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Aberrant expression and association of VEGF and Dll4/Notch pathway molecules under hypoxia in patients with lung cancer

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Summary. Tumor angiogenesis plays important roles in the pathogenesis and prognosis of lung cancer. Both vascular endothelial growth factor (VEGF) and Dll4/Notch pathways are critical for angiogenesis, whereas their relationship under hypoxia in lung cancer remains unknown. Thus, in the present study, we evaluated the expression of VEGF and Dll4/Notch signaling molecules, and assessed their association with the microvessel density (CD31) and hypoxia (HIF1a) in lung cancer and normal lung tissues using immunohistochemical and Real-time RT-PCR techniques. Then, we investigated the biological function of Dll4 by transfecting Dll4 into HUVECs. In lung cancer tissues, Notch pathway molecules (HES1) and VEGF pathway molecules (VEGFR1 and VEGFR2) were significantly up-regulated, while the ratio of VEGFR1/VEGFR2 was decreased. CD31 and HIF1a were also found to be elevated in lung cancer. VEGFR1 was negatively correlated with Notch1 while positively correlated with Dll4. CD31 was positively correlated with HIF1a but negatively correlated with VEGFR1. Moreover, HIF1a was nearly positively correlated with HES1 in lung cancer tissues. After transfection, Dll4, Notch1 and VEGFR1 were up-regulated while VEGF and VEGFR2 were down-regulated in Dll4-transfected HUVECs compared with controls. Also, our findings suggest that the expression of VEGF and VEGFR2 increased gradually with the disease progression of lung cancer. In summary, VEGF and Notch signaling pathway molecules were overexpressed in lung cancer, which positively correlates with hypoxia (HIF1a) and angiogenesis (CD31). There might be a negative feedback loop between VEGF and Dll4/Notch signaling pathway in lung tumor angiogenesis.

Key words: VEGF, Dll4, Notch, Angiogenesis, Lung cancer

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Introduction

Lung cancer is one of the most common cancers and has become the leading cause of cancer death. Its development is positively correlated with tumor vascular proliferation under hypoxia. It is acknowledged that vascular endothelial growth factor (VEGF) is a very important factor in promoting cancer vascular proliferation, and VEGF blockers have shown a good effect on suppressing tumor vascular proliferation (Kramer and Lipp, 2007). However, some tumors easily become resistant to VEGF blockers, and produce gastrointestinal reactions, bleeding and other side effects (Jain et al., 2006). Therefore, it is of great importance to explore other angiogenesis signaling pathways as therapeutic targets.

Delta-like ligand 4 (Dll4)/Notch signaling pathway is recognized as another important pathway in angiogenesis (Iso et al., 2003; Shawber and Kitajwski, 2004; Li and Harris, 2005). Membrane Dll4 binds with Notch receptors on adjacent cells leading to Notch receptor cleavage and releasing its intracellular domain (Notch-IC), which is translocated into the nucleus. In the nucleus, Notch-IC forms a ternary complex with a highly conserved transcription factor, CSL

(CBF1/Suppressor of Hairless/ Lag1) and transcriptional coactivators of the mastermind-like (MAML) family. This complex activates target gene transcription including the hairy/enhancer-of-split (Hes1) and Hey1 and regulates vascular endothelial cell differentiation and proliferation (Ehebauer et al., 2006). Some studies have shown that the expression of Dll4 is increased in tumor blood vessels of xenograft tumor in mice, human breast cancer and kidney cancer, while there is little or no expression in normal blood vessels. Patel (Patel et al., 2005) showed that Dll4-inhibition can induce tumor vascular endothelial cell cycle arrest, apoptosis increase and tumor angiogenesis reduction, which further confirmed the promoting effect of Dll4 in tumor angiogenesis. However, another study showed that Dll4 may play an inhibitory effect in tumor angiogenesis. When Dll4 signaling is blocked by antibodies or siRNA, it may promote tumor vascular endothelial cell proliferation, sprouting and branching, and the tumor vascular density has a significant increase (Noguera-Troise et al., 2006). However, the effect of the Dll4/Notch pathway in the angiogenesis of lung cancer remains to be elucidated.

