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SHORT REPORT

# Genomic imprinting of *DIO3*, a candidate gene for the syndrome associated with human uniparental disomy of chromosome 14

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**Individuals with uniparental disomy of chromosome 14 (Temple and Kagami–Ogata syndromes) exhibit a number of developmental abnormalities originating, in part, from aberrant developmental expression of imprinted genes in the *DLK1–DIO3* cluster. Although genomic imprinting has been reported in humans for some genes in the cluster, little evidence is available about the imprinting status of *DIO3*, which modulates developmental exposure to thyroid hormones. We used pyrosequencing to evaluate allelic expression of *DLK1* and *DIO3* in cDNAs prepared from neonatal foreskins carrying single-nucleotide polymorphisms (SNPs) in the exonic sequence of those genes, and hot-stop PCR to quantify *DIO3* allelic expression in cDNA obtained from a skin specimen collected from an adult individual with known parental origin of the *DIO3* SNP. In neonatal skin, *DLK1* and *DIO3* both exhibited a high degree of monoallelic expression from the paternal allele. In the adult skin sample, the allele preferentially expressed is that inherited from the mother, although a different, larger *DIO3* mRNA transcript appears the most abundant at this stage. We conclude that *DIO3* is an imprinted gene in humans, suggesting that alterations in thyroid hormone exposure during development may partly contribute to the phenotypes associated with uniparental disomy of chromosome 14.**
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## INTRODUCTION

Genomic imprinting (GI) refers to an epigenetic modification of the DNA that results in differential gene expression between both alleles depending on their parental origin.[1,2] GI originates in germ cells, where DNA is differentially methylated between sexes in critical genomic regions of imprinted loci. After fertilization, these differential epigenetic marks are maintained and ultimately translated into preferential allelic expression from one of the alleles.[1] Although the evolutionary etiology for GI is unclear,[2] it is a phenomenon that occurs in many mammals, but affects only a small subset of genes. However, appropriate expression of these imprinted genes appears critical for proper developmental and physiological outcomes. In humans, abnormal GI, typically due to mutations and epimutations in regions of the imprinted domain that regulate allele-specific expression, leads to severe phenotypes, like those associated with Prader–Willi, Angelman and Beckwith–Wiedemann syndromes.[3,4] Altered expression of imprinted genes also occurs in patients with uniparental disomy (UPD) of whole chromosomes or chromosomal regions containing imprinted loci. These individuals carry the same set of epigenetic marks in both copies of the imprinted domains leading to aberrant allelic expression of the genes in the cluster resulting in abnormal developmental and physiological outcomes.[5]

The imprinted *DLK1–DIO3* region is located in the distal arm of human chromosome 14.[6] Patients with maternal or paternal UPD of human chromosome 14 (UPD14; named Temple and Kagami–Ogata syndromes, respectively) present with distinct developmental abnormalities, including hydrocephalus, hypotonia, abnormal growth, mental retardation, craniofacial dismorphisms, altered puberty onset, abnormal rib cage and others.[7,8] Several genes within this region, including *DLK1* and *MEG3*, are subject to genomic imprinting, that is, they exhibit a high degree of monoallelic expression.[6] However, it is uncertain whether *DIO3* is imprinted. Studies on the placentas of infants with UPD of chromosome 14 and related epimutations suggest that *DIO3* is not imprinted in this tissue in humans.[9] In the mouse, *Dio3* is considered an imprinted gene, as fetal tissues exhibit marked preferential *Dio3* expression from the paternal allele.[10,11] In the mouse placenta, *Dio3* is preferentially expressed from the paternal allele, although the contribution of the maternal allele to overall *Dio3* expression is significant.[12] We thus hypothesized that human *DIO3*, like its mouse homolog, is also imprinted in certain issues. Here we report that this is the case in skin.

