Lymphocyte Antigen 6 Family Member D (LY6D) affects stem cell phenotype and progression of pancreatic adenocarcinoma

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Abstract

The membrane-bound protein Lymphocyte Antigen 6 Family Member D (LY6D) has been implicated in stemness and is associated with various tumors. LY6D is specifically expressed in cancer cells, with little expression in normal cells, but its physiological function in cancer progression remains poorly understood. Here, we investigated how LY6D was associated with pancreatic ductal adenocarcinoma (PDAC), and involved in stemness. We conducted functional analysis of LY6D to evaluate its contribution to impacting PDAC cells in vitro. Using our in-house developed stem cell separation technique, which can isolate cells with low proteasome activity and high stem cell properties, we successfully enriched cells with extremely high tumorigenic ability. We then used these cells, to perform in vitro functional assays by manipulating the LY6D expression. Immunohistopathological analysis was also conducted to reveal the clinical significance of LY6D in PDAC. In vitro functional assays demonstrated that LY6D was crucially involved in the cancer malignant phenotype including invasive ability, drug resistance, migration abilities, and cancer stemness. Immunohistopathological analysis revealed that high LY6D expression levels were associated with high recurrence rate and poorer prognosis in PDAC. Our present findings demonstrated that LY6D plays a key role in regulation of cancer stem cells. LY6D is not only a prognostic indicator but also could be a promising therapeutic target in PDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is among the most common cancers, with over 400,000 new cases emerging every year worldwide.[1] Despite advances in treatment, PDAC remains a leading cause of cancer-related death in many industrialized countries.[2] Its poor prognosis is partly due to the fact that approximately 90% of tumors are diagnosed at an advanced stage where the tumor spread has already expanded beyond the pancreas and systemic metastases are present in over 50% of patients.[3,4] PDAC treatment requires a multidisciplinary approach, including both chemotherapy and radiotherapy. Even with such treatment, patients often experience treatment resistance and cancer recurrence, largely due to the existence of cancer stem-like cells (CSCs).[5,6]

CSCs are defined as cancer cells with a self-renewal capacity and multilineage potency, which constitute a small population within bulk tumors and play critical roles in tumorigenicity, cancer progression, metastasis and recurrence.[7] The CSC model has attracted significant attention because it provides an explanation for the clinical observation that even when cancer treatments initially seems to eliminate cancer cells, the cancer can later reoccur.

LY6D is a membrane-bound protein that attaches to the cell surface through a C-terminal glycosylphosphatidylinositol (GPI) anchor.[8] It exhibits lineage-specific expression, and is thus commonly used as a surface marker to identify leukocyte subsets, however, its physiological function in cancer progression is poorly understood. Genes of the Ly6 family are located on chromosome 8q24 alongside c-Myc. Somatic copy number gain in the 8q region is the most common type of copy number gain in several types of cancer.[9,10] Within this gene family, LY6D is located in the 8q24.3 region, which is frequently amplified in various types of human malignancies. Recent studies show that LY6D is associated with distant

metastasis in breast cancer and poor prognosis in lung cancer.[11] Moreover, it has been suggested that LY6D may be involved in cancer stemness in lung cancer and laryngeal cancer.[11]

At this time, only limited evidence links LY6D to pancreatic cancer. To explore the role of LY6D in cancer stemness, here we used the ornithine decarboxylase (ODC) degron system to identify cells having characteristics resembling cancer stem cells. In this system, 26S proteasome directly recognized the degron sequence, resulting in degradation of the implicated protein. Under a microscope, cancer cells with low-proteasome-activity are visualized using the stable expression of green fluorescence (ZsGreen) fused to the ODC degron, albeit at a low level. These cells are likely to display CSC-like traits, including strong sphere-forming ability, increased tumorigenicity, and elevated expression of CSC markers.[12] In the present study, to explore how LY6D was involved in stemness, we used cells characterized by low proteasome activity and CD44v9 positivity, which were flow-sorted from Panc-1 cells with higher stemness using the ODC degron system. Our aim was to investigate the possible role of LY6D in cancer stemness and its clinical significance as a prognostic factor in PDAC. We also explored the therapeutic implications of targeting this gene in PDAC.

Methods

Ethics Statement

The study was approved by the Institutional Review Board for Studies in Humans at Osaka University (approval number: 15144-6).). All assays were performed in accordance with

the committee's guidelines and regulations.

Cell lines and culture conditions.

The cell culture and quality maintenance techniques have been previously described.[13,14] The human pancreatic cancer cell line Panc-1 was obtained from American Type Culture Collection (Manassas, VA). Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, 08456–36; Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA). 100 U/mL penicillin, and 100 mg/mL streptomycin at 37C in a humidified incubator with 5% CO2. All experiments were performed with cells passaged <8 times.

Transduction of the degron reporter into pancreatic cancer cells.

The low-proteasome activity cell (LPAC) isolation system was established by engineering cells that stably expressed ZsGreen fused to the carboxyl terminal degron of ornithine decarboxylase (ODC), as previously described.[15] The degron sequence of ODC is directly degraded by proteasomes. Consequently, cells with low proteasome activity accumulate the fluorescent fusion protein and can be detected by fluorescent microscopy or flow cytometry (FITC channel).

Flow cytometry.

We used FACSAria II (BD Biosciences) for cell sorting and FACSDiva software (BD Biosciences) for analysis. Cells were washed with PBS containing 2% FBS, and then incubated with the primary antibody (Ab), anti-CD44v9 (Cosmo Bio, Tokyo, Japan) at 4 °C for 20 min. To detect CD44v9, PE mouse anti-rat IgG2a (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was used as a secondary Ab. Degron^{+}, cells were detected using Cell Sorter SH800Z (Sony Biotechnology Inc. San Jose, CA). The cell population with high fluorescence intensity was collected by the EGFP channel as we previously described.[12,16]

Clinical tissue samples.

PDAC tissue samples (*n* = 75) were collected during surgeries performed between 2007-2012, at the Department of Gastroenterological Surgery, Osaka University. All patients had clear diagnoses with PDAC, according to the clinicopathological criteria described by the Japanese Society for pancreatic cancer. Samples were fixed in buffered formalin at 4℃ overnight, processed through graded ethanol solutions, and embedded in paraffin. The specimens were appropriately used, with approval by the Ethics Committee at the Graduate School of Medicine, Osaka University.

Construction of the LY6D-shRNA lentivirus.