VEGF induces significantly higher Dll4 expression to promote tumor angiogenesis, and blocking the expression of VEGF causes sharp decline of Dll4 and endothelial cell growth inhibition, which indicates that Dll4 expression is regulated by VEGF (Noguera-Troise et al., 2006). However, studies have shown that a high expression of Dll4 may inhibit VEGF. Overexpression of Dll4 in vascular endothelial cells could decrease the expression of VEGFR2, weaken vascular endothelial cell response to VEGF-induced angiogenesis and inhibit angiogenesis (Williams et al., 2006), while expression of VEGFR3 was increased and angiogenesis was promoted after Dll4 was inhibited (Siekmann and Lawson, 2007). Therefore, it is speculated that there may exist a negative feedback loop between VEGF and Dll4/Notch pathway. However, their exact mechanism under hypoxia in lung cancer remains unclear.

To gain insight into their roles in the pathogenesis, progression and prognosis of lung cancer, we examined VEGF and Dll4 signaling pathway molecules expression and evaluated their clinical relevance.

Materials and methods

Patients

A total of 36 patients with newly-diagnosed lung cancer (9 females and 27 males, age range 42-83 years, median 63 years) were enrolled in this study. The clinical characteristics of those subjects were summarized in Table 1. Thirty-five lung cancer specimens and 27 normal specimens at the margin of tumor sections were obtained after lung cancer operation in Qilu Hospital of Shandong University, Jinan, China. Cancer and normal lung tissues were examined by a certified pathologist, and the normal lung samples were confirmed to be free from tumor deposits. The study was approved by the Institutional Review Boards of Qilu Hospital of Shandong University. Informed consent was obtained from each patient before being included in the study.

Immunohistochemical analysis

Formalin-fixed, paraffin-embedded tissue sections were deparaffined in xylene, rehydrated in grade alcohols, and briefly microwaved in citrate buffer to optimize antigen retrieval. Nonspecific binding sites were blocked with diluted goat serum for 30 minutes at room temperature. Slides were incubated with rabbit polyclonal primary antibodies raised against human Notch1 intra (ab8387), Hes1 (ab49170), Dll4 (ab7280), VEGFR1 (ab2350), VEGFR2 (ab2349), HIF1 alpha (ab1) from Abcam, or VEGF (ZA-0509) and CD31 (ZM-0044) from Zhongshan Co. Ltd, China at a 1:200 dilution at 4°C overnight. Phosphate buffered saline was used for all subsequent washes and for antiserum

Table 1. Clinical characteristics of lung cancer patients.

Patients	Clinical stages	Pathological type	Differentiation degree	Lymph node metastasis
P1	Ia	Squamous cell carcinoma	low-grade	no
P2	Ia	Adenocarcinoma	moderate	no
P3	IIIa	Adenocarcinoma	moderate	no
P4	Ib	Adenocarcinoma	low-grade	no
P5	Ib	Squamous cell carcinoma	low-grade	no
P6	IIIa	Adenocarcinoma	low-grade	yes
P7	IIb	Adenocarcinoma	moderate	yes
P8	Ia	Adenocarcinoma	moderate	no
P9	IIb	Sarcoma kind cancer	-	yes
P10	Ib	Squamous cell carcinoma	moderate	no
P11	IIIa	Squamous cell carcinoma	low-grade	yes
P12	IIa	Adenocarcinoma	low-grade	yes
P13	Ia	Bronchioloalveolar carcinoma	-	no
P14	IIIa	Squamous cell carcinoma	moderate	yes
P15	IIIa	Large cell carcinoma	-	yes
P16	Ia	Gland squamous cell carcinoma	moderate	no
P17	IIIa	Large cell carcinoma	-	yes
P18	IIa	Squamous cell carcinoma	low-grade	yes
P19	Ib	Squamous cell carcinoma	low-grade	no
P20	IIa	Squamous cell carcinoma	moderate	yes
P21	Ib	Adenocarcinoma	low-grade	no
P22	IIIa	Adenocarcinoma	moderate	yes
P23	IIa	Adenocarcinoma	moderate	yes
P24	Ia	Adenocarcinoma	-	no
P25	Ia	Adenocarcinoma	moderate	no
P26	IIIb	Squamous cell carcinoma	low-grade	yes
P27	Ia	Bronchioloalveolar carcinoma	-	no
P28	IIIa	Adenocarcinoma	low-grade	yes
P29	Ia	Squamous cell carcinoma	low-grade	no
P30	Ib	Squamous cell carcinoma	moderate	no
P31	IIb	Squamous cell carcinoma	high-grade	no
P32	IIb	Squamous cell carcinoma	-	yes
P33	Ib	Squamous cell carcinoma	low-grade	no
P34	IIa	Squamous cell carcinoma	high-grade	yes
P35	IIa	Squamous cell carcinoma	moderate	no
P36	IIa	Squamous cell carcinoma	low-grade	yes

