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ORIGINAL ARTICLE

Delta-like protein (DLK) is a novel immunohistochemical marker for human hepatoblastomas

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Abstract Delta-like protein (DLK) is a membrane protein with mostly unknown function. It is expressed by several embryonic tissues among others by the hepatoblasts of rodent and human fetal livers. We have investigated in the present study if this protein is expressed in human hepatoblastomas. The presence of DLK has been studied by standard immunohistochemistry in 31 hepatoblastomas and in several differential diagnostically related tumours: hepatocellular carcinomas and in undifferentiated childhood neoplasms. All the hepatoblastomas were positive for DLK; the surrounding liver tissue remained negative. The reaction was present in the epithelial component of the tumours. The staining pattern was mostly membranous, occasionally cytoplasmic. The other studied tumours were negative for DLK, except one hepatocellular carcinoma and the differentiating cells of two ganglioneuroblastomas. Therefore, DLK seems to be a highly sensitive and specific marker for hepatoblastomas.

Keywords Immunohistochemistry · Hepatoblastoma · DLK

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Abbreviations

DLK	delta-like protein
AFP	alpha-fetoprotein
TLCT	transitional liver cell tumour
MPNST	malignant peripheral nerve sheath tumour

Introduction

The DLK1 gene codes for an EGF-like homeotic protein called delta-like protein (DLK) [12]. Since DLK is expressed in a wide variety of embryonic tissues in the human, it has several alternative names: Preadipocyte factor 1 (Pref-1), pG2, SCP-1, ZOG or FA1 [23, 6, 7]. DLK is a transmembrane protein containing six EGF-like repeats, a signal sequence in the extracellular domain, a unique transmembrane domain and a short intracellular region [20]. The cysteine spacing and the amino acid sequence within the individual EGF-like repeats are similar to those of delta. Delta is a ligand of the Notch receptor in *Drosophila* [1]. The function of the DLK molecule is largely unknown.

Recently, DLK has been shown to be an important marker for the oval cells in rat liver [9, 22]. The oval cells are hepatic progenitor cells sharing phenotypic markers with the embryonic hepatoblasts [19]. DLK is also highly expressed in the hepatoblasts of the human embryonic and fetal liver [6]. In the present study, we wanted to investigate if DLK could be used as a marker for the primitive human childhood liver tumour, hepatoblastoma. DLK expression could be demonstrated in all the examined hepatoblastomas by standard immunohistochemistry, while it was not present in the majority of differential diagnostically related tumours. Therefore, we suggest that DLK can be used as a highly specific immunohistochemical marker for hepatoblastoma.

Materials and methods

Formalin-fixed, paraffin-embedded tissue was used for immunohistochemical staining. The most important parameters of the investigated tumours are listed in Tables 1, 2 and 3. In brief, after blocking the endogenous peroxidase activity in methanol and hydrogen peroxide, the antigens were retrieved by heating in 10 mmol citrate buffer (pH 7.0) in a microwave oven for 10 min. The sections were then blocked in pre-immune serum diluted in phosphate-buffered saline before overnight incubation with the primary antibodies [DLK, R&D Systems, catalog number AF1144, dilution 1:100, Minneapolis, MN, USA; alpha-fetoprotein (AFP), DAKO, catalog number A0008, dilution 1:100, Glostrup, Denmark; and CK19, Biogenex, catalog number MU246-UC, dilution 1:50, San Remon, CA, USA] at 4°C. The Vectastain ABC Elite Kit (Vector Laboratories, Burlingname, CA, USA) was used for developing the reactions with diaminobenzidine as chromogen. The pre-immune serum was not replaced with primary antibody in the negative control sections. Rat liver tissue with oval cells was used as positive control for DLK and AFP.

Statistical analysis was performed by Fisher's exact test using 3×3 and 3×4 contingency tables.

