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RESEARCH

Overexpressions of DLL4 and CD105 are Associated with Poor Prognosis of Patients with Pancreatic Ductal Adenocarcinoma

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Abstract The aim of this study was to investigate the expression of Delta-like ligand 4(DLL4) and Endoglin(CD105) labeled microvessel density(MVD) in pancreatic ductal adenocarcinoma (PDAC) and evaluate their correlation with major clinicopathologic features and patients' survival. Forty-two pancreatic cancer and 20 normal pancreatic tissues were included in the study. Immunohistochemical staining was employed to assess the expression level of DLL4 both in tumor cells and stromal vascular endothelial cells, as well as CD105 which was used to determine MVD. The relationships of DLL4 and CD105 expression with clinicopathologic parameters and clinical outcome were evaluated. Both DLL4 and CD105-labeled microvessel were observed highly immunostained in PDAC cases, and high expression of DLL4 was positively correlated with MVD. Moreover, the high expression of DLL4 was significantly associated with histological grade, node stage and TNM stage in not only the cancer cells but also stroma; while high expression of CD105 was associated with histological grade, TNM stage, node stage and distant metastasis. In univariant analysis, patients with high expression of DLL4 and CD105 tended to significantly poorer overall survival. Both DLL4 and CD105 were overexpressed in a large proportion of patients with PDAC. The expression of DLL4 was positively correlated with CD105-labeled MVD, indicating DLL4 may involved in angiogenesis. In addition, high DLL4 and CD105 expression correlated with the poor clinical outcome and overall survival in patients with PDAC.

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Introduction

The incidence of pancreatic cancer has steadily increased and now ranks as the fourth leading cause of cancer death in the last two decades [1]. It has a high mortality rate, and the prognosis remains poor despite recent advances in surgery, with a 5-year survival rate of less than 5 % [2].

Angiogenesis, the formation of new blood vessels from the existing vascular bed, is a crucial process in tumor progression. When tumors reach a size of 1–2 mm, they become dependent on neovascularization, not only to provide them with nutrients and oxygen, but also as an exit route for metabolic waste products, further growth of the primary tumor, and eventually, metastatic spread [3]. Angiogenesis as reflected in tumor microvessel density has been associated with poor prognosis in several neoplasms such as rectal [4], breast [5], gastric [6], lung cancer [7], as well as pancreatic cancer [8]. While some studies suggested that prognosis in pancreatic cancer does not seem to be correlated with angiogenesis [9]. Thus the role of angiogenesis in the development of pancreatic cancer still needs to be explored. Recent study has proven that, with high specificity, CD105 was limited to angiogenic vessels, while other vessel markers such as CD31 existed in both old and new blood vessels in tumor tissue [10].

Many of the processes were involved in tumor angiogenesis. Evolutional studies of cell interaction mechanisms and downstream genes have been providing evidence that Notch signaling is pivotal in vasculature development [11, 12]. In humans, the Notch signaling pathway is evolutionarily conserved, and consists of four receptors (Notch 1–4) and five transmembrane ligands (Jagged 1, 2, Delta like ligands 1, 3,

4). DLL4 was once found mainly expressed in endothelial cells lining the tumor vasculature [13, 14], and progress has been made in understanding the role of DLL4 in regulation of tumor vascularization. But recent study has reported the widespread DLL4 expression was observed in the cytoplasms of tumor cells from majority of tumors, including pancreatic ductal adenocarcinoma. Therefore, the present study was designed to determine whether high DLL4 expression in tumor cells and stromal vascular endothelial cells is correlated with stromal CD105-labeled microvessel density. Furthermore, we correlated the expression of DLL4 and CD105-labeled MVD with the clinicopathological features and prognosis of PDAC.

Materials and Methods

Ethics Statement

The research protocol was reviewed and approved by the Research Ethics Committee of Second Affiliated Hospital, School of Medicine, Zhejiang University. All participants or their guardians gave written consent of their tissue samples and medical information to be used for scientific research.

