Int J Clin Exp Pathol 2016;9(3):2989-2997
www.ijcep.com /ISSN:1936-2625/IJCEP0020342

Original Article

High level of Delta-like ligand 4 suppresses the metastasis of hepatocellular carcinoma

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Received November 23, 2015; Accepted January 25, 2016; Epub March 1, 2016; Published March 15, 2016

Abstract: This study aims to explore the role of Delta-like ligand 4 (DLL4) in human hepatocellular carcinoma (HCC), we examined the expression of DLL4 both in protein and ribonucleic acid levels. Additionally, the lentiviral vector over expression of DLL4 (Lv-DLL4-OE) was to assess the HCC cells biological behavior. 150 HCC tissues were detected by immunohistochemistry. Hepatocellular carcinoma, paracancer and normal liver samples were analyzed by reverse transcriptase polymerase chain reaction (RT-PCR). SMMC-7721 and HepG2 with Lv-DLL4-OE were investigated cells biological behavior by MTT and Transwell. In protein level, DLL4 expression in HCC was significantly different from paracancer tissues ($P<0.05$). DLL4 positive rate in HCC was 41.7% (61/150) and 81.3% (122/150) in paracancer tissues. The association of DLL4 with clinical parameters indicated that patients with low DLL4 protein levels were prone to metastasize ($P<0.05$). Kaplan-Meier analysis showed that patients with DLL4 positive expression was not statistically different with those negative in overall survival time ($P>0.05$). RT-PCR analysis showed DLL4 mRNA in tumor tissue was the lowest of three groups ($P<0.05$). The relationship between clinical features and DLL4 mRNA levels was consistent with protein levels except serum alpha fetal protein (AFP). Cell cultures confirmed that over expression DLL4 could significantly inhibit the migration capacity of HepG2 and SMMC-7721 ($P<0.05$), but did not affect cells proliferation. Our findings indicate that DLL4 was low express in HCC tissues, and over expression of DLL4 might suppress the migration ability in vitro. Therefore, DLL4 might be as a negative regulator involved in the development of HCC.

Keywords: DLL4, human hepatocellular carcinoma, lentiviral vector, migration, proliferation

Introduction

Human hepatocellular carcinoma (HCC) is one of the most frequently diagnosed cancers worldwide but the fifth leading cause of cancer death in the United States [1]. Despite new treatment has developed in the last decade, the prognosis for patients with hepatocellular carcinoma has remained poor, the 5-year relative survival rate is only 16% secondly to pancreatic carcinoma [2]. Obviously, surgical resection is the optimal method for hepatocellular carcinoma up to now, but chemotherapy and targeted therapy as the principal adjuvant therapies which contribute to improve its survival rate is so limited. Therefore, we need to seed effective and identified therapy for HCC as adjunctive therapy.

Delta-like ligand 4 (DLL4), a member of Notch family, has been recognized as an important factor for tumor angiogenesis [3], and has been showed to be aberrantly expressed in several types of tumors and play different biological function. For example, over expression of DLL4 in ovarian, colorectal carcinoma are associated with tumorigenesis but not related with clinical outcomes [4, 5]. On the other hand, elevated DLL4 expression of breast cancer, pancreatic cancer, clear cell renal carcinoma and nasopharyngeal carcinoma predicts poor prognosis [6-9]. These may be suggested that the role of DLL4 in different cancer is cancer type specific. But, there are a few reports about DLL4 expression in human hepatocellular carcinoma tissues till now. In order to explore whether DLL4 expression in HCC associated with the tumori-

