells.

The up-regulated expression of DLGAP5 was closely related to the poor prognosis of ccRCC, indicating that its expression may promote the tumor development. The wound-healing and matrigel assays were therefore performed to assess the effects of DLGAP5 knockdown by RNAi on cell migration and invasion. The wound-healing assay revealed that cell migration was inhibited as a result of DLGAP5 knockdown in
786-O and CAKI-1 cells (Fig. 8a-b). In comparison with the cells that were transfected with siRNA-NC, the
cells of both cell lines that were transiently transfected siRNA2 also showed a significant inhibition of cell
invasion through a Matrigel barrier when fibronectin was used as an attractant (Fig. 8c-d).

Figure 8 The knockdown of DLGAP5 levels significantly inhibits migration and invasion. (a) 786O and (b)
CAKI1 cells Monolayer wound healing assay . (c) 786O and (d) CAKI1 cells Transwell assay demonstrated
a significant decrease in the number of migration and invasion cells in siDLGAP51 and siDLGAP52-
transfected human clear cell renal cell carcinoma compared with that observed in siNC-transfected cells.
Scale bar, 100 µm. (*P < 0.05; **P < 0.01; ***P < 0.001; vs. control. DLGAP5, DLG Associated Protein 5; si,
small interfering; NC, negative control.)

Discussion

DLGAP5/HURP, a K-MT plus-end binding protein gene during mitosis, plays a key role in proliferation and
cell cycle progression through spindle assembly [7, 10–12]. The previous studies have shown that an
increased expression is detected in many cancer types and the tumor cell lines [7, 16, 21–23]. In this
study, based on the analysis of multiple databases, we found that DLGAP5 is Upregulated in at least 26
common cancers, of which the prognosis and immune infiltration levels of several cancer types are also
related to its upregulated. Renal carcinogenesis, including ccRCC, seems to be particularly sensitive to
this gene expression.

To exactly evaluate the expression patterns of DLGAP5 between various cancer tissues or cancer cell
lines, firstly, the GTEx data set was analyzed to explore DLGAP5 transcript levels in a wide spectrum of
human normal tissues. The results showed that very little expression of DLGAP5 transcripts were found in
31 normal tissues, including kidney tissue, except for tissues that the cells divide vigorously, such as bone
marrow, testis (Fig. 1a), Those findings are well consistent with the previous experimental results [7],
which report its expressions are very low or undetectable in most normal tissues. Furthermore, analysis of
data obtained from CCLE released that the similar mRNA levels of DLGAP5 was observed among 21
types of tumor cell lines, while kidney cancer cell lines showed lowest expression (Fig. 1b). Altogether,
DLGAP5 transcript levels were to be showed very low in both kidney normal tissues and their respective
cancer cell lines compared with other normal tissues or cancer cell lines.

An increased number of studies have confirmed that DLGAP5 Upregulated occurs in many cancer tissues
as mentioned in the introduction. DLGAP5/HURP is initially found to be upregulated in HCC by
bioinformatics analysis, and the results subsequently confirmed by experiments [7], In a similar way, its
Upregulated in many cancers is found by analysis of database or microarray data and experimental
verification, such as prostate cancer [16, 17], breast cancer cells [19], lung cancer [20, 21], and endometrial
carcinoma [23]. In the present study, our TCGA pan-cancer analysis clearly showed significant
upregulated of DLGAP5 in at least 26 types of cancer tissues compared with the respective adjacent
tissues (P < 0.001), including ccRCC, and 7 types mentioned above. Surprisingly, among 26 types of
cancers tested, all 3 types of kidney-related cancers (KICH, KIRC/ ccRCC and KIRP) exhibited almost
minimum upregulated (P < 0.001). The analysis further released 2 types of kidney-related cancers (KIRC/ccRCC and KIRP), as well as LGG show a worse prognosis in relation to the upregulated at a very significant level (P < 1 × 10^{-10} for OS, and P < 1 × 10^{-8} for DSS and PFI Fig. 3) when compared with other 23 types of cancer. To explore markers of glioblastoma based on bioinformatics analyses, Zou et al found that DLGAP5 is one of 10 hub gene significantly associated with the development of malignant glioblastoma [24]. They also provided the expression profiles of DLGAP5 in 31 types of tumor and corresponding normal tissues, and showed the similar expression pattern to our results [24]. Overall, the results of this study indicate that the occurrence and progression of kidney cancer, including ccRCC, is extremely sensitive to the upregulated of DLGAP5, albeit the extents of upregulation are minor. This tissue-specific differential sensitivity to the same oncogene is well illustrated in a recent other comprehensive study [35].

Unexpectedly, the analysis of TIMER database displayed DLGAP5 upregulated also closely relating to the level of invasion of 3 types of cancer, of which an extremely significant level of immune infiltration was found in KIRC/ccRCC. These results provide further support for the worse prognosis and an association between DLGAP5 expression and aggressive ccRCC, as showed that DLGAP5 expression levels in tumorous tissues was significantly (P < 0.05) positive correlated with clinical TNM stage and Fuhrman grade of patients with ccRCC based on TCGA data set.

