Epigenetic alteration at the DLK1-GTL2 imprinted domain in human neoplasia: analysis of neuroblastoma, phaeochromocytoma and Wilms’ tumour

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Epigenetic alterations in the 11p15.5 imprinted gene cluster are frequent in human cancers and are associated with disordered imprinting of insulin-like growth factor (IGF)2 and H19. Recently, an imprinted gene cluster at 14q32 has been defined and includes two closely linked but reciprocally imprinted genes, DLK1 and GTL2, that have similarities to IGF2 and H19, respectively. Both GTL2 and H19 are maternally expressed RNAs with no protein product and display paternal allele promoter region methylation, and DLK1 and IGF2 are both paternally expressed. To determine whether methylation alterations within the 14q32 imprinted domain occur in human tumorigenesis, we investigated the status of the GTL2 promoter differentially methylated region (DMR) in 20 neuroblastoma tumours, 20 phaeochromocytomas and 40 Wilms’ tumours. Hypermethylation of the GTL2 promoter DMR was detected in 25% of neuroblastomas, 10% of phaeochromocytoma and 2.5% of Wilms’ tumours. Tumours with GTL2 promoter DMR hypermethylation also demonstrated hypermethylation at an upstream intergenic DMR thought to represent a germline imprinting control element. Analysis of neuroblastoma cell lines revealed that GTL2 DMR hypermethylation was associated with transcriptional repression of GTL2. These epigenetic findings are similar to those reported in Wilms’ tumours in which H19 repression and DMR hypermethylation is associated with loss of imprinting (LOI, biallelic expression) of IGF2. However, a neuroblastoma cell line with hypermethylation of the GTL2 promoter and intergenic DMR did not show LOI of DLK1 and although treatment with a demethylating agent restored GTL2 expression and reduced DLK1 expression. As described for IGF2/H19, epigenetic changes at DLK1/GTL2 occur in human cancers. However, these changes are not associated with DLK1 LOI highlighting differences in the imprinting control mechanisms operating in the IGF2-H19 and DLK1/GTL2 domains. GTL2 promoter and intergenic DMR hypermethylation is associated with the loss of GTL2 expression and this may contribute to tumorigenesis in a subset of human cancers.

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Genomic imprinting is a process whereby imprinted genes demonstrate parent-of-origin differences in allelic expression. Although only a minority of genes are imprinted, many of those identified to date have a role in the regulation of cell growth and differentiation and aberrant imprinting frequently leads to abnormal pre- and/or postnatal growth (Reik and Walter, 2001). Furthermore, disordered imprinting has been implicated in the pathogenesis of paediatric and adult cancers. Imprinted genes frequently occur in clusters. In particular, the imprinted gene cluster at 11p15.5, which contains the paternally expressed insulin-like growth factor (IGF)2 and the maternally expressed CDKN1C and H19 genes has been implicated in disorders of growth and in neoplasia (Maher and Reik, 2000; Feinberg et al, 2002). For example, germ-line-inactivating mutations in CDKN1C and epigenetic alterations leading to the loss of imprinting (LOI) (biallelic expression) of IGF2 and silencing of CDKN1C or H19 cause Beckwith–Wiedemann syndrome, a congenital overgrowth disorder associated with susceptibility to embryonal tumours (Maher and Reik, 2000; Weksberg et al, 2003). In addition, epigenetic alterations at IGF2 and H19 have been implicated in the pathogenesis of sporadic childhood (e.g. Wilms’ tumour) and adult (e.g. colorectal) cancers (Cui et al, 2001; Nakagawa et al, 2001). IGF2 and H19 are closely linked and reciprocally imprinted, and epigenetic alterations in human cancers at an intergenic differentially methylated region (DMR) are associated with LOI of IGF2 and silencing at H19.
In the past decade, the imprinted gene clusters at 11p15.5 and 15q13 (Prader-Willi and Angelman syndrome region) have been the subject of intense investigation. Recently, an imprinted gene cluster at 14q32 (distal mouse chromosome 12) was identified. The first imprinted 14q32 gene identified was GTL2/Gtl2, which, like H19, is a maternally expressed RNA with no apparent open reading frame (Miyoshi et al., 2000). Subsequently, a paternally expressed gene, DLK1/Dlk1, which encodes an EGF-like membrane protein similar to the Drosophila Delta gene was identified upstream of GTL2/Gtl2 (Kobayashi et al., 2000; Schmidt et al., 2000; Takada et al., 2000; Wylie et al., 2000). There are a number of similarities between the IGF2/H19 and the DLK1/GTL2 domains. In both cases, two closely linked genes are similarly organised, reciprocally imprinted and developmentally regulated; H19 and GTL2 do not encode known proteins, and paternally methylated DMRs associated with both genes have been implicated in the regulation of their imprinting (Paulsen et al., 2001; Takada et al., 2002).