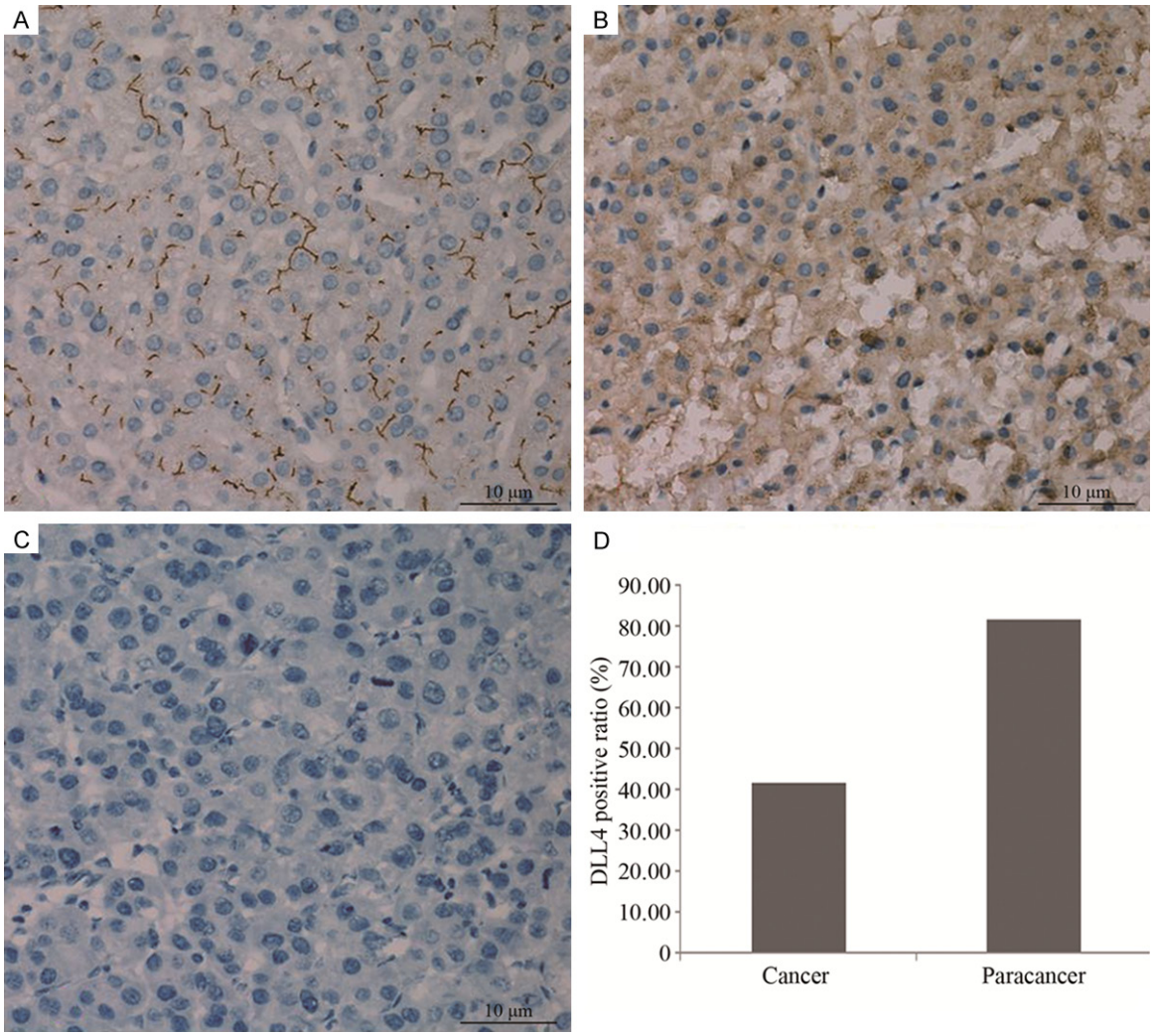

Figure 1. Immunohistochemical staining for DLL4 in human hepatocellular carcinoma and peritumoral liver tissue. A. DLL4 was mainly expressed in cytomembrane of peritumoral liver tissue; B. DLL4 was expressed most in cytoplasm and few in cytomembrane of HCC tissue; C. DLL4 was no staining in negative control (Original magnification, ×200). D. DLL4 in HCC tissue was significantly lower than that in adjacent noncancerous liver tissue ($P<0.05$).

genesis or with clinical outcomes, we examined the expression of DLL4 in hepatocellular tumor tissues and adjacent noncancerous liver tissues, and drew survival curve to analyze the relationship of DLL4 expression and prognosis of HCC patients to clarity the possible role of DLL4 in HCC. Furthermore, we selected cell cultures to further investigate that enforced expression of DLL4 how to effect on human liver cancer cell functions.

