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Original Article

Delta-like ligand 4 (Dll4) predicts the prognosis of clear cell renal cell carcinoma, and anti-Dll4 suppresses tumor growth *in vivo*

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Abstract: The Delta-like ligand 4 (Dll4) and Notch signaling pathway plays a key role in embryonic vascular development and tumor growth. In this study, we measured the expression of Dll4 in clear cell renal cell carcinoma (ccRCC) and explored the correlation between Dll4 and ccRCC. We used sh-Dll4 treatment in a nude mouse model to observe the effect that inhibition of the Dll4/Notch pathway had on angiogenesis and vasculogenesis. We found up-regulation of Dll4 to be closely correlated with distant metastasis and worse overall survival. Cox regression analysis showed that Dll4 might be a prognostic marker of ccRCC. Blockade of Dll4/Notch signaling inhibited tumor growth in the mouse model via anti-angiogenesis and anti-vasculogenesis effects. We concluded that Dll4 might be a novel therapeutic target for the treatment of ccRCC.

Keywords: Angiogenesis, anti-Dll4, clear cell renal cell carcinoma, Delta-like ligand 4, prognosis, tumor growth

Introduction

The Notch signaling pathway is a conserved pathway that participates in diverse cellular processes including cell differentiation, apoptosis, and stem cell maintenance [1]. The mammalian Notch receptors include four heterodimeric transmembrane receptors, Notch1-4, and five transmembrane ligands, Delta-like ligand 1, 3, and 4 (Dll1, 3, and 4), and Jagged 1 and 2.

Canonical Notch signaling has been clearly illustrated. The Notch pathway is activated by ligand binding, which induces two cleavage events. Two sequential proteolytic cleavages release the Notch intracellular domain (NICD), which then translocates to the nucleus, where it first combines with Recombination Signal Binding Protein 1 for J-κ (RBP-Jκ, also known as CSL) and then with one of three Mastermind-like proteins (MAML1-3). The RBP-Jκ/MAML complex recruits other co-activators, including p300, and releases co-repressors to induce transcription of downstream Notch effectors [2]. The Notch effectors are themselves basic helix-loop-helix transcription factors, including Hairy and Enhancer of Split (Hes) and Hairy-related (Hey) family members [3]. Abundant studies have indicated that irregular Notch activities are involved in the etiology of human cancers, such as melanoma, breast cancer, glioma, pancreatic cancer, cervical cancer, medulloblastoma, and mucoepidermoid carcinomas [4-7].

Delta-like ligand 4 (Dll4) is essential to normal vessel formation [8]. Previous studies have associated Notch/Dll4 signaling with regulation of arterial identity and angiogenic sprouting, which determines arteriogenesis and collateral vessel formation [9, 10]. Dll4 has such a critical role in vascular development that in mice of most genetic backgrounds Dll4 heterozygosity causes embryonic lethality due to "profound vascular defects" [10, 11].

Dll4 is over-expressed in the endothelial cells and pericytes/vascular smooth muscle cells (pericytes/VSMC) in most tumors [12-14]. Dll4/Notch signaling has been shown in multiple mouse models to be involved in tumor growth

Table 1. Clinical Characteristics with ccRCC Patients

Parameter	No. of Patients
Age; Mean±SD	61±10.9
Gender	
Male	21
Female	13
Furham grade	
1	7
2	17
3	10
4	0
Pathologic tumor classification	
pT1	2
pT2	9
pT1+pT2	11
pT3	16
pT4	7
pT3+pT4	23
Lymph node metastasis	
Negative	21
Positive	13
Distant metastasis	
Negative	16
Positive	18
Overall survival	
Alive	7
Dead	25
Recurrence	
No, n=14	12
Yes, n=20	22

through mediating angiogenesis and vasculogenesis. Therapies that target might be an effective approach for the treatment of cancer.

However, it is not presently known whether inhibition of Dll4 can inhibit tumor growth, and the detailed function of Dll4 in ccRCC has not been clarified. We explored the association between Dll4 and ccRCC, and investigated the effect of Dll4 inhibition in a mouse model of ccRCC.