The data analysis results were also verified by the experiments with RCC cell lines 786-O and CAKI-1 through western blotting (Fig. 6), RNAi (Fig. 7), and cytological analysis (Fig. 7, 8), as well as immunohistochemistry (Fig. 6), showing DLGAP5 knockdown significantly inhibited the cells viability and proliferation, as well as reduction of invasion and migration potential in RCC cell lines. Unlike HK-2 cell line, a slight elevated expression of DLGAP5 was detected in the 786-O and CAKI-1 cells line (Fig. 5), the knockdown, however, resulted in significant effect on the cell malignancy (Fig. 6, 7). This result combining with bioinformatics analysis led us to conclude that renal carcinogenesis is sensitive to expression DLGAP5, which may be as an independent prognostic biomarker in ccRCC.

Both sensitivity of ccRCC cells to DLGAP5 and its low-level upregulated in renal carcinomic tissues are reminiscent of whether its expression is controlled by hypoxia or HIF in kidney tissues, especially in renal cancer tissues. This highly speculative hypothesis may be reasonable for two reasons. First, hypoxia is an inherent feature of the tumor microenvironment (TME), and a key player governing various cancer hallmarks [36, 37]. Additionally, it has been well documented that over 90% of ccRCC is closely associated with VHL mutations, which results in stabilization of hypoxia inducible factors (HIF) [4, 38]. Second, DLGAP5/DLG7 has been identified as a downregulated gene that respond to HIF by an integrative genomics [26]. In other study, it is found that DLGAP5 protein is one of top ten oxygen-sensitive proteins in prostate cancer [16]. In addition, several putative HIF binding sites are found to be located at upstream of DLGAP5 promoter, most of which are highly methylated region [16, 25]. It is well known that the highly methylated regions are commonly inversely correlated with gene expression [39]. Indeed, the increased methylation of HURP promoter is associated with a reduction in protein levels in the prostate cancer cell lines, and hypoxia accelerated the rate of decrease of the expression, although an
increased expression is observed in this tumor tissues [25]. However, in HCC tissues that show DLGAP5 upregulated, DNA methylation status of the DLGAP5 promoter is reduced [40]. Additionally, our present pan-cancer analysis showed that the expression patterns of DLGAP5 in both prostate adenocarcinoma (PRAD) and KIRC/ccRCC are very similar. Both cancer tissues show that the upregulated is minor, but with a very significant statistical difference compared with corresponding adjacent normal tissues (P < 0.001 Fig. 1). This may imply that DLGAP5 expression in ccRCC may also be controlled by hypoxia, just like it is in prostate cancer [16, 25]. The future studies are necessary to explore whether DLGAP5 expression in ccRCC is really regulated by hypoxia or HIF. These studies will undoubtedly expand and deepen our understanding of its functions in cancer tissues for potential targeted therapy.

**Conclusion**

In conclusion, the special role of DLGAP5 in spindle assembly during cell division led to speculation that it may function as an oncogene in many cancers when upregulated. In the present study, based on pan-cancer analysis, we found that DLGAP5 is overexpressed in at least 27 cancer tissues, including ccRCC and a number of cancers studied previously. In addition, its upregulated is association with the immune penetration and poor prognosis in several cancers tested, especially in 3 types of renal cancers, including KIRC/ccRCC. Surprisingly, DLGAP5 upregulated in 3 types of kidney cancer display a minor but statistically significant difference compared with the adjacent normal tissues. These findings were confirmed by the results obtained from experiments with kidney cancer lines and immunohistochemistry. Taken together, our results show that DLGAP5 upregulated occur in common human cancers, and ccRCC seems to be particularly sensitive to its expression. DLGAP5, therefore, may be as an independent prognostic biomarker in ccRCC diagnosis.

**Abbreviations**

ccRCC: clear cell renal cell carcinoma; TCGA: The Cancer Genome Atlas; DLGAP5: Discs large-associated protein 5; KM: Kaplan–Meier; GTEx: Genotype-Tissue Expression; CCLE ;Cancer Cell Line Encyclopedia OS: overall survival; DSS: disease-specific survival; PFI :disease-free interval ; 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; 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; OV: Ovarian serous cystadenocarcinoma; PAAD: Pancreatic adenocarcinoma; PRAD: Prostate adenocarcinoma; READ: Rectum adenocarcinoma; SKCM: Skin Cutaneous Melanoma; STAD: Stomach adenocarcinoma; TGCT: Testicular Germ Cell Tumors; THCA: Thyroid carcinoma; UCEC: Uterine Corpus Endometrial Carcinoma; UCS: Uterine Carcinosarcoma.
Declarations

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Authors’ information

Yun Feng, Fang Li and Xianli Guo contributed equally to this work.

