DLK1 and the Notch pathway in the liver
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DLK1,
A SERUM MARKER FOR
HEPATOBLASTOMA IN YOUNG INFANTS

CHAPTER 3
Abstract

Background
Hepatoblastoma is the most common malignant liver tumor in children. Currently the only useful diagnostic serum marker for hepatoblastoma is α-fetoprotein (AFP). However, AFP levels are not always reliable in infants presenting with hepatoblastoma due to the physiologically elevated levels of AFP in this age group. In this report, we explored whether Delta-like 1 homolog (DLK1), a protein highly expressed during fetal development, but almost completely absent after birth, and an established liver-stem cell marker, is a suitable biochemical marker of hepatoblastoma, specifically in young infants.

Procedure: We collected serum samples from 7 hepatoblastoma patients and 48 pediatric controls and measured DLK1 and AFP serum levels in these samples.

Results: We show that serum DLK1 levels in pediatric controls decline with age, similar to but more rapidly than AFP. DLK1 serum levels were significantly elevated in hepatoblastoma patients compared to age-matched controls, with one exception, which was also an AFP-negative tumor. However, especially in the youngest patients, the AFP serum levels in infants with hepatoblastoma, were within the control range, whereas DLK1 serum levels in these patients were ~10-fold higher than controls.

Conclusions: These findings make DLK1 a candidate serum marker to diagnose hepatoblastoma in the young infant age group.

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Keywords
DLK1, hepatoblastoma, serum marker

List of abbreviations
CI: confidence interval; DLK1; delta-like 1 homolog
Part I

Introduction

Hepatoblastoma is the most common malignant liver tumor in children, but its incidence is low, comprising only 1% of all pediatric malignancies [1]. Hepatoblastoma mostly affects children up to 5 years of age [1, 2], with a maximum incidence between 6 and 36 months, but with ~10% percent of cases presenting before the age of six months [2, 3]. Prognosis is variable, mostly depending on the risk stratification (stage, resectability, metastases, vascular involvement, α-fetoprotein level) of the tumor at presentation [1, 4-6]. Chemotherapy followed by complete surgical resection with or without liver transplantation is essential for a favorable outcome and results in a 5-year survival of 70-90% [1, 7]. The most important diagnostic serum marker for hepatoblastoma is α-fetoprotein (AFP) [8]. However, 5-10% of hepatoblastomas are AFP-negative [2, 9]. Furthermore, AFP levels are not always reliable in young infants presenting with hepatoblastoma due to the physiologically elevated levels of AFP in this age group [3, 10-13]. Therefore, a biopsy is essential to establish the correct diagnosis in these patients. However, histological examination can still be difficult in differentiating between fetal hepatoblastoma and benign liver tumors like hemangioendothelioma and needle biopsy may not yield enough material for a reliable diagnosis and often necessitates open biopsy [10]. Thus, there is a need for additional biochemical markers to differentiate hepatoblastoma from benign liver lesions specifically in young infants and in AFP-negative hepatoblastomas.

Delta-like 1 homolog (DLK1) belongs to the EGF-like family of proteins, and is believed to signal through the Notch pathway, a developmentally highly conserved signaling pathway involved in cell-to-cell interactions and cell-fate decisions [14]. DLK1 is a transmembrane protein with a signal sequence and six EGF-repeats in its extracellular region, a unique transmembrane domain and a short intracellular region [15]. Because DLK1 expression in the fetal liver is high and the extracellular domain of DLK1 can be cleaved and secreted in serum, it was previously identified as Fetal Antigen1 (FA1) and found to be highly elevated in serum during the 2nd and 3rd trimester of pregnancy [16]. DLK1 is also known as preadipocyte factor 1 (Pref-1), because it can function as an inhibitor of adipogenesis [17].

The precise role of DLK1 in mammalian development is unclear, but the strong decline of its expression during development and its almost complete absence after birth, also in the liver [15, 16, 18, 19], suggests that it can be used to identify the retention of a fetal phenotype in DLK1-producing tissues. In agreement, DLK1 has been used as a marker for liver stem cells [18]. Previous reports showed DLK1 upregulation in hepatoblastomas, both at the mRNA and protein level [20-22] in up to 100% of hepatoblastomas [21, 22], while expression was absent in normal liver tissue and benign liver tumors like infantile hemangioendothelioma and mesenchymal hamartoma [20-22]. It has, therefore, been proposed that DLK1 can be used as an additional immunohistochemical tissue marker for hepatoblastomas [21]. In the present study, we explored whether DLK1 is a reliable serum marker for the presence of hepatoblastoma, especially in the early postnatal period when AFP levels are still physiologically elevated. We found that, similar to AFP, serum DLK1 levels in control
Introduction

infants and children decline with age to reach adult levels after 6 months of age. Serum DLK1 levels were significantly elevated in almost all hepatoblastomas studied. Unlike AFP, however, DLK1 levels in hepatoblastoma patients were, with one exception, never in the physiological range. This finding makes DLK1 a promising candidate marker for the detection of hepatoblastomas in young infants.
Materials and methods

