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Dlk-1, a cell surface antigen on foetal hepatic stem/progenitor cells, is expressed in hepatocellular, colon, pancreas and breast carcinomas at a high frequency

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Delta-like 1 protein (Dlk-1), also known as preadipocyte factor 1 (Pref-1), is a transmembrane and secreted protein with epidermal growth factor (EGF)-like repeats. Dlk-1 is known to be expressed in foetal liver, but absent in neonatal and adult liver in mice and rats. Dlk-1 is also expressed in a subpopulation of hepatic oval cells, which are considered as stem/progenitor cells in rat adult liver. In this study, we generated monoclonal antibodies against human Dlk-1 (hDlk-1) and investigated hDlk-1 expression in human liver and hepatocellular carcinoma (HCC). Like rodent livers, hDlk-1 was detected in foetal liver, but not in adult liver. In HCC, hDlk-1 was positive for 20.5% of the cases examined and was localized in both cytoplasm and cell membrane, whereas hDlk-1 was undetected in viral hepatitis, nodular cirrhosis. Interestingly, hDlk-1 positive HCC was found more frequently in younger patients and its expression was correlated with alpha-fetoprotein expression. Furthermore, hDlk-1 was also detected frequently in colon adenocarcinomas (58%), pancreatic islet carcinoma (50%), and small cell lung carcinoma (50%). Thus, hDlk-1 is a cell surface protein expressed in many carcinomas including HCC and may be a potential target for monoclonal antibody therapy for carcinomas.

Keywords: cell surface protein/colon adenocarcinoma/hDlk-1/hepatocellular carcinoma (HCC)/small cell lung carcinoma.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence activated cell sorter; His, histidine; MEM, minimum essential medium; mRNA, messenger RNA; PBS, phosphate-buffered saline.

Hepatocellular carcinoma (HCC) is one of the most common malignant tumours in the world (*1*). While the occurrence has been unusually high in Asia and Africa, it is recently increasing in United States and the incidence and mortality rates are anticipated to double over the next 10–20 years (*2*, *3*). HCC is often diagnosed at an advanced stage when curative therapies are of limited efficacy. In order to reduce morbidity and mortality of HCC, it is of prime importance to develop a system for early diagnosis, novel systemic therapies for the advanced disease as well as means to prevent HCC development.

Dlk-1 protein, also known as Pref-1, foetal antigen 1 (FA1), pG2 and ZOG, is a transmembrane and secreted protein, which is a member of the epidermal growth factor (EGF)-like family including Notch/Delta/Serrate (*4*–*8*). Dlk-1 is strongly expressed in foetal tissues such as liver, pancreas and skeletal muscle, but its expression is restricted in adult tissues such as placenta and adrenal grand (*9*–*12*). As there are many receptors and ligands in the EGF-like family proteins, which regulates cell fate and differentiation during development in many organisms, Dlk-1 may also play a role in development and differentiation (*12*–*14*). In fact, there are several reports showing the involvement of Dlk-1 in adipogenesis (*15*), hematopoiesis (*16*, *17*) and development of pancreas (*18*, *19*), placenta (*20*) and adrenal gland (*21*, *22*). In addition to the normal tissues, Dlk-1 was also shown to be expressed in several tumours, such as neuroblastoma (*23*), glioma (*24*), small cell lung carcinoma (*25*), myelodysplastic syndrome, acute myelogenous leukaemia (*26*), etc. (*27*, *28*). These results suggest that Dlk-1 may play an important role in tumourigenesis as well as organogenesis.

Previously, we demonstrated that Dlk-1 is strongly expressed on the cell surface of hepatoblasts in murine foetal liver from embryonic day (ED) 10.5–16.5 and Dlk-1+ cells isolated from foetal liver showed high-proliferative activity and bi-potentiality (*10*). Its expression is down-regulated in late gestation and completely absent after birth. In liver injury under conditions that limit proliferation of hepatocytes, immature cells with oval shaped nucleus called hepatic oval cells appear around the portal vein. As they are proliferative and express markers of hepatocytes and cholangiocytes, hepatic oval cells have been considered as adult liver progenitors (*29*). Similar cells were also shown to be present in severe hepatitis and implicated in tumourigenesis (*30*). The expression of Dlk-1 was also observed in a subpopulation of rat oval cells induced by the 2-acetylaminofluorene/partial hepatectomy model (*31*). These data suggest that Dlk-1 is a cell surface antigen of foetal/adult hepatic stem/progenitor cells.

It has become clear that tumour, in many cases, is a heterogeneous cell population and only a small fraction of the cells possess the potential to self-renew. Cancer stem cell or tumour initiating cell, which was first documented in haematological malignancies, has subsequently been discovered in many solid tumours, including breast, brain, prostate, liver, lung, melanoma, pancreas and colon tumours (*32–36*). While it was shown that CD133, known as a stem cell marker, is expressed in cancer stem cells in many tumours including HCC (*37–41*), the relation between normal tissue stem cells and cancer stem cells is not clear in most of the cases.