In sheep, a single-nucleotide (nt) substitution is associated with altered postnatal expression of GTL2 and DLK1, although their imprinting is not perturbed. This mutation results in the Callipyge muscle hypertrophy phenotype (Charlier et al., 2001; Freking et al., 2002; Georges et al., 2003). In human mutations or epigenetic alterations of DLK1 and GTL2 have not been described, although distinct phenotypes associated with maternal and paternal disomy for chromosome 14 are reported (Georgiades et al., 2002; Georges et al., 2003). In addition, downregulation of GTL2 has been reported in pituitary adenomas with ectopic expression of IGF2/H19 and the DLK1/GTL2 domains. In both cases, two closely linked genes are similarly organised, reciprocally imprinted and developmentally regulated; H19 and GTL2 do not encode known proteins, and paternally methylated DMRs associated with both genes have been implicated in the regulation of their imprinting (Paulsen et al., 2001; Takada et al., 2002).

We have investigated neuroblastoma and other tumours to determine whether epigenetic alterations within the DLK1-GTL2 imprinted domain are a feature of human neoplasia. We investigated neuroblastoma and phaeochromocytoma as DLK1 is highly expressed in the adrenal medulla. Neuroblastoma may also demonstrate 14q allele loss and Wilms’ tumour was of interest as epigenetic alterations at the IGF2/H19 locus are common in this tumour.

MATERIALS AND METHODS

Multiplex methylation polymerase chain reaction (mPCR) assay

Methylation-specific PCR with oligonucleotide primers specific for the methylated and unmethylated copies of the GTL2 promoter on chromosome 14q52 were performed using previously published primers (Murphy et al., 2003): MS3M-UF (5'-GAG GAT GTG TAG TTG GGG T3-); nt position 65912 (GenBank accession no. AL117190); MS3M-UR (5'-CCA TAA CCA ACC ACC ATAT CAC A3-); nt 65931–66004; MS3M-MF (5'-GTT AGT CAA CCG GTG TTT GGG C3-); nt 66450–66471) and MS3M-MR (5'-AAT CAT AAC TCC GAA ACC CGG CGG CGG-3'; nt 65487–66409). The multiplex mPCR conditions used were initial denaturation at 95°C for 3 min; five cycles of 95°C for 30 s, 70°C for 30 s, 72°C for 30 s; five cycles of 95°C for 30 s, 65°C for 30 s, 72°C for 30 s; followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s and a final extension of 72°C for 5 min. PCR amplification were performed using 0.05 μl-1 Taq polymerase in (NH4)2SO4 buffer with 3.0 mM MgCl2 (MBI Fermentas, St Leon-Rot, Germany). PCR products (160 bp for methylated allele and 120 bp for unmethylated) were separated on a 3% agarose gel, stained with ethidium bromide and visualised under UV illumination.

Analysis of DNA methylation using bisulphite sequencing

Genomic DNA was treated with sodium bisulphite using previously published method (Herman et al., 1996). DNA (0.5–1 μg) was denatured at 37°C for 10 min in 0.3 M NaOH followed by sulphonation of unmethylated cytosines by incubation in 5.12 M sodium bisulphite containing 1 M hydroquinone (pH 5) at 95°C for 30 s and 50°C for 15 min for 20 cycles. The resulting sulphonated DNA was purified using the Wizard DNA clean-up system (Promega, Southampton, UK) according to the manufacturer’s instructions and DNA was eluted with 50 μl of distilled water. Following elution, DNA was desulphonated in 0.3 M NaOH for 5 min at room temperature, ethanol precipitated and resuspended in 50 μl distilled water.