## MATERIALS AND METHODS

### Collection of human samples, processing and analysis

Foreskin samples were collected from neonatal circumcisions performed by the Department of Pediatrics Newborn Service at Maine Medical Center (Portland, ME, USA). No personal information was collected from these infants. For a set of 14 samples, a protocol for research on Human Subjects was approved by the Internal Review Board at Maine Medical Center. For this set of samples, parental consent was obtained and, in addition to a foreskin specimen from the infant, buccal swabs were collected from the mother or both parents for genotypic analysis. No personal or clinical information was collected in relationship with these infants. In addition, an adult skin specimen

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was obtained from a healthy, 41-year-old male volunteer, and buccal swabs from him and his relatives were collected using a kit from Epicentre Technologies (Madison, WI, USA). DNA and total RNA were isolated from the skin specimens using RNAeasy and DNA kits from Qiagen (Germantown, MD, USA). Genotyping of single-nucleotide polymorphisms (SNP; NCBI rs1802710, chr14.hg19:g.101200645T>C and rs945006, chr14.hg19:g.102029277T>G, for the *DLK1* and *DIO3* genes, respectively) was performed by DNA sequencing at the Protein, Nucleic Acid and Cell Imaging Core at MMCRI and confirmed by pyrosequencing (EpigenDX Hopkinton, MA, USA). Reverse transcription of total RNA (1 ug) was performed with MMLV reverse transcriptase (Life Technologies, Carlsbad, CA, USA) using standard protocols. A genomic DNA *DIO3* fragment containing the *DIO3* SNP was amplified by PCR from buccal swabs, using taq DNA polymerase (Denville Scientific, South Plainfield, NJ, USA) and primers 5′-GGTTC CCTGTTGCTTTTGTG-3′ (forward) and 5′-CCCTCAAGGTTTAGGT GCTG-3′ (reverse). *DIO3 and DLK1* alelle quantification in cDNAs from foreskin samples was performed by pyrosequencing (EpigenDX Hopkinton). In the cDNA from adult skin, *DIO3* allele quantification was performed using a variation of the hop-stop PCR method[13] (The description and validation of this method are included in Supplementary Figure 1). The data pertaining to the abundance of the SNP variants in the samples studied have been submitted to dsSNP Database at NCBI, and the submission is being processed (http://www.ncbi.nlm.nih.gov/SNP/snp_viewTable.cgi?handle=MMCRIHERNANDEZ).

Commercial human fetal and adult total RNA was obtained from Origene (Rockville, MD, USA), and subjected to northern blot analysis using standard protocols using as a probe a 0.7 kb Sal I/Sac I genomic fragment containing exonic *DIO3* sequence. This probe was labeled with $^{32}$P using $^{32}$P-dCTP (MP Biomedicals, Santa Ana, CA, USA) and the random primer labeling kit from Pfizer (New York, NY, USA). Statistical significance between two groups was determined by the Student's *t*-test. $P<0.05$ was considered significant.

## RESULTS

We genotyped the *DLK1* and *DIO3* SNPs (GenBank rs1802710 and rs945006, respectively) in 52 neonatal foreskin samples. Genotyping results are shown in Figure 1a. The T/C genotype of the *DLK1* SNP was present in 56.1% of the individuals, whereas the T/G genotype of the *DIO3* SNP was identified in 36.4% of the individuals. According to GenBank databases, the prevalence of the G variant of *DIO3* is very low, low and high in individuals of Asian, European and African descent, respectively.