Patients

Forty-two formalin-fixed and paraffin-embedded pancreatic ductal adenocarcinoma and 20 adjacent normal pancreatic tissue specimens were obtained from pancreatic cancer patients in the Second Affiliated Hospital, School of Medicine, Zhejiang University between 2008 and 2013. All cases were confirmed by pathological diagnosis and were analyzed according to 7th edition of UICC 2010 TNM classification, any cases of other pancreatic malignancies were excluded from this study. For each case, one block containing tumor tissue was selected and 2–3 μm sections were cut. The normal pancreatic tissues were obtained at least 2 cm away from the cancer and used as normal control.

Immunohistochemistry

Immunohistochemical staining was carried out using the biotin-streptavidin method. Briefly, paraffin-embedded tissue sections were dewaxed and rehydrated through an alcohol series, and then endogenous peroxidase activities were blocked. After non-specific sites were saturated with a rabbit polyclonal antibodies (Santa Cruz Biotechnology Inc Santa Cruz, CA, USA), the sections were incubated sequentially in the primary antibodies, a biotinylated secondary antibody and biotin-peroxidase complex. Finally, the sections were counterstained with hematoxylin. staining without primary antibody in parallel served as negative control. The primary antibodies used were DLL4 (monoclonal, 1:100, Abcam, UK) and CD105 (monoclonal, 1:200, Abcam, UK).

Evaluation of DLL4 Staining and Microvessel Density

The scoring procedure was carried out twice by two independent pathologists without any knowledge of the clinical data. The number of tumor exhibiting DLL4 staining was counted under a light microscope at a magnification of×400. Five random fields were examined for each tumor specimen. The dominant staining intensity in both tumor cells and stromal cells was scored as: 0 indicates negative; 1 indicates weak; 2 indicates intermediate; and 3 indicates strong staining intensity. The cell density of the stroma was scored as: 1 indicates low density; 2 indicates intermediate density; and 3 indicates high density. High expression in tumor cells was defined as score≥1.5. Stromal expression was calculated by adding density score (1–3) and intensity score (0–3) before categorizing into low and high expression. High expression in stroma was defined as score≥3.

MVD was counted under a light microscope at a magnification of×400 of CD105-labelled microvessels in areas showing the most intense vascularization, initially located at low magnification. Each positive endothelial cell or group of cells was counted as an individual vessel. The mean vessel count from three fields was adopted as the number of microvessels.

Statistical Analysis

The data were compiled with the software package SPSS, version 19.0. Data were presented as mean±standard error (SE). Fisher's exact and $\chi 2$ tests were used to assess the associations between DLL4, CD105 expressions and clinicopathological parameters. Spearman correlation was used to analysis the correlation between DLL4 and CD105 expressions. Univariate survival analysis was performed according to Kaplan-Meier, and differences in survival curves were assessed with the log-rank test. Multivariate survival analysis was performed using the Cox regression model. P value<0.05 was considered as statistically significance.

Results

Expression of DLL4 in PDAC and Normal Pancreatic Tissues

The results from immunohistochemistry showed that DLL4 proteins were mainly expressed in the cytoplasm and membrane of cancer cells, regardless of tumor histology, as well as in the stromal vascular endothelial cells (Fig. 1). No or weak