In this study, we established many hybridoma clones which produced anti-hDlk-1 monoclonal antibodies (mAb). Among them, we selected three independent clones usable for immunohistochemistry and characterized these antibodies by flow cytometry. Using these mAbs which recognized a different epitope, we investigated the expression of human Dlk-1 (hDlk-1) during liver development by immunohistochemistry. The expression of hDlk-1 showed a pattern similar to mouse Dlk-1 during liver development, suggesting that hDlk-1 is also a marker of hepatic stem/progenitor cells in embryo. We then examined hDlk-1 expression in human neoplastic liver lesions. About a half of HCC specimens from under 40-years-old patients expressed hDlk-1, whereas the positive ratio of hDlk-1 over 50-years-old patients was ~10%. Our study indicates the possibility that hDlk-1 is a common cell surface antigen both in human foetal liver stem/progenitor cells and in a part of HCC. Moreover, hDlk-1 was also frequently expressed in colon, breast, pancreas and lung carcinoma. These observations suggest that hDlk-1 is a potential target for monoclonal antibody-based therapy in those carcinomas.

Materials and Methods

Plasmid constructs

Full length hDlk-1 and its derivatives (EGF1-3 and EGF 4-6) were amplified by PCR. The sequences of primers were as follows: Fw1: 5′-cgcgtccgcaaccagaagccc-3′, Rv1: 5′-aagcttgatctcctcgtcgccggcc-3′ (for full length hDlk-1), Fw2: 5′-gcggccgcgctgaatgcttcccggcc-3′, Rv2: tctagaggcccgaacatctctatcac-3′ (for hDlk-1 EGF1-3), Fw3: 5′-gcggccgcgcctgctcctcggccccc-3′, Rv3: 5′-gcgtatagtaagctctgcgg-3′ (for hDlk-1 EGF4-6). All PCR products were verified by DNA sequencing. Full-length hDlk-1 cDNA was cloned in pcDNA3 vector (Invitrogen, Carlsbad, CA) with Flag tag. hDlk-1 EGF1-3 cDNA was subcloned in pME18SNeo carrying the signal sequence of CD8, His tag, and transmenbrane and cytoplasmic domains of FXYD5, which was kindly gifted by Dr Tanaka, M. (University of Tokyo, Tokyo, Japan). hDlk-1 EGF4-6 cDNA was subcloned in pME18SNeo containing the signal sequence of CD8, His tag.

Antibodies

Mouse monoclonal antibodies against hDlk-1 (clone DI-6, DI-2-20 and DI-4-22) were generated by the DNA immunization method (Nosan Corp., Kanagawa, Japan). To prepare purified monoclonal antibodies, hybridoma clones (3×10^6 cells) were intraperitoneally administered to BALB/c Slc-nu/nu mice (Japan SLC, Shizuoka, Japan), which received 2,6,10,14-tetramethylpentadecane (Sigma Aldrich Japan K.K., Tokyo, Japan) 7 days before injection of hybridoma. After collection of ascites, the antibodies were purified with a protein G column (GE Healthcare, Buckinghamshire, England). Rabbit polyclonal antibodies against hDlk-1 were prepared by immunizing with peptides containing the extracellular domain of hDlk-1 except for the putative signal sequence. Polyclonal antibodies were purified by affinity chromatography using columns conjugated with the peptides used for immunization.

Cell culture, transfection and flow cytometry

COS7 cells, HEK-293 cells, Huh-7 cells and SK-N-FI cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum. HepG2 cells and C3A/HepG2 cells were maintained in MEM supplemented with 10% foetal bovine serum. COS7, HEK-293, Huh-7 and HepG2 cells were from Human Science Research Resource Bank (Osaka, Japan). SK-N-FI and C3A/HepG2 cells were purchased from American Type Culture Collection (Rockville, MD). Transfection was performed using Lipofectoamine and Plus reagent (Invitrogen). To establish HEK-293 cells stably expressing hDlk-1 (293-hDlk-1), HEK-293 cells were transfected with pcDNA3 vector containing full-length hDlk-1 cDNA and selected with G418 (Invitrogen). COS7 cells were transiently transfected with expression constructs containing either hDlk-1 EGF1-3 or hDlk EGF4-6, and two days after transfection, these cells were harvested and subjected to fluorescence activated cell sorter (FACS) analysis. 293-hDlk-1 cells were cultured to subconfluency and harvested for flow cytometry by FACSCalibur (Nippon Becton Dickinson, Tokyo, Japan).