We used two short hairpin RNA (shRNA) sequences specifically targeting LY6D (GeneBank ID:8581; 5'-ATCTGGTGAAGAAGGACTGTG-3' and 5'-CCAGCAACTGCAAGCATTCTG-3'). Control cells were generated by transfecting cell with empty vector. The LY6D gene was cloned into the enhanced green fluorescent protein (eGFP) containing pReceiver-Lv193x lentivector (iGene Biotechnology Co., Ltd.) using FastDigest KpnI (cat. no. FD0524) and XhoI (cat. no. FD0694) restriction endonucleases (both Thermo Fisher Scientific, Inc.) to produce plasmids termed LY6D-shRNA-1, and LY6D-shRNA-2.

Cell transfection.

We purchased pCMV6-XL5-LY6D and pCMV-XL5-vector (empty vector) from ORIGENE (USA). For transfection experiments, lipofectamine 3000 (Invitrogen, USA) was used to transfect LY6D or empty vector into Panc-1 cells. Transduction efficiency was analyzed by PCR and western blot.

Quantitative real-time reverse transcriptase–polymerase chain reaction.

Total RNA was extracted from cultured cells using TRIzol^{R} RNA Isolation Reagents (Thermo Fisher Scientific) as previously described.[17] Complementary DNA (cDNA) was synthesized from 10 ng of total RNA using a High Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was performed in a Light Cycler^{TM} 2.0 System (Roche Applied Science) using the Thunderbird® SYBR® quantitative PCR (qPCR) mix (Toyobo Life Science, Osaka, Japan). For each experiment, data were normalized to the expression of a control gene (GAPDH). The following primers were used: LY6D: forward, 5'-CATTGCTGCTCCTTGCAG-3' and reverse, 5' -ATGCTTGCTTGCAGTTGCTGGAG-3' ; GAPDH: forward: 5' -AGCCACATCGCTCAGACAC-3' and reverse, 5' -GCCCAATACGACCAAATCC-3'.

Immunohistochemical staining.

Immunohistochemical staining was performed as previously described.[18] Slides were incubated with the anti-LY6D rabbit antibody (1:500 dilution, HPA024755; Sigma-Aldrich, Tokyo, Japan), overnight at 4℃. As a positive control for LY6D, we used human esophageal squamous tissue, based on the database of the Human Protein Atlas (https://www.proteinatlas.org). Groups that were slightly stained were considered positive, and those that were not stained at all were considered negative.

Cell proliferation assay.

We counted the number of living cells using Cell Counting Kit8 (DOJINDO) according to the manufacturer's instructions. After a 2-hour preincubation in the assay solution, we determined the viable cell number in each well, based on the absorbance at 450 nm (OD 450) as measured by a microplate reader (BIO-RAD Model 680 XR).

Scratch wound healing assay.

Cells were seeded at a density of 5×10^5 cells per well in 6-well plates, and grown to confluence under standard conditions. The scratch assay was performed by running a 200μL pipette tip though the well and then containing to culture the cells under standard conditions, except with DMEM containing 1% FBS, to prevent proliferation. Next, the plates were washed with fresh DMEM with 1% FBS, to remove non-adherent cells before each photography session. Cell migration was evaluated by measuring the average distances between the wound edges at 9 random areas.

Invasion assay.

The cell invasion assay was performed in a 24-well Corning Matrigel invasion chamber (Corning, NY) with 8-micron pores. To the top chamber, we added 200 μL of cell suspension (5×10^4 cells/mL) in serum-free media. Next, to the lower chamber, we added 500 μL of FBS-supplemented media to serve as a chemoattractant. After 72 h, the supernatant was discarded, the cells in the upper chamber were removed using a cotton swab, and the cells on the lower surface were fixed and stained with hematoxylin eosin. The number of invaded cells was counted under a microscope, assessing six high-power fields per group. Cells were counted using ImageJ software.

Sphere formation assay.

The sphere formation assay was performed as previously described.[12] Briefly, sorted cells were plated in 96-well ultralow attachment plates (Corning Inc.) at a density of 100 cells per well, These cells were grown in tumorspheric culture medium (DMEM/F-12) supplemented with 20 ng/mL human platelet growth factor, 20 ng/mL epidermal growth factor, G418, and 1% antibiotic–antimycotic solution, at 37℃ in a humidified atmosphere of 95% air and 5% CO2. We counted the numbers of spheres in all wells, and evaluated differences in the average number per well.

Drug sensitivity assay.

LP/CD44$^+$ Panc-1, cells were seeded at a density of 2.0 × 10^3 cells/well in 96-well plates, and pre–cultured for 24 hours. The cells were exposed to various concentrations of oxaliplatin and gemcitabine, and the cytotoxic effects were evaluated using a Cell Counting Kit-8 (Dojindo), according to the manufacturer's protocol.

Animal experiments.

Animal experiments were performed in 8-week-old male BALB/cAJcl-nu/nu immunodeficient mice (CLEAJapan, Tokyo). To produce tumorsin vivo, cells were mixed with Matrigel (BD Biosciences) and medium at a 1:1 ratio (vol:vol). Mice were subcutaneously injected with approximately 1.0 × 10^3 cells in 100 μL medium/Matrigel solution, in both sides of the lower back regions. On week 6, the mice were sacrificed and the tumors were excised. Collected tissues were homogenized using a TissueLyser II (QiagenInc, Valencia, CA).

Western blotting.

Total protein (60 μg) was extracted from cultured cells using RIPA buffer containing protease inhibitor and phosphatase inhibitor (Thermo Fisher Scientific). Protein was electrophoresed on 10 % SDS-PAGE gels and electroblotted onto PVDF membranes (Merck Millipore, Darmstadt, Germany) at 100V for 90 min. As a loading control, we used β-actin, and β-actin antibodies (A2066; Sigma-Aldrich). After blocking with 5 % skim milk for 1 h, these membranes were incubated with primary antibodies at the appropriate dilutions (1:1000 for LY6D, 1:1000 for CD24 and 1:2000 for β-actin) overnight at 4°C. After incubation with secondary antibodies, the protein bands were detected with the Amersham ECL Detection System (Amersham Biosciences, Piscataway, NJ).

Prognostic analysis of LY6D.

To examine LY6D expression in normal and tumor tissues, we used clinical information about tumors downloaded from the TCGA database.[19] We analyzed the correlation between LY6D expression and PDCA prognosis, using overall survival (OS).

Gene mutation analysis of LY6D.