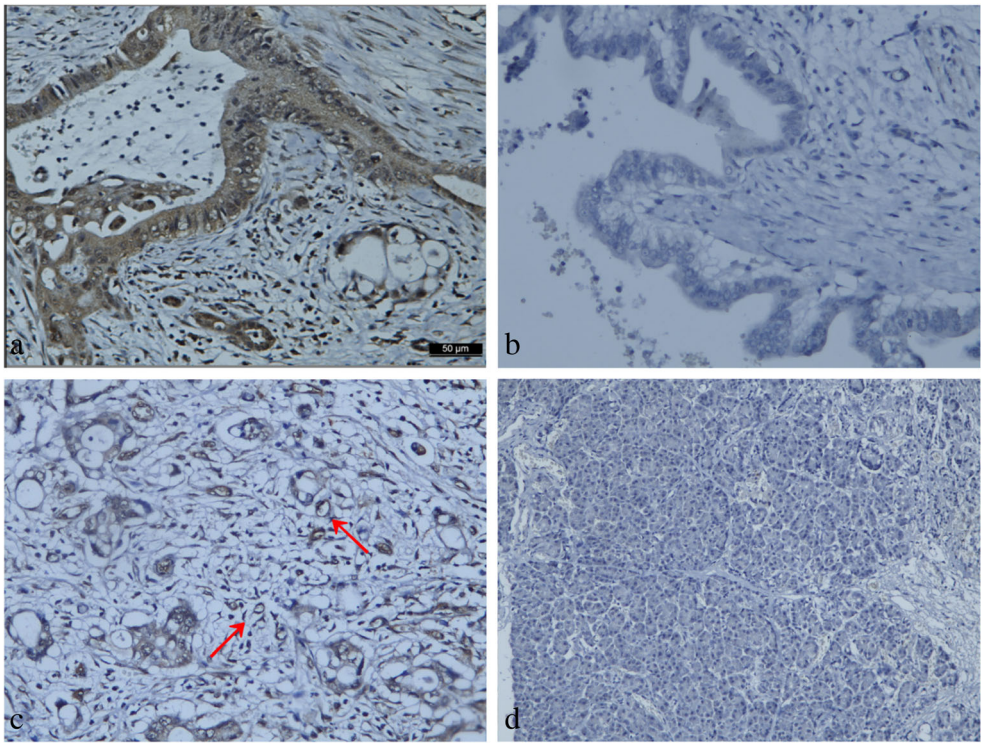

Fig. 1 Immunohistochemical staining for DLL4 in pancreatic ductal adenocarcinoma. A-B: DLL4 was expressed in the cytoplasm and membrane of cancer cells, **a**: score 3; **b**: score 0 magnification×200. **c**: DLL4 was expressed in the stromal vascular endothelial cells (*red arrow*), score density, 3; intensity, 3; magnification×200. **d**: DLL4 was negatively expressed in normal pancreatic tissue; magnification×100

expression was observed in normal pancreatic tissue. High DLL4 expression in tumor cells and stroma was observed in 27(64.3 %) and 22(52.4 %) patients, respectively, higher than that in normal pancreatic tissue (20 %, $\chi 2=8.931$, $P<0.01$; 20 %, $\chi 2=5.8341$, $P=0.026$, respectively).

MVD of CD105-labeled Vessels were Calculated in PDAC

The immunohistochemical expression of CD105 was analyzed in each patient. CD105 expression was mainly observed in endothelial cells of small tumor-associated blood vessels (Fig. 2),