Immunohistochemistry

Tissue arrays and sections of tumours used in this study were purchased from Cybrdi (Rockville, MD), Shanghai Outdo Biotech Co. (Shanghai, China), Super Bio Chips (Seoul, Korea), ISU ABXIS (Seoul, Korea), US Biomax (Rockville, MD). Clinical information of patients (age, sex, grade and pathology diagnosis) is described in their homepage and data sheets. Foetal liver specimens were purchased from Biochain (Hayward, CA).

Paraffin embedded tissue sections and arrays were deparaffinized, and then autoclaved for 5 min in citrate buffer (pH 6.0) or TE buffer (pH 9.0). Slides were treated with methanol containing 0.3% H_2O_2 to inhibit endogenous peroxidase activity, and incubated with anti-hDlk-1 mAbs (10 µg/ml) at 4°C over night. After washing with phosphate-buffered saline (PBS), sections were stained with Vectastain ABC Elite kit (Vector, Burlingame, CA) and then counterstained with haematoxylin (Wako, Osaka, Japan). HCC sections with more than 10% immunopositive cells, either cell membrane or cytoplasmic stainin, were considered as positive.

Result

Characterization of anti-hDlk1 monoclonal antibodies

We established over 100 hybridoma clones producing anti-hDlk-1 mAb. Among them, three independent clones usable for immunohistochemistry in paraffin-embedded tissue sections were selected. First, we evaluated the reactivity and specificity of these antibodies by flow cytometry. Three mAbs against hDlk-1, DI-6, DI-2-20 and DI-4-22, specifically recognized HEK-293 cells stably expressing hDlk-1 (Fig. 1B), but not parent HEK-293 cells (not shown). On the other hand, these antibodies failed to recognize mouse Dlk-1, which shares ~90% similarity with hDlk-1 at the amino acid level (data not shown). These results confirmed that anti-hDlk-1 mAbs, DI-6, DI-2-20 and DI-4-22, specifically recognize hDlk-1. We then mapped the region of hDlk-1 to which the antibodies bind using deletion mutants of hDlk-1 (Fig. 1A). As shown in Fig. 1D, DI-6 recognized the EGF repeats 1–3 (amino acid 24–129), whereas DI-2-20 and DI-4-22 recognized EGF repeats 4–6 (amino acid 126–382). DI-2-20 and DI-4-22 recognized the same epitope because they competed each other (results not shown).