RNA was extracted from seven foreskin samples carrying the informative variation of the *DLK1* SNP, and subjected to reverse transcription. The obtained complementary DNA (cDNA) was used as a template for PCR and subsequent allele quantification by pyrosequencing (Figure 1b). All seven samples analyzed exhibited a very high degree (>95%) of monoallelic expression for *DLK1*. This is consistent with the published data on the genomic imprinting of *DLK1* in other developing tissues.[6] Likewise, allelic quantification of *DIO3* was performed in eight samples carrying the *DIO3* G/T genotype (Figure 1c). On average, the degree of monoallelic expression for *DIO3* was 74% (Figures 1c and d; Representative pyrograms for *DLK1* and *DIO3* allele quantification in two of the samples analyzed are shown in Supplementary Figure 2). Allelic contribution to *DIO3* expression was significantly biased towards one of the alleles when compared to the allelic contribution to genomic DNA as quantified by the same method (Figure 1d). Mean *DIO3* allelic cDNA ratio was higher than 3, and significantly different to that in genomic DNA (Figure 1e). These results demonstrate strong preferential allelic expression of the human *DIO3* gene. To ascertain the parental origin of the preferentially expressed *DIO3* allele, we collected an additional number of foreskin specimens from children whose mothers (or both parents) consented to provide a buccal swab for *DLK1* and *DIO3* genotyping. From these sets of samples, we identified two sets for each of the genes that proved informative as to the parental origin of each allele. Pyrosequencing evaluation of *DLK1* and *DIO3* cDNAs obtained from these samples confirmed preferential allelic expression in both genes (Figure 1f). On the basis of the mother and/or father genotype we concluded that, in newborn foreskin, both *DLK1* and *DIO3* are preferentially expressed from the allele inherited from the father.

We also performed *DIO3* allelic quantification studies on RNA isolated from a skin biopsy obtained from an adult male volunteer heterozygous for the T/G *DIO3* polymorphism. His father and one of his sons were also heterozygous for the T/G *DIO3* polymorphism, but not his mother, who was homozygous for the T variant of *DIO3* (Figure 2a). cDNA containing the *DIO3* SNP was prepared from a skin biopsy of this individual (Figure 2b). Sense and antisense Sanger sequencing of the amplicon indicated the presence of the polymorphisms, with comparable peaks for both variants when the amplicon originated from genomic DNA (Figure 2c, top). However, the T peak (and the A peak for the antisense strand) markedly predominated in the amplicon originated from cDNA (Figure 2c, bottom).

*DIO3* allele quantification in this cDNA sample was performed using a variation of the hot-stop PCR method[13] (see Materials and Methods). Quantification of the contribution of each allele of the subject to the genomic DNA was comparable, but in the cDNA the contribution from the T allele was much higher than that from the G allele (Figure 2d). Densitometry quantification in four different replicates indicated an ~50% presence of each of the variants in genomic DNA, but an 80% presence of the T variant (versus 20% of the G variant) in the cDNA pool. These results indicate strong preferential expression of *DIO3* from the T allelic variant in this individual. As this variant was inherited from his mother, the results demonstrate that the maternal *DIO3* allele is preferentially expressed in the skin of this adult individual. The *DLK1* genotype of this sample (T/T) was not informative about the paternal origin of the *DLK1* alleles.

Northern blot analysis of RNA from fetal and adult human skin reveals different abundance of *DIO3* mRNA transcripts at these developmental stages. The characterized 2.1–2.2 kb transcript is the most abundant in fetal skin, but a larger 2.5–2.6 kb transcript is the only one detected by this method in adult skin (Figure 2f). This observation indicates a marked shift from fetal to adult life in the predominant *DIO3* mRNA species in this tissue.

## DISCUSSION

Our results demonstrate that human *DIO3* is preferentially expressed from the paternally inherited allele in neonatal foreskins. This is also applicable to *DKL1*. For both *DLK1* and *DIO3*, there is not a particular polymorphic variant that is preferentially expressed, suggesting that the results observed are truly the result of genomic imprinting and not due to the presence of genetic or epigenetic factors that might be specifically associated to one of the polymorphic variants. These findings are consistent with observations showing preferential allelic expression of the paternal *DLK1* allele in human fetal tissues,[6] and also with those showing that both *Dlk1* and *Dio3* genes are preferentially expressed from the paternal allele in the mouse fetus.[10,11]

In this context, our additional observation of preferential maternal-allele expression of *DIO3* in adult skin is not a fully anticipated result. The most likely explanation is that the larger *DIO3* transcript present in the adult skin exhibits opposite imprinting. This larger