Methylation status of GTL2 and DLK1 DMRs

Following multiplex PCR methylation screening (see above), the methylation status of three areas of the CpG-rich region upstream of GTL2 were analysed in detail (G1, position 66591–66697; G2, position 66541–66920; G3, position 67541–67910; GeneBank accession no. AL117190; relative to GTL2 transcription start site, G1 = –363 bp → –161 bp upstream; G2 = +293 bp → +673 bp downstream; G3 = +1213 bp → +1583 bp downstream) GTL2 transcription start site; GeneBank accession no. AY314975) and a region upstream DLK1 promoter (D1, position 140551–140810; GeneBank accession no. AL132711) and two downstream regions (D2, position 141271–141420; D3, position 141571–141894; GeneBank accession no. AL13271). Except for D1 and D2 regions, bisulphite-modified DNA was amplified using two rounds of nested PCR (95°C for 15 min followed by 30 cycles of 95°C for 30 s, 55–58°C for 30 s, 72°C for 30 s and a final extension of 72°C for 5 min) using HotStar Taq DNA polymerase (Qiagen, Crawley, UK). Primer sets were designed to amplify DNA fragments containing both methylated and unmethylated CpG dinucleotides. The primer sequences are: G1F (5'-TTA GTG TGT GGG TTT GTT TGA TAG TT-3'); G1R (5'-TAA AAA AAT CAA TTT CTT CTA ACR TCA ACR CAT TCT ACT A3-); G1FN (5'-GTT TAT TGG TTY TTT GAG GAY GGT TAG TT-3'); G2F (5'-TTA GTG TTT TTT TTT GGA GGG TTT AGT TT-3'); G2R (5'-AAA ACT AAT CCA TAA AAA CAA CTA CTA ACA AAT CAA-3'); G3F (5'-AGA GGG ATT GGT TTT TTT TTT TTT YGG ATT TTA TTT-3'); G3R (5'-ATC TAA CCA AAC AAC CAC TCA TCA CCA CCT ATA TTA A3-3'); G3MN (5'-ATA ATC TCR AAA CRA AAA ACA ACC CCT ATA A3-3'); D1F (5'-TAT ATA GTT GTG ATT ATT TTT GTC TTG TT-3-'); D1R (5'-TAA AAA GCA AAA CCC ATA AAC ATC CCC AAA A3-3'); D2F (5'-TTG GTA ATT GTT ATT TTT TTT TTT TA-3'); D2R (5'-ACT TTT TTT AAC AAT AAC ATA AT CAA AAT AAA A3-3'); D3F (5'-TTG TTT ATT ATG TTT GAG TAT GAT-3'); D3R (5'-TAA AAC ACC AAA AAC TAC AAT CAA-3'); D3FN (5'-TTT AAG GTT ATT GAG ATG TGT TTT G-3'); and D3RN (5'-TAT ACC AAC CCT CAT AAT AAA AAC ACA AA-3'). Amplification products were purified from agarose gel using QiaQuick gel extraction kit (Qiagen, Crawley, UK), cloned into pGEM T-Easy vector system (Promega, Southampton, UK). In all, eight to 10 individual clones for each DMR regions were sequenced using BigDye terminator cycle sequencing kit V 1.1/3.1 (Perkin-Elmer/Applied Biosystems, Warrington, UK) and run on an ABI377/3730.

Methylation status of the intergenic germline-derived differentially methylated region (IG-DMR)

A seminested PCR was performed to amplify the IG-DMR (nt position 51021–51180; GeneBank accession no. AL117190). The primer sequences are: IG-F (5'-TTT TGA GAT GTA TAT TTT TAG TTT TAT T3-'); IG-R (5'-ATA AAC TAC ACT ACT AAA AAC TAC ATT TAA A3-3'); and IG-Fnes (5'-TTA TGT GAA TGT AAT GGT TTT AAG GTT TTG AAT GG TTT CTT-3').
A TAA GAG TTT GTG GAT T-3). PCR was performed as previously described using HotStar Taq DNA polymerase (Qiagen) with an annealing temperature of 53°C and 1.5 mM MgCl₂ for both first-round (primer set IG-F/IG-R) and second-round (primer set IG-F/ primers: IG-IG) PCRs. PCR products were then cloned and sequenced as described above.