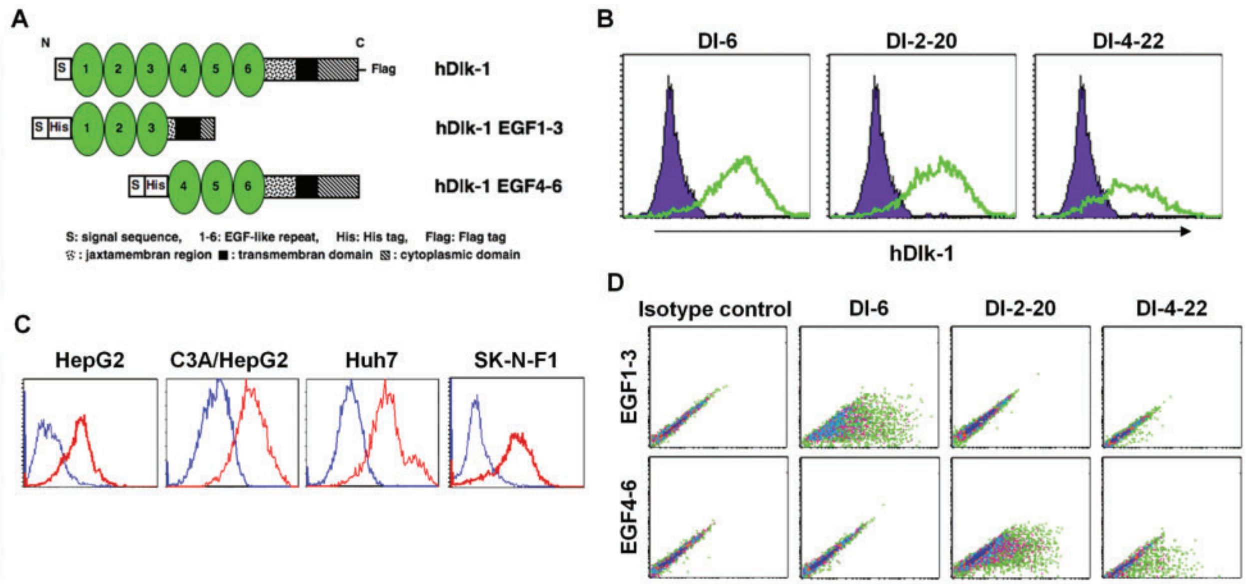

Fig. 1 Characterization of anti-hDlk-1 monoclonal antibodies by FACS analysis. (A) Schematic representation of various hDlk-1 constructs used in this study. (B) Three monoclonal antibodies used in this study specifically recognize hDlk-1 expressing cells. HEK-293 cells stably-expressing hDlk-1 were harvested, dispersed in a single cell suspension, and analysed by flow cytometry using monoclonal antibodies against hDlk-1, DI-6, DI-2-20, DI-4-22 (green line). Blue area: isotype control (mouse IgG1). (C) Anti-hDlk-1 mAb DI-2-20 also recognizes endogenous hDlk-1 in human cancer cell lines. Cancer cell lines indicated here were harvested and subjected to FACS analysis by using DI-2-20, respectively. Blue line: isotype control (mouse IgG1), red line: anti-hDlk-1 mAb (DI-2-20). Another monoclonal antibody, DI-6, showed similar result (data not shown). (D) Identification of the region of hDlk-1 to which anti-hDlk-1 mAbs bind. COS7 cells were transiently transfected with plasmids containing a various domain of hDlk-1 represented in (A). Two days after transfection, these cells were harvested and subjected to FACS analysis with anti-hDlk-1 mAb, DI-6, DI-2-20 and DI-4-22, respectively. DI-2-20 and DI-4-22 recognize EGF repeat 4–6, whereas DI-6 recognizes EGF repeat 1–3.

As reported previously, hDlk-1 was expressed early in liver development, but not in adult liver (*9*). To confirm that selected mAbs were suitable for immunohistochemistry in paraffin-embedded tissue sections, the expression pattern of hDlk-1 protein in liver was examined by immunohistochemical staining using DI-2-20 mAb (Fig. 2). A foetal liver tissue at 22 weeks (22w) showed membrane and cytoplasmic staining of hDlk-1 in hepatocytes. However, hDlk-1 was not detected in foetal liver at 38 weeks (38w) and adult liver. Northern blot analysis showed that hDlk-1 mRNA was strongly expressed in foetal liver from 6 to 12 weeks of gestation (not shown). The expression of hDlk-1 showed a pattern similar to mouse Dlk-1 during liver development, suggesting that hDlk-1 is also a marker of hepatic stem/progenitor cells in embryo. Similar results were obtained with either DI-6 or DI-4-22 mAb, though less sensitive than DI-2-20 (not shown). These results suggest that DI-2-20 was also useful for detecting endogenous hDlk-1 by immunohistochemistry. Therefore, we used mainly DI-2-20 mAb for further analysis.

Expression of hDlk-1 in HCC cells

We then examined the cell surface expression of hDlk-1 in a number of cancer cell lines originated from HCC by flow cytometry by using DI-6 and DI-2-20 antibodies. Among them, significant cell surface expression of hDlk-1 was detected in HepG2, C3A/HepG2 and Huh-7 cells (Fig. 1C). These results suggest that hDlk-1 is the cell surface antigen of HCC

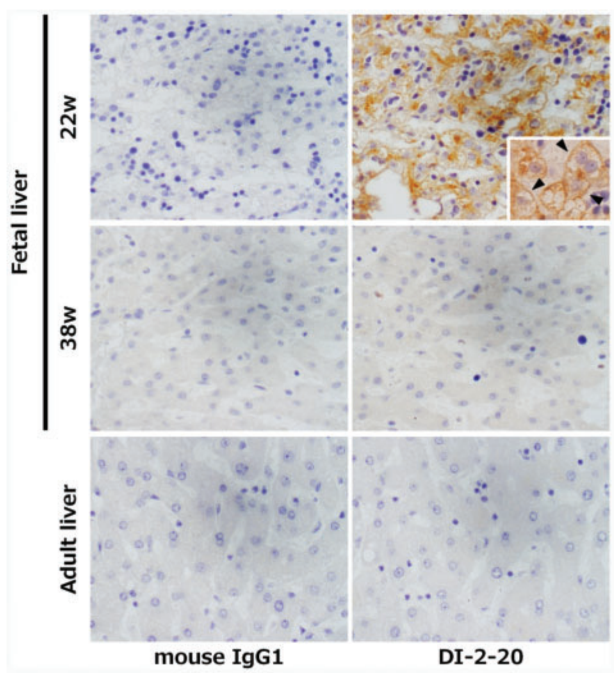

Fig. 2 Immunohistochemical analysis of hDlk-1 in foetal and adult liver. Each specimen was stained with anti-hDlk-1 mAb DI-2-20 (right panels). Foetal liver tissue at 22w (upper) shows membrane (arrow heads) and cytoplasmic staining with DI-2-20 monoclonal antibody. hDlk-1 staining was not observed in foetal liver at 38w (middle) and adult liver (lower). Mouse IgG1 used as isotype control was negative (left panels). Magnification is ×400.