Materials and methods

Patient samples

Formalin-fixed, paraffin-embedded samples were taken from 150 HCC patients at the Liver Center, the First Affiliated Hospital of Fujian Medical University, from November 2008 to April 2011. All HCC tissue samples and corresponding noncancerous liver tissue samples were obtained from patients who had undergone surgical hepatectomy (130 men and 20 women; age range 25-81 years old, median 52 years old). All patients were followed up from 1 month to 28 months, and we gained 80 consummate data from patients to survival analysis (31 deaths and 49 survivals). None of the patients received preoperative chemotherapy or radiation therapy. Fresh tissue samples of HCC and the paired adjacent noncancerous liver tissue, normal liver tissue adjacent to hepatic hemangioma as the control group were obtained from the same Liver Center, between

Table 1. Correlation of DLL4 expression with clinicopathological parameters in HCC

Parameters		n	DLL4 expression		*P*-value
			Negative	Positive	
Gender	Male	130	79	51	0.361
	Female	20	10	10	
Age (years)	≤50	68	39	29	0.653
	>50	82	50	32	
Tumor size	≤5 cm	57	29	28	0.099
	>5 cm	93	60	33	
AFP (ng/ml)	≤400	78	48	30	0.567
	>400	72	41	31	
HBV	Negative	17	11	6	0.632
	Positive	133	78	55	
Cirrhosis	No	38	22	16	0.835
	Yes	112	67	45	
Microvascular invasion	No	77	45	32	0.819
	Yes	73	44	29	
Extrahepatic metastasis	No	105	58	47	0.038
	Yes	45	33	12	
Histological classification	I-II	103	60	43	0.690
	III-IV	47	29	18	
BCLC stage	0/A	38	17	21	0.099
	B	78	51	27	
	C/D	34	21	31	

September 2009 and October 2013. The three paired tissue samples marked respectively and were immediately frozen and stored at -80°C for reverse transcriptase polymerase chain reaction (RT-PCR) analysis. All samples were collected and used in accordance with the ethical rules of our institution and with the patients' consents. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Fujian Medical University. Written informed consent was obtained from all participants.

Immunohistochemistry

Immunohistochemistry was performed using the Elivision™ Plus Two-step System (Maixin Incorporation, Fuzhou, China) according to the manufacturer's instructions. The paraffin sections were blocked with 10% normal goat serum at 37°C for 30 min and incubated with a 1:200 dilution of rabbit polyclonal anti-human DLL4 antibody (HPA023392, Sigma, USA) for 1 h at 37°C followed by three washes with phosphate buffer saline. The slides were incubated with polymerized HRP-Anti Ms/RbIgG (Maixin Incorporation, Fuzhou, China), followed by 3-3' diaminobenzidine, and counterstained with hematoxylin. Negative control sections were incubated with preimmune serum. The percentage of tumor cells showing cytomembrane/cytoplasmic staining was scored for each case. Moderate to strong cytomembrane/cytoplasmic staining observed in >10% of tumor cells was regarded as positive. Faint cytomembrane/cytoplasmic staining or moderate to strong observed in <10% of tumor cells were regarded as negative. All stained sections were evaluated by two independent pathologists in a blind manner.

Reverse transcription and real-time RCR

Total RNA was isolated from the liver tissue following the TriPure Isolation Reagent (Roche, CH) protocol and reverse transcribed using Prime Script™ RT reagent kit (TaKaRa, Dalian). The cDNA was then amplified with primers for the DLL4 gene (Forward 5'-CTGGAGGTGAGGTGAGTGCT-3', Reverse 5'-GGGTAGACGGACATTCTTGC-3', 115 bp) and the human β-actin gene (Forward 5'-GCGTGACATTAAGGAGAAGC-3', Reverse 5'-CCACGTCACACTTCATGATGG-3', 230 bp). Real-time PCR was done using ABI Step-One system (Applied Biosystems, US) and SYBR Green qPCR premix (TaKaRa, Dalian), and β-actin for standardization. The thermal cycling conditions composed of an initial denaturation step at 95°C for 3 minutes followed by 40 cycles of PCR using the following profile: 95°C for 5 seconds, 60°C for 34 seconds. There is inverse relationship between threshold cycle (Ct value) and gene starting copy numbers, the Ct value was estimated after adjustment of baseline cycles and calculation of the Ct value.