Expression analysis: reverse transcriptase (RT) and quantitative real-time PCR

RNA (1 µg) was reverse transcribed using Reverse Transcription Systems and oligo-dT primers (Promega) according to the manufacturer’s protocols. cDNA (1 µl) obtained was then used as a template for RT–PCR amplification. A single-nucleotide polymorphism (SNP) previously identified in exon 5 of GTL2 and an SNP in exon 5 of DLK1 (Wylie et al, 2000) were used to analyse GTL2 and DLK1 gene expression. GTL2 and DLK1 cDNA was amplified using oligonucleotide primers described by Wylie et al (2000), except that for GTL2 reverse primer, GTL2FK (5'-AGG TTT GTG GAT T-3) and GTL2FS (5'-AGG CAC CTG GGT GGA TGA T-3). For quantitative real-time PCR, total RNA from cell lines was extracted with the RNAzol B (Biogenesis, Poole, UK) according to the manufacturer’s instruction and treated with DNase 1 (In vitrogen, Paisley, UK). Total RNA (1 µg) was reverse transcribed as described previously. The cDNA was used in triplicate in a real-time PCR analysis using a real-time Thermal Cycler Model 7900 (Applied Biosystems). The primer sets are: DLK1 (5'-GGC AGG ATG ACA ATG TTT GC-3') (forward) and 5'-AGG AGC CAA GCT TCT TGG AA-3') (reverse)). Beta-actin was used as a control for normalisation. PCR was performed in a 25 µl volume that included 12.5 µl of 2 × Cyber green (Applied Biosystems), 25 ng of cDNA template and 0.0125 – 0.025 pmol of each primer. Relative RNA quantification was performed using the 2^-ΔΔCt method. The fold differences in RNA expression are the mean value from three independent observations.

Treatment of cell lines with 5-aza-2’-deoxycytidine (5-aza-dC)

5-Aza-dC (Sigma, Gillingham, Dorset, UK) was freshly prepared in ddH₂O at 2 mg ml⁻¹ and filter sterilised. A total of 1 x 10⁶ cells were plated in 75 cm² flask in RPMI 1640 medium supplemented with 10% FCS and left to settle for 24 h (day 0). Cells were treated with 2 µM of 5-aza-dC at day 1 and 4 and harvested at day 5. The culture medium was changed before each treatment and 24 h after treatment.

RESULTS

Epigenetic analysis of DLK1/GTL2 domain in neuroblastoma tumours and cell lines

The methylation status of the GTL2 promoter DMR was assessed in four normal control blood DNA samples and all four cases demonstrated both methylated and unmethylated allele products. We then analysed tumour DNA from 20 primary neuroblastomas. We found that five of 20 (25%) neuroblastoma tumours demonstrated only products from a methylated allele at the GTL2 promoter DMR. The other 15 tumours showed a normal result with methylated and unmethylated allele products. In four of five tumours with hypermethylation of the GTL2 promoter DMR, heterozygosity at a DLK1 SNP (c.564T>C) (n = 2) or at closely linked microsatellite markers (D14S598, D14S1426, D14S749 and D14S1006) excluded 14q32 maternal allele loss as a cause of the GTL2 promoter DMR hypermethylation. In order to investigate whether GTL2 promoter DMR hypermethylation in tumours was associated with alterations in DLK and GTL2 expression and imprinting, we examined the GTL2 promoter DMR methylation status in four neuroblastoma cell lines (SK-N-F1, SK-N-AS, SK-N-DZ, Kelly). All four neuroblastoma cell lines demonstrated GTL2 promoter DMR hypermethylation (Figure 1A and B). One cell line, SK-N-AS, was heterozygous for a transcribed DLK1 SNP, thus enabling us to (a) exclude maternal allele loss as the cause of 5’ GTL2 DMR hypermethylation and (b) perform allele-specific expression studies to investigate DLK1 imprinting status. We also analysed DLK1 and GTL2 expression before and after treatment with the demethylating agent 5-azacytidine (5-AzaC) in the four neuroblastoma cell lines with GTL2 promoter DMR hypermethylation. In all cases, there was silencing of GTL2 expression in the untreated cell line and reactivation of GTL2 expression after treatment with 5-AzaC (Figure 1C and Table 1). In addition, 5-AzaC treatment resulted in a reduction in DLK1 mRNA expression. Analysis of DLK1 allelic expression in the informative SK-N-AS neuroblastoma line demonstrated monoalectic (allele C) DLK1 expression pre- and post-treatment with 5-AzaC (Figure 2). Thus, hypermethylation of the GTL2 promoter DMR in the SK-N-AS