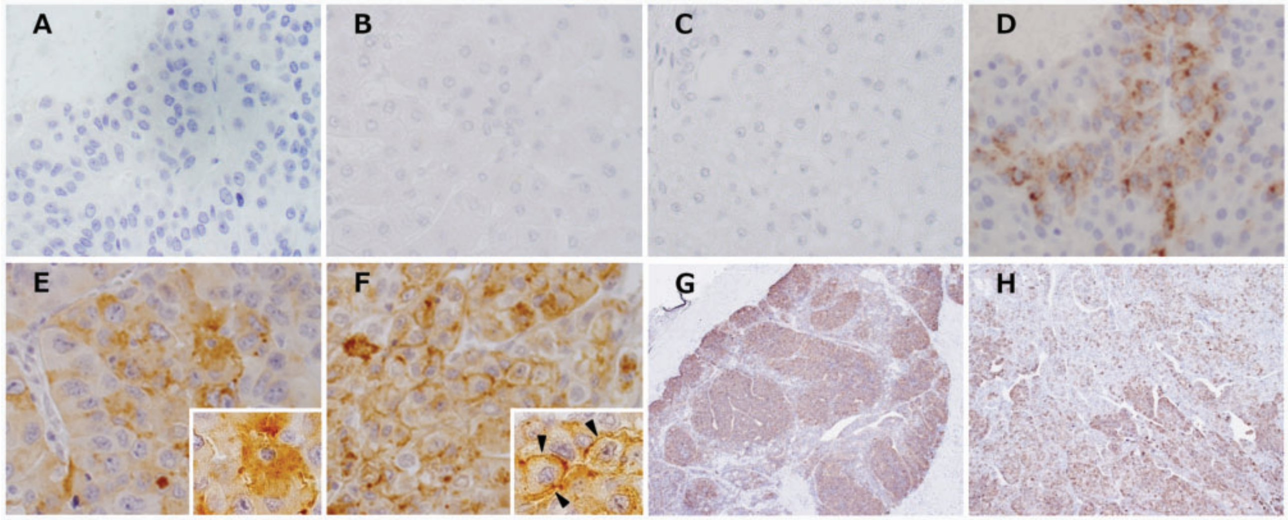

Fig. 3 hDlk-1 expression was observed only in hepatocellular carcinoma. Immunohistochemical staining with anti-hDlk-1 monoclonal antibody DI-2-20 was performed using tissue microarrays of liver tumour (388 malignant and 19 benign), nodular cirrhosis (40), viral hepatitis (11) and normal liver (26). The expression of hDlk-1 was detected only in HCC sections (D–F), not in adult liver (not shown), viral hepatitis (B), nodular cirrhosis (C), cavernous hemangioma (not shown) and intrahepatic cholangioma (not shown). hDlk-1 immunoreactivity in HCC cells was localized in either cytoplasm (E, inset) or cell membrane (F, inset). Membranous staining of hDlk-1 was shown by arrow heads. hDlk-1 expression was also examined in some samples of conventional tissue section corresponding to hDlk-1 positive spots in tissue array (G and H). Mouse IgG1 was used as negative control (A). Magnification is ×400 (A–F), ×1000 (inset in E and F) and ×40 (G and H).

cells. Additionally, hDlk-1 was also expressed in the cell surface of SK-N-F1 cells, a neuroblastoma cell line (Fig. 1C).

To evaluate the expression of hDlk-1 in non-neoplastic and neoplastic liver lesions, immunohistochemical analysis was performed by using tissue arrays. Typical staining profiles are shown in Fig. 3 and the results are summarized in Table I. hDlk-1 expression was undetectable at all in normal adult liver (0/26, not shown), non-neoplastic liver lesions, viral hepatitis (0/11, Fig. 3B) and nodular cirrhosis (0/40, Fig. 3C). hDlk-1 was also not found in cavernous hemangioma (0/19, not shown) and intrahepatic cholangioma (0/2, not shown). In contrast, hDlk-1 expression examined by DI-2-20 antibody was specifically observed in HCC and was positive for 79 out of 386 cases (20.5%, Fig. 3D–F). The pattern of hDlk-1 staining varied among individual tumours, e.g. hDlk-1 signal exhibited a uniform distribution within the tumour in one case (Fig. 3E, F), whereas it showed mosaic-like pattern in another case (Fig. 3D). Similar results were obtained with the same tissue array by using DI-6 antibody that recognizes a different epitope from DI-2-20 (not shown). Although hDlk-1 is a type I transmembrane protein, the immunoreactivity in HCC cells was mainly observed in the cytoplasm (Fig. 3E, inset), whereas hDlk-1 was expressed in cell surface in some cases (Fig. 3F, inset). We also examined some of conventional tissue slides corresponding to hDlk-1 positive spots in tissue arrays for hDlk-1 expression. The staining of hDlk-1 was not uniform, but covered more than 10% of the tumour in all tissue sections that we studied (Fig. 3G and H). No immunoreactivity was observed in normal tissues adjacent to the tumour (not shown).

