**ORIGINAL ARTICLE**

Imprinting, expression, and localisation of DLK1 in Wilms tumours

R Fukuzawa, R W Heathcott, I M Morison, A E Reeve

**BACKGROUND:** Loss of imprinting (LOI) of the H19/IGF2 domain is a common feature of Wilms tumour. The GTL2/DLK1 domain is also imprinted and is structurally similar to H19/IGF2. The question arises as to whether DLK1 also undergoes LOI in Wilms tumour, or whether the LOI mechanism is restricted to the H19/IGF2 domain.

**AIM:** To investigate the imprinting status of DLK1 in Wilms tumours with IGF2 LOI. The cellular localisation of DLK1 in the tumours was also examined.

**MATERIAL AND METHODS: Sample collection and histology**

Thirty Wilms tumours used for our study were selected from a collection of tumours obtained with informed consent from their corresponding paternally expressed, protein encoding genes, DLK1 and IGF2. 6, 15

(2) Similar to IGF2 and H19, DLK1 and GTL2 are transcribed in the same orientation and are reciprocally imprinted. 6, 15

(3) Both H19 and GTL2 have DMRs containing CTCF binding sites for the formation of a chromatin boundary. 6, 15

(4) Similar enhancer consensus sequences are located approximately 8 kb downstream from the H19 and GTL2 transcription start sites. 5

**RESULTS:**

Loss of imprinting (LOI) is an epigenetic error associated with tumorigenesis, particularly in Wilms tumour. 15, 17 The aim of our study was to investigate whether tumours with IGF2 LOI also have LOI of DLK1, or whether the LOI mechanism is restricted to the H19/IGF2 domain. Imprinting status, expression, and cellular localisation of DLK1 were examined in 30 Wilms tumours. We also investigated whether the expression of DLK1 in Wilms tumours is associated with rhabdomyogenic differentiation because mutations in a regulatory locus of DLK1 lead to muscle hypertrophy in callipyge sheep. 14

**CONCLUSION:** LOI does not occur at the GTL2/DLK1 domain in Wilms tumour. This finding suggests that LOI at 11p15.5 does not reflect non-specific disruption of a shared imprinting mechanism. DLK1 expression in Wilms tumour might reflect the presence of myogenic differentiation, rather than an alteration of its imprinting status.

**REFERENCES:**

1. The maternally expressed, non-coding GTL2 and H19 transcripts are both separated by approximately 100 kb from their corresponding paternally expressed, protein encoding genes, DLK1 and IGF2. 6, 15

2. Similar to IGF2 and H19, DLK1 and GTL2 are transcribed in the same orientation and are reciprocally imprinted. 6, 15

3. Both H19 and GTL2 have DMRs containing CTCF binding sites for the formation of a chromatin boundary. 6, 15

4. Similar enhancer consensus sequences are located approximately 8 kb downstream from the H19 and GTL2 transcription start sites. 5

5. Loss of imprinting (LOI) is an epigenetic error associated with tumorigenesis, particularly in Wilms tumour. 15, 17
from North Health ethics committee, Auckland, New Zealand. From this collection, all tumours with IGF2 LOI and WT1 mutations (17 and eight, respectively) were included in this study, and five tumours with 11p15.5 loss of heterozygosity (LOH) were chosen at random. IGF2 LOI and WT1 mutations were measured as described previously. LOH at 11p15.5 was assessed either by Southern blotting with Ha-Ras VNTR (variable number of random repeats), INS VNTR, and globin probes or by DNA microsatellite analysis with tyrosine hydroxylase. The tumours were examined histologically for the presence of myogenic differentiation; that is, the presence of muscle cells with striation (rhabdomyogenesis). Thirteen of the 30 tumours and two intralobar nephrogenic rest (ILNR) lesions were also available for immunohistochemical study. Fetal kidneys (8 and 21 weeks) and fetal skeletal muscle samples (8 weeks) were also used for immunohistochemistry.

