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ARTICLE

The epsilon-sarcoglycan gene (*SGCE*), mutated in myoclonus-dystonia syndrome, is maternally imprinted

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Myoclonus-dystonia syndrome (MDS) is a non-degenerative neurological disorder that has been described to be inherited in an autosomal dominant mode with incomplete penetrance. MDS is caused by loss of function mutations in the epsilon-sarcoglycan gene. Reinvestigation of MDS pedigrees provided evidence for a maternal imprinting mechanism. As differential methylated regions (DMRs) are a characteristic feature of imprinted genes, we studied the methylation pattern of CpG dinucleotides within the CpG island containing the promoter region and the first exon of the *SGCE* gene by bisulphite genomic sequencing. Our findings revealed that in peripheral blood leukocytes the maternal allele is methylated, while the paternal allele is unmethylated. We also showed that most likely the maternal allele is completely methylated in brain tissue. Furthermore, CpG dinucleotides in maternal and paternal uniparental disomy 7 (UPD7) lymphoblastoid cell lines show a corresponding parent-of-origin specific methylation pattern. The effect of differential methylation on the expression of the SGCE gene was tested in UPD7 cell lines with only a weak RT–PCR signal observed in matUPD7 and a strong signal in patUPD7. These results provide strong evidence for a maternal imprinting of the SGCE gene. The inheritance pattern in MDS families is in agreement with such an imprinting mechanism with the exception of a few cases. We investigated one affected female that inherited the mutated allele from her mother. Surprisingly, we found the paternal wild type allele expressed whereas the mutated maternal allele was not detectable in peripheral blood cDNA.
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Introduction

Myoclonus-dystonia syndrome (MDS or DYT11; MIM 159900) is characterised by bilateral, alcohol-sensitive myoclonic jerks involving mainly the arms and axial muscles and dystonia. In addition, patients often show psychiatric abnormalities, including panic attacks and obsessive–compulsive behaviour.

We recently reported that MDS is caused by heterozygous loss-of-function mutations in the gene encoding epsilon-sarcoglycan (*SGCE*) on chromosome 7q21.3.[1] Because not all obligate carriers show symptoms of the disease, MDS has originally been regarded to have a reduced penetrance. Reinvestigation of MDS pedigrees suggested a maternal imprinting mechanism which has previously been reported for the mouse *Sgce* gene.[2] We found that 49 affected individuals inherited the disease allele from their father and only

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four from their mother. By contrast, we found a maternal origin of the mutated allele in 14 of 17 clinically asymptomatic carriers whereas paternal transmission occurred in only three cases.[1]

Differential methylation of the parental alleles is a hallmark of imprinted genes, and thus methylation assays are widely used to support the identification of novel imprinted genes. We determined the methylation pattern of a fragment within the potential promoter region of *SGCE* in leukocyte and brain DNA and in lymphoblastoid cell lines from patients with uniparental disomy 7 (UPD7) using bisulphite genomic sequencing.[3,4] Sodium bisulphite catalyses the conversion of cytosine to uracil residues in single-stranded DNA, whereas methylated cytosines remain unreactive under these conditions. Upon PCR amplification of the genomic region of interest, the converted uracil residues are replicated as thymines instead of cytosines. A remaining cytosine in the sequence of the PCR product therefore demonstrates that this site was originally methylated in the template DNA. The sequences upstream of the SGCE gene showed extensive methylation of the maternal alleles in both leukocytes and brain tissue. Accordingly, differential expression could be demonstrated in the UPD7 cDNAs and in leukocytes of MDS patients.

Materials and methods

Families

We investigated six families with a total of 37 living affected patients (14 women and 23 men). Pedigrees and a summary of clinical characteristics are described elsewhere.[1,5–7] Family MD7 and MD9 contained five and four living affected individuals, respectively. One of the affected individuals in both families inherited the mutation from the mother. The female patient in MD7 presented with a typical MDS phenotype with onset of myoclonus in the left arm at about 6 years and writer's cramp of her right hand. In MD9, symptoms of the male patient started in early childhood with mild generalised myoclonic jerks recognised by the patient's family as 'clumsiness of movements'. At school age the patient developed writer's cramp of his right hand.

Nucleic acid sources

Cell lines A control EBV-transformed lymphoblastoid cell line was established by standard procedures.[8] EBV-transformed lymphoblastoid UPD7 cell lines were obtained from the Max-Planck-Institute for Molecular Genetics, Berlin.[9]

DNA and RNA isolations Genomic DNA from peripheral blood was prepared following standard protocols. Brain tissue was obtained from the German Brain Bank 'BrainNet'. Genomic DNA from frozen human brain tissues and lymphoblastoid cell lines was isolated with the QIAamp DNA Mini Kit (QIAGEN). Total RNA from fresh blood samples of MDS patients was extracted with Trizol (Life Technologies). Total RNA from the lymphoblastoid cell lines were isolated with the RNeasy Mini Kit (QIAGEN) and treated with DNase I.