We analyzed mutations of the LY6D gene in all tumor tissues using the cBioPortal platform.[20,21] The identified alterations included mutations, amplifications, deep deletions, and multiple alterations

Statistical analysis.

Statistical analyses were performed using JMP Pro 16.0.0 (SAS Institute Inc., Cary, NC, USA). We conducted an overall survival analysis, including all patients (n = 75), using the Kaplan–Meier method. The log-rank test was used to test differences between the survival curves. Data are reported as mean ± standard error of the mean (SEM). Variables that were significantly correlated with survival in univariate analysis were entered into a Cox proportional hazards regression model for multivariate analysis. A P value of < 0.05 was considered to indicate a statistically significant difference.

Data availability

Raw data of RNA-Seq are available and ready to be uploaded to the public structured data depository. The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Results

LY6D is specifically upregulated in tumor tissues.

We employed TCGA to study LY6D expression in tumors and found that LY6D expression was up-regulated in almost all tumors compared with corresponding normal tissues (Figure 1A). We also analyzed the genetic alteration status of LY6D in different tumor samples from the cBioPortal platform. Amplification of LY6D constituted for the largest proportion among all mutation types, observed in various cancerous lesions, including pancreatic cancer. (Figure 1B).

Overexpression of LY6D-enhanced malignant potential of PDAC cells.

To elucidate how LY6D is involved in cancer progression, we performed in vitro functional assays with manipulation of LY6D gene expression. We observed that LY6D overexpression at both the mRNA and protein levels significantly enhanced cell proliferation (Figure 2A,B). LY6D overexpression also enhanced the invasion and migration ability of Panc-1 cells (Figure 2C,D). Notably, LY6D expression in Panc-1 cells was significantly enhanced under 3D stem cell culture conditions compared to in 2D monolayer cultures (Figure 3A). Sphere formation assays revealed that LY6D overexpression significantly increased the number of spheroids (Figure 3B). Representative stem cell markers of PDAC including CD24, CD44, CD44v9, and CXCR4 were significantly enriched in LY6D overexpressing cells (Figure 3C).

The correlation between LY6D and stemness.

Using the ODC-degron system, we previously identified that cells exhibiting low proteasome activity possess high stem cell properties.[16] Here we used this ODC-degron system to conduct a more detailed investigation into the association of LY6D with stemness. Subsequently, these cells were gated using flow cytometry with the CD44v9 antibody, a representative PDAC stem cell marker. (Figure S1A,B). This led to the successful enrichment of Panc-1 cells with an extremely high tumorigenic ability, which we termed LP/CD44$^+$.

Stem cell properties of LP/CD44$^+$ cells.

The sphere formation ability was significantly increased in LP/CD44$^+$ cells compared with parent cells (Figure S2A). We also further examined the expressions of various cancer stem cell (CSC) markers in LP/CD44$^+$ cells and parent cells. Representative stem cell surface markers in PDAC (CD24, CD133, and CXCR4) were significantly enriched in LP/CD44$^+$ cells compared with parent cells (Figure S2B). Sphere formation assay and CD markers results indicated the stemness of LP/CD44$^+$ cells. Furthermore, compared to parent cells, LP/CD44$^+$ cells showed enhanced drug resistance to the anticancer drugs oxaliplatin and gemcitabine (Figure S2C). We additionally analyzed the potential effect of LY6D on Panc-1 cell migration by

performing a wound-healing assay, which demonstrated that LP/CD44$^+$ cells displayed increased migration ability (Figure S3A). We confirmed a significant elevation of LY6D expression in LP/CD44$^+$ cells (Figure 4A). Furthermore, LY6D expression was significantly enhanced under 3D stem cell culture conditions compared to in 2D monolayer cultures, among both parent and LP/CD44$^+$ cells (Figure 3A, Figure S3B).

The effects of LY6D knockdown on cancer stem cells.

Knockdown experiments were performed in LP/CD44$^+$ cells, which showed high LY6D expression levels. RNA interference (RNAi)-mediated gene silencing suppressed the gene expression by 50% in sh1 cells and by 70 % in sh2 cells (Figure 4B). Sphere formation assays revealed that LY6D knockdown significantly decreased the number of spheroids formed (Figure 4C). LY6D knockdown also inhibited the invasion and migration ability of LP/CD44$^+$ cells (Figure 5A,B). Furthermore, LY6D knockdown suppressed the cells' resistance to the anticancer drug gemcitabine to lower than in NC cells (Figure 5C). To investigate whether LY6D played a role in tumor formation in vivo, we subcutaneously injected LP/CD44$^+$ cells with stable LY6D knockdown or NC cells into the bilateral hind legs of nude mice. LY6D knockdown significantly decreased the volume of PDAC tumors in this xenograft mouse model (Figure 5D).

Immunohistochemical analysis for LY6D expression in PDAC.

LY6D expression was not detected in normal tissues (Figure 6A). In contrast, 26 of 75 PDACs (34.6%) exhibited positive staining for the LY6D protein, and the majority of tumors exhibited heterogeneous LY6D expression (Figure 6A). Moreover, LY6D expression was positively correlated with the expression of CD24 (Figure 6B).

High LY6D expression was associated with poor prognosis in PDCA from the TCGA datasets (Figure 6C). We also showed that positive LY6D expression was significantly associated with lower overall survival rate (P = 0.03) and lower relapse-free survival rate (P = 0.005) (Figure 6D,E). Univariate analyses indicated that LY6D expression was a significant prognostic factor for overall survival (P = 0.0092) and relapse-free survival (P = 0.0156) (Table 1). In multivariate analysis, LY6D expression remained an independent prognostic factor for overall survival (P = 0.0012) and relapse-free survival (P = 0.0025) (Table 1). Clinicopathological analyses indicated no significant association between LY6D expression and various clinicopathological features of PDAC (Table 2)

Discussion

In addition to conventional therapy, there is growing demand for novel targeted therapies for pancreatic cancer. Despite increasing evidence in this field**,** the currently available results are not satisfying in terms of evaluating therapeutic strategies to target CSCs.[22] CSCs are responsible for sustaining the tumorigenicity of the tumor and generating the diverse cell types that comprise the tumor.[23] Here we established a population of cancer cells with extremely high tumorigenic potential, with specific focus on immune-related LY6D gene. Our findings revealed that LY6D plays a crucial role in maintaining the stem cell properties of these cells.