Figure 1 (A) Methylation-specific multiplexed PCR of the DMR upstream GTL2 promoter in neuroblastoma cell lines showing the presence of only methylated allele of 160 bp. M+ is in vitro methylated DNA. (B) Normal blood DNA was used as control showing both unmethylated (120 bp) and methylated (160 bp) allele. (C) RT–PCR analysis of DLK1 and GTL2 in four neuroblastoma cell lines before (−) and after (+) treatment with demethylating agent 5-AzaC. DLK1 expression was reduced, while GTL2 expression was activated following 5-AzaC treatment. Molecular weight marker was a 100-bp ladder (Invitrogen).
was associated with the upregulation of DLK1 expression and silencing of GTL2 expression, but not LOI of DLK1.

Hypermethylation of 5′ GTL2 promoter was observed in five of 20 (25%) of neuroblastoma tumours. RNA was available from seven of the 20 tumours (see Figure 3). A low level of GTL2 expression was detected in the tumour with hypermethylation of the 5′ GTL2 promoter DMR (possibly caused by normal tissue contamination).

To establish the precise epigenetic status of individual CpGs within the GTL2 promoter DMR region, bisulphite sequencing was undertaken in four normal control blood DNA samples and in the SK-N-AS hypermethylated neuroblastoma cell line, which is heterozygous for DLK1 SNP. A total of 54 CpGs were analysed in three subregions of the CpG-rich region upstream of GTL2 (G1 n = 12 CpGs, G2 n = 19 and G3 n = 21). Between eight and 10 clones were sequenced from each of the normal blood samples and the methylation status of each of the 54 CpGs determined (see Figure 4C). Comparison of the CpG methylation status of the GTL2 promoter DMR subregions in controls and in the neuroblastoma cell line revealed that within subregions G1 and G2, there was extensive methylation of CpGs in SK-N-AS compared to normal control DNAs. However, in subregion G3, there was little difference between the CpG methylation status of normal control DNAs and the SK-N-AS cell line DNA.

| Table 1 | DLK1 and GTL2 expression in neuroblastoma cell lines following 5-AzaC treatment analysed by real-time PCR. |
|-----------------|--------------------------------------------------------------------------------|
| **Cell line**   | **Fold change (± s.d.)a** after 5-AzaC treatment | **Fold change (± s.d.)a** after 5-AzaC treatment |
| SK-N-DZ         | 0.282 ± 0.056 | 5.437 ± 0.237 |
| SK-N-AS         | 0.219 ± 0.097 | 3.953 ± 0.724 |
| SK-N-F1         | 0.365 ± 0.259 | 2.773 ± 0.507 |
| Kelly           | 0.173 ± 0.015 | 2.693 ± 0.082 |

5-AzaC = 5-azaCytidine; PCR = polymerase chain reaction; s.d. = standard deviation.

The fold differences in RNA expression are the mean ± s.d. of three independent observations.