Materials and methods

Tissue samples

Samples were taken from 34 patients (21 men and 13 women) with pathologically confirmed ccRCC (Shanghai 10th People's Hospital, Shanghai, China). The mean age was 61±10.9 years (**Table 1**). The patients were classified according to World Health Organization criteria and were staged according to the tumor–lymph node–metastasis (TNM) classification system. The pathology for all patients was clear cell renal carcinoma. All patients gave their written informed consent before samples were taken at the Shanghai 10th People's Hospital of Tongji University.

Cell culture and mouse models

CAKI-1 human renal cell carcinoma cells were grown in McCoy's 5a Medium (Gibico, 16600-082, US) supplemented with 10% fetal bovine serum and 1 mmol/L penicillin–streptomycin (Thermo Scientific, Inc., Shanghai, China). All animal procedures were approved by the Institutional Animal Care and Use Committee at Shanghai 10th People's Hospital of Tongji University. For CAKI-1 transplant experiments, BALB/c nude mice were purchased from B&K Universal Group Limited (Shanghai, China).

CAKI-1 cell transplant

Nude mice were subcutaneously injected with 2×10^7 CAKI-1 cells (CAKI-1 cells in 100 μL PBS) subcutaneously injected with 2×10^7 CAKI-1 cells (CAKI-1 cells in 200 μL PBS) on the right flank and 2×10^7 CAKI-1 cells (CAKI-1 cells in 200 μL PBS) on the left flank. Short hairpin RNA (shRNA) targeting mouse Dll4 (sh-Dll4) (Shanghai Integrated Biotech Solutions Co., Ltd., Shanghai, China) was made by annealing the DNA oligomer 5′-GATCCAGTCACTTGGGTGC-AGTGTTTCAAGAGAACACTGCACCCAAGTGACTT-TTTTTGGAAA-3′ to its complimentary sequence. Polyethylenimine (PEI) was purchased from Polyplus (*in vivo*-jetPEI, catalog number: 201-50G; ILLKIRCH, France). PEI/shRNA complexes were prepared at a PEI:DNA phosphate ratio of 1:8 (PEI: 2.5 μL *vs.* Nucleic acid: 20 μg). The PEI/sh-RNA was diluted in 5% glucose so that the final volumes (200 μL/each subcutaneous tumor) were the same for each solution. Multiple 20 μL injections of the PEI/shRNA complexes were performed at different sites of the tumor to avoid reflux.

shRNA treatment in vivo

Six days after nude mice had been subcutaneously injected with 2×10^7 CAKI-1 cells (CAKI-1 cells in 200 μL PBS) on the right flank and 2 ×

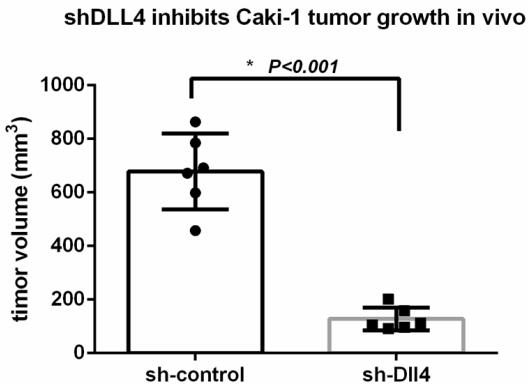

Figure 1. Dll4 up-regulated in the enrolled ccRCC tissues confirmed by Quantitative Reverse Transcription PCR. Dll4 was up-regulated in the enrolled ccRCC tissues. Dll4 expression fold changes in clear cell renal cell carcinoma compared with adjacent normal tissues: DLL4 significantly up-expression in ccRCC (*P=0.0003).*

10^7 CAKI-1 cells (CAKI-1 cells in 200 μL PBS) on the left flank. (N=6)., tumors were palpable, and intratumoral injections were begun (4 injections per week for 4 weeks). Left side tumors were injected with PEI/sh-control, while right side tumors were injected with PEI/sh-Dll4. Each tumor received a total of 200 μL PEI/RNA mixture (20 μg RNA) per treatment. Four weeks later (12 total treatments), final tumor volumes were recorded, mice were killed, and tumors were harvested for examination by immunohistochemical staining.