LY6 family genes were initially identified in mice as lymphocyte differentiation antigens and as membrane-bound proteins with GPI-anchors.[24,25] The original member of the LY6 protein family, CD59, was discovered in human lymphoid cells, where it plays roles in the complement membrane attack complex and T cell activation.[26] Currently, 20 human LY6 proteins have been identified, with molecular weights ranging from 11_36 kDa, and they have been categorized as either transmembrane or secretory based on the presence of a GPI-anchored signal sequence.[27] LY6 family genes are located on chromosome 8q24 alongside c-Myc. Somatic copy number gain in the 8q region is the most common type of copy number gain in multiple types of cancer.[9,10] LY6D is located on 8q24.3, a region that is frequently amplified in various types of human malignancies.

The precise function of LY6D in carcinogenesis remains largely unknown. Cell invasion and migration are crucial processes for cancer cell metastasis. Our vitro studies showed that LY6D expression was closely associated with drug resistance, migration, and invasion ability of pancreatic cancer cells. Furthermore, LY6D knockdown inhibited pancreatic stem-like cancer cell treatment resistance, migration, and invasion. On the other hand, LY6D overexpression promoted invasion and migration ability. These findings suggested that LY6D is involved in PDAC cell progression and invasion. Previous studies have demonstrated that elevated LY6D expression is significantly correlated with increased risk of tumor recurrence, and poor prognosis in breast and lung cancers.[28] In our present study, we showed that LY6D positivity group was related to poor prognosis and high relapse rate in PDAC. Multivariate analysis indicated that LY6D was an independent prognostic factor for overall survival. Our present findings suggest that abnormal LY6D expression may represent a promising new predictive biomarker and therapeutic target for pancreatic cancer.

Overall, our present findings showed that LY6D is highly expressed in pancreatic cancer stem cells and is associated with tumor malignancy. Further research is needed to reveal the detailed regulatory mechanisms of LY6D, and to explore the potential development of cancer drugs targeting LY6D.

Declarations

Disclosure

Conflict of interest

The authors declare no conflicts of interest associated with this manuscript.

Informed Consent: Informed consent was waived owing to the retrospective nature of the study. The opt-out recruitment method was applied to all patients, with an opportunity to decline to participate.

Registry and the Registration No. of the study/trial: N/A

Animal Studies: This study was approved by the institutional review board of our institution (Permission No. #15218).

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Tables

Table 1. Survival analyses.

Univariate and multivariate analyses for overall survival (Cox regression model)

Clinicopathological factors	Univariate			Multivariate		
	RR	95% CI	P value	RR	95% CI	P value
Gender (male/female)	0.7758	0.4510-1.3342	0.3614			
Lymph node metastasis (positive/negative)	1.0001	0.5764-1.7390	0.9965			
Lymphatic duct invasion (positive/negative)	2.1192	1.1460-3.9188	0.0119 *	2.6488	1.4027_5.0018	0.0003 *
Venous invasion (positive/negative)	1.5837	0.9059-2.7686	0.1145			
Neural invasion (positive/negative)	1.4379	0.7007-2.9506	0.3033			
LY6D expression (positive/negative)	2.1786	1.2352-3.8426	0.0092 *	2.7745	1.5285_5.0363	0.0012 *

Abbreviations: RR relative risk; CI, confidence interval.

* $P < 0.05$.

Univariate and multivariate analyses for relapse free survival (Cox regression model)

Clinicopathological factors	Univariate			Multivariate		
	RR	95% CI	P value	RR	95% CI	P value
Gender (male/female)	0.8255	0.4748_1.4350	0.4984			
Lymph node metastasis (positive/negative)	0.9819	0.5555_1.7357	0.9501			
Lymphatic duct invasion (positive/negative)	2.1486	1.1563_3.9924	0.0112*	2.6280	1.3868_4.9801	0.0018*
Venous invasion (positive/negative)	1.6227	0.9100_2.8936	0.1102			
Neural invasion (positive/negative)	1.4399	0.6994_2.9644	0.3040			
LY6D expression (positive/negative)	2.0906	1.1078_3.7330	0.0156*	2.6353	1.4349_4.8398	0.0025*

Abbreviations: RR, relative risk; CI, confidence interval.

* *P* 0.05.

Table 2. Relationship between LY6D expression and clinicopathological features in pancreatic ductal adenocarcinoma.

		Positive	Negative	P value
Age	Average±SD	68	67	0.1068
Gender	Male	18	27	0.2050
	Female	8	22	
Lymphatic duct invasion	Positive	16	34	0.4947
	Negative	10	15	
Venous invasion	Positive	10	16	0.6162
	Negative	16	33	
Neural invasion	Positive	22	39	0.5907
	Negative	4	10	
Lymph node metastasis	Positive	6	22	0.0579
	Negative	20	27	
Distant metastasis	Positive	0	2	0.1882
	Negative	26	47	
Stage	A, B	6	13	0.7431
	A_	20	36	
Total	Ly6D expression	26	49	-