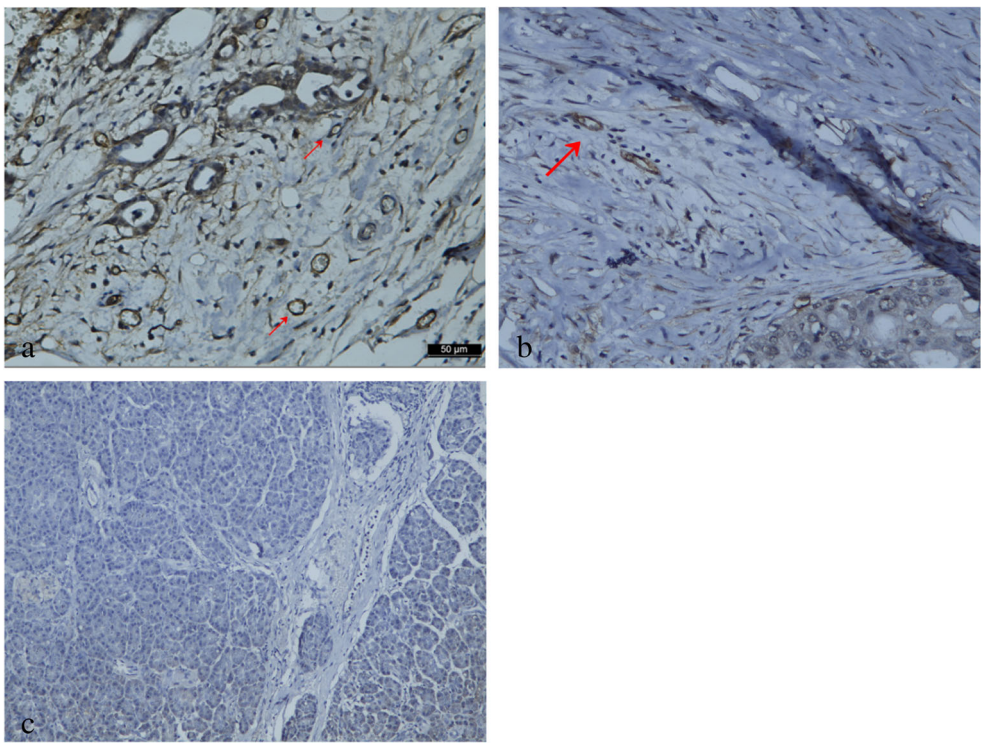

Fig. 2 Immunostaining of CD105 in pancreatic ductal adenocarcinoma. CD105 expression was mainly observed in endothelial cells of small tumor-associated blood vessels (*red arrows*). **a**: high CD105 expression in PDAC; magnification×200. **b**: low CD105 expression in PDAC; magnification×200. **c**: negative CD105 expression in normal pancreatic tissue; magnification×100

whereas its expression in normal, non-tumorous tissue was weak or negative. MVD was also elevated in PDAC (12.78±5.77) compared to normal pancreatic tissue (1.2±0.768)(t=8.90, $P<0.001$).

Clinicopathological Significance of DLL4 and CD105 Expressions in PDAC

To elucidate the significance of DLL4 and CD105 in PDAC, we correlated their expression with major clinicopathological features, respectively. As shown in Table 1, we observed a positive relationship between DLL4 expression and histological grade ($p<0.05$, $p<0.01$), node stage ($p<0.05$, $p<0.01$) and TNM stage ($p<0.05$, $p<0.05$ respectively) in not only the cancer cells but also stroma. While CD105-labeled MVD was significantly correlated with histological grade ($p<0.01$), node stage ($p<0.01$), TNM stage ($p<0.05$) and distant metastasis ($p<0.05$). No associations were observed between CD105-labeled MVD with age, gender, tumor size, tumor stage or tumor size (Table 2).

Correlation between the Expressions of DLL4 and CD105 in PDAC

Sequential sections of pancreatic cancer tissues were subjected to HE staining and immunostaining with DLL4 and CD105 antibodies. The CD105-labeled MVD was found to be significantly higher where DLL4 was highly expressed (Fig. 3). According to the tumor cell DLL4 expression, the pancreatic cancer tissues were divided into two groups, the MVD in DLL4 high expression group was significantly higher than that in DLL4 low expression group (14.75±5.59 vs 9.247±4.32, $P<0.01$). Besides, by spearman correlation test, it was proved that the expression of DLL4 both in tumor cells and stromal vascular endothelial cells was significantly correlated with CD105-labeled MVD (r=0.530, $P<0.01$; r=0.721, $P<0.01$, respectively).

Table 1 Correlation of expression of DLL4 with major clinicopathological parameters

parameters	(n)	Tumor cell DLL4 expression		χ^2	*P* value	Stromal DLL4 expression		χ^2	*P* value
		low	high			low	high		
Age									
<60	11	4	7	0.00	1.00	5	6	0.003	0.839
≥60	31	11	20			13	18		
Sex									
Male	19	8	11	0.617	0.432	9	10	0.288	0.591
Female	23	7	16			9	14		
Tumor site									
Head	19	5	14	0.769	0.381	8	11	0.008	0.929
Body + tail	23	9	14			10	13		
Histological grade									
Poor	12	1	11	3.94	0.047*	1	11	9.236	0.005*
Moderate + well	30	14	16			18	12		
Tumor size (cm)									
≤2.5	14	8	6	4.20	0.040*	8	6	1.750	0.186
>2.5	28	7	21			10	18		
Tumor stage									
T1~T2	9	7	2	6.65	0.01*	6	3	2.652	0.139
T3~T4	33	8	25			12	21		
Node stage									
N0	20	11	9	4.69	0.030*	13	7	7.644	0.006*
N1	22	4	18			5	17		
TNM stage									
I~II	26	13	13	4.54	0.033*	15	11	6.133	0.024*
III~IV	16	2	14			3	13		
Distant metastasis									
M0	34	13	21	0.086	0.770	16	18	1.287	0.431
M1	8	2	6			2	6		