Methylation analysis of the GTL2 promoter DMR in phaeochromocytoma and Wilms’ tumour

Methylation analysis of the GTL2 DMR was then undertaken in 20 phaeochromocytomas and 40 Wilms’ tumours. GTL2 promoter DMR hypermethylation was detected in two phaeochromocytomas (10%) and one Wilms’ tumour (2.5%). Genotyping with 14q32 microsatellite markers (D14S598, D14S1426, D14S1006, D14S749) excluded 14q32 allele loss in all three tumours.

Methylation analysis of IG-DMR

Recently, an IG-DMR was demonstrated to function as an imprinting control element for all imprinted genes on the maternal chromosome only (Lin *et al.*, 2003). To determine whether GTL2 promoter DMR hypermethylation was associated with epigenetic alterations at the upstream IG-DMR, we analysed the methylation status of the eight CpGs within the IG-DMR in four normal control DNAs and in four neuroblastoma cell lines with 5′ GTL2 DMR methylation. Eight to 12 individual clones for each were analysed for IG-DMR CpGs methylation status. In the four normal control samples, 32% of IG-DMR CpGs sequenced were methylated, while in the SK-N-AS neuroblastoma cell line with GTL2 promoter DMR hypermethylation, all eight CpGs (n = 12 clones) were methylated. In addition, the neuroblastoma cell lines with 5′ GTL2 DMR hypermethylation (but uninformative for DLK1 imprinting status) showed almost complete methylation at all IG-DMR CpGs analysed (see Figure 4B). Similarly, analysis of the IG-DMR CpG methylation status in four primary tumours (two neuroblastoma, one phaeochromocytoma and one Wilms’ tumour) with GTL2 promoter DMR hypermethylation demonstrated heavy CpG methylation (although less complete than in the neuroblastoma cell lines, possibly because of normal tissue contamination). In contrast, cell lines and tumours with GTL2 promoter DMR and IG-DMR hypermethylation did not show any differences from normal controls at the CpG-rich region upstream of the DLK1 promoter.

DISCUSSION

We detected epigenetic changes within the GTL2 promoter DMR (and the IG-DMR) in 25% of neuroblastoma tumours, 10% of phaeochromocytoma and 2.5% of Wilms’ tumours analysed. In most cases, allele loss was excluded as a cause of the apparent hypermethylation. All cases with GTL2 promoter DMR methylation also demonstrated hypermethylation at the upstream IG-DMR region, but epigenetic alterations were not detected in the three
CpG-rich regions close to DLK1. Analysis of DLK1 and GTL2 expression in four neuroblastoma cell lines with GTL2 DMR hypermethylation demonstrated repression of GTL2 transcription and re-expression of GTL2 after treatment with 5-AzaC. In most cell lines, re-expression of GTL2 after treatment with 5-AzaC was associated with downregulation of DLK1 expression. Furthermore, in an informative neuroblastoma cell line (SK-N-AS), there was no evidence of LOI of DLK1. The absence of LOI of DLK1 is consistent with other reports of monoallelic DLK1 expression in neuroblastoma, brain tumours and lymphoma (van Limpt et al., 2003; Yin et al., 2004). The DLK1/GTL2 imprinted domain has similarities to the IGF2/H19 gene at 11p15.5. In both cases, two closely linked genes are reciprocally imprinted, and both H19 and GTL2 do not appear to encode a protein. Like H19 and Igf2, Gt2l and Dlk transcripts can be found in the same tissues during development (Takada et al., 2000). In Beckwith–Wiedemann syndrome and sporadic Wilms’ tumour, hypermethylation of the H19 promoter DMR (and the upstream CTCF box DMR) is associated with silencing of H19 expression (Cui et al., 2001; Nakagawa et al., 2001). We identified hypermethylation of the GTL2 DMRs in tumours and cell lines, so there are apparent similarities between epigenetics at the H19 and GTL2 loci in human neoplasia, although the frequency of GTL2 hypermethylation in Wilms’ tumour is much less than that at H19. However, whereas de novo hypermethylation of the H19 promoter and CTCF box DMRs is associated with H19 silencing and LOI of IGF2, LOI of DLK1 was not associated with GTL2 promoter DMR and IG-DMR hypermethylation. In mice, deletion of the unmethylated copy of the IG-DMR leads to bidirectional LOI of all genes in the imprinted cluster after maternal inheritance, but imprinting is unaltered after paternal transmission of the deleted methylated copy (Lin et al., 2003). This asymmetric regulation of imprinting distinguishes the imprinting control mechanisms in the 11p15.5 and the 14q32 imprinted gene clusters. The Callipyge phenotype demonstrates complex inheritance patterns. Thus, while paternal inheritance of the Callipyge mutation is associated with muscle hypertrophy, and maternal transmission is not, surprisingly, homozygotes do not demonstrate the phenotype. This unusual inheritance pattern (known as polar overdominance) has been attributed to the Callipyge mutation affecting a long-range control element resulting in enhanced expression in cis of all genes in the heterozygote sheep (but with no change in their imprinting) (Charlier et al., 2001; Freking et al., 2002; Georges et al., 2003). Furthermore, the absence of a mutant phenotype in the homozygotes suggests that overexpression of imprinted genes on the maternal chromosome abrogates the functional defect caused by the paternal chromosome in trans. Hence, when the Callipyge mutation is paternally inherited, DLK1 transcription is upregulated, while GTL2 is upregulated after maternal transmission (Charlier et al., 2001; Freking et al., 2002; Georges et al., 2003). In homozygotes, it is postulated that GTL2-associated transcripts negatively regulate DLK1 transcripts; however, the precise mechanism is not known. Our finding that hypermethylation of the GTL2 promoter and upstream DMR is associated with silencing of GTL2 and upregulation of DLK1 without LOI would be consistent with a model, whereby GTL2-associated transcripts negatively regulate DLK1 transcription and further analysis of neuroblastoma cell lines with GTL2 hypermethylation and silencing may provide important insights into the mechanisms of imprinting control in the 14q32 imprinted domain.