Bisulphite genomic sequencing

Bisulphite treatment Genomic DNA (1–3 μg) was denatured for 15 min at 37°C by adding sodium hydroxide to a final concentration of 0.3 M. For deamination of the DNA 30 μl 10 mM hydroquinone and 520 μl 2 M sodium bisulphite (Sigma, adjusted to pH 5.2) were mixed with the denatured DNA and incubated for 16 h at 55°C. The treated DNA was recovered from the bisulphite solution by using the QIAamp DNA Mini Kit (QIAGEN). Desulphonation was performed for 15 min at 37°C by adding sodium hydroxide to a final concentration of 0.3 M and afterwards neutralised with 90 μl 3 M sodium acetate. Subsequently, the DNA was ethanol precipitated, washed with 70% ethanol, dried and resuspended in 30 μl 1 mM Tris.

PCR The methylation patterns of all sequences were determined for one strand. 3–5 μl of bisulphite-treated DNA (~500 ng) were subjected to PCR amplification carried out in a 25 μl volume containing 1× PCR Gold Buffer, 5 mM dNTP, 1.5 mM $MgCl_2$, 25 pmol of each forward and reverse primer, 1 U AmpliTaq Gold polymerase (ABI) under the following cycle conditions: 95°C for 5 min, for 1 cycle; 95°C for 30 s, 56°C for 30 s and 72°C for 40 s, for 40 cycles; final extension 72°C for 5 min. Methylation-unspecific primers complementary to bisulphite-treated DNA were used to amplify a fragment of 375 bp in the *SGCE* promoter region (Figure 1) (BSTx_F: 5′-GTGTTATGTTTTATAAATAGATAAG-3′, BSTx_R: 5′-AACTCATATACCTCTACAATTC-3′).

Cloning and sequencing PCR products were gel-extracted by using the QIAquick Gel Extraction Kit (QIAGEN), ligated into pGEM-T vector system I (Promega) and transformed into competent *E. coli XL1blue* cells. Recombinant plasmid DNA was isolated from overnight cultures of white colonies (QIAquick Spin Prep Kit, QIAGEN). Sequencing was performed using a BigDye Terminator Cycle sequencing kit (ABI) and universal sequencing primers (SP6 and T7).

Expression analysis

RT–PCR For expression analysis by RT–PCR, first-strand cDNA was synthesised from 3 μg of total RNA using a cDNA synthesis first strand kit (Amersham Pharmacia Biotech) with oligo-$(dT)_{12}$ primers. Subsequently, 1 μl of the resulting cDNA was subjected to PCR. The cDNA-primers were designed for the following genes located on chromosome 7q21 (primers are available upon request): *TFPI2* (426 bp), *PEG10* (429 bp), *SGCE* (389 bp), *BET1* (283 bp), *PON3* (200 bp), *PON2* (443 bp), *SLC25A13* (373 bp) and *DSS1* (360 bp). *GAPDH* cDNA primers (282 bp) served as a control. Expression analysis was performed following the

AmpliTaq Gold protocol (ABI) as described under bisulphite genomic sequencing. PCR reactions were carried out at 95°C for 5 min, for 1 cycle; 95°C for 30 s, 54–58°C for 30 s and 72°C for 45 s, for 35–40 cycles; final extension 72°C for 5 min.

Multiplex RT-PCR Multiplex RT–PCR was performed using the Advantage 2 PCR Kit (BD Clontech). 0.8 μl of each cDNA was subjected to PCR amplification carried out with two pairs of primers, one for *SGCE* (SGCE_cF: 5′-GGTGCAGATGTCCCGTTTTC-3′, SGCE_cR: 5′-GGATGTCT-GGTGTTTGCATG-3′, 389 bp) or *PEG10* (PEG10_cF: 5′-GA-AAGCTGAACCTGTGCCTC-3′ and PEG10_cR: 5′-TCAAAT-GACAGCACCTCTCG-3′), the other for GAPDH (GAPDH_cF: 5′-CTGCACCACCAACTGCTTAG-3′, GAPDH_cR: 5′-AGGTC-CACCACTGACACGTT-3′, 282 bp) serving as an internal control. PCR reactions were carried out at 95°C for 1 min, for one cycle; 95°C for 30 s, 59°C for 30 s and 68°C for 30 s, for 30 cycles; final extension 68°C for 1 min.