Figures

Figure 1

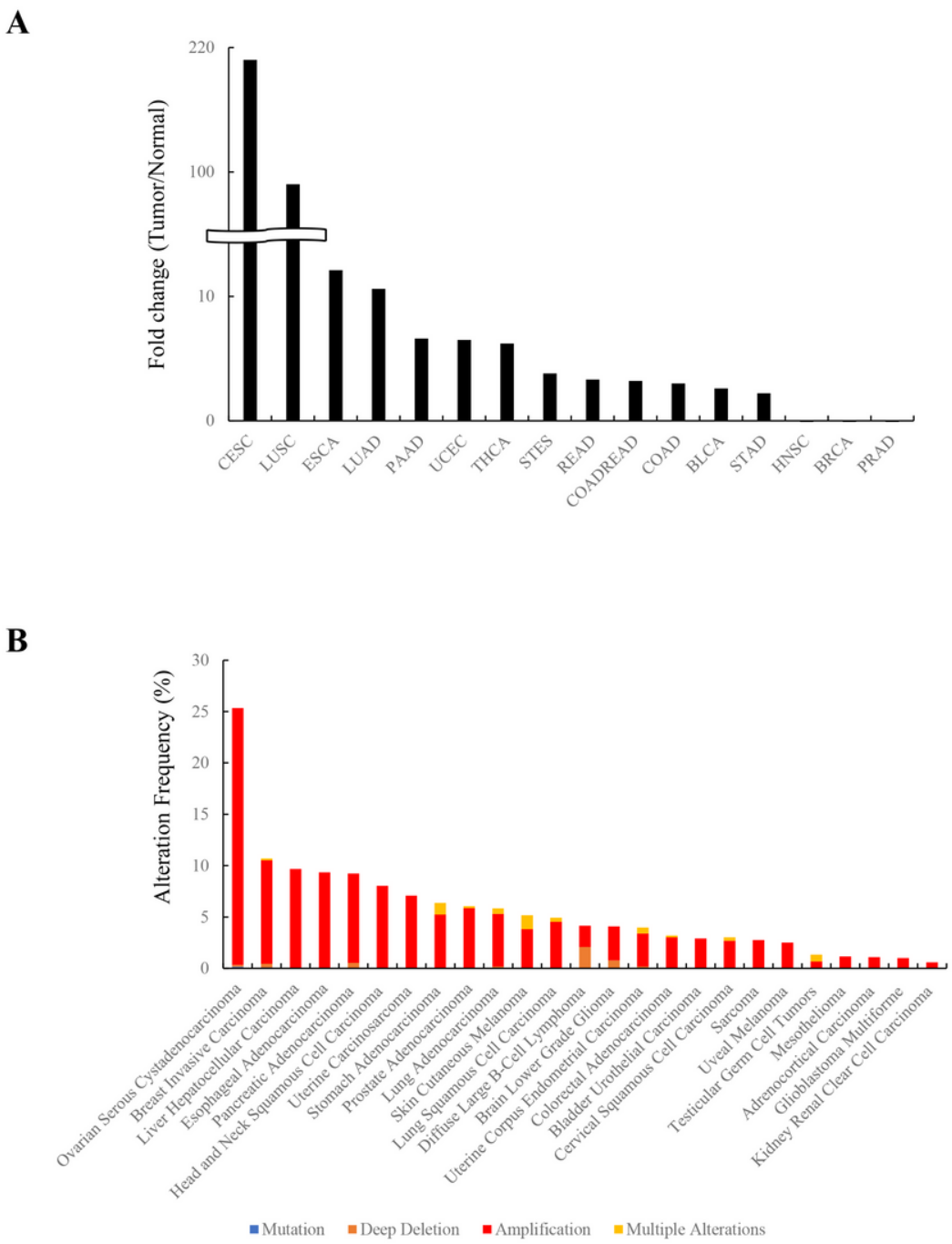

Figure 1

LY6D expression overview.

(A) LY6D mRNA expression is significantly higher in human cancers than in normal tissues.

(B) Alteration frequency of LY6D.

Figure 2

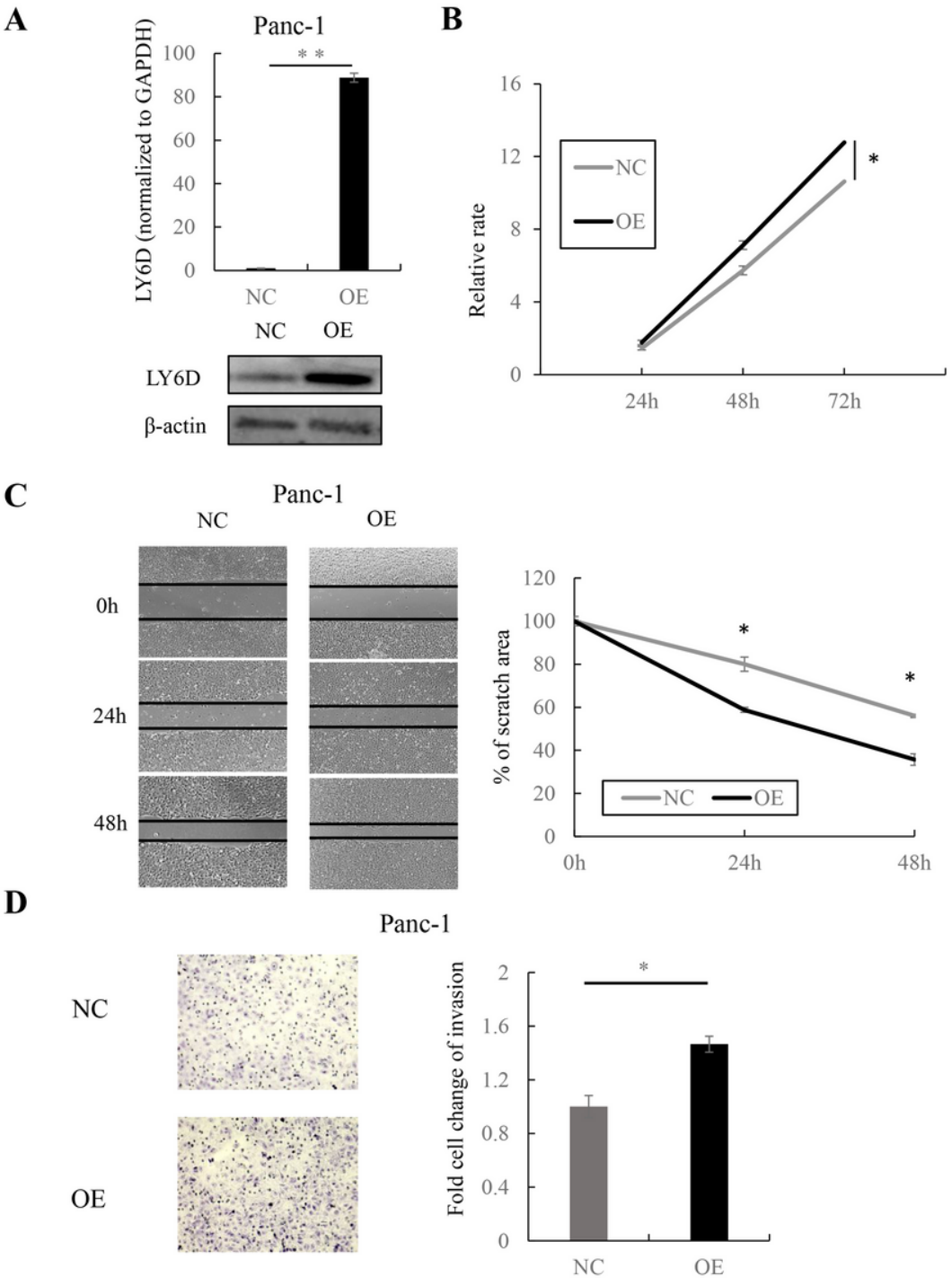

Figure 2

Effect of LY6D overexpression on pancreatic ductal adenocarcinoma (PDAC) cells.