* $P<0.05$ was considered significant

Table 2 Correlation of expression of CD105-labeled MVD with major clinicopathological parameters

parameters	MVD	t	*P* value
Age			
<60	11.9±6.37	−0.585	0.562
≥60	13.10±5.62		
Sex			
Male	12.34±4.58	−0.442	0.661
Female	13.14±6.68		
Tumor site			
Head	13.95±6.61	1.198	0.238
Body+tail	11.817±4.93		
Histological grade			
Poor	17.13±7.09	3.475	0.001*
Moderate+well	11.045±4.14		
Tumor size (cm)			
≤2.5	12.10±8.13	−0.537	0.594
>2.5	13.123±4.29		
Tumor stage			
T1~T2	10.03±4.66	−1.645	0.108
T3~T4	13.532±5.88		
Node stage			
N0	9.91±3.39	3.469	0.001*
N1	15.40±6.29		
TNM stage			
I~II	10.49±3.71	−3.76	0.001*
III~IV	16.503±6.66		
Distant metastasis			
M0	11.88±4.37	−2.17	0.036*
M1	16.60±9.20		

* $P<0.05$ was considered significant

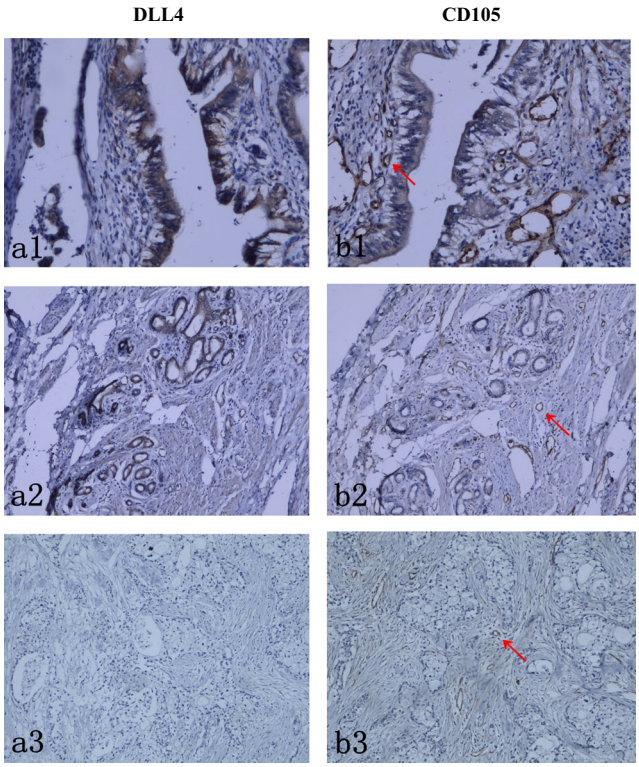

Fig. 3 Sequential sections of pancreatic cancer tissues were subjected to immunostaining with DLL4 (a1-3) and CD105 (b1-3) antibodies. DLL4 expression in tumor cells was positively correlated with CD105-labeled microvessel. a1,a2: strong DLL4 expression in the tumor cells;b1,b2:high microvessel density in the tumor stroma. a3: weak DLL4 expression in the tumor cells;b3: low microvessel density in the tumor stroma

DLL4 and CD105 Expressions are Important Predictive Markers for Poor Prognosis of PDAC

We analyzed the prognostic effects of DLL4 expression and CD105-labeled MVD in PDAC using Kaplan–Meier method and log-rank test. The patients were divided into high/low expression groups according to the expression of DLL4 or using the median CD105-MVD (>12.8 or <12.8 vessels per field, $n=21$ in each group). Patients with high DLL4 expression (both in tumor cell and stroma) and CD105-MVD were correlated to shorter overall survival ($\chi^2=9.19$, $P<0.01$; $\chi^2=7.511$, $P<0.01$; $\chi^2=6.205$, $P=0.013$, respectively Fig. 4).