MDS patients *SGCE* expression in MDS patients was analysed by RT–PCR and sequencing using the following primers: sgceF: 5′-CCATCAGCAGGTGTCCTCT-3′ and sgceR: 5′-CTCACTGGCCAACATTTC-3′.

Results

CpG island

The *SGCE* gene consists of 12 exons and extends over 71 kb of genomic DNA. The potential promoter region, the first exon, and the beginning of intron 1 is embedded within a typical CpG island. This region of approximately 1.7 kb extends from 1117 bp upstream to 552 bp downstream of the initiation codon. It is harbouring 114 CpG dinucleotides with an overall G+C content of 60.4%, and a ratio of observed *vs* expected CpGs of 0.70 (Figure 1). This sequence does not contain high copy number repeats. In the following, all basepair positions are given relative to the start codon of *SGCE*.

Bisulphite genomic sequencing

We determined the methylation pattern for the following DNAs: (i) two maternal UPD7 lymphoblastoid cell lines; (ii) one paternal UPD7 lymphoblastoid cell line; (iii) female leukocyte DNA; (iv) two brain DNAs; (v) leukocyte DNA from a female MDS patient who is affected although she inherited the mutation from her mother. The bisulphite treated DNA was used as template for PCR amplification of the forward strand with primers unspecific with regard to the methylation status. The amplified product comprised 375 bp containing 25 CpG dinucleotides. It extends from position −773 to −1148. The PCR products were cloned and randomly picked clones were sequenced. The results of the bisulphite genomic sequencing analysis are summarised in Figure 2.

UPD7 lymphoblastoid cell lines

Sequencing of 15 clones each of two maternal UPD7 lymphoblastoid cell lines (15/96, 100/98) revealed that CpG dinucleotides are methylated to an extent between 94 and 96% (Figure 2a,b). We did not observe PCR products

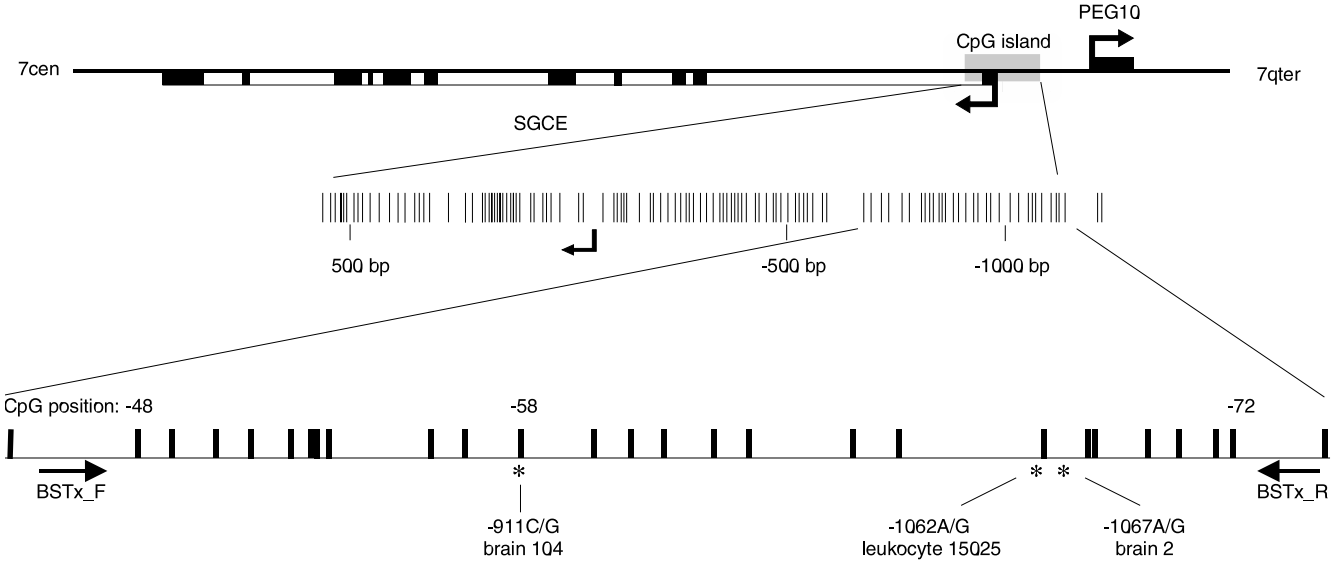

Figure 1 Genomic structure of the *SGCE* and PEG10 genes on human chromosome 7q21. The transcription starts are indicated by bowed arrows, the exons by black boxes. The middle part of the figure shows a CpG density plot of the CpG island. The lower part shows the region amplified for bisulphite genomic sequencing. The CpG dinucleotides are indicated by vertical bars, the primers by horizontal arrows. The polymorphic sites found in leukocyte and brain DNAs are indicated by asterisks.