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Authors’ contributions

YF initiated the study and organized; YF, FL, XG, JZ, XW and CH designed and carried out bioinformatics analyses, statistical analyses, drew figures and drafted the manuscript; YF, YG, DL, JY, performed the experiments; FW, HS, JD, HZ, YY, WH, MZ, and RD participated in modifying the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated and analyzed during the current study are available in the The Cancer Genome Atlas (TCGA), https://portal.gdc.cancer.gov/, Genotype-Tissue Expression (GTEx) database (https://commonfund.nih.gov/GTEx/data), Cancer Cell Line Encyclopedia (CCLE) database (https://portals.broadinstitute.org/ccle), and Tumor Immune Estimation Resource (TIMER): (https://cistrome.shinyapps.io/timer/)

Ethics approval and consent to participate

Not applicable.
Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

**Figure 1**

(a) Kruskal-Wallis test $p=0$

(b) Kruskal-Wallis test $p=3\times10^{-17}$
DLGAP5 expression in normal human tissues and tumor cell lines (a) GTEx database analysis of the expression in normal human tissues (K-W test, P=0). (b) CCLE database analysis of the expression in cancer cell lines (K-W test, P=3e-17)

Figure 2

DLGAP5 expression in Pan-cancer (a) The expression of DLGAP5 in different human tumors within TCGA. (b) DLGAP5 expression in 33 human tumors was analyzed by integrating normal tissue data from GTEx
The survival forest map for prognostic analysis of DLGAP5 expression in pan-cancer. (a) HR (95% CI) for OS. (b) HR (95% CI) for DSS. (c) HR (95% CI) for PFI. (Using a single-factor survival analysis, DSS, disease-specific survival; OS, overall survival; PFI, progression-free interval; *P < 0.05, **P < 0.01, ***P < 0.001).
Association of DLGAP5 expression with immune infiltration in Pan-cancer. (a) Scores of six immune-infiltrating cells of 33 cancers were downloaded from TIMER database, and the correlation between DLGAP5 expression and scores of these immune cells was analyzed respectively. The expressions in three cancers (KIRC, LGG, and LIHC) were significantly associated with the Scores. (b) The Stromal
Scores, Immune Scores, and ESTIMATE Scores for THCA, KIRC, STAC, and HUSC, with showing a significant correlation between the gene expression and Scores.

Figure 5

The association of DLGAP5 expression with clinical stage in TCGA dataset. (a) topography (T1, T2, T3 and T4), (b) lymph node metastasis (N0, N1), (c) distant metastasis (M0 and M1), (d) G stages (I, II, III and IV) and (e) pathological stage (I, II, III and IV) of patients with ccRCC. (f-h) Kaplan-Meier survival curves of
patients with ccRCC based on DLGAP5 expression levels (f: DSS; g: OS; h: PFI). (DSS, disease-specific survival; OS, overall survival; PFI, progression-free interval; *P<0.05. TCGA, The Cancer Genome Atlas; DLGAP5, DLG Associated Protein 5; ccRCC, clear cell renal cell carcinoma)

Figure 6

Up-regulated expression of DLGAP5 in ccRCC. (a) Data from TCGA database demonstrated a significant upregulation of DLGAP5 expression in ccRCC tumor samples (n=531) compared with normal tubular tissue (n=72). (b) Data from GTEx database and TCGA tumor tissue demonstrated a significant upregulation of DLGAP5 expression in ccRCC tumor samples (n=531) compared with normal tubular tissue (n=98). (c) Western blotting results revealed that DLGAP5 protein levels were higher in the ccRCC cell lines 786-O and CAKI-1 compared with the normal renal epithelial cells HK-2. (d) Representative images of DLGAP5 immunohistochemistry in paired tumor and normal tissue samples. (*P<0.05; **P<0.01 vs. control. DLGAP5, DLG Associated Protein 5; ccRCC, clear cell renal cell carcinoma; TCGA, The Cancer Genome Atlas.)
The knockdown of DLGAP5 levels inhibits the proliferation capability. (a) Reverse transcription qPCR and (b) western blotting were performed and revealed a significant decrease in the mRNA and protein levels of DLGAP5 in siDLGAP51 and siDLGAP52-transfected human ccRCC, compared with those observed in siNC transfected cells. The band intensities of DLGAP5 were quantified relative to β-actin and normalized to the siNC sample. (c) CCK8 results showed that DLGAP5 knockdown significantly inhibited the proliferation of...
786 O and CAKI 1 cells. (d) Cell proliferation monitoring 786 O and CAKI 1 cells inhibited the proliferation. DLGAP5 knockdown significantly decreased the number of (e) 786 O and CAKI-1 cell colonies compared with the control group. (*P<0.05; **P<0.01; ***P<0.001; vs. control. DLGAP5, DLG Associated Protein 5; si, small interfering; NC, negative control.)

**Figure 8**

The knockdown of DLGAP5 levels significantly inhibits migration and invasion. (a) 786 O and (b) CAKI 1 cells Monolayer wound healing assay . (c) 786 O and (d) CAKI 1 cells Transwell assay demonstrated a significant decrease in the number of migration and invasion cells in siDLGAP51 and siDLGAP52-transfected human clear cell renal cell carcinoma compared with that observed in siNC-transfected cells. Scale bar, 100 µm. (*P<0.05; **P<0.01; ***P<0.001; vs. control. DLGAP5, DLG Associated Protein 5; si, small interfering; NC, negative control.)

**Supplementary Files**

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