Discussion

Since Folkman proposed the theory of tumor angiogenesis and noted that tumor cells cannot develop in the absence of neovascularization [3], research on this subject has increased rapidly. Pancreatic carcinoma is one of the most aggressive types of cancer, highly invasive, rapidly reaching the stage of metastasis, but studies of angiogenesis in pancreatic cancer are rare [15]. Previously, expression of DLL4 in cancerous tissue was thought to be limited to the stromal vascular endothelial cells. However, recent reports have shown a notable expression of DLL4 in the tumor cells [16]. To better understand the role of DLL4 in angiogenesis, DLL4 protein expression both in tumor cells and stroma was analyzed in our study, and high expressions were observed in 64.3 and 52.4 % patients of PDAC. It is a little higher than that in gastric cancer reported by Sumiya Ishigami et al. [17]. Recent findings suggested that DLL4 expression played a key role in tumor angiogenesis, which was assessed by CD105 immunostaining in our study, considering its specific expression in new blood vessels. Patricia Kuiper et al. analyzed the expression of CD105 and CD31 in gastroenteropancreatic neuroendocrine tumors, and they observed that CD105 staining was limited to angiogenic vessels, whereas CD31 stained both old and new blood vessels in tumor tissue [10].

By observing the sequential sections of pancreatic cancer tissues which were subjected to immunostaining with DLL4 and CD105 antibodies, we found that the CD105-labeled MVD was significantly higher where DLL4 was highly