dilution. After extensive washing (3x5 min) to remove excess antibody, the slides were incubated with diluted HRP-labeled goat anti-rabbit antibody (Jingmei Co. Ltd, Beijing, China) for 1 hour at room temperature. All the slides were then processed by the SP method (Zhongshan Co. Ltd, Beijing, China) for 30 minutes at room temperature. Non-immune IgG was used as negative controls instead of the primary antiserum. For measurement and scoring of each sample, all slides were stained in a single batch and thus received equal staining. All the sides were imaged digitally and evaluated by Image Pro Plus (IPP), a digitalized immunohistochemistry scoring program (Media Cybernetics, San Diego, CA).

RNA extraction and reverse transcription

Total RNA was isolated by Trizol (Invitrogen) according to the manufacturer's instructions. Approximately, one μg of total RNA from each sample was subjected to first-strand cDNA synthesis using RevertAid™ First Strand cDNA Synthesis Kit (MBI, Fermentas, USA). Reverse transcription reaction was done at 42°C for 1 h, followed by 95°C for 5 min.

Real-time quantitative polymerase chain reaction (RQ-PCR) was performed using an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. The real-time PCR contained, in a final volume of 20 μL, 10 μL of 2xSYBR Green Real-time PCR Master Mix, 1 μL of cDNA, and 1 μL of the forward and reverse primers. The primers are shown in Table 2. The thermal cycling profile consisted of a 95°C denaturation step for 5min, then 40 cycles at 95°C for 15 sec, 65°C for 15 sec and 72°C for 45 sec. All experiments were conducted in triplicate. The PCR products were analyzed by melt curve analysis and agarose gel electrophoresis to determine product size and to confirm that no by-products were formed. The results were expressed relative to the number of GAPDH transcripts used as an internal control.

Human umbilical vein endothelial cells (HUVECs) culture

HUVECs were isolated from fresh human umbilical vein according to methods previously published (Marin et al., 2001). Briefly, the umbilical vein was filled with collagenase. Then we incubated the cord for 7 min at 37°C.After incubation, the cord was kneaded gently to help cell detachment. We centrifuged the cells to obtain HUVECs. HUVECs were cultured in M199 media supplemented with 20% fetal bovine serum (FBS) (Gibco) at 37°C and 5% CO_2. HUVECs were used for transfection at passages 3 to 7.

Dll4 transfection into HUVECs

Transfection was performed in 24-well plates using Lipofectamine 2000 according to the manufacturer's instructions. Briefly, HUVECs ($5x10^5$) in 500ul of growth medium without antibiotics were seeded into plates. Twenty-four hours later, 8 ug plasmid DNA pDll4-IRES2-EGFP or its backbone control vector p-IRES2-EGFP (kindly provided by Dr. Andreas Fischer, University of Heidelberg, German) in 50 ul of opti-MEM I Medium was mixed with diluted Lipofectamine 2000 for twenty minutes. Then, the complexes were added into plates. The transfection media were removed and replaced by culture media 8 h later. HUVECs were harvested 3 days after transfection, and used for real-time PCR detection.

Statistical analysis

Statistical analyses were performed using SAS version 9 software. For data with nonnormal distribution or heterogeneity of variance, median (range) was shown. Comparison between groups was analyzed by Wilcoxon rank-sum test or Student t test. Spearman's test was used for correlation analysis. P-value <0.05 was considered statistically significant.

Results

Aberrant expression profile of Dll4/Notch and VEGF pathway molecules in lung cancer patients

To examine whether Dll4/Notch and VEGF pathway molecules are involved in lung cancer, their expression patterns were investigated using the immunohisto-chemical method. As shown in Figures 1 and 2, though both lung cancer tissues and normal lung tissues were

Table 2. Primers and annealing temperatures (temp) for Notch and VEGF pathway molecules.