Quantitative reverse transcription polymerase chain reaction

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed in triplicate with an Applied Biosystems Prism 7900 Fast Sequence Detection System using TaqMan universal PCR master mix according to the manufacture's protocol (Applied Biosystems Inc., Foster City, CA, USA). TaqMan probes and primers were purchased from Applied Biosystems Inc. Dll4 primers: forward: 5′ACTCACCACTCTCCGTGCAAGAAT3′; reverse: 5′ TGTGTAACAGCCGGTTCACTCCTT3′. Human β-actin was used as an endogenous control (forward: 5′ACCCAGCACAATGAAGATCA3′; reverse: 5′CGATCCACACGGAGTACTTG3′). Levels of RNA expression were determined by using the 7900 Fast System SDS software package (version 1.3.1; Applied Biosystems Inc.).

Western blot analysis

Total cell protein (40 μg) was used for western blot analysis. Samples were resolved in 4% to 20% Precise Protein Gels (catalog:P0012A, Beyotime, Shanghai, China) and transferred to nitrocellulose membranes. The membranes were immersed for 1 h in 0.3% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween 20, and were probed overnight at 4°C with primary polyclonal and monoclonal antibodies against Notch1 (catalog number: ab65297; Abcam, UK), Hes1 (catalog number: 2922-1; Epitomics, Burlingame, CA, USA), Hes2 (catalog number: ab134685; Abcam), Hey1 (catalog number ab22613; Abcam), Hey2 (catalog number: ab25404; Abcam), and internal control β-actin (catalog number 1854-1; Epitomics).

To confirm the expression of Dll4 in the mouse model following sh-Dll4 treatment, we used anti-Dll4 antibody (catalog number: ab7280; Abcam). Blots were washed in TBS that contained 0.1% Tween 20, and were labeled with horseradish peroxidase-conjugated secondary anti-rabbit antibody (Cell Signaling Technology, Shanghai, China). Proteins were enhanced by chemiluminescence for visualization. The reported protein expression levels are expressed relative to β-actin levels.

Immunohistochemistry

Frozen slides of specimens of the tumors harvested from the xenograft models were permeabilized by submersion in acetone for 10 min followed by incubation in PBS. Paraffin-embedded sections were dewaxed, rehydrated, and incubated with the anti-CD31 monoclonal antibody QBEnd10 (Novocastra, Newcastle, UK), anti-Dll4 antibody (catalog number: ab7280; Abcam), and anti-alpha smooth muscle Actin antibody (catalog number: ab5694; Abcam).

Statistical analysis

RT-PCR results were analyzed by using the ddCt method (Applied Biosystems, User Bulletin No. 2). Chi-square test (χ^2 test) and Spearman's rank correlation coefficient were used to analyze the associations between Dll4 expression and ccRCC clinical features. The Cox regression model was used to assess the influence of Dll4 on overall survival of patients with ccRCC.

Table 2. Activation of Dll-4 mRNA in clear cell Renal cancer Patients

Parameter	ccRCC (n=27)	Normal tissues (n=7)	P
Age; Mean±SD	62.8±10.8	64.7±9.1	0.67
Gender			
Male, n=21	16	5	0.68
Female, n=13	11	2	
Furham grade			
1	6	1	
2	13	4	0.82
3	8	2	
4	0	0	
Pathologic tumor classification			
pT1, n=2	1	1	
pT2, n=9	8	1	
pT1+pT2	9	2	Reference
pT3, n=16	13	3	
pT4, n=7	5	2	
pT3+pT4	18	5	0.52
Lymph node metastasis			
Negative, n=21	16	5	Reference
Positive, n=13	11	2	0.56
Distant metastasis			
Negative, n=16	10	6	Reference
Positive, n=18	17	1	0.02*
Overall survival			
Alive, n=9	4	5	Reference
Dead, n=25	23	2	0.002**
Recurrence			
No, n=12	8	4	Reference
Yes, n=22	19	3	0.175

Activation of Dll4 was not associated with grade and pTNM of clear cell renal cell carcinoma, while correlated with distant metastasis and overall survival (*P<0.05, **P<0.01).