Cell culture

HepG2 and SMMC-7721 cell lines were obtained from the cell bank of the Chinese Academy of Science (Shanghai, China). Reagents for cell culture were purchased from HyClone

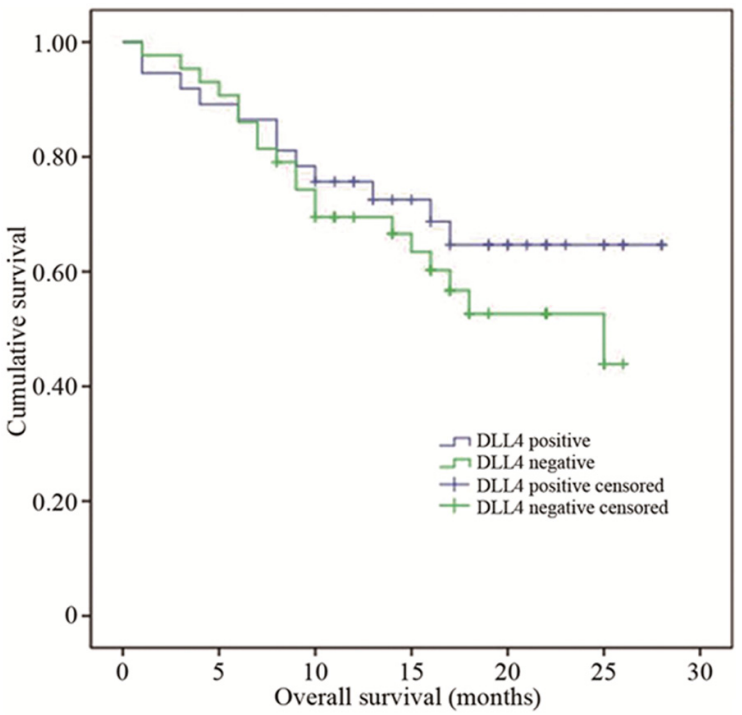

Figure 2. Kaplan-Meier estimates overall survival time according to DLL4 expression levels in HCC. No significant difference was observed between the positive and negative DLL4 expression subgroups (P>0.05).

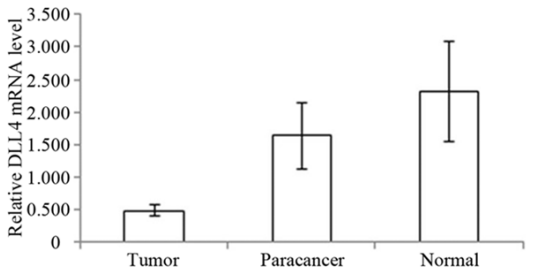

Figure 3. Relative DLL4 mRNA level of HCC tumor, paracancer liver tissue and normal liver tissue. HCC tumor tissue is the lowest one of that three groups in DLL4 mRNA level (P<0.05).

Biochemical Products Co., Ltd. (Beijing, China). Cells were grown in complete culture medium (high-glucose), consisting of DMEM supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin, at 37°C in a humidified incubator with 5% CO_2.

Construction of vectors and transfection

Based on the DLL4 gene sequence in GenBank (NM_019074) and design principles, over expression of DLL4 mRNA were designed. A lentiviral vector, GV287-GFP, (Genechem Incorporation, Shanghai, China) was used to construct the over expressing vector. The human DLL4 over expression vectors (Lv-DLL4-OE) and corresponding empty vector (Lv-NC) are purchased from Genechem Incorporation (Shanghai, China). Using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), 293T cells were cotransfected with the Lv-DLL4-OE, pHelper1 vector, and pHelper2 vector, for 48 h to generate lentivirus. The viral titers were determined, and the lentiviral particles were used to infect HepG2 and SMMC-7721 cells respectively. Transfected cells, expressing green fluorescent protein (GFP), were selected and expanded in cell cultures for further investigation.

Transwell assay

HepG2 and SMMC-7721 cells were digested in the logarithmic growth phase. Cell migration assay was carried out using Boyden chambers containing Transwell (Corning Costar Corp., Cambridge, MA) membrane filter inserts with pore size of 8 μm. Cells were transfected with plasmids as indicated above. 1×10^5 cells were then seeded on Boyden chambers (upper chamber) in 100 μl DMEM containing 0.1% BSA. At the same time, 5×10^5 cells were seeded on 96 pore plates and measured OD_{490}. The lower chambers were filled with DMEM containing 10% FBS. After 16 hours of migration at 37°C. Transwell chamber was taken out, washed, fixed by paraformaldehyde, and then cells were stained with Giemsa. 10% acetic acid was used to decolorize, then the absorbance of eluent was measured at 570 nm using a microplate reader. OD_{570}/OD_{490} represents the ability of cells to migrate.