Patient sera
Blood samples from 7 patients who presented with hepatoblastoma on the Oncology ward of the Emma Children’s Hospital of the Academic Medical Center (AMC), Amsterdam, The Netherlands, were obtained between December 2004 and May 2009 (see Table 1 for patient characteristics), with signed consent from the parents. Hepatoblastoma was diagnosed histologically by a specialized pathologist in tissue obtained by needle biopsy and resected liver. From one patient who died before surgical resection was performed, only needle biopsy material was available. As control pediatric blood samples, residual venous or capillary blood samples (0.1-1 mL) were collected from 48 children ranging in age from neonatal day 1 to 16 years, who were admitted to the general pediatric wards of the Emma Children’s Hospital AMC, in October 2009. The obtained sera were frozen and stored at -20°C until analysis. The study was approved by the Medical Ethics Committee of the AMC. Liver sections of hepatoblastoma patients were obtained from the AMC pathology department.

<table>
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<tr>
<th>Patient no.</th>
<th>Age (yr)</th>
<th>Gender</th>
<th>Classification</th>
<th>Risk group</th>
<th>AFP ng/ml</th>
<th>DLK1 ng/ml</th>
<th>Outcome</th>
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<td>149139</td>
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<td>CR</td>
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</tbody>
</table>

Table 1 Characteristics of hepatoblastoma patients
Risk stratification according to the SIOPEL study group:
SR standard risk, HR high risk
DOD dead of disease, CR complete remission

Serologic concentration of DLK1 and AFP
DLK1 and AFP levels in the same serum samples were determined using commercially available ELISA kits for the quantitative determination of human DLK1 and AFP in serum (R&D systems, Abingdon Science Park, UK). Hepatoblastoma samples were all analysed in duplicate. Accuracy and precision were checked by standard dilution series supplied in the kit.
Immunohistochemistry
Four µm sections of paraffin embedded hepatoblastoma tissue were deparaffinized, hydrated in graded alcohols, heated for 10 min at 120º C, 1 kPa in 10 mM sodium citrate (pH 6.0) to retrieve antigens, blocked in TENTGT (10mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.025% (w/v) gelatin, 0.05% (v/v) Tween-20) and incubated overnight with goat polyclonal DLK1-C19 antibody (Santa Cruz Biotechnology, Santa Cruz, USA) 1:500 diluted in TENTGT. After washing 3 times in Phosphate-Buffered Saline (PBS), sections were incubated with alkaline phosphatase-labeled rabbit-anti-goat secondary antibody (Sigma, Zwijndrecht, The Netherlands), diluted 1:50 in TENTGT, for 1.5 hour. After incubation, sections were washed 3 times in PBS, followed by visualization of alkaline phosphatase with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoly-phosphate (NBT/BCIP 1:50; Roche Woerden, The Netherlands). After dehydratation in graded alcohols, the sections were mounted with Entellan (Merck, Darmstadt, Germany) and photographed with a Leica DMRA2 microscope equipped with a DC300 camera.

Statistical analysis
Significance of a difference between groups was determined by the Mann-Whitney U test. The level of significance was set at P <0.05.
**Results**

**DLK1 serum levels decline with age**

Similar to serum AFP control levels (Figure 1B, black squares), DLK1 serum levels (Figure 1A; black dots) decline with increasing age. DLK1 serum levels decline from ~10 ng/mL (highest level 12.6 ng/mL, 95% CI: 6.9-10.5 ng/mL) in the first 3 months till 6 months of age, to reach adult levels afterwards (95% CI: 0.4-2.4 ng/mL).

![Figure 1](image)

**Figure 1 Serum levels of DLK1 and AFP in hepatoblastoma patients and pediatric controls**

Panel A shows serum DLK1 levels in 48 pediatric controls (black dots) and 7 hepatoblastoma patients (red dots) in ng/mL, while panel B shows serum AFP levels in the same samples (black and red squares, respectively). Serum DLK1 levels decline with age to reach adult levels after 6 months, similar to AFP levels. In 6 out of the 7 hepatoblastoma patients, serum DLK1 levels were ~10-fold above the control range, including during the first 3 postnatal months, whereas serum AFP levels are within the range of controls during this time period. Only in a 7 months old patient with small-cell undifferentiated hepatoblastoma, serum DLK1 (and AFP) level was within the normal range.
DLK1 serum levels in hepatoblastoma patients, including the young infant age group, are significantly elevated

DLK1 serum levels in the hepatoblastoma patient group (Figure 1A, red dots) were significantly higher than the pediatric control group ($P = 0.001$). Serum DLK1 levels in hepatoblastoma patients in the age group younger than 6 months ($n=3$) remained significantly elevated compared to age-matched controls ($n=18$; $P = 0.006$; Figure 2A), whereas serum AFP levels in these patients were not significantly different from controls (Figure 1B and 2B). Illustrative, our series included one patient with congenital hepatoblastoma, where the AFP level was within the physiological range on postnatal day 1 (178,554 ng/mL). In this patient, the serum DLK1 level was 87.3 ng/mL, or ~10x higher than the normal range for serum DLK1 concentration in this age group. Our series included one AFP-negative hepatoblastoma patient (7 months old), whose serum DLK1 level of was within the control range. This patient had a small cell undifferentiated hepatoblastoma, an uncommon histological subtype (Table 1). The lowest serum DLK1 level in the 6 other hepatoblastoma patients (9.3 ng/mL) was measured in a 7 year old boy, whereas age-matched DLK1 control levels were ~0.8 ng/mL, that is, ~10-fold lower.