**Analysis of heterozygosity for DLK1**

Wilms' tumours were examined for a polymorphism at exon 5 (rs1802710) in the DLK1 gene by direct sequencing using primers DLK-F9 and DLK-R7 (see below). Analysis of DLK1 imprinting was performed for the informative cases. To confirm DNA heterozygosity and allele specific expression, parental DNA was also analysed.

**Analysis of DLK1 imprinting**

Total RNA (2 µg) was DNase treated and cDNA was synthesised using Superscript TMII RNase H− reverse transcriptase according to the manufacturer’s protocol (Life Technologies, Gaithersburg, Maryland, USA). To eliminate the possibility of contamination by genomic DNA, cDNA was amplified using primers (F9 and R7) spanning exons 4 and 5. DLK1 genomic DNA and cDNA were amplified (35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds) and PCR products were purified using a Qiagen kit (Qiagen, Hilden, Germany). Sequence reactions were performed with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, California, USA) and run on an ABI 3100 sequencer. To confirm the sequencing results, all samples were sequenced from both directions using the forward and reverse primers (F9 and R7). The primers have been reported previously (DLK-F9, AAC AAC GGG ACC TGC GTG; DLK-R7, GCT TGC ACA GAC ACT CGT AGC TCA CC).

**Quantitative real time PCR**

Quantitative real time PCR (Q-PCR) was performed with an ABI PRISM 7900HT Sequence Detection System using ABI TaqMan probes, primers for DLK1 (Hs00171584, ABI), and Absolute QPCR ROX mix. The expression of DLK1 in each tumour sample was normalised to an endogenous control gene UBE2G2 (ubiquitin conjugating enzyme E2G2) and expressed relative to a pooled reference RNA sample.

**Immunohistochemistry**

Paraffin wax embedded sections (4 µm thick) were treated with 3% hydrogen peroxide for 15 minutes. Slides were autoclaved at 121°C for 15 minutes in 10 mM citrate buffer. Sections were incubated for 30 minutes in 5% normal rabbit
serum in phosphate buffered saline (PBS) with 0.1% bovine serum albumin (BSA). The sections were incubated with anti-DLK1 antibody (SC-8623; Santa Cruz Biotechnology, California, USA; 1/20 dilution) in PBS containing BSA at room temperature for one hour, followed by overnight incubation at 4˚C. As a negative control, adjacent sections were incubated without primary antibody. Tissue sections were washed in PBS and reincubated for 30 minutes with horseradish peroxidase conjugated rabbit antigoat IgG (H + L) (Zymed, South San Francisco, California, USA), diluted 1/200 in PBS with BSA. Sections were incubated with diamino-benzidine substrate (Rockland, Gilbertsville, Pennsylvania, USA) and counterstained with haematoxylin.

# RESULTS

## Expression of DLK1 RNA and histological correlation in Wilms tumour

DLK1 RNA expression was examined by Q-PCR (table 1). The highest expression levels were detected in the tumours with myogenic differentiation (rhabdomyogenesis); that is, five WT1 mutant tumours (WT1-1, WT1-2, WT1-3, WT1-5, and WT1-8) and two IGF2 LOI tumours with myogenic differentiation (LOI-15 and LOI-16). Although all eight WT1 mutant tumours had rhabdomyogenesis, three tumours (WT1-4, WT1-6, and WT1-7) showed low DLK1 expression. Most tumours with IGF2 LOI were composed predominantly of blastemal cells and showed low expression of DLK1, whereas two tumours without myogenic differentiation (LOI-9 and LOI-11, both of which showed mixed epithelial and blastoidal histology) had moderate to high expression of DLK1. The tumour (LOI-11) with the highest DLK1 expression had blastemal predominant histology with focal anaplasia, and contained a large area of necrosis surrounded by granulation tissue with aggregated foamy macrophages. Moderate DLK1 expression was detected in three tumours with 11p15.5 LOH (LOH-1, LOH-2, and LOH-3). One of these tumours (LOH-1) contained myogenic differentiation, but the remaining two tumours showed blastemal predominant histology without myogenic differentiation.