Table I. Summary of immunohistochemical analysis.

	hDlk-1 staining	
	−	+
(A) *hDlk-1 expression in HCC (386 cases)*		
CS03-01-002 (Cybrdi)	40	15
CC03-01-001 (Cybrdi)	43	12
CC03-01-003 (Cybrdi)	46	10
CC03-02-001 (Cybrdi)	14	3
A204 (ISU ABXIS)	29	6
A204(II) (ISU ABXIS)	29	6
BC03013 (Biomax US)	49	10
OD-CT-DgLiv02-002 (Outdo bio.)	25	7
CS3 (HCC only) (Super Biochips)	32	10
Total	307 (79.5%)	79 (20.5%)
(B) *hDlk-1 expression in normal liver, non-neoplastic liver lesions, benign liver tumour and cholangiocarcinoma*		
Normal liver	23	0
Viral hepatitis	11	0
Nodular cirrhosis of liver	40	0
Cavernous hemangioma of liver	19	0
Intrahepatic cholangicarcinoma	2	0

As summarized in Table II, there was no clear correlation between hDlk-1 expression and pathological grade, gender, or aetiology such as HBV or HCV infection ($P > 0.05$ by χ^2 test). In contrast, hDlk-1 expression was clearly correlated with age or expression of alpha-fetoprotein (AFP). Interestingly, hDlk-1 expression was detected at higher frequency in HCC under 50 years old (51 out of 162 specimens, 31.5%), whereas the hDlk-1 positive HCC was dramatically decreased over 50 years old (28 out of 220 specimens, 12.7%). Especially, the hDlk-1 positive HCC was 43.1% (22 out of 51 specimens) under 40 years old. AFP is a well-established marker for HCC and was

Table II. Relationship of hDlk-1 expression and clinical features.

	hDlk-1			
	−	+	hDlk-1+ ratio	
Grade				
I	46	9	16.40%	
II	158	54	25.50%	
III	50	14	21.90%	$P = 0.347$
Gender				
Male	245	59	19.40%	
Female	58	20	25.60%	$P = 0.225$
Age				
40>	29	22	43.10%	
40–49	82	29	26.10%	
50–60	99	17	14.70%	
>60	93	11	10.60%	$P < 0.01$
AFP				
−	179	25	12.30%	
+	26	25	49.00%	$P < 0.01$
Aetiology				
HBV+	44	12	21.40%	
HCV+	6	0	0.00%	
−	7	1	12.50%	$P = 0.393$

present in 51 of 255 cases (20.0%). hDlk-1 was detected in 25 of 51 AFP-positive HCCs (49.0%), whereas 25 of 204 AFP-negative HCCs (12.3%) were positive for hDlk-1. These results indicated that the hDlk-1 was expressed more frequently in a patient under 50 years old and in AFP-positive HCC.

Expression of hDlk-1 in other carcinomas

We then examined the expression of hDlk-1 in various carcinomas and found that hDlk-1 was highly and frequently expressed in colon adenocarcinoma (58.6%), breast carcinoma (39.0%), pancreatic carcinoma (30.8%) and lung carcinoma (30.2%), but not in ovarian carcinoma (13.2%) and gastric carcinoma (3.33%). Interestingly, in pancreatic carcinomas, hDlk-1+ cells were found more frequently in islet carcinoma (50.0%) than duct carcinoma (28.3%). In the lung carcinoma, hDlk-1 was expressed in small cell lung carcinoma (52.5%), but only few non-small cell carcinoma (8.9%) expressed hDlk-1 (Fig. 4, summarized in Table III). These results suggested that hDlk-1 was expressed in various carcinomas.

As described above, hDlk-1 was more frequently expressed in AFP-positive HCC. Therefore we examined the expression of hDlk-1 in other AFP positive cancer, AFP-producing gastric cancer. hDlk-1 was rarely expressed in gastric carcinoma (3.33%), but was positive for two out of 10 cases in AFP-producing gastric cancer (20.0%, Fig. 4 G and H, summarized in Table III). Previously, Dezso *et al.* (*42*) reported that hDlk-1 was highly expressed in hepatoblastoma, AFP-positive liver cancer occurring in childhood. Together with our result, it was suggested that hDlk-1 was frequently expressed in AFP-positive cancers.

Discussion

Previously, we demonstrated that Dlk-1 is strongly expressed in hepatoblasts in mouse foetal liver, down-regulated in late gestation, and completely disappeared in neonatal and adult liver. Single Dlk-1+ cell isolated from ED14.5 liver exhibited high proliferating activity and was able to differentiate into both hepatocyte and biliary epithelial cell lineages (*10*). These findings suggested that Dlk-1 is a cell surface antigen of foetal hepatic stem/progenitor cells in the mouse. In this study we prepared mAbs against hDlk-1 and showed that the expression pattern of hDlk-1 is similar to mouse Dlk-1/Pref-1 during liver development, i.e. hDlk-1 is expressed in foetal liver but not in adult liver and hDlk-1 is present in both cell membrane and cytoplasm (Fig. 2). Thus, hDlk-1 may be an excellent marker of foetal hepatic stem/progenitor cells in human as well.