Cell proliferation assay

SMMC-7721 and HepG2 cells were plated in 96 well plates at 3×10^3 cells per well after transfection with DLL4 for 48 h. Cells were cultured for 24 h, and then 10 μl MTT (thiazolyltetrazolium bromide) was added in per well. After 4 h, absorbance at 490 nm was measured after incubation with 150 μl of DMSO. Each assay was performed in triplicate.

Table 2. Correlation of DLL4 relative mRNA level with clinicopathological parameters in HCC

Parameters		n	DLL4 relative mRNA level	*P*-value
Gender	Male	57	1.423	0.378
	Female	5	4.120	
Age (years)	≤50	27	1.380	0.406
	>50	35	1.841	
Tumor size	≤5 cm	22	1.635	0.987
	>5 cm	40	1.643	
AFP (ng/ml)	≤400	29	1.032	0.043
	>400	33	2.175	
HBV	Negative	4	1.430	0.733
	Positive	58	1.655	
Cirrhosis	No	8	1.014	0.102
	Yes	54	1.733	
Microvascular invasion	No	31	1.249	0.172
	Yes	31	2.032	
Metastasis	No	43	2.758	0.041
	Yes	19	1.374	
Histological classification	I-II	45	1.304	0.053
	III-IV	17	2.530	
BCLC stage	0/A	17	1.640	0.092
	B	33	1.596	
	C/D	12	1.575	

Statistical analysis

Statistically significant differences between and among groups were assessed using X^2 test, Student's t-test or one-way ANOVA. Overall survival curve were calculated using the Kaplan-Meier method and compared by the log-rank test. All statistical analyses were performed using the SPSS version 17.0 software package (SPSS Inc., Chicago, IL, USA). *P*-values less than 0.05 were considered statistically significant.

Results

Expression of DLL4 protein and its relation with clinicopathological parameters

Lower expression of DLL4 in HCC tissues and its association with extrahepatic metastasis. Immunohistochemistry was performed on TMAs containing 150 HCC tissue samples and corresponding adjacent noncancerous liver tissues to assess the expression of DLL4. Expression of DLL4 was strong in adjacent noncancerous liver tissues with cytomembrane or cytoplasm staining (**Figure 1A**), while the tumor cells of the HCC tissue samples showed absent or moderate expression of DLL4 that was observed staining most in cytoplasm and few in cytomembrane (**Figure 1B**). This staining was specific for DLL4 as immunoreactivity was lost when the DLL4 antiserum was preincubated with a DLL4-blocking peptide (**Figure 1C**). The presence of DLL4 in HCC tissue samples (41.7%) was significantly lower than in adjacent noncancerous liver tissue samples (81.3%) ($P<0.05$, **Figure 1D**).

According to statistical analysis of the association of DLL4 with clinicopathological parameters, we found that patients with low DLL4 protein levels were prone to extrahepatic metastasis ($P<0.05$). While none of gender, age, tumor size, alpha fetal protein (AFP), HBV, liver cirrhosis, microvascular invasion, histological classification, BCLC stage was correlated with the expression of DLL4 (**Table 1**).

The correlation of DLL4 expression levels and overall survival time were evaluated by Kaplan-Meier analysis with log-rank statistic. As shown in **Figure 2**, patients with DLL4 positive expression had longer overall survival time than those of DLL4 negative after surgery for ten months. But the difference of that two groups was not significant in statistics ($P=0.328$).

Expression of DLL4 mRNA and its relation with clinicopathological parameters

Lower expression of DLL4 mRNA in HCC tissues associated with extrahepatic metastasis and serum AFP. As shown in **Figure 3**, DLL4 gene was detected by real-time PCR in normal liver tissue, HCC and paracancer liver tissue. The lowest level was recorded in HCC, and it was significantly lower than another two groups, but there was no statistically significant association between paracancer liver tissue and normal liver tissue. The relative quantity of DLL4 mRNA of paracancer liver tissue is 3.36 fold of HCC tumor tissue, and that of normal liver tissue is 4.76 folds of HCC tumor tissue.