Results

Thirty-one formalin-fixed, paraffin-embedded hepatoblastomas were collected from our archives (Table 1). All of the hepatoblastomas were positive for DLK protein; that is, the

Table 1 Important characteristics of hepatoblastomas

Number	Age (year)	Gender	Classification		Positive reaction			Remarks
			Type	Dominant epithelial component	%	Intensity	Pattern	
1	3	Male	W	F	11–40	+	Ca	
2	5	Female	M	F	1–10	+	Cy	
3	5	Male	W	E	1–10	+	M	
4	1	Female	W	E	1–10	+	Cy	
5	0.5	Male	M	F	>40	+	M	
6	1	Male	M	F	>40	+	M	
7	1	Male	M	E	11–40	++	Cy	
8	2	Female	W	F	1–10	++	M	
9	0.5	Female	M	E	11–40	+	Ca	
10	1	Female	W	F	1–10	+	Cy	
11	7	Male	W	F	1–10	+	M	
12	1	Female	W	F	11–40	+	M	
13	4	Male	W	E	11–40	++	Ca	Splenic metastasis
14	1	Female	M	E	11–40	+	Ca	
15	1	Male	M	E	1–10	++	Cy	
16	10	Male	W f	F	1–10	+++	D	
17	3	Male	W	E	11–40	++	Cy	
18	3	Male	W	E	1–10	+	Ca	
19	3	Male	W	F	>40	++	M	Lung metastasis
20	2	Male	W	F	1–10	+	M	
21	5	Female	W	E	11–40	+	M	
22	1	Female	W	F	1–10	+	M	
23	2	Male	W	F	11–40	++	M	
24	14	Male	W f	F	1–10	+	Cy	
25	1	Female	M	F	1–10	+	M	
26	4.5	Female	W	E	1–10	+	Cy	
27	1	Female	W	E	1–10	+	M	
28	4	Male	M	F	1–10	+	Cy	
29	5	Female	W f	F	11–40	++	M	
30	5	Female	W f	F	>40	++	Cy/M	
31	0.5	Male	W	F	11–40	++	Ca	

Classification according to SIOPEL: *W* wholly epithelial, *M* mixed, *Wf* purely fetal, *E* embryonal, *F* fetal
Staining pattern: *Cy* cytoplasmic, *M* membranous, *Ca* canalicular, *D* dot like

Table 2 Important characteristics of hepatocellular carcinomas

Number	Age (year)	Gender	Histological type	Grade	AFP	CK19	DLK
1	56	Male	PG	II	+	–	–
2	73	Male	So	III	+	–	–
3	64	Male	Tr	III	+	+	–
4	57	Male	PG	II	–	–	–
5	77	Male	So	II	+	–	–
6	57	Male	So	III	+	–	–
7	70	Male	PG	III	+	+	–
8	53	Female	Tr	III	+	–	–
9	54	Male	PG	III	–	+	–
10	50	Male	PG	II	–	–	–
11	62	Male	Tr	II	+	+	+
12	54	Male	PG	III	+	–	–
13	78	Male	PG	II	–	–	–
14	53	Female	PG	II	+	–	–
15	59	Male	Tr	II	–	–	–
16	49	Male	So	III	+	–	–
17	51	Male	Tr	III	–	–	–
18	49	Male	Tr	II	–	–	–
19	68	Male	So	II	+	–	–
20	64	Male	PG	II	+	–	–
21	68	Male	So	III	+	–	–
22	68	Female	So	III	+	+	–
23	60	Male	Tr	II	–	+	–
24	79	Female	PG	II	–	+	–

Grading according to Edmondson and Steiner [4]
PG Pseudoglandular, *So* solid, *Tr* trabecular

sensitivity of this antibody on these tumours was 100%. The staining was usually focal, covering 5–50% of the tumour, while the surrounding liver always was negative. The DLK protein was exclusively located on the epithelial components of the tumours, while the mesenchymal part of the mixed hepatoblastomas stained negative. Within the epithelial part, there was no strictly consequent preferential staining. Although the reaction was usually stronger over the more differentiated fetal components compared to the embryonal areas (Fig. 1a–c). Two of our samples represented metastatic tumours, where the DLK staining was preserved in the metastases (Fig. 1d). The staining pattern at the cellular level was mostly membranous (Fig. 2a). Occasionally, the membrane staining was confined to some poles of the cells showing a canalicular-like pattern (Fig. 2b). Sometimes, the cytoplasm was decorated (Fig. 2c), or dot-like positive areas were present in the cells (Fig. 2d), but the nucleus always remained negative. The cellular distribution and the extent and intensity of the DLK staining did not show significant correlation with the histological subtype or any other parameters. All of the studied tumours were also positive for AFP, the established immunohistochemical