In adult liver, hepatic progenitor cells (HPCs) appear around the portal vein when liver is severely injured. These cells are known as hepatic oval cells in rodents and express markers of both hepatocytes and biliary epithelium (*29*). HPCs in chronic liver diseases are suggested to contribute to liver regeneration as well as hepatocarcinogenesis (*43–45*). Dlk-1 is not expressed in normal liver and was found in a subpopulation of hepatic oval cells induced in rats treated with 2-acetylaminofluorene and partial hepatectomy, a well-established rat model of hepatic oval cell induction (*31*). However, in a mouse model of hepatic oval cell induction by 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet, Dlk-1 is not expressed in hepatic oval cells (*46*). In the present study, hDlk-1 immunoreactive cells were not found in the specimens of viral hepatitis and nodular cirrhosis. In contrast, it was expressed frequently in HCC, but not in intrahepatic cholangioma, cavernous hemangioma and non-neoplastic liver lesions. These results demonstrate that hDlk-1 is expressed in HCC at high frequency, but do not exclude the possibility that Dlk-1 is expressed in some of adult hepatic stem/progenitor cells in chronically injured liver, which can lead to tumourigenesis.

It still remains unclear whether liver tumour is derived from hepatic stem/progenitor cells or mature hepatocytes. Recently, Lee *et al.* (*47*) reported that two subtypes (HB and HC) of HCC were categorized by analysis of gene expression patterns, and suggested that they may reflect the origin of tumour cells. The HB subtype shared a gene expression pattern with foetal hepatoblasts, whereas the HC subtype shared with adult hepatocytes, suggesting that the HB subtype may arise from hepatic stem/progenitor cells. The HB subtype of HCC showed poor prognosis compared to HC subtype. Interestingly, the HB subtype accounts for ~20% of HCC examined, similar to the frequency of hDlk-1 positive cells. In addition, the expression of hDlk-1 was more frequently found in HCC patients younger than 50 years old. Because HCC develops after a long latency period of chronic infection with HBV, HCV or both, the incidence of HCC is relatively high over 50 years old. Therefore, our finding that HCC patients under 50 years old expressed hDlk-1