Genes	Forward (5´to 3´)	Reverse (5´to 3´)	Size (bp)	Temp (°C)
Notch1	TCAGCGGGATCCACTGTGAG	ACACAGGCAGGTGAACGAGTTG	104	62
Dll4	CCCTGGCAATGTACTTGTGAT	TGGTGGGTGCAGTAGTTGAG	74	58
Hes1	TGATTTTGGATGCTCTGAAGAAAGATA	GCTGCAGGTTCCGGAGGT	99	65
VEGF	CCTGGTGGACATCTTCCAGGAGTACC	GAAGCTCATCTCTCCTATGTGCTGGC	196	61
VEGFR1	CTGGACTGACAGCAAACCCAAG	CCACAGCTGGAATGGCAGAA	117	58
VEGFR2	AGCCAGCTCTGGATTTGTGGA	CATGCCCTTAGCCACTTGGAA	133	58
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA	138	62

found to express all the tested molecules at protein level, there were statistical differences for some of these molecules. VEGFR1, VEGFR2 and HES1 of lung cancer tissues were statistically higher than those of controls (Fig. 2A-C), while the ratio of VEGFR1/VEGFR2 was significantly decreased in lung cancers (Fig. 2D). Though other molecules (Notch1, Dll4 and VEGF) were marginally higher in lung cancers, no statistical difference was observed (Table 3).

RQ-PCR method was performed to confirm the alternation of protein level of Notch1-IC and Dll4. In concordance with the immunohistochemical analysis, the mean levels of Notch1-IC and Dll4 mRNA in lung cancer group [2.49e-4 (4.23e-5 ~ 2.80e-3) or 1.7e-3 (3.75e-4 ~ 4.45e-2)] were marginally higher than those in control group [8.05e-5 (1.09e-5-2.35e-2) or 7.3e-4 (1.34e-5~5.6e-1)].

Spearman correlation coefficients have been calculated between these factors. VEGFR1 was negatively correlated with Notch1 (r=-0.43356, p=0.0117) (Fig. 3A) and positively correlated with Dll4 (r=0.35809, p=0.0442) (Fig. 3B) in lung cancer tissues, while VEGFR2 was positively correlated with Dll4 in lung cancer tissues(r=0.25427, p=0.1405) (Fig. 3C). Besides that, there were no correlations among other indicators.

Elevated HIF1a and CD31 in lung cancer tissues

To determine whether hypoxia and high vessel density exist in lung cancer tissues, we determined the levels of CD31 and HIF1a using the immunohistochemical method. The results demonstrated that HIF1a and CD31 were significantly higher in lung tumor tissues compared with controls (Fig. 2E,F).

In lung cancer tissues, CD31 was negatively correlated with VEGFR1 (r=-0.38055, p=0.0289) (Fig. 3D) and positively correlated with HIF1a (r=0.35437, p=0.0340) (Fig. 3E). Moreover, HIF1a and HES1 nearly reached positive correlation in lung cancer tissues