## Imprinting status of DLK1 in Wilms tumours

Ten of the 30 tumours were heterozygous for the T/C polymorphism at exon 5 (rs1802710), namely: five tumours with IGF2 LOI, three tumours with WT1 mutations, and two tumours with 11p15.5 LOH. Imprinting (monoallelic expression) of DLK1 was seen in all heterozygous tumours, irrespective of the imprinting status of IGF2 and/or level of DLK1 expression. Heterozygosity and parental specific expression of DLK1 in the tumours were confirmed by analysing the parental DNA (fig 2).

## Cellular localisation of DLK1

The localisation of DLK1 was first examined in the developing kidney because Wilms tumour often recapitulates early kidney epithelial development. Expression of DLK1 is probably required for the later stages of epithelial kidney development rather than the early stages, because DLK1 immunostaining was faintly positive or negative in the uninduced mesenchyme, condensed mesenchyme, renal vesicles, comma shaped bodies, S shaped bodies, presumptive podocytes, and glomerular epithelia (fig 3A), and weakly positive in the cytoplasm of ureteric buds and ureteric bud ampullae (fig 3A). DLK1 was moderately to highly expressed in the cytoplasm of developing tubules (future proximal and distal convoluted tubules) (fig 3A). DLK1 was also expressed in the apical membrane of distal tubules or Henle’s loop and

![Cellular localisation of DLK1](image-url)
localised in the cytoplasm and cell membrane in collecting ducts. Three normal kidney tissues adjacent to the tumours (WT1-1, WT1-2, and WT1-3) were available for immunohistochemistry. Moderate to strong expression of DLK1 was seen in the nucleus and cytoplasm of collecting ducts, Henle’s loops, proximal and distal tubules (fig 3B). Weak expression of DLK1 was sporadically seen in the nuclei of glomerular capillary endothelia in both immature and mature glomeruli. DLK1 was also weakly expressed in the smooth muscles of small arteries and nuclei in endothelia. Skeletal muscles in a fetus (8 weeks) showed DLK1 expression (fig 3C), indicating that DLK1 is associated with myogenic differentiation.

Thirteen Wilms tumours were analysed for DLK1 using immunohistochemistry (table 2): three IGF2 LOI tumours, eight WT1 mutant tumours, and two 11p15.5 LOH tumours that had detectable DLK1 expression by Q-PCR. Moderate to strong expression of DLK1 was seen in the cytoplasm and cell membrane of the myogenic elements in all tumours (fig 4A), including three WT1 mutant tumours that showed low expression of DLK1 in the Q-PCR assay. Weak to moderate expression of DLK1 was localised to the cytoplasm and nucleus of the epithelial components (fig 4A, B). Immunostaining for DLK1 was occasionally seen in the nucleus and cytoplasm of the blastemal component in all three molecular classes (fig 4A, B). The tumour with the highest DLK1 expression by Q-PCR (LOI-11) showed strong expression of DLK1 in the cytoplasm of foamy macrophages, suggesting that the high number of macrophages may have contributed to the high expression of DLK1 RNA in this tumour. The expression pattern of DLK1 in ILNRs (WT1-1 and WT1-2) was similar to that of Wilms tumours in that they had focal expression of DLK1 in the blastemal and epithelial components (fig 4C). Although myogenic elements were not seen in ILNRs, the non-polarised epithelial structures, sometimes referred to as “central and stromal tubules” that correspond to the collecting duct system, showed high DLK1 staining, similar to that seen in normal kidney.

DISCUSSION

We have shown that LOI in Wilms tumour is restricted to the IGF2/H19 domain, even though the DLK1/GTL2 domain shares structural and epigenetic similarities with the IGF2/H19 domain. This finding suggests that IGF2 LOI does not reflect non-specific disruption of a shared imprinting mechanism. DLK1 has been shown to be expressed in neuroendocrine cancers, such as neuroblastoma, phaeochromocytoma, small cell carcinoma of the lung, and pancreatic islet tumours. Analysis of DLK1 imprinting has recently been reported in neuroblastoma, lymphoma, and brain tumours. However, none of these tumours showed loss of imprinting of DLK1.