DLK1 encodes a transmembrane protein with six EGF-like repeats that shows homology to the Delta gene in Drosophila melanogaster, which is involved in the Notch signalling pathway. The precise function of the DLK1 protein is unclear, but DLK1 expression is upregulated in myelodysplastic syndrome, a slowly progressing haematological malignancy, and in uterine leiomyomata.
(compared to normal myometrium) (Miyazato et al., 2001; Tsibris et al., 2002). In addition, overexpression of the DLK1 protein is reported to prevent adipocyte differentiation of 3T3-L1 cells in response to IGF1 or insulin. These latter effects were attributed to changes in the activation levels and kinetics of extracellular-regulated kinase/mitogen-activated protein kinase (Ruiz-Hidalgo et al., 2002). However, although it might be postulated that increased DLK1 expression might be pro-oncogenic, there is no direct evidence for this. Indeed, van Limp et al. (2003) suggested that neuroblastomas DLK1 expression increases during chromaffin cell lineage differentiation and high DLK1 expression in neuroblastoma cell lines may merely indicate that the neuroblastoma has developed from a later stage chromaffin cell precursor. Hence, upregulation of DLK1 expression associated with GTL2 promoter and intergenic DMR hypermethylation may be coincidental to tumourigenesis. GTL2 promoter and intergenic DMR hypermethylation was associated with transcriptional silencing of GTL2 and treatment with 5-AzaC resulted in re-expression of GTL2. Recently, an isoform of GTL2, MEG3a, was reported to be expressed highly in normal pituitary and brain and in other tissues including adrenal (Zhang et al., 2003), but not in many pituitary tumours. Furthermore, transfection of MEG3a into a HeLa cell line inhibited cell proliferation in the in vitro colony formation and growth rate assays. Hence, although neither GTL2 nor MEG3a have been shown to encode an expressed protein, GTL2 promoter and intergenic DMR hypermethylation might promote tumorigenesis by downregulating GTL2 expression. These findings are similar to those reported for H19 (Hao et al., 1993), although the precise role of H19 silencing per se in Wilms’ tumour and tumour susceptibility in Beckwith–Wiedemann syndrome has not been unequivocally established (Tycko, 2000). Thus, further investigations of the potential roles of GTL2, MEG3a and epigenetic changes in the DLK1/GTL2 domain are indicated.

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