**a**

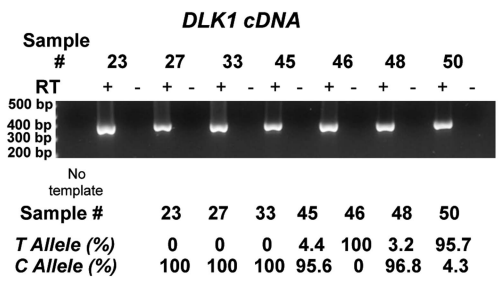
Genotype Distribution of *DLK1* rs1802710 and *DIO3* rs945006 in the 66 samples collected

| Gene | Genotype | Number (%) |
|---|---|---|
| | T/T | 17 (25.8%) |
| *DLK1* | T/C | 37 (56.1%) |
| | C/C | 12 (18.2%) |
| | T/T | 41 (62.1%) |
| *DIO3* | T/G | 24 (36.4%) |
| | G/G | 1 (1.5%) |

*Combined Genotypes*

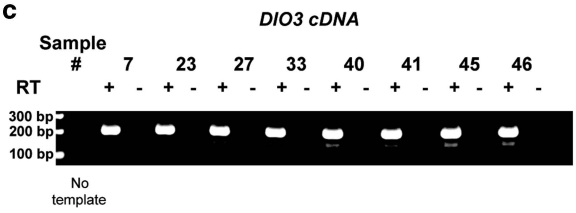
| *DLK1* | *DIO3* | Number (%) |
|---|---|---|
| T/T | T/T | 14 (82%) |
| T/T | T/G | 3 (18%) |
| T/T | G/G | 0 (0%) |
| T/C | T/T | 22 (59%) |
| T/C | T/G | 14 (38%) |
| T/C | G/G | 1 (3%) |
| C/C | T/T | 5 (42%) |
| C/C | T/G | 7 (58%) |
| C/C | G/G | 0 (0%) |

**b**



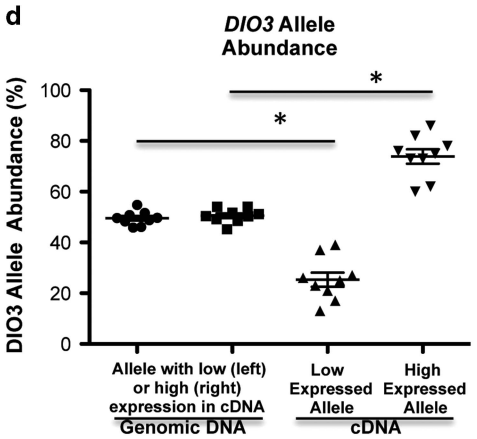

| Sample # | 23 | 27 | 33 | 45 | 46 | 48 | 50 |
|---|---|---|---|---|---|---|---|
| *T Allele (%)* | 0 | 0 | 0 | 4.4 | 100 | 3.2 | 95.7 |
| *C Allele (%)* | 100 | 100 | 100 | 95.6 | 0 | 96.8 | 4.3 |

**c**

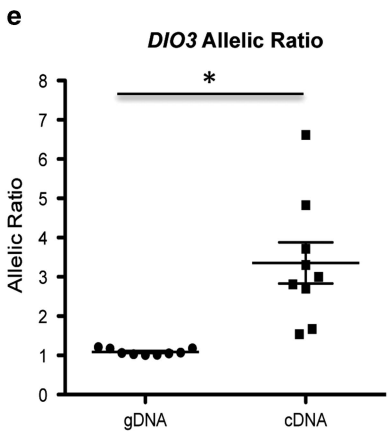



| Sample # | 7 | 23 | 27 | 33 | 40 | 41 | 45 | 46 |
|---|---|---|---|---|---|---|---|---|
| *T Allele (%)* | 62.3 | 25 | 13.7 | 60.3 | 73.9 | 27 | 17.8 | 21.5 |
| *G Allele (%)* | 37.7 | 75 | 86.3 | 39.8 | 26.1 | 73 | 82.2 | 78.6 |