Table 3 Other tumours tested for DLK

Tumour type	Age range (years)	Mean age	Gender M/F	Number	Remarks
Germ cell tumour[a]	20–51	22.8	6/0	6	AFP+
Wilms tumour	1–11	5.7	4/1	5	
Ganglio/Neuroblastoma	0.5–17	3.2	3/6	9	
Medulloblastoma	1–19	7.6	3/2	5	
Rhabdoid tumour	1	–	1/0	1	
Infantile haemangioendothelioma	1–3[b]	2	2/0	2	
Mesenchymal hamartoma	0.5	–	0/1	1	
Liver MPNST	23	–	0/1	1	

[a] Two yolk sac tumours, one teratocarcinoma, one seminoma, two immature teratomas
[b] Months

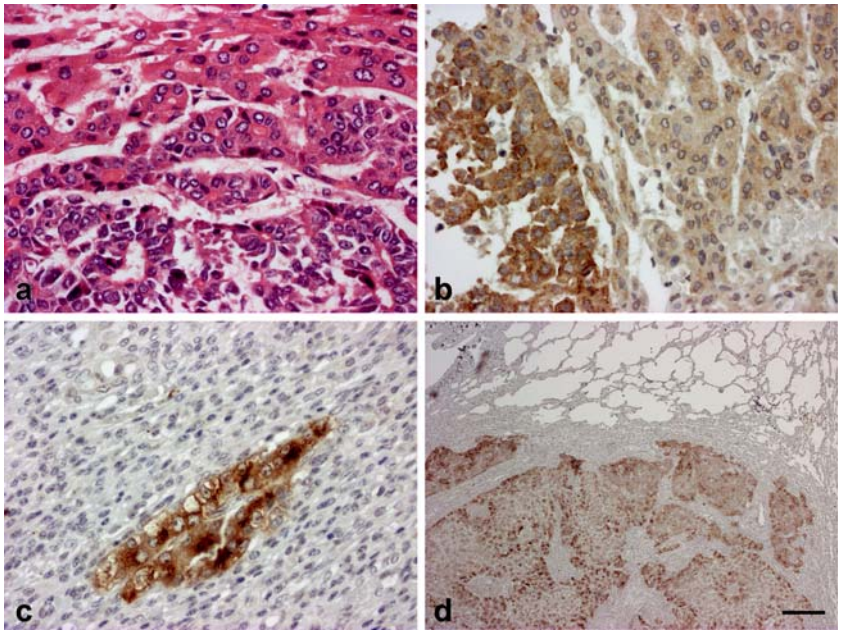

Fig. 1 **a** A hepatoblastoma with fetal (*lower part*) and embryonal components (H&E stain); **b** DLK stains preferentially the fetal component of the tumour shown in **a**. **c** Exclusive DLK staining in the epithelial part of a mixed hepatoblastoma; the mesenchymal component is negative. **d** Metastatic tumour in the lung, DLK is present only in the tumour tissue. *Scale bar* for **a**–**c**, 25 μm; **d**, 200 μm

marker of hepatoblastoma [5] (data not shown). The distribution of AFP and DLK occasionally overlapped, but this was not consistent.