“DLK1 might provide a useful marker for other tumours that show rhabdomyogenesis, such as rhabdomyosarcoma and pleuropulmonary blastoma”

| Table 2 Immunohistochemical localisation and staining intensity of DLK1 in Wilms tumours |
|---------------------------------|------|------|------|------|
| Tumour group        | Tumour no | Epi  | Bl   | Str  |
| LOI tumours with myogenesis | LOI-15 and LOI-16 | -/+  | -/+  | ++/+ (myogenic) |
| LOI tumours without myogenesis | LOI-11 | -/+  | -/+  | ++/+ (macrophage) |
| WT1 mutant tumours   | WT1-1 to WT1-8 | -/+  | -/+  | ++/+ (myogenic) |
| ILNRs               | WT1-1 and WT1-2 | ++/+ | -/+  | - |
| LOH tumours         | LOH-2 and LOH-3 | -/+  | -/+  | - |

Each column shows the range of expression: no detectable expression, -; mild, +; moderate, ++; high expression, +++; Bl, blastemal; Epi, epithelial; ILNRs, intralobar nephrogenic rests; LOH, loss of heterozygosity; LOI, loss of imprinting; Str, stromal.
Expression of DLK1 is associated with muscular growth and development, because DLK1 is highly expressed without alteration of its imprinting status in the callipyge phenotype in sheep that have an inherited skeletal muscular hypertrophy. Overexpression of DLK1 in the callipyge phenotype is seen preferentially in skeletal muscle, although it has been shown to be expressed in uterine leiomyomata, which are derived from smooth muscle cells. In our immunohistochemical study, expression of DLK1 was strong in fetal skeletal muscles, but weak in the smooth muscles of small arteries of tumour bearing kidneys. Similar to the observation of DLK1 overexpression in the callipyge phenotype in sheep, high amounts of DLK1 mRNA were found predominantly in Wilms tumours with abundant rhabdomyogenesis, many of which carried WT1 mutations. The localisation of DLK1 in the myogenic elements was confirmed by immunohistochemistry. The expression of DLK1 did not mimic that seen in early kidney epithelial development because expression of DLK1 was weak or absent in the developing kidney, whereas it was variable in the blastemal and epithelial components.

Our immunohistochemical study suggests that DLK1 might provide a useful marker for other tumours that show rhabdomyogenesis, such as rhabdomyosarcoma and pleuropulmonary blastoma. The specificity of loss of imprinting within the H19/IGF2 domain suggests that future studies into the mechanism of abnormal imprinting in Wilms tumour should focus on the specific genetic and epigenetic changes within the H19/IGF2 domain, rather than the abnormal function of a generalised imprinting mechanism.

ACKNOWLEDGEMENTS
We thank Dr M Sano, Department of Pathology, Nihon University School of Medicine, Japan, for helpful advice on immunohistochemistry. This work was supported by the Health Research Council of New Zealand, the Cancer Society of New Zealand, and the NZ Lottery Health Grants Board.

Authors’ affiliations
R Fukuzawa, R W Heathcott, I M Morison, A E Reeve, Cancer Genetics Laboratory, Department of Biochemistry, University of Otago, PO Box 56, Dunedin, New Zealand

REFERENCES
Imprinting, expression, and localisation of DLK1 in Wilms tumours

R Fukuzawa, R W Heathcott, I M Morison and A E Reeve

doi: 10.1136/jcp.2004.021717

Updated information and services can be found at:
http://jcp.bmj.com/content/58/2/145

These include:

References
This article cites 24 articles, 5 of which you can access for free at:
http://jcp.bmj.com/content/58/2/145#BIBL

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections
Articles on similar topics can be found in the following collections

- Paediatric oncology (111)
- Molecular genetics (355)
- Immunology (including allergy) (1664)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/