Immunohistochemistry results were analyzed with mean integrated optical density (IOD) by Image Pro-Plus 6.0. All statistical analyses were performed using IBM SPSS Statistics (Version 20.0, IBM Corp., Almonk, NY, USA) and Graphpad Prism 6.0 software (GraphPad Software, Inc., California, USA).

Results

Dll4 was up-regulated in the samples of 27 out of 34 patients

We obtained specimens from 34 patients with ccRCC, and performed quantitative qRT-PCR to detect Dll4 mRNA expression. These specimens included primary and metastatic tumor samples. Dll4 mRNA expression was higher in renal cancer tissues than in matched normal kidney tissues in 27 of 34 paired tissue specimens (79.4%) of normal kidney and renal cancer (**Figure 1**).

Correlation between Dll4 expression level and clinical characteristics

Dll4 mRNA expression was classified into two categories based on qRT-PCR results: the higher expression group and the lower expression group. We investigated the relation between Dll4 mRNA expression and clinical parameters, including gender, Fuhrman grade, tumor (pT), lymph node status (N), metastasis status (M) classification, and patients' survival. Results demonstrated that Dll4 expression was significantly associated with distant metastasis and worse overall survival (**Table 2**). There was no significant correlation between Dll4 expression and gender, Fuhrman grade, and lymph node metastasis.

High expression of Dll4 predicted worse prognosis

Results of overall survival (OS) are listed in **Table 3**. Significantly higher expression of Dll4 (HR: 5.50; 95% CI: 1.29-23.48, *P*=0.021) was associated with decreased OS compared with lower Dll4 expression (**Figure 2**). Using the propensity adjustment method in the multivariable analysis of OS, the relationships remained similar, with a decrease in OS with up-regulation of Dll4 (HR: 5.49; 95% CI: 1.21-24.87, *P*=0.027). A high expression of Dll4 may predict a worse prognosis (**Table 3**, **Figure 2**).

shDll4 inhibited tumor growth in vivo

Tumor volume and weight was measured immediately before mice were killed. Tumors treated

Table 3. Univariate and multivariate models for overall survival. Both univariate (HR: 5.50; 95% CI: 1.29-23.48, *P=0.021<0.05*) and multivariate Cox proportional model (HR: 5.49; 95% CI: 1.21-24.87, *P=0.027<0.05*) revealed up-regulation of Dll4 were associated with decreased OS. A high expression of Dll4 may predict a bad prognosis

	Univariate		Multivariate			
	P value	HR	*P* value	HR	95.0% CI	
					Lower	Upper
sex	0.271	0.611	0.467	0.712	0.285	1.780
age	0.138	0.968	0.178	0.972	0.932	1.013
recurrence	0.801	1.108	0.977	1.013	0.428	2.397
Distant metastasis	0.922	0.961	0.770	0.876	0.361	2.125
Dll4	*0.021**	5.490	*0.027**	5.487	1.213	24.815

HR = hazard ratio; CI = confidence interval; *indicates statistically significant *p* value of <0.05.