We also examined several other tumour types for DLK expression. Twenty-four hepatocellular carcinomas were studied (Table 2). There was weak focal staining in one of these tumours; it is interesting to note that this hepatocellular carcinoma (HCC) was positive also for AFP and cytokeratin 19. In addition, the liver tumour of a 16-year-old girl showing transitional features between hepatoblastoma and hepatocellular carcinoma stained strongly for DLK (this tumour is not listed in the tables). Several other tumour samples have been stained with DLK antibody (Table 3), including six AFP-positive germ-cell tumours, a few undifferentiated childhood (Table 3) tumours covering neuroblastomas, Wilms tumours and medulloblastomas. The "small round cell" component of the tumours was consistently negative, but the enlarged, differentiating cells of two ganglioneuroblastomas reacted with the antibody. A few rare pediatric liver tumours (infantile haemangioendothelioma, mesenchymal hamartoma, malignant peripheral nerve sheath and rhabdoid tumour) were also examined, without any positive reactions. The specificity of DLK in the relation of all the tumours described above was 94%.

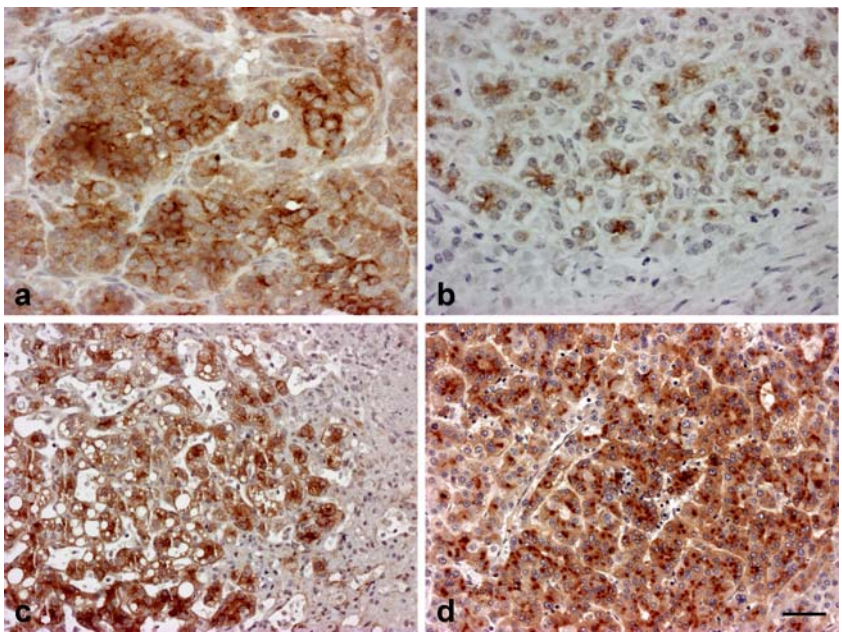

Fig. 2 Different staining pattern of DLK in hepatoblastomas: **a** membranous, **b** canalicular, **c** dot-like, **d** cytoplasmic. *Scale bar* for **a** and **b**, 25 μm; **c** and **d**, 50 μm

Discussion

Hepatoblastoma is the most common malignant liver tumour in children [17]. Its histological appearance is rather variable, but AFP is a stable hallmark for this tumour. In this paper, we report the finding of a novel reliable marker protein, namely DLK, which was detected in all the 31 studied hepatoblastomas. DLK was always expressed in the epithelial component, but its distribution did not show statistical correlation with the histological subtype. The staining pattern of DLK on the cells of hepatoblastomas is not uniform, but it is mostly associated with the cell surface. This is in agreement with the fact that DLK is a transmembrane protein with a large extracellular domain, but cytoplasmic staining of DLK also has been reported [8].