Figure 2 DLK1 and AFP serum levels in young infants with hepatoblastoma and age-matched controls
Panels A and B show serum DLK1 and AFP levels, respectively, in 3 hepatoblastoma patients less than 6 months of age, together with 18 age-matched controls. Serum DLK1 levels in these young hepatoblastoma patients were significantly elevated ($P = 0.006$), whereas serum AFP levels in hepatoblastoma patients of this age group are within the range of controls.
Results

DLK1 expression in hepatoblastoma is confined to epithelial components

In agreement with previous reports, DLK1 protein showed an irregular expression pattern in examined hepatoblastomas, with expression mainly confined to epithelial components of the tumor [21], as shown in Figure 3B-D. As expected, adjacent normal liver tissue stained negative for DLK1 protein (Figure 3D).

Figure 3 DLK1 protein expression in hepatoblastoma
Panel A shows a Hematoxylin Eosin stained liver section of a 2 year old hepatoblastoma patient with both mesenchymal (upper part) and epithelial components (lower part). Panel B shows a DLK1 stained serial section of the same hepatoblastoma with DLK1 staining mainly confined to the epithelial part. Panels C and D show different parts of the same hepatoblastoma with DLK1 staining in epithelial components only and no staining in adjacent normal liver tissue (panel D). Scale bar in A is applicable for B-D.
Chapter 3 DLK1, a serum marker for hepatoblastoma in young infants
Discussion

In children with hepatoblastoma, AFP is the most important diagnostic serum marker [8]. However, in infants younger than 6 months old presenting with hepatoblastoma, AFP is not a reliable marker, due to the physiologically elevated AFP levels in this age group [3, 10-13]. Furthermore, 5-10% of hepatoblastomas are AFP-negative [2, 9]. In this study, we show that DLK1, a protein highly expressed during fetal development and a liver stem-cell marker [14-16, 18], is a candidate serum marker to diagnose hepatoblastoma in the young infant age group. First, we created a reference curve for serum DLK1 levels in control pediatric patients (Figure 1) and found that serum DLK1 levels decline with age. Highest serum DLK1 levels are present in the first three months (CI: 6.9-10.5 ng/mL), these decline to adult levels from 6 months onwards (CI: 0.4-2.4 ng/mL). Serum AFP levels which were measured in the same plasma control samples, showed a pattern similar to DLK1, consistent with previous reports [12, 13].

In 6 out of the 7 hepatoblastomas studied, serum DLK1 levels were ~10-fold higher than age-matched control levels. In a 7th patient, 7 months old, serum levels of both DLK1 and AFP were within the control range. This patient had a small cell undifferentiated hepatoblastoma, which suggests that AFP- and DLK1-negative hepatoblastomas belong to a separate subgroup. Importantly, in the young infant age group (0-6 months) we found that serum DLK1 levels in hepatoblastomas were significantly elevated compared to age-matched control group. Serum DLK1 levels in controls of this age group varied between 0.8-12.6 ng/mL, whereas the levels in the 3 hepatoblastoma patients we investigated varied between 23.7 and 87.2 ng/mL. For serum AFP levels in the same age group, no significant difference between hepatoblastoma patients and controls was found, due to the fact that in this age group the range of serum AFP levels in hepatoblastoma patients was within the same range of control levels. Therefore, despite the small number of patients studied, DLK1 seems to be a superior marker compared to AFP to diagnose hepatoblastoma in the young infant age group.

A previous study reported the presence of both DLK1 and AFP protein expression in 31 hepatoblastomas, and absence of DLK1 expression in non-malignant liver tumours [21]. Together with our findings, this further strengthens our conclusion that DLK1 can be used as a sensitive serum marker for hepatoblastomas.

In summary, we showed that serum DLK1 levels in pediatric controls decline with age similar to serum AFP levels. DLK1 serum levels were significantly elevated in hepatoblastoma patients compared to age-matched controls, with one exception. Unlike AFP, which is unreliable as a serum marker in infants younger than 3 months, serum DLK1 levels are significantly elevated in hepatoblastoma patients of this age group, which makes DLK1 a candidate marker to diagnose hepatoblastoma in young infants. While these results are promising, clinical validation relies on the collection of many serum samples of hepatoblastoma patients, more age-matched controls and samples from patients with other liver tumors.
We thank Prof. H.N. Caron (AMC) for his help with the study design and Dr. H. Klinkspoor (AMC) for making the pediatric aged-matched control samples available. We thank Dr. Bras (AMC) for his help with the interpretation of DLK1 stainings on hepatoblastomas.
Reference list