(A) The efficacy of LY6D overexpression (OE) in Panc-1 cells, compared to in negative controls (NC), was determined by real-time PCR and western blotting (mean ± SD; ** $P < 0.01$, two-tailed Student's t-test).

(B) Effect of LY6D OE on PANC-1 cells proliferation. (mean ± SD; * $P < 0.05$, two-tailed Student's t-test).

(C) Representative images of the scratch wound healing assay, using LP/CD44^{+} cells transfected with NC and LY6D OE (Magnification: 100×). Average distance between wound edges in six different areas at the indicated time-points (relative change from the distance at 0 h) (mean ± SD; *P< 0.05, two-tailed Student's t-test).

(D). Representative image of NC and OE cells in Matrigel invasion assay for 72h (mean ± SD; *P< 0.05, two-tailed Student's t-test).

Figure 3

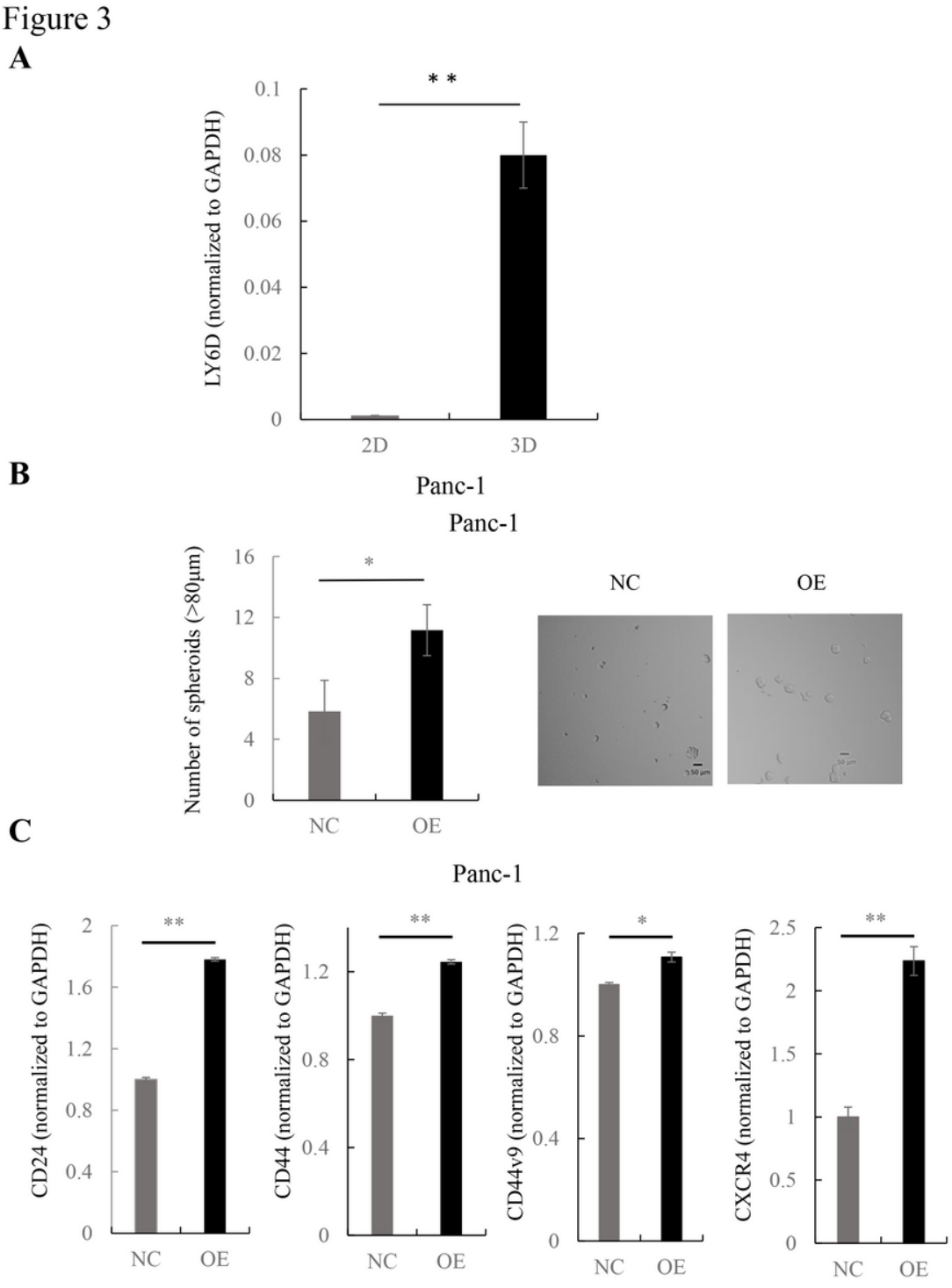

Figure 3

Overexpression of LY6D affects involvement in stemness.

(A) Panc-1 cells in 3D culture had greater LY6D upregulation than Panc-1 cells in 2D culture. (mean ± SD; $^{**}P < 0.01$, two-tailed Student's t-test).

(B) Overexpression (OE) cells had a stronger sphere-forming ability than negative control (NC) cells. The y-axis shows the average number of spheres per well.

(C) Cancer stem cell (CSC) marker analysis showed that OE cells had upregulated CSC markers. (mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, two-tailed Student's t-test).