**d**




**e**




**f**

| *Gene* | *Sample #* | *Mother Genotype* | *Father Genotype* | *Infant Genotype* | *Foreskin cDNA Allele quantification* |
|---|---|---|---|---|---|
| *DLK1* | 54 | C/C | Unknown | T/C | C (13%), T (87%) |
| | 58 | C/C | Unknown | T/C | C (18%), T (82%) |
| *DIO3* | 62 | T/G | T/T | T/G | G (31%), T (69%) |
| | 65 | T/G | T/T | T/G | G (22%), T (78%) |

**Figure 1** Preferential allelic expression of *DLK1* and *DIO3* in human newborn foreskins. (**a**) Summary of *DLK1* and *DIO3* SNP genotypes in the samples collected. (**b** and **c**) Agarose gels of *DLK1* and *DIO3* cDNAs in selected samples with its corresponding allelic quantification by pyrosequencing. (**d**) Pyrosequencing data on the abundance of *DIO3* alleles in genomic DNA and mRNA-derived cDNA. (**e**) Higher abundance/lower abundance *DIO3* allelic ratio in genomic DNA and mRNA-derived cDNA. (**f**) Genotype data and foreskin cDNA allele quantification in the sample sets that were informative as to the parental origin of each allele. *$P<0.01$ as determined by the Student's *t*-test. RT, reverse transcription.

*DIO3* mRNA species has been identified in skin cancer cells, and appears to be transcribed by an unidentified upstream promoter.[14] It is thus possible that different epigenetic factors control its transcription. Furthermore, preferential expression from the maternal *Dio3* allele has been described in the adult rat hippocampus,[15] a tissue in which the larger *Dio3* transcript accounts for the vast majority of *Dio3* expression.[16] It is thus plausible that the larger *DIO3* transcript, more abundant in adult tissues,[17] exhibits an allelic pattern of expression that is opposite to that observed for the shorter, development-associated *Dio3* transcript. Opposite imprinting between transcripts from the same gene, or as a result of different developmental stages, has been observed for other imprinted genes.[18–20] In this regard, we have recently described tissue-, developmental stage- and transcript-specific variations in the allelic expression of *Dio3* in the mouse.[21] Recently, Kagami *et al.*[22] have not found sufficient evidence of *DIO3* imprinting when analyzing human placental tissue which, as in the mouse, expresses exclusively the short *DIO3* transcript.[23] That result is probably due to a low or inexistent degree of preferential allelic expression of *DIO3* in the human placenta, a finding that is consistent with the very relaxed pattern of *Dio3* imprinting observed in the same tissue of the mouse.[12]