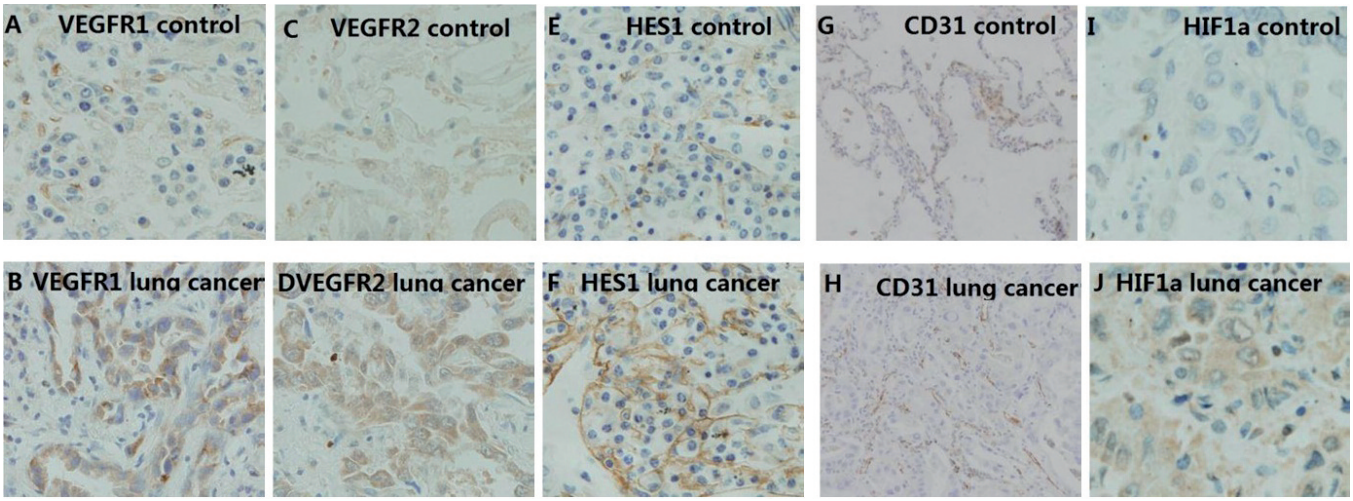

Fig. 1. Expression of VEGF and Dll4/Notch signaling pathway molecules in lung cancer and normal lung tissues. Sections of lung cancer tissue were stained with antibodies that recognized VEGFR1, VEGFR2, HES1, CD31 and HIF1a. All five proteins were more highly expressed in lung tumor tissues (**B, D, F, H and J**) than those in normal lung tissues (**A, C, E, G and I)**. A-F, I, J, x 400; G, H, x 100.

Table 3. VEGF and Notch pathway molecules protein levels of lung cancer patients and controls.

molecules	Median(range)		p Value
	Patients	Controls	
VEGF	71.129 (10.248~126.329)	45.515 (1.925~82.300)	0.1274
VEGFR1	66.218 (3.426~158.093)	50.602 (2.149~89.326)	0.0273
VEGFR2	102.475 (5.078~396.853)	41.739 (1.198~86.042)	0.008
VEGFR1/VEGFR2	0.701 (0.0473~12.223)	1.248 (0.208~1.794)	0.038
Notch1	53.268 (20.157~133.879)	30.245 (3.642~108.176)	0.2053
HES1	65.014 (21.857~138.159)	36.134 (0.501~51.574)	0.0036
Dll4	58.775 (2.247~184.100)	37.333 (25.241~75.576)	0.1997
HIF1a	63.032 (18.843~169.406)	29.350 (0.307~55.050)	0.0015
CD31	28.6 (3.2~73.6)	1.4 (0~2)	0.0004