Both DLK and AFP mostly show focal staining pattern, but there was only partial overlap between the marked areas. Therefore, we believe that the application of both antibodies may increase the diagnostic sensitivity in case of limited availability of tumour sample, e.g. core biopsy or fine-needle aspiration biopsy. The divergent distribution of the two markers predicts that the regulation of the two genes is not identical. This is supported by the lack of DLK staining in all of the studied AFP-expressing germ-cell tumours. In addition, AFP can be present in HCCs, but we have detected focal staining for DLK only in one of the 24 examined HCCs. This particular tumour was positive for AFP and cytokeratin 19 as well. Lee et al. [14] defined recently a novel HCC subtype, which shares gene expression pattern with fetal hepatoblasts. This tumour type can be identified by microarray-based gene-expression profiling, but cytokeratin 19 and AFP co-immunostaining is characteristic for most of them. It is interesting to note that the only DLK-positive HCC sample is one of the three CK19+/AFP+ tumours, further supporting the relationship between these tumours and hepatoblasts. However, the great majority of the HCCs were negative for DLK. Consequently, DLK can be applied for the histological distinction between HCC and hepatoblastoma. DLK seemed to be useful, although with less stringent criteria for this distinction by a microarray-based global transciptome analysis [15]. In this study, elevated DLK expression was found only in a subset of hepatoblastomas. The partial disagreement between these and our results might be explained by the different methodology. Focally increased DLK expression was found in most of the hepatoblastomas by immunohistochemistry. The dilution effect of a sampling error might be responsible for the negative cases if the gene expression is analyzed on isolated RNA.

Recently, a malignant hepatocellular tumour developing in older children and adolescents have been termed transitional liver-cell tumour (TLCT) [16]. As its name implies, this highly aggressive neoplasm has a morphotype between hepatoblasts and hepatocytes. We have examined one liver tumour of a 16-year-old girl, which morphologically would fit into this category. This tumour was positive for hepatocyte-specific antigen and showed strong nuclear β-catenin reaction. However, differently from all the published TLCT cases, AFP could not be detected in the tumour by immunohistochemistry, and the serum value was also not elevated. This tumour showed strong, diffuse staining with DLK antibody. We are still uncertain on how to call this neoplasm, and thus it was not included in the listed cases in the tables. Nevertheless, we found DLK very useful to distinguish it from typical hepatocellular carcinoma.

DLK expression was conserved in the two hepatoblastoma metastases we examined, indicating that this immunophenotypic marker can be useful for the recognition of metastatic tumours. A few other undifferentiated childhood tumours were also tested for this marker, but they were negative except for the large ganglion cells of ganglioneuroblastomas (data not shown) in accordance with a previous report [8]. This is in line with the observation that DLK expression correlates with maturation along the chromaffin lineages [24].

In the adult rat liver, transcription and translation of the AFP and DLK1 genes are the only known reliable markers (which are not present in the biliary cells) for the bipotential oval/progenitor cells [19]. The human counterpart of the oval cells is still debated, but most results point to the ductular reaction which occurs in a wide variety of human liver diseases [18]. However, unlike murine oval cells [10], the cells of the human ductular reactions are consistently negative for AFP [11] and DLK (CH Jensen and HC Bisgaard, unpublished results) as well as the canals of Hering (P Nagy, unpublished results), which are the most likely candidates for the hepatic progenitor cell niche. These observations could indicate that oval cells expressing DLK and AFP in rat may recapitulate certain features of embryonic and fetal-liver development; this may not be the case in humans. Although the histogenesis of the hepatoblastomas is not clear, the presence of two embryonic liver-cell markers AFP and DLK supports the present notion about their hepatic embryonic or fetal progenitor-cell origin [25]. The function of DLK is not known. It is thought to play a role in cell-to-cell interactions controlling cell-fate determination during embryonic and fetal development and in adult organism [13]. DLK has been reported to interact with Notch1 in a specific manner using the yeast two-hybrid system [2, 3], and it may participate in the regulation of differentiation processes modulated by Notch1 activation and signalling [21]. If this is so, DLK may play a role in the growth and differentiation regulation of hepatoblastomas and may be a potential aim of molecularly targeted therapy.

In conclusion, DLK, a membrane protein of hepatoblasts and hepatocytes during embryonic and fetal development,

can be demonstrated by immunohistochemistry in hepatoblastomas but not in the majority of hepatocellular carcinomas or other related tumours. Therefore, DLK is a highly sensitive and specific immunophenotypic marker of this childhood liver tumour.

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Conflict of interest statement We declare that we have no conflict of interest.

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