According to the data in **Table 2**, DLL4 gene level was significant with extrahepatic metasta-

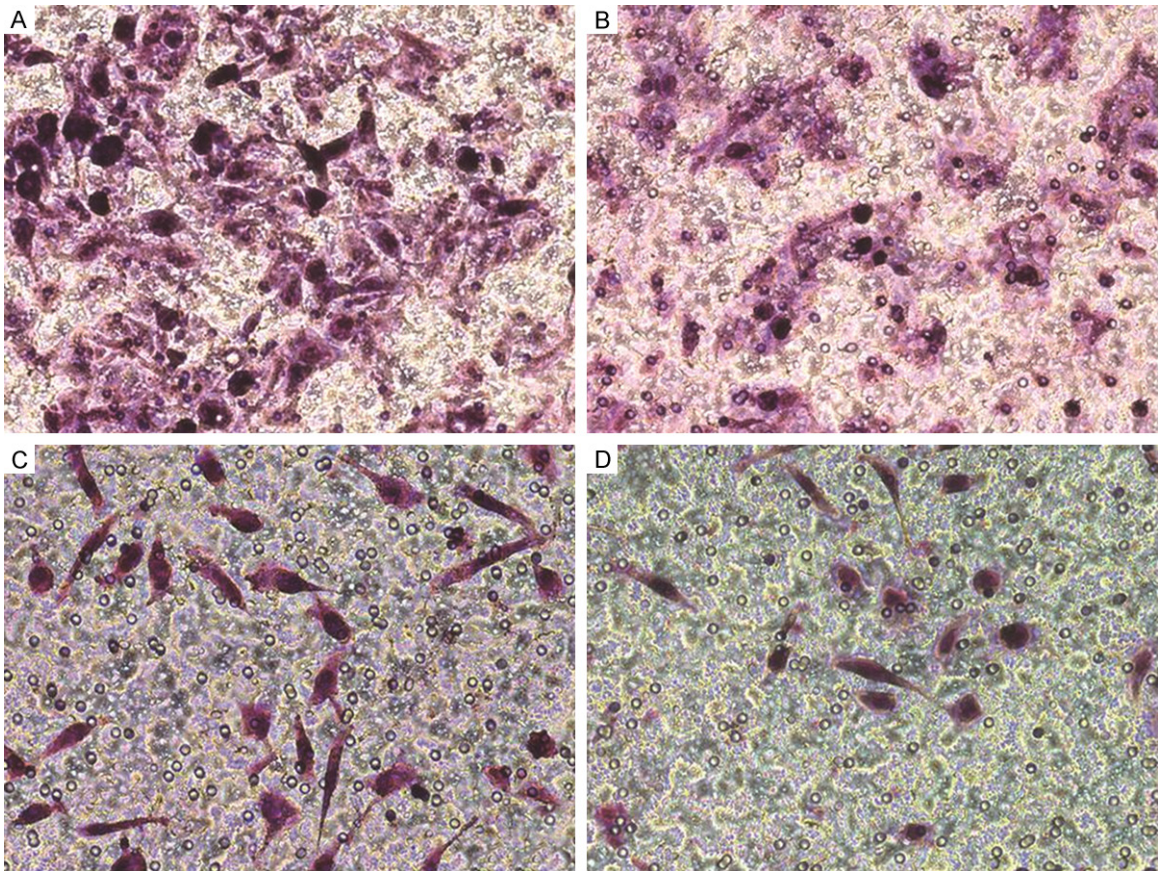

Figure 4. HepG2 and SMMC-7721 migration by transwell assay. The quantity of migrated HepG2 cells in corresponding empty vector (A) was more than DLL4 over expression vectors (B) subgroup. The quantity of migrated SMMC-7721 cells in corresponding empty vector (C) was more than DLL4 over expression vectors (D) subgroup (Original magnification, ×200).

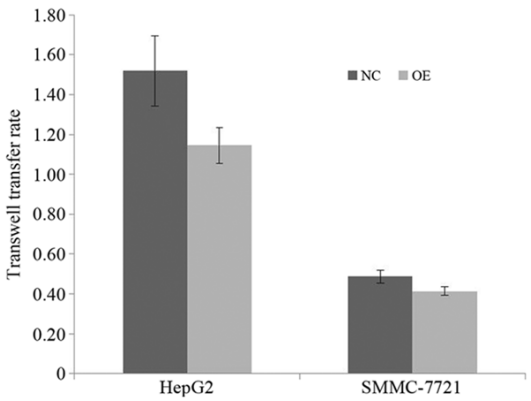

Figure 5. Transfer rate of migration cells in HepG2 and SMMC-7721 are show in bars. The transfer rate of negative control cells were significantly higher than that of DLL4 over expression both HepG2 and SMMC-7721 ($P<0.05$).

sis and serum AFP level ($P<0.05$), but was no significant association with other clinicopathological features in HCC patients, such as patient genders, age, tumor size, HBV, liver cirrhosis, microvascular invasion, histological classification, and BCLC stage. This result demonstrated that the alteration of mRNA levels of DLL4 in HCC was consistent with the change of its protein levels described above but serum AFP.