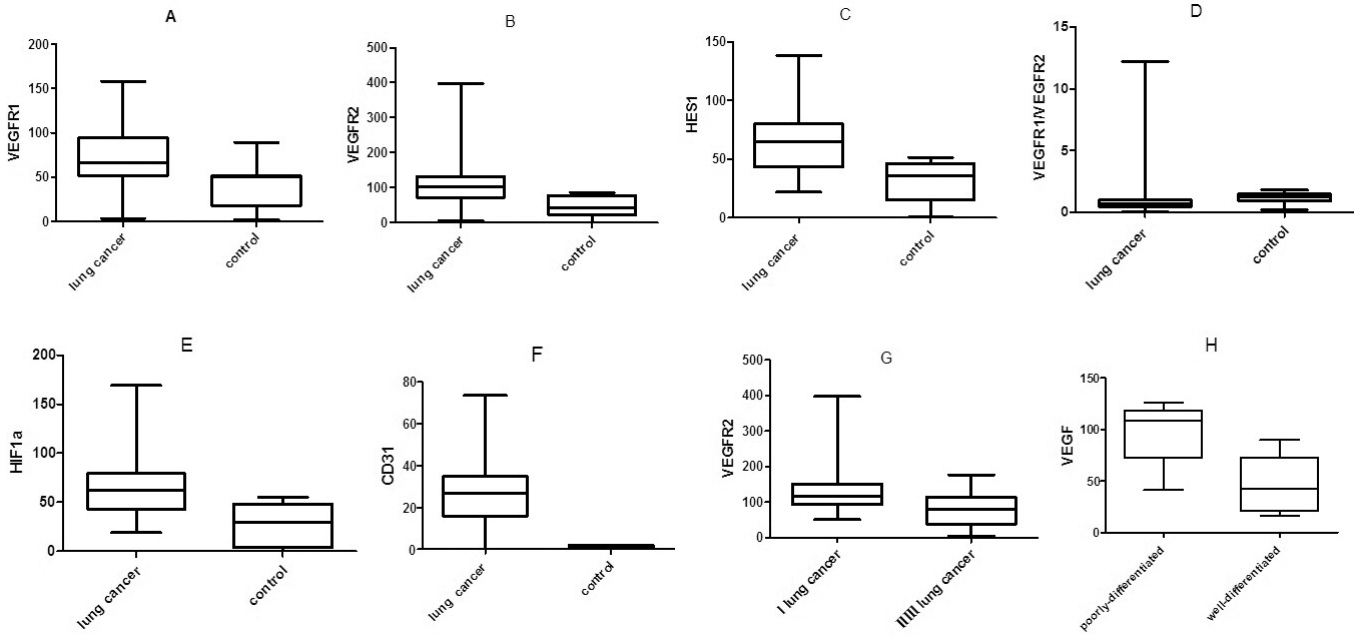

Fig. 2. Results of immunohistochemical analysis. **A-C.** VEGFR1, VEGFR2 or HES1 was significantly up-regulated in lung cancer tissues compared with normal lung tissues. **D.** The ratio of VEGFR1/VEGFR2 was significantly down-regulated in lung cancer tissues compared with normal lung tissues. **E and F.** HIF1a and CD31 were significantly up-regulated in lung cancers compared with normal lung tissues. **G.** The expression of VEGFR2 was significantly elevated in stage I lung cancer compared with stage II and III lung cancer. **H.** The expression of VEGF was significantly increased in poorly-differentiated lung cancer compared with well-differentiated lung cancer.

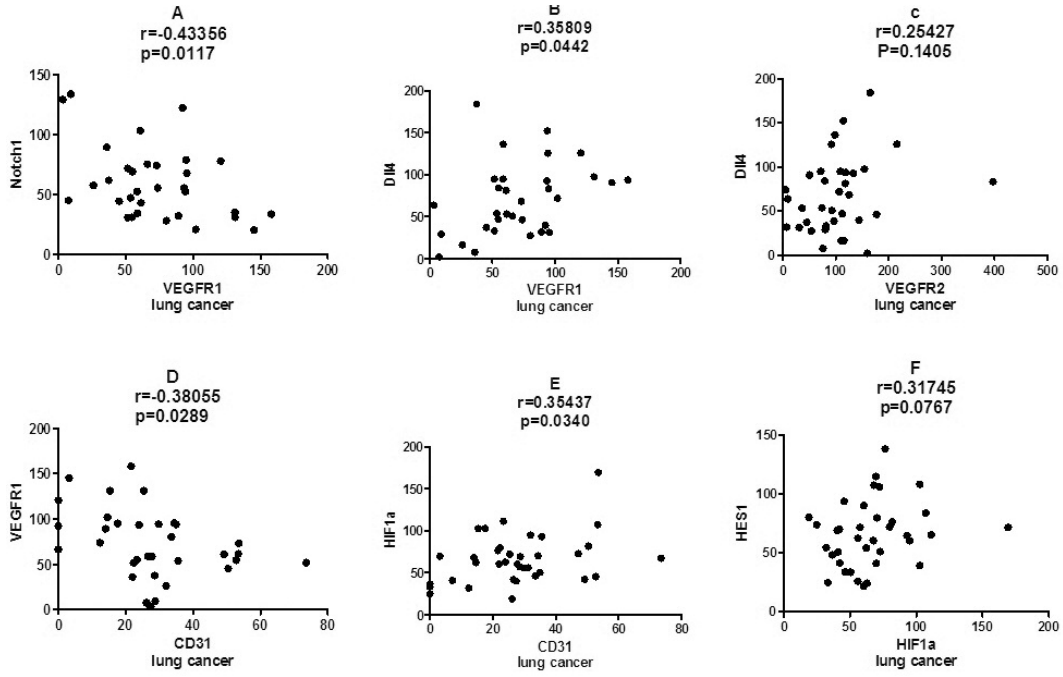

Fig. 3. Correlation analysis in lung cancer tissues. **A.** VEGFR1 was negatively correlated with Notch1. **B.** VEGFR1 was positively correlated with Dll4. **C.** VEGFR2 was positively correlated with Dll4, but no statistical significance was observed. **D.** CD31 was negatively correlated with VEGFR1. **E.** CD31 was positively correlated with HIF1a. **F.** HIF1a was nearly positively correlated with HES1.

(r=0.31745, p=0.0767) (Fig. 3F). No significant differences were found between other factors.