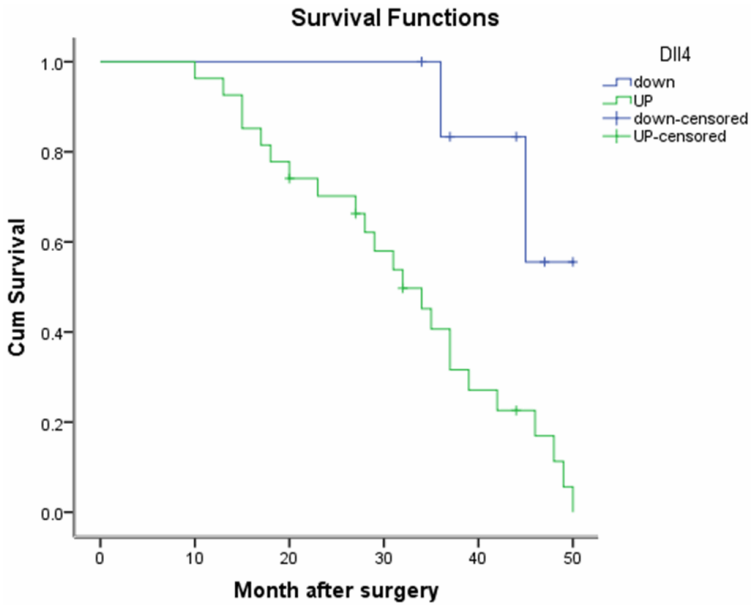

Figure 2. Survival curve of high vs. low Dll4 expression ccRCC patients. High expression of Dll4 is associated with worse prognosis (Log-rank (Mantel-Cox) test: *P=0.0096*). Cox proportional hazards models: Overall survival by Dll4 expression, adjusted for covariates in multivariate. (HR: 5.49; 95% CI: 1.21-24.87, *P=0.027*).

with PEI/sh-Dll4 were significantly smaller (127.5 mm^3) and lighter than those treated with PEI/sh-control (687.3 mm^3, *P*<0.001) (**Figure 3**).

Intratumoral PEI/sh-Dll4 inhibited expression of Dll4 on tumor vessels

We used intratumoral injections of the nonviral vector polyethylenimine (PEI) carrying shRNA against Dll4 (sh-Dll4) or a non-targeting shRNA control (sh-control) to inhibit expression of Dll4 on tumor vasculature. Nude mice were subcutaneously injected with CAKI-1 cells on both the left and right flanks. When tumors were palpable (day 6), intratumoral injections of sh-Dll4 (right side) or sh-control (left side) were performed 4 times a week for 4 weeks. The final tumor volumes were measured, mice were killed, and tumors were harvested for immunohistochemical detection.

We first confirmed that Dll4 was inhibited following injection of PEI/sh-Dll4. In tumors treated with PEI/sh-Dll4, Dll4 was dramatically reduced compared to control (**Figure 4**). In the PEI/sh-Dll4 treated tumors, very little Dll4 (IOD; sh-control *vs.* sh-Dll4, *P*=0.009<0.05) and endothelial cell marker CD31 (IOD; sh-control *vs.* sh-Dll4, *P*=0.005< 0.01) was observed surrounding the vessels. To determine whether the loss of Dll4 correlated with an overall reduction in the number of pericytes/VSMC, we examined the expression of α-SMA. α-SMA expression was significantly reduced in the PEI/sh-Dll4 treated tumors (IOD; sh-control *vs.* sh-Dll4, *P*<0.0001).

Inhibition of Dll4 down-regulated downstream effectors of Notch signaling: Notch1, Hes1, and Hey1

Western blot analysis was used to determine which key components of the Notch pathway were influenced by inhibiting Dll4. Key downstream proteins including Notch1, Hes1, Hey1, Hes2 and Hey2 proteins were detected. Silencing of Dll4 decreased the levels of Notch1, Hes1 and Hey1 (**Figure 5**). There was no significant change in the expression of Hes2 and Hey2 protein (**Figure 5**).

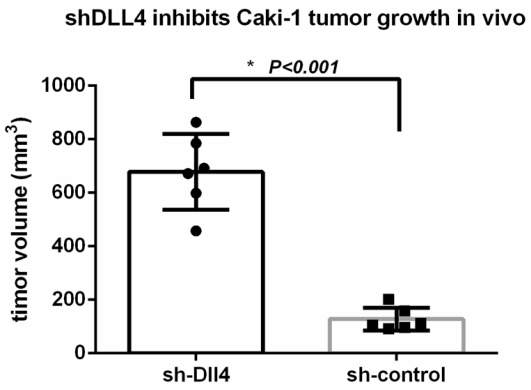

Figure 3. Tumor growth after sh-Dll4 treatment. Nude mice were injected with bilateral subcutaneous CAKI-1 cells. When tumors were palpable, every other day intratumor injections of PEI/shDLL4 (right side tumors) or PEI/sh-control (left side tumors) were performed. 4 weeks later, tumor volumes were measured and mice were sacrificed. Tumor volumes are displayed as matched pairs of right and left side tumors from the same mouse. Student's paired t-test, t=10.2, **P<0.0001*.