Transwell assay

Over expression of DLL4 inhibit hepatic tumor cells migration. We also examined the effects of DLL4 on metastasis and invasion capacity of liver cancer cells. The Transwell assays were applied in HepG2 and SMMC-7721 cells. As **Figure 4A**, B shown, enforced expression of DLL4 notably inhibited migration of HepG2 cells on the surface of the tissue culture plate, significantly decreased area compared with controls in Transwell assay. Similarly, data of SMMC-7721 cells showed enforced expression of DLL4 apparently repressed the invasion capacity of HCC (**Figure 4C**, **4D**). The transfer

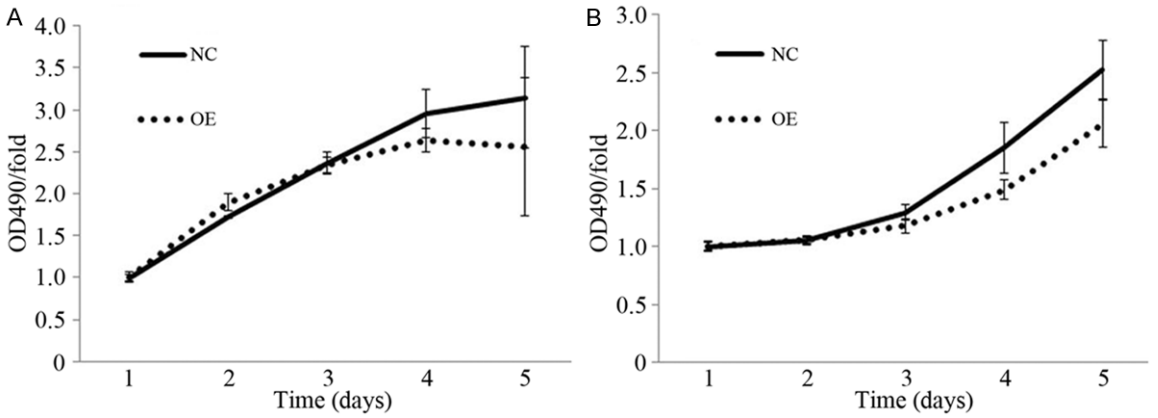

Figure 6. SMMC-7721 and HepG2 proliferation by MTT assay. A. DLL4 over expression cells gained lower proliferation capacity than negative control cells after the third day in SMMC-7721, but it was no significance (P>0.05); B. DLL4 over expression cells gained less proliferation capacity than negative control cells after the second day in HepG2, but it was no significance (P>0.05).

rate of migration cells in HepG2 and SMMC-7721 are shown in bars (**Figure 5**). The transfer rate of negative control cells were significantly higher than that of DLL4 over expression both HepG2 and SMMC-7721 (P<0.05). These data suggest DLL4 plays a depressive role in migration of hepatic tumor cells.

MTT assay

Over-expression of DLL4 did not inhibit hepatic tumor cells proliferation. The proliferation ability of liver cancer cells was also been detected by MTT assay. Compared to negative control, DLL4 over-expression cells did not significantly affect liver cells proliferation either SMMC-7721or HepG2 (P>0.05) (**Figure 6A**, **6B**).

Discussion

HCC as a most common malignant cancer has been study for long, many molecules and signaling pathways are proved to be involved in the progresses of it. In recent years, Notch signaling had been showed to play an important role in hepatic tumors, and four Notch receptors are differently expressed in hepatocellular carcinoma. For example, Notch1 and Notch4 are up regulated in HCC tissues; Notch2 is down regulated in HCC, while Notch3 showed no difference in HCC compared with adjacent noncancerous liver [10]. As for cell lines, Some reports declared that Notch1 could inhibit the HCC cell growth through induction of cell cycle arrest and apoptosis [11], but another showed that Notch3, Jaggd1, and HES-1 are all expressed in HepG2 cell to take a positive effect [12]. For above mentioned, the role of Notch receptors were so equivocation that should be further elucidated.