Dll4 up-regulated Notch and down-regulated VEGF expression in HUVECs

To confirm the interaction of Dll4/Notch and VEGF pathway, HUVECs cultured on dishes were transfected with Dll4-expressing plasmid. Compared with HUVECs transfected with GFP control plasmid, Dll4, Notch1 and VEGFR1 were up-regulated while VEGF and VEGFR2 were down-regulated in Dll4 transfected HUVECs (P<0.05) (Fig. 4).

Clinical relevance of Dll4/Notch and VEGF pathway molecules in lung cancer patients

The relationship between lung cancer clinical stage status and signaling molecules expression was analyzed. VEGFR2 was significantly higher in stage I (Median: 116.59, range: 50.512~396.853) than that in stage II and stage III (Median: 79.413, range: 5.078~176.993; p=0.0193) (Fig. 2G). As for the differentiation status, VEGF expression was higher in poorly-differentiated lung cancers tissues (Median: 92.188, range: 10.248~126.330) than that in well-differentiated lung cancers (Median: 51.266, range: 16.745~109.059; p=0.0225) (Fig. 2H). No significant relationship was found between the other molecules and clinical parameters in lung cancer tissues.

Discussion

Angiogenesis is a crucial step in the development and progression of numerous solid tumors, including lung cancer. In our study, the level of microvessel density (CD31) was shown to be higher in lung cancer tissues than in controls, which further demonstrated the increasing angiogenesis in lung tumor growth. VEGF plays an important role in tumor angiogenesis through binding its receptors, and inhibition of the VEGF pathway inhibits tumor growth in some preclinical tumor models. VEGFR1 and VEGFR2 are two primary receptors of VEGF, and are prominently expressed by vascular endothelial cells. VEGF released by tumor cells up-regulates the expression of VEGFR1/VEGFR2. Consistent with these reports, we found that the level of VEGF increased in lung cancer, though no statistical significance was observed. The levels of VEGFR1 and VEGFR2 were elevated in lung cancer tissues compared with normal lung tissues. Many data showed that VEGFR1 was a negative regulator of VEGF activity while VEGFR2 was the main mediator of VEGF biological effect. In our study, we demonstrated that the ratio of VEGFR1/VEGFR2 was lower and the expression of VEGFR1 was negatively correlated with CD31 in lung cancer tissues. Also, our findings suggest that the expression of VEGF and VEGFR2 increased gradually with the disease progression of lung cancer. All these indicated the angiogenesis-promoting effect of the VEGF pathway in our tested lung cancer tissues. Therefore, our results further demonstrate the importance of the VEGF pathway as a target in lung cancer therapy. However, several studies suggest that expression of VEGF/VEGFR1/VEGFR2 in lung cancer does not necessarily mean a functional VEGF signaling pathway (Brekken et al., 1998). Not all lung cancers are responsive to VEGF blockers, and some of them that are responsive initially may become resistant during the course of treatment. Thus, it is necessary to explore other angiogenesis signaling pathways, including those that interact with the VEGF pathway.