Discussion

Notch signaling has been proven to play a significant role in tumor cell proliferation *in vitro* and tumor growth *in vivo*. Because Dll4 is one of the Notch signal ligands, inhibition of Dll4 could possibly destroy the angiogenesis in tumors; however, the function of Dll4 in ccRCC was unknown. In the present study, we confirmed that Dll4 was up-regulated in ccRCC and was correlated with distant metastasis and overall survival. High expression of Dll4 predicted a worse prognosis. In addition, inhibition of Dll4 *in vivo* significantly suppressed ccRCC growth.

ccRCC is the most common type of malignant renal tumor, accounting for 70% to 80% of cases, and occurs in both hereditary and sporadic forms. ccRCC is resistant to chemotherapy and radiotherapy [15]. Anti-angiogenic therapy has been found effective in ccRCC; however, some patients are inherently resistant to these treatments, and most patients acquire resistance over time [16].

The Notch pathway deeply influences stem cell maintenance, development, and cell fate [1], and recently, Notch signaling has been found to be involved in angiogenesis in numerous cancers, promoting cell survival and anti-angiogenic resistance [17, 18]. The key components of the pathway in mammals include four single-pass transmembrane receptors (Notch1-4) and five canonical DSL (Delta/Serrate/Lag2) ligands (Dll1, 3, and 4; Jagged 1 and 2) [2]. Therefore, the Notch pathway depends on direct cell–cell interactions for signal propagation. Proteolytic activation of the receptor is triggered through ligand binding to the extracellular domain of Notch. Gamma-secretase complex-mediated cleavage and ADAM metalloproteinase sequentially generate a juxtamembrane region cleavage of Notch. The Notch intracellular domain (NICD) then translocates to the nucleus where it interacts with the RBP-Jκ transcription factor and induces downstream Notch target genes such as those for basic helix-loop-helix proteins Hes and Hey [3].

Abundant studies in humans and mice have demonstrated that Dll4 is strongly expressed by the tumor vasculature and generally not by the tumor cells themselves. In various mouse models, high Dll4 expression was observed in the majority of tumor vessels, compared with significantly lower vascular expression in adjacent normal tissues [19, 20]. Dll4 expression is up-regulated in the renal cell cancer endothelium but not in the normal renal vasculature [12]. Strong Dll4 expression is generally not detected in the tumor parenchyma, although sporadic tumor cell expression is observed in colorectal and brain tumor samples [21, 22].

In our study, high expression of Dll4 predicted poor prognosis in ccRCC, which is accordant with reports for nasopharyngeal carcinoma, pancreatic cancer, and breast cancer [23-25]. Our study is the first to demonstrate that elevated Dll4 expression is implicated in poor prognosis in renal cancer. Strong expression of Dll4 significantly correlated with distant metastasis and reduced overall survival. A limit of our study is the small number of patients enrolled; a large-scale cohort will be required for future studies.

Previous studies have identified the Dll4/Notch pathway as being involved in angiogenesis and vasculogenesis. In the present study, inhibition of Dll4 with shRNA led to decreased expression of Hey1, which is vital for endothelial cell proliferation, migration, and network formation [12]. Neutralization of Dll4 leads to hyper-proliferation of endothelial cells, and causes defective