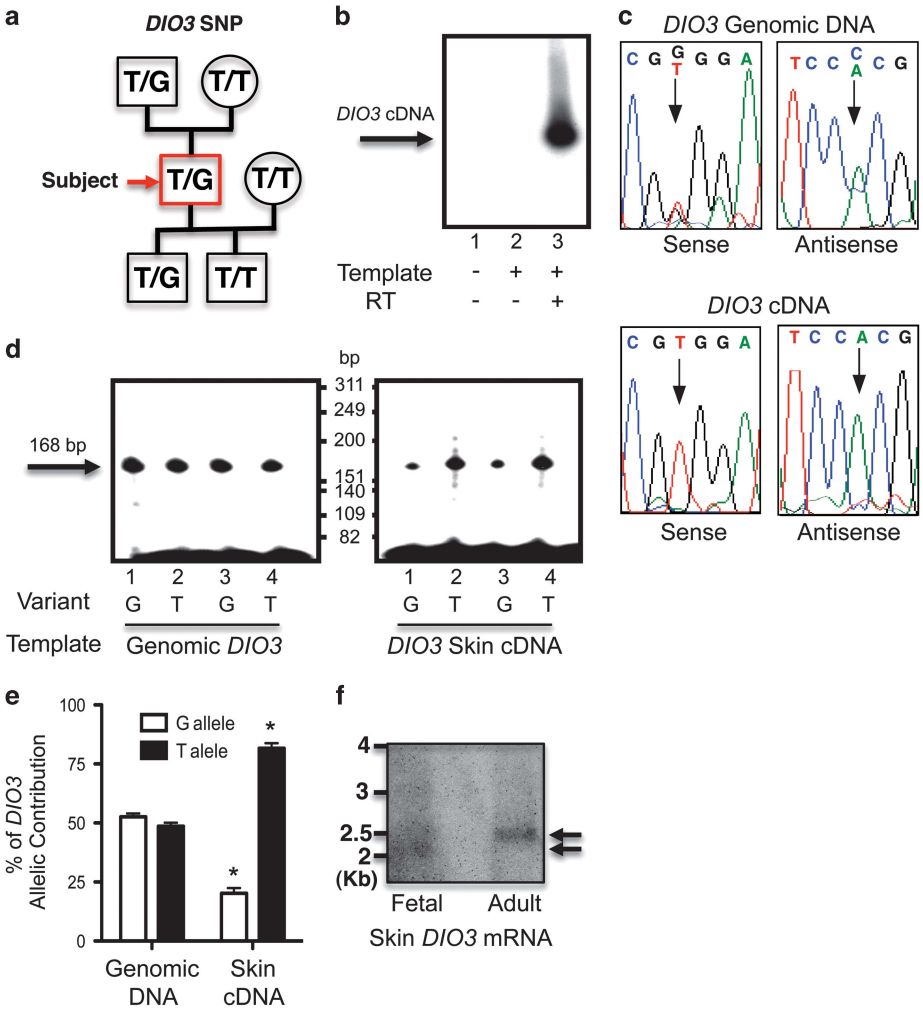



**Figure 2** *DIO3* imprinting in adult human skin. (**a**) Pedigree of the subject (highlighted in red) regarding the single-nucleotide polymorphism and *DIO3* rs945006; (**b**) mRNA-specific origin of the *DIO3* cDNA fragment analyzed; (**c**) Sequence of *DIO3* rs945006 in the genomic DNA and in RNA-derived cDNA from the adult skin sample; (**d**) Agarose gels of allele-specific *DIO3* PCR products from genomic DNA and cDNA from the adult skin sample. (**e**) Quantification of the results shown in **d**; (**f**) Northern blot analysis of human fetal and adult skin mRNA. Arrows indicate the two transcript that are most abundant at each age. *$P<0.01$ as determined by the Student's *t*-test ($n=4$).

The genomic imprinting of *DIO3* suggest that abnormal thyroid hormone levels may occur in patients with Temple and Kagami–Ogata syndromes, but no such abnormalities have been reported. Observations in a mouse model of genetic inactivation of the paternal *Dio3* allele, a model comparable to Temple syndrome in humans, indicate elevated levels of thyroid hormone at neonatal stages,[21] but not in adulthood.[24] However, owing to timing differences in brain developmental processes, the hypothalamic–pituitary–thyroid axis is already mature in human newborns, whereas it is still immature in newborn rodents. It is thus possible that abnormal thyroid hormone exposure occurs *in utero* in patients with the above syndromes.

Our results demonstrate that the human *DIO3* is an imprinted gene preferentially expressed from the paternal allele in neonatal skin, and suggest that *DIO3* imprinting is transcript-specific. Thus, abnormal thyroid hormone exposure of developing tissues as a result of altered thyroid hormone metabolism may contribute to the phenotypic anomalies observed in Temple and Kagami–Ogata syndromes. Our results warrant additional studies on the genomic imprinting status of *DIO3* in other developing human tissues, especially those relevant to those syndromes.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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