Figure 4

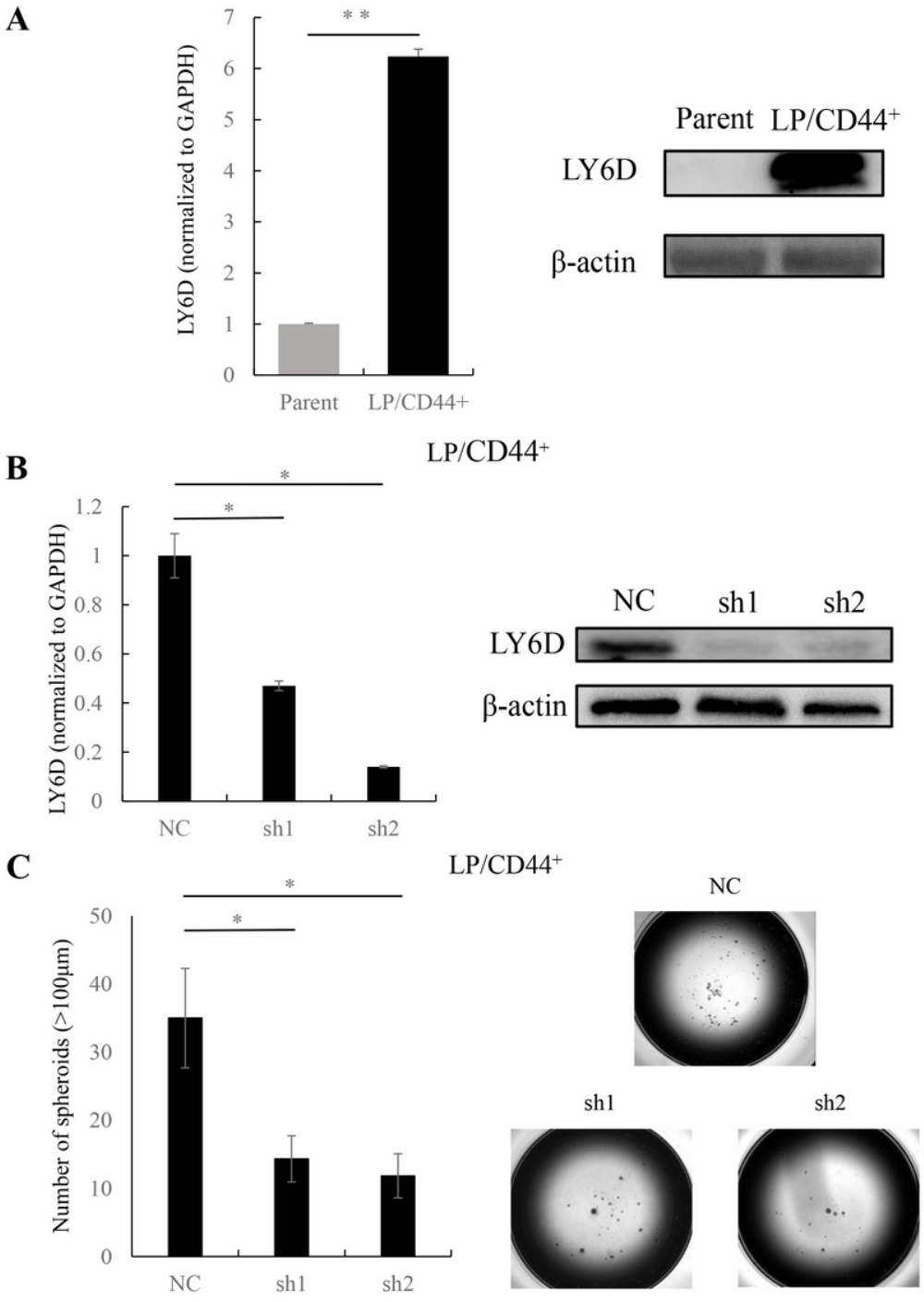

Figure 4

Knockdown of LY6D affects sphere formation

(A) LY6D expression in LP/CD44$^+$ cells was determined by real-time PCR and western blotting. (mean ± SD; $^{**}P < 0.01$, two-tailed Student's t-test).

(B) LY6D knockdown in LP/CD44$^+$ cells was detected by real-time PCR and western blotting.

(C) LP/CD44$^+$ cells had stronger sphere-forming ability than shNC cells. The y-axis show the average number of spheres per well. (mean ± SD; *P< 0.05, two-tailed Student's t-test).