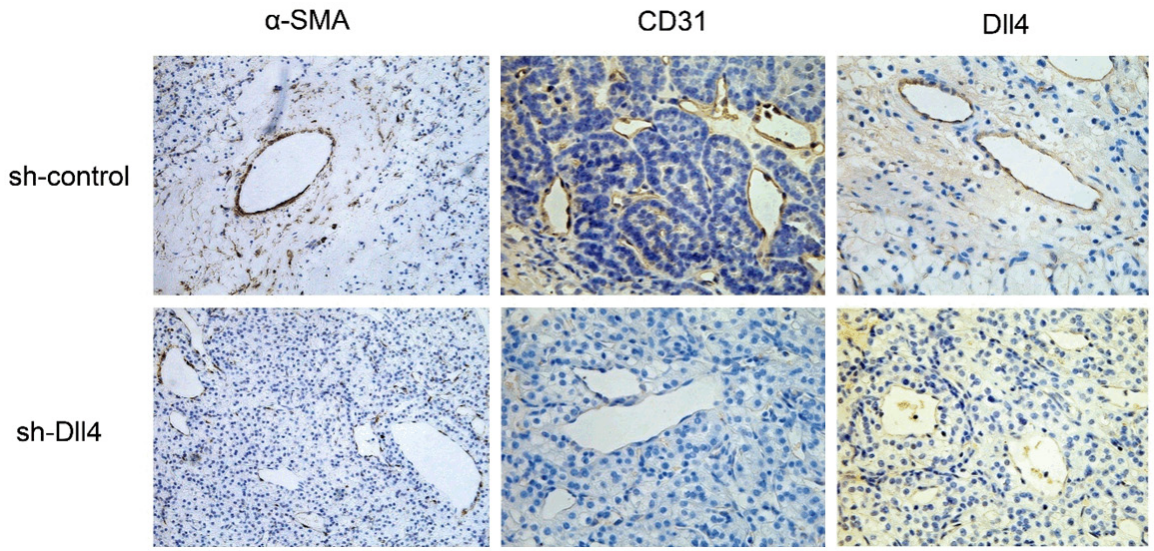

Figure 4. Intratumor PEI/shDLL4 inhibits expression of DLL4 on tumor vessels, endothelial cell markers CD31 and the pericytes/vSMC markers α-SMA are reduced in shDLL4 treated tumors (magnification, × 200); CAKI-1 cells were implanted bilaterally in nude mice. Tumors were treated with intratumoral injections of PEI/shDLL4 (right side tumors) or PEI/sh-control (left side tumors) for 4 injections per week for 4 weeks. Tumors were then harvested and examined for the presence of CD31 to identify endothelial cells in combination with α-SMA, Dll4 to identify endothelial cells and pericytes/vSMC.

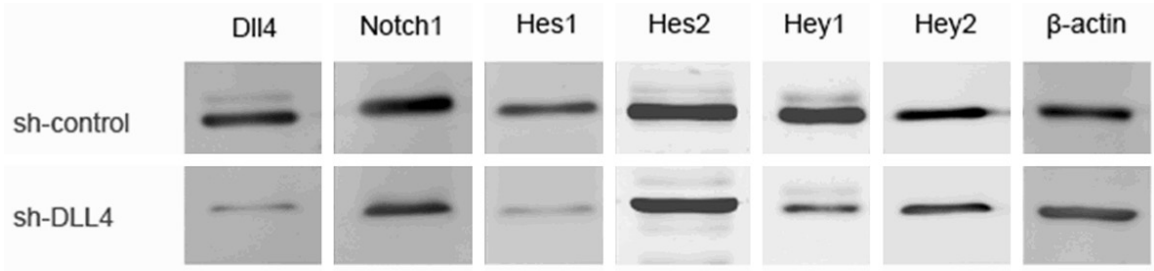

Figure 5. Downstream target protein of Dll4/Notch pathway were detected by Western blot. Inhibition of DLL4 by sh-RNA decreased levels of the Notch1, Hes1 and Hey1. There were no significant changes in the expression of Hes2 and Hey2 protein.

cell fate specification or differentiation, finally causing increased vascular density and vascular sprouting in tumors [8, 12, 20]. Surprisingly, the vascular overgrowth phenotype is associated with a low viable tumor cell content, consistent with poor vascular function, and results in tumor growth inhibition in a variety of human and rodent tumor models [8, 20, 26]. Vessel perfusion studies have demonstrated that the hyper-sprouting tumor vasculature is non-functional; hence, inhibition of Dll4 results in increased levels of hypoxia [27]. Renal cell cancer growth inhibition was also observed in our research due to *in vivo* interference the Dll4 mRNA.