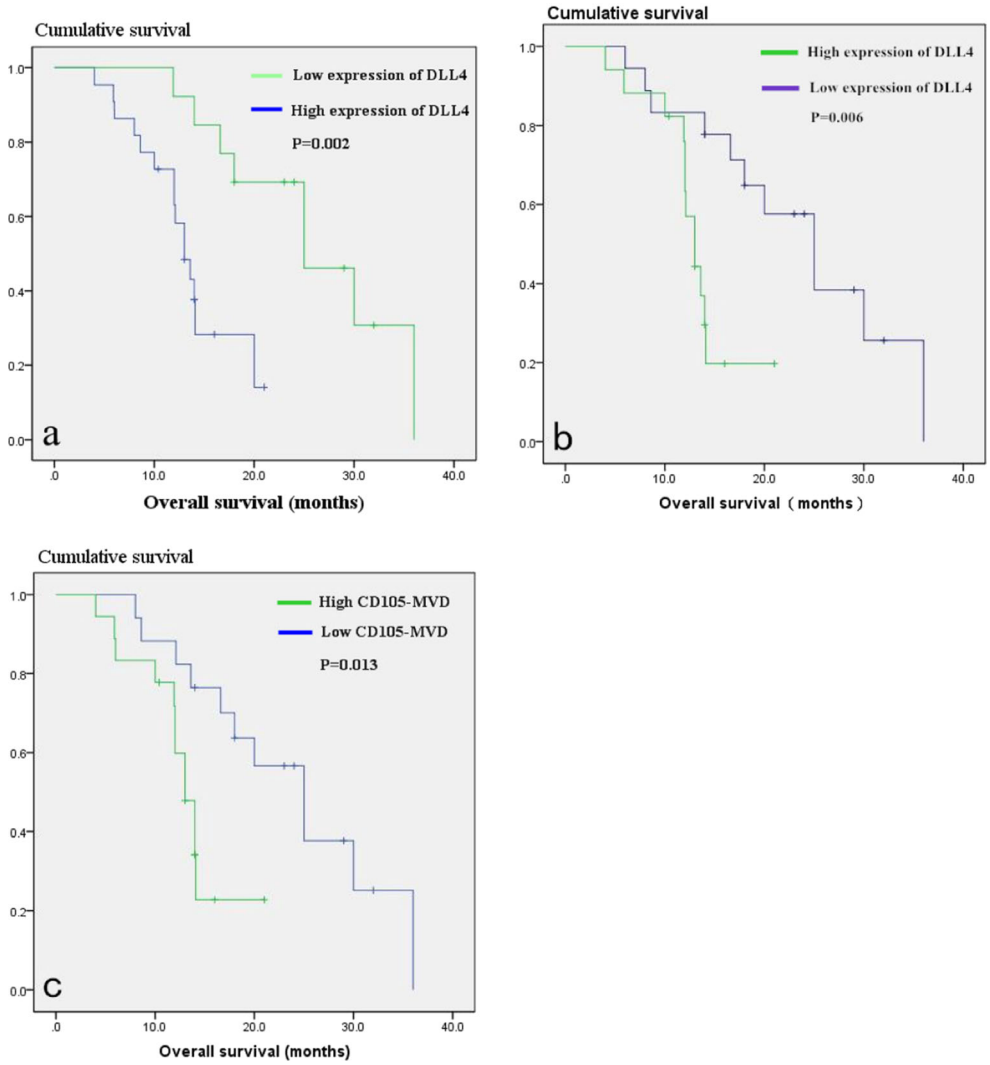

Fig. 4 Kaplan–Meier survival curves of PDAC were stratified by:**a**. DLL4 expression of tumor cells; **b**. DLL4 expression of stromal vascular endothelial cells; **c**. CD105-labeled MVD

expressed (Fig. 3). Furthermore, high expressions of both DLL4 and CD105 were significantly correlated with TNM stage, histological grade and node stage of PDAC, and the survival analysis revealed that patients with high DLL4 and CD105 expressions had significantly poorer overall survival when compared with low DLL4 and CD105 expression patients. Considering the consistent expression of DLL4 and CD105, it is reasonable to explore the positive correlation between them, which was confirmed by Spearman correlation test. However, by multivariate analysis, DLL4 and CD105 were not found to be an independent prognostic marker, which may be influenced by the strong association with lymph node metastasis. Besides, we should note that DLL4 expression in other cancers is not always associated with a poor clinical prognosis. For example, Donnem et al. demonstrated that DLL4 positivity was a good prognostic marker in lung adenocarcinoma [18]. So organ specificity in the evaluation of DLL4 expression of the tumor tissues should be considered.

Notch signaling is an evolutionarily conserved intercellular signaling pathway that plays numerous crucial roles in vascular development and physiology. Natalie M. Kofler et al. suggested a role for Notch1 signaling in “tip” versus “stalk” cell differentiation, thus leading to the maturation of endothelial cells and formation of functional blood-carrying vessels, although the vascular density was reduced [19]. So it is rational to treat malignant tumor with a method of blocking Notch signaling by inducing dysfunction of blood vessels [20]. However, it is still rarely studied how overexpression of DLL4 in tumor cells affect the growth of endothelial cells. In addition to classic pathway that is mediated by cell-cell contact, Helen Sheldon et al. recently came up with a new mechanism for Notch signaling to endothelium by DLL4 incorporation into exosomes [21]. As a newly found vesicle, exosome plays an important role in intercellular communication. Research has presented evidence that exosomes can transfer the Dll4 protein to endothelial cells and incorporate it into their cell membrane. Interestingly, the transference results in an inhibition of Notch

signaling, and followed by the induced tip cell phenotype and a significant increase in vessel density. Besides, Thomas L et al. revealed that overexpression of endogenous DLL4 in Drosophila inhibited Notch signaling by making the signal-receiving cell refractory to the signal [22], which was further demonstrated by others that Notch receptor is degraded rather than internalized and recycled to the surface at a later time point [21]. Consequently, the accumulation of DLL4 in tumor cells and endothelium can confer a tip cell phenotype on the endothelial cell and induce a high vascular density. In combination with previous studies, our findings suggest the positive correlation between DLL4 expression and CD105-labeled MVD.

In conclusion, we demonstrated that both DLL4 and CD105 were overexpressed in a large proportion of patients with PDAC, and the expression of DLL4 was positively correlated with CD105-labeled MVD. In addition, high DLL4 and CD105 expression correlated with the poor clinical outcome and overall survival in patients with PDAC. Therefore, targeted inhibition of DLL4/Notch signaling might be a new idea for therapy of PDAC. Certainly, further strong supports from basic investigations are still needed.

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Conflict of Interest Authors declare that they have no conflict of interest.

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