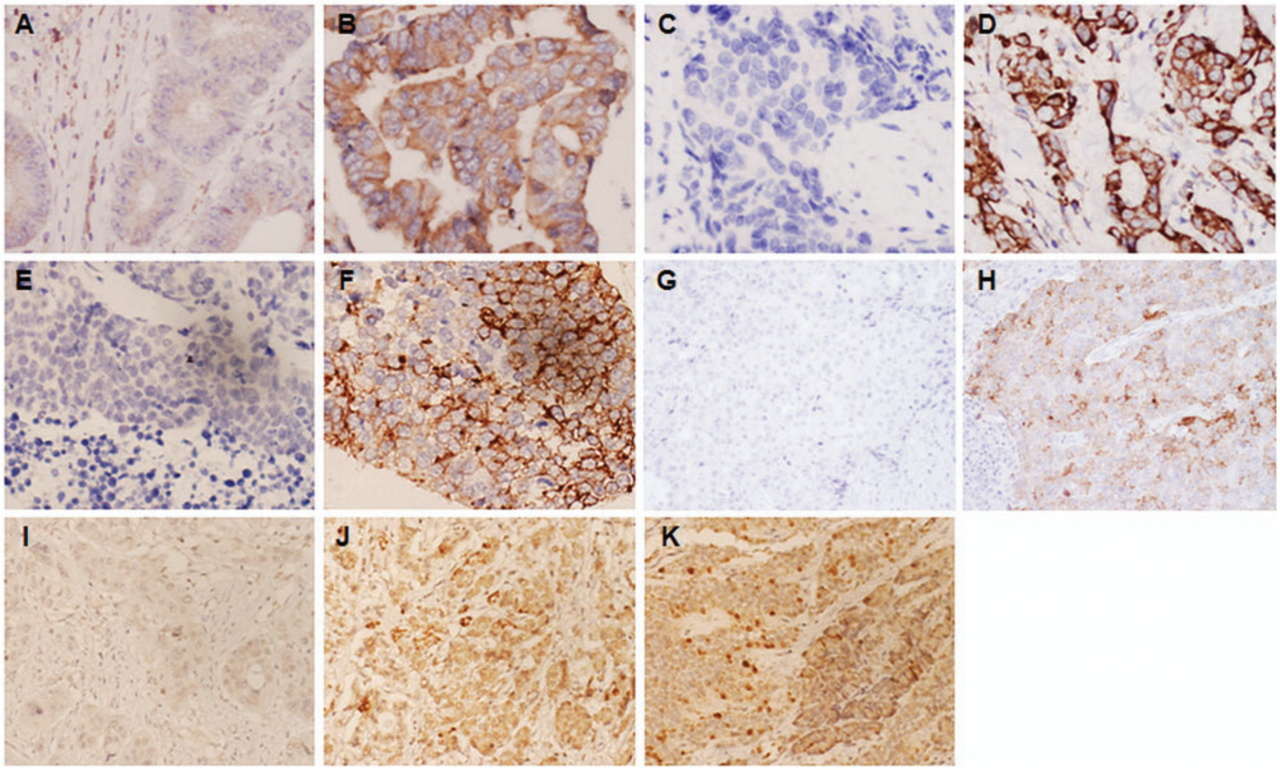

Fig. 4 hDlk-1 expression in various tumours. hDlk-1 expression in various tumours were examined by immunohistochemistry using anti-hDlk-1 mAb, DI-2-20. (A and B) Colon adenocarcinoma, (C and D) breast carcinoma, (E and F) small cell lung carcinoma, (G and H) AFP-producing gastric cancer, (I and J) pancreatic adenocarcinoma, (K) islet cell carcinoma. Tumour cells showed strong staining in cytoplasm (J and K), and cytoplasm and cell membrane (B, D, F and H). A, C, E, G and I showed hDlk-1 negative tumour cells. Magnification is ×200.

Table III. Summary of hDlk-1 staining in various tumours.

Tumour		Dlk−	Dlk+	Total
Colon adenocarcinoma		24 (41.4%)	34 (58.6%)	58
Ovarian carcinoma		59 (86.8%)	9 (13.2%)	68
Pancreatic carcinoma	Duct adenocarcinoma	33 (71.7%)	13 (28.3%)	46
	Islet cell carcinoma	3 (50.0%)	3 (50.0%)	6
Breast carcinoma		36 (61.0%)	23 (39.0%)	59
Lung carcinoma	NSCLC	51 (91.1%)	5 (8.9%)	56
	SCLC	19 (47.5%)	21 (52.5%)	30
Gastric carcinoma		29 (96.7%)	1 (3.33%)	30
AFP-producing gastric cancer	8 (80%)	2 (20%)	10	

more frequently is unexpected and intriguing. However, there was so far no clear correlation between hDlk-1 positive tumours in patients under 50 years old and specific aetiologies such as gender, pathological grade and stage. Recently, Huang *et al.* (*48*) also reported that hDlk-1 expression in HCC showed no significant correlation with HBV infection, tumour size and serology of AFP. Thus, our finding suggests that hDlk-1+ HCC develops in a relatively short latency period and may have an origin different from other HCC with a longer latency period. Alternatively, considering the recent finding that albumin positive hepatocytes can be converted into induced pluripotent stem cells (iPS) by transient expression of c-Myc, Sox2, Oct3/4 and Klf4 (*49*), conversion of mature hepatocytes to an immature stage with hDlk-1 expression may occur during chronic liver injury. Thus, it is tempting to speculate that hDlk-1 may be a hallmark of HCC originated from hepatic or cancer stem/progenitor cells. The origin and mechanism of tumourigenesis of HCC still need extensive investigation.

While Dlk-1, also known as Pref-1, was originally described as an inhibitor of adipogenesis (*8*), the precise function still remains unknown. In this study, we showed that hDlk-1 is expressed in not only HCC but also many carcinomas such as colon, breast, pancreatic and lung carcinomas. As previously reported, colony formation, cell growth and tumourigenicity of HCC cell lines were significantly decreased when the endogenous hDlk-1 was knocked down by RNAi (*48*), and hDlk-1 promoted proliferation of glioblastoma cell line (GBM cells) (*24*) and erythroid leukemia cell line (K562 cells) (*26*). Furthermore, Dlk-1 has been reported to interact with Notch 1, and modulate

Notch signalling as a negative regulator (*50*). Notch 1 signalling prevented HCC cells to proliferate by induction of cell cycle arrest and apoptosis (*51*). Thus, hDlk-1 may contribute to tumourigenesis by enhancing tumour growth. However, precise molecular mechanism of Dlk functions is still unknown, and requires further studies. Because hDlk-1 is a cell surface molecule expressed in many HCCs and also other carcinomas, but neither in normal adult liver nor most of the tissues, it may be an attractive target for antibody therapy. In this study, we established many monoclonal antibodies against hDlk-1, and now we are developing monoclonal antibodies against hDlk-1 that block proliferation of HCC in a xenograft model.

Conflict of interest
None declared.

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