Gene targeting studies demonstrate that Notch1 is the primary Notch receptor during developmental angiogenesis [28], and it seems that Notch1 is also the predominant mediator of Dll4/Notch signaling in the tumor vasculature. Notch1-specific inhibitory antibodies exhibited similar effects on tumor vasculature as seen following blockade of Dll4 [29]. In our study, *in vivo* inhibition of Dll4 by shRNA down-regulated Notch1 and suppressed the downstream signaling through Hes1, Hey1.

Dll4 expression has also been analyzed in the formation of bone marrow-derived pericytes/vascular smooth muscle cells. Dll4 gene expression silenced by shRNA is associated

with decreased numbers of bone marrow-derived cells in tumor vessels and decreased numbers of α-SMA(+), desmin(+), and NG2(+) pericyte markers, as well as increased tumor hypoxia *in vivo* [30]. Immunohistochemistry demonstrated that the endothelial marker CD31 and perivascular marker α-SMA were significantly decreased by inhibition of Dll4. Thus, silencing of Dll4 could suppress renal cell carcinoma growth *in vivo* by inhibition of angiogenesis and vasculogenesis. Dll4 might be a promising therapeutic target for the inhibition of vasculogenesis and angiogenesis.

It is noteworthy that some studies have demonstrated that Dll4 expression in tumor vessels appears to be directly regulated by VEGF and bFGF, and that Dll4 expression levels in tumors correlates with expression levels of VEGF [13, 20]. It has been found that Dll4 and Notch4 are activated by VEGF in the determination of arterial endothelial cell fate during arterial endothelial differentiation in zebra fish [31]. bFGF is also produced by renal cancers. Furthermore, the combination of VEGF and bFGF leads to a synergistic induction of Dll4, Notch1, and Notch4 in human umbilical vein endothelial cells [12, 32]. Thus, Dll4/Notch signaling critically regulates endothelial and smooth muscle cells induced by VEGF, and Dll4 in particular has been identified as a novel therapeutic target in tumor angiogenesis.

A variety of inhibitory agents of Dll4/Notch signaling (including anti-Dll4 antibodies, DNA vaccination, soluble Dll4-Fc, Notch-Fc decoys, Notch antibodies, and gamma-secretase inhibitors), each of which yields different effects, have been studied in preclinical tumor models [8, 20, 33-36].

Recent studies have found that Dll4-Fc can suppress liver metastasis of small cell lung cancer cells in a mouse model through the down-regulation of the NF-κB activity [36]. Specific targeting of Dll4 with anti-Dll4 antibodies did not induce apparent weight loss or animal death in tumor-bearing mice, unlike with gamma-secretase inhibitors that broadly block all Notch signaling and lead to gastrointestinal toxicity [37].

It is also noted that high expression of Dll4 is involved with resistance to chemotherapy and radiotherapy [17, 34]. Dll4/Notch blockade by Dll4-specific blocking monoclonal antibodies in combination with ionizing radiation might suppress tumor growth by promoting nonfunctional tumor angiogenesis and extensive tumor necrosis [34].

In spite of the confirmed aberrant expression of Dll4 in many tumor samples, including bladder, colon, brain, and breast cancers, it is currently not known which types of cancer would benefit from anti-Dll4 therapy. Most studies have shown that inhibition of Dll4 has a potent growth inhibitory effect on tumors that are resistant to anti-VEGF therapies [8, 20]. Inhibition of Dll4 might provide an important approach to tackling angio-therapy resistance in ccRCC. However, the efficacy, side-effects, and toxicity of a combination of anti-Dll4 and anti-VEGF need to be carefully examined.

In conclusion, Dll4 expression is up-regulated in ccRCC patients, and elevated Dll4 expression predicts poor prognosis. In a mouse model of ccRCC, blockade of Dll4 with shRNA impaired tumor growth by anti-angiogenesis and anti-vasculogenesis. Dll4 might be an attractive therapeutic target for the treatment of ccRCC.

Disclosure of conflict of interest

None.

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