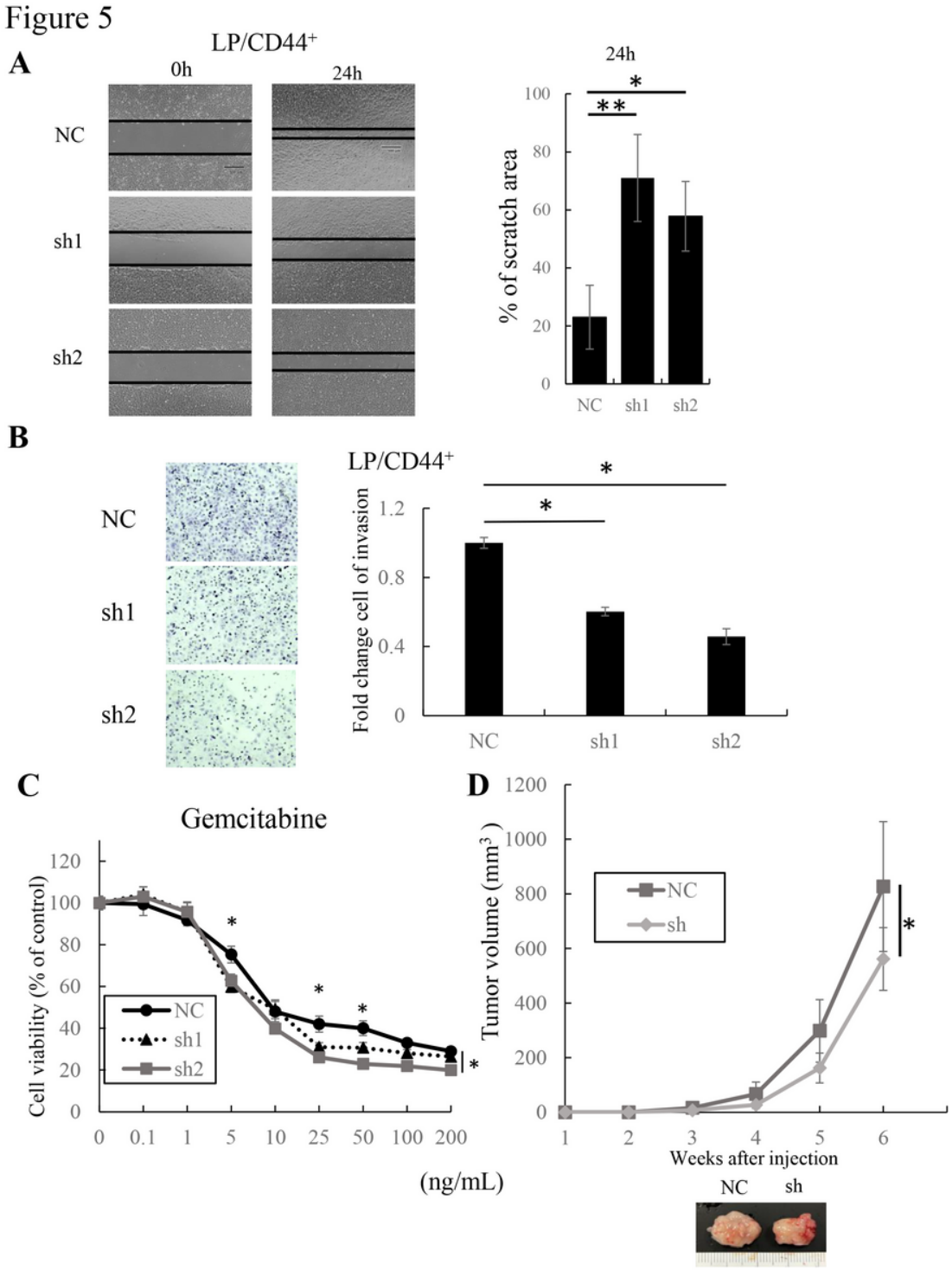

Figure 5

Knockdown of LY6D affects on pancreatic ductal adenocarcinoma (PDAC) cells.

(A) Representative images of the scratch wound-healing assay using LP/CD44$^+$ cells transfected with negative control (NC) and LY6D shRNA (Magnification: 100×). Average distance between wound edges

determined for six different areas at the indicated time points (relative change from the distance at 0 h). (mean ± SD; *P< 0.05, **P< 0.01, two-tailed Student's t-test).

(B) Representative image of cells in Matrigel invasion assay of NC and LY6D shRNA cells for 72h.

(C) Dose response curve after exposure to gemcitabine.

(D) In vivo analysis using a xenograft model. Tumor size in Panc-1, LP/CD44$^+$, NC and LY6D shRNA cells. (mean ± SD; *P< 0.05, two-tailed Student's t-test).

Figure 6

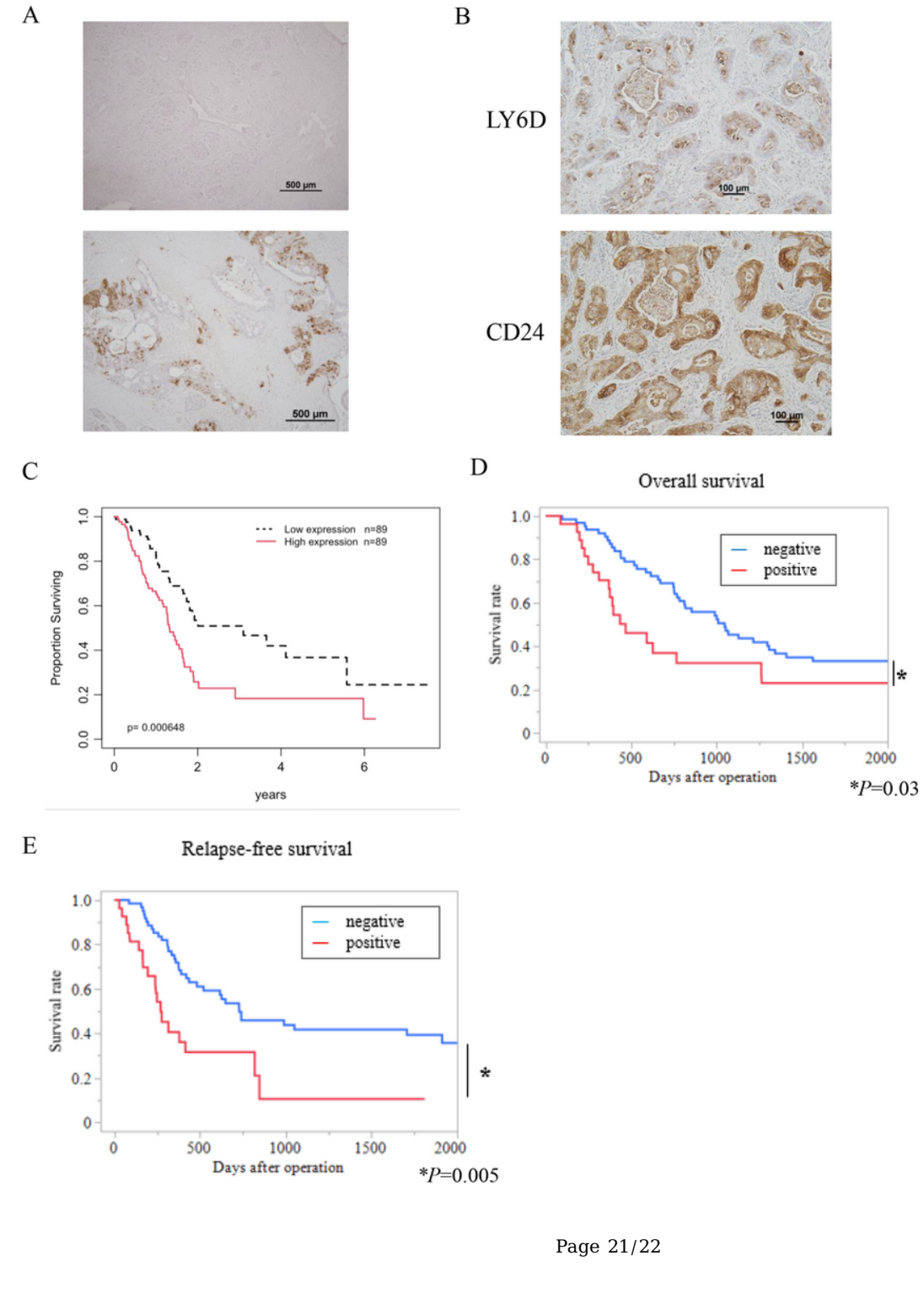

Figure 6

Predominant expression of LY6D in cancer tissues and its clinical significance.

(A) Representative images of immunohistochemical staining of LY6D, showing heterogenic expression in cancer tissues, and negative results in normal tissues (Magnification ×4; scale bar indicates 500μm). PDAC, pancreatic ductal adenocarcinoma.

(B) LY6D and CD24 markers are co-expressed in PDAC.

(C) Kaplan–Meier overall survival curves of PDAC patients according to TCGA of LY6D.

(D) Kaplan–Meier overall survival curves of PDAC patients (*n* = 75) according to immunohistochemical staining of LY6D.

(E)Kaplan–Meier relapse-free survival curves of PDAC patients (*n* = 75).

Supplementary Files

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- supplement.pptx