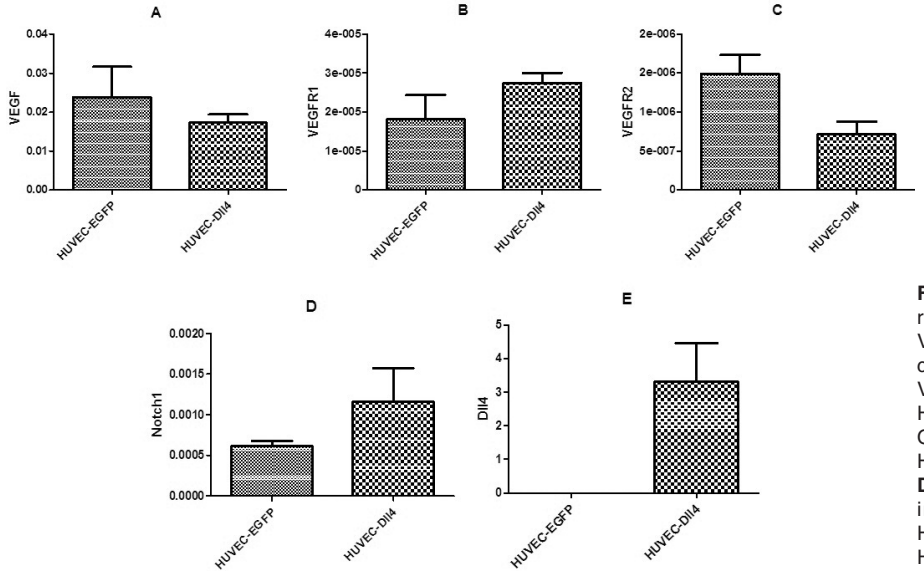

Fig. 4. Dll4 up-regulated Notch and down-regulated VEGF expression in HUVECs. **A.** VEGF in Dll4-transfected HUVECs decreased compared with non-transfected HUVECs. **B.** VEGFR1 was higher in Dll4-transfected HUVECs than in non-transfected HUVECs. **C.** Overexpression of Dll4 in Dll4-transfected HUVECS down-regulated the level of VEGFR2. **D.** Notch1 in Dll4-transfected HUVECs increased compared with non-transfected HUVECs. **E.** Dll4 was overexpressed in HUVECs after transfection.

Dll4/Notch signaling pathway has recently been proved to be involved in vascular development and tumor angiogenesis (Benedito and Duarte, 2005). In particular, Dll4 is significantly expressed in tumor vessels (Mailhos et al., 2001; Gale et al., 2004; Patel et al., 2005). In order to determine the effects of the Dll4/Notch signaling pathway in lung cancer angiogenesis, we studied the signaling pathway in lung tumors using several approaches. The expression of Dll4 and Notch1 was found to be elevated in lung cancer, though did not reach statistical difference. Moreover, HES1, the Notch signaling pathway effector molecule, was demonstrated to be strongly expressed in lung tumor tissues, which indicated that the Notch pathway was activated in lung cancer. Also HES1 was positively correlated with CD31, though no statistical difference was observed, which indicates that high activity of the Dll4/Notch signaling pathway is a critical positive regulator of lung tumor angiogenesis. All the findings suggest that increased Dll4/Notch activity might result in increased tumor vascular density, which needs to be further investigated.

Separate studies have shown that there is a crosstalk between Dll4/Notch and VEGF signaling pathways. Down-regulation of VEGFR2 can result in activation of Notch in cultured endothelial cells (Taylor et al., 2002), and blocking Dll4 can increase VEGFR2 expression (Suchting et al., 2007). Recent studies suggest that inhibition of Notch activity can result in decreased VEGFR1 expression, but increased VEGFR2 expression (Roca and Adams, 2007). Consistently, in our study, we found a negative correlation between VEGFR1 and Notch1 in lung cancer tissues. In cultured HUVECs cells, we found that up-regulation of Dll4 can result in increased Dll4 and Notch1 expression, but causes decreased VEGF and VEGFR2, and increased VEGFR1 expression. Thus, we further deduce that there may be a negative feedback loop between VEGF pathway and Dll4/Notch pathway in lung cancer angiogenesis.

Hypoxia has been another regulator of neo-angiogenesis and a hallmark in a number of solid tumors (Harris, 2002). Here we demonstrated that the level of HIF1a, a transcription factor that regulates many genes involved in the response of hypoxia, was elevated in lung cancers. Moreover, HIF1a was shown to be positively correlated with CD31 level in lung cancers. All these results suggest that lung cancers are in a hypoxia environment and the hypoxia may contribute to the neo-angiogenesis in lung cancer. Recent studies show that there is an important interaction between Dll4/Notch signaling pathway and HIF1a. Several groups have demonstrated that the level of Dll4 is increased in low-O_2 tension (0.1%). Notch1 appears to up-regulate HIF1a expression, and HIF1a binds and stabilizes activated Notch1 leading to enhanced Notch1 signaling (Gustafsson et al., 2005). Our data showed that HIF1a was positively correlated with HES1 in lung cancer tissues, which indicated a positive interaction between hypoxia and the Dll4/Notch pathway in lung cancer angiogenesis.

In conclusion, there exists overexpression of the VEGF and the Notch signaling pathway molecules in lung cancers, which positively correlate with hypoxia (HIF1a) and angiogenesis (CD31). There might be a negative feedback loop between VEGF and the Dll4/Notch signaling pathway in lung tumor angiogenesis. The specific mechanism of the two signaling pathways in lung cancer angiogenesis will be the focus of our ongoing work.

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