In this study, both DLL4 protein and gene expression were significantly decreased in HCC tissue compared to adjacent noncancerous tissues. The relative quantity of DLL4 mRNA of paracancer liver and normal liver tissues were 3.36 and 4.76 folds of HCC tumor tissues respectively. In addition, DLL4 expression and its correlation with malignant clinicopathological characteristics in HCC samples was also estimated, patients with low levels of DLL4 protein and gene tended to transfer out of liver. Furthermore, patients with higher serum AFP Level are up regulated expression DLL4 mRNA. As we all known, serum AFP is a famous biomarker for HCC, so we guess that high expression of DLL4 mRNA may be as a potential biomarker for HCC. More interestingly, Ishii's study demonstrated that AFP producing cells in cholangiocarcinoma possessed properties mimic cancer stem cell (CSC), and these cells expressed Notch1 [13]. The AFP producing cells were suggested to be CSCs, so our research hinted that DLL4 might play an important role in maintaining the CSC characteristics. Besides, Lv-DLL4-mediated enforced expression of DLL4 inhibited HepG2 and SMMC-7721 cells to immigrate, but it did not observably inhibit the proliferation of both hepatic cell lines. In a word, these results indicated that DLL4 may

serve as a tumor suppressor during human hepatocellular oncogenesis and metastasis.

DLL4 is one of the most significant ligands of the Notch signaling pathway considered as a promising target in angiogenesis based cancer therapy [14]. Not only DLL4 plays a pivotal role in determination and regulation of vasculogenesis during embryonic development, but also its activity and expression pattern suggest a significant role in the control of endothelial cell biology [15, 16]. In addition, DLL4 was the only ligand exhibited on tip cells at the end of growing vascular sprouts. It was also present in stalk cells, capillaries, arterial endothelium and in mural cells of mature arteries [17]. More interestingly, DLL4 expression in human adult vasculature is low but in the tumor vessels is up regulated [14, 18-20]. For example, the expression of DLL4 within the vasculature of clear cell renal cell carcinoma was up-regulated almost 9 fold more than in normal kidney tissue and was correlated with the level of VEGF. DLL4 was also significantly up-regulated expression in the case of superficial and invasive bladder cancer and was clearly correlated with CD34 and VEGF expression [21]. It is worth noting that down-regulation of DLL4 may have led to inhibition of endothelial cell proliferation, migration and network formation [22]. In clinical breast cancer samples, high intensity of DLL4 endothelial expression was a statistically significant adverse prognostic factor, which may suggest that breast tumors with high DLL4 expression progressed more rapidly [23]. Similarly, Hu et al. reported that DLL4 overexpression in ovarian cancer was an independent predictor of poor survival [24]. Inversely, endothelial expression of DLL4 in colon cancer should not be considered as a prognostic factor [25]. Moreover, the expression of DLL4 may be as a factor of differential diagnosis in thyroids. Immunohistochemical staining of DLL4 was highly variable in thyroid benign lesion and tumor. Expression of DLL4 in carcinomas was detected in large vessels with intensely and homogeneous, but in Graves' patients only capillary endothelial cells were positive [26]. Obviously, our study has suggested that the expression of DLL4 protein may be also as a factor of differential diagnosis from tumor to noncancerous liver, but not a factor of prognosis because of the positive expression of DLL4 and the negative expression of DLL4 has no statistically significant difference in liver. The reason may be depended on the tumor type. Contribute to above results, we may be can use DLL4 as different diagnose for HCC and none liver tumor. As known all, immunohistochemistry is a cheap, fast and wildly accessible method in diagnosis pathology.

In summary, DLL4 could be an anti-oncogene in the hepatocellular carcinoma, and high level of DLL4 is as a negative regulator associated with hepatocellular carcinoma metastasis in tissues and cells. DLL4 may be as a prospective biomarker in HCC for different diagnoses by immunohistochemistry. Further research must be carried on to probe the possible mechanisms that high level DLL4 suppresses the metastasis of HCC in future.

Acknowledgements

The present study was supported by grants from the key project of Science and Technology Department of Fujian Province (Grant No. 2014Y0027), The Scientific Research Project of Education Department of Fujian Province (Grant No. 2013B009) and the Affiliation Project of Health and Education of Fujian Province (Grant No. WKJ-FJ-04), and the Natural Science Foundation of Fujian Province (No. 2013J01331 and No. 2016J01531).

Disclosure of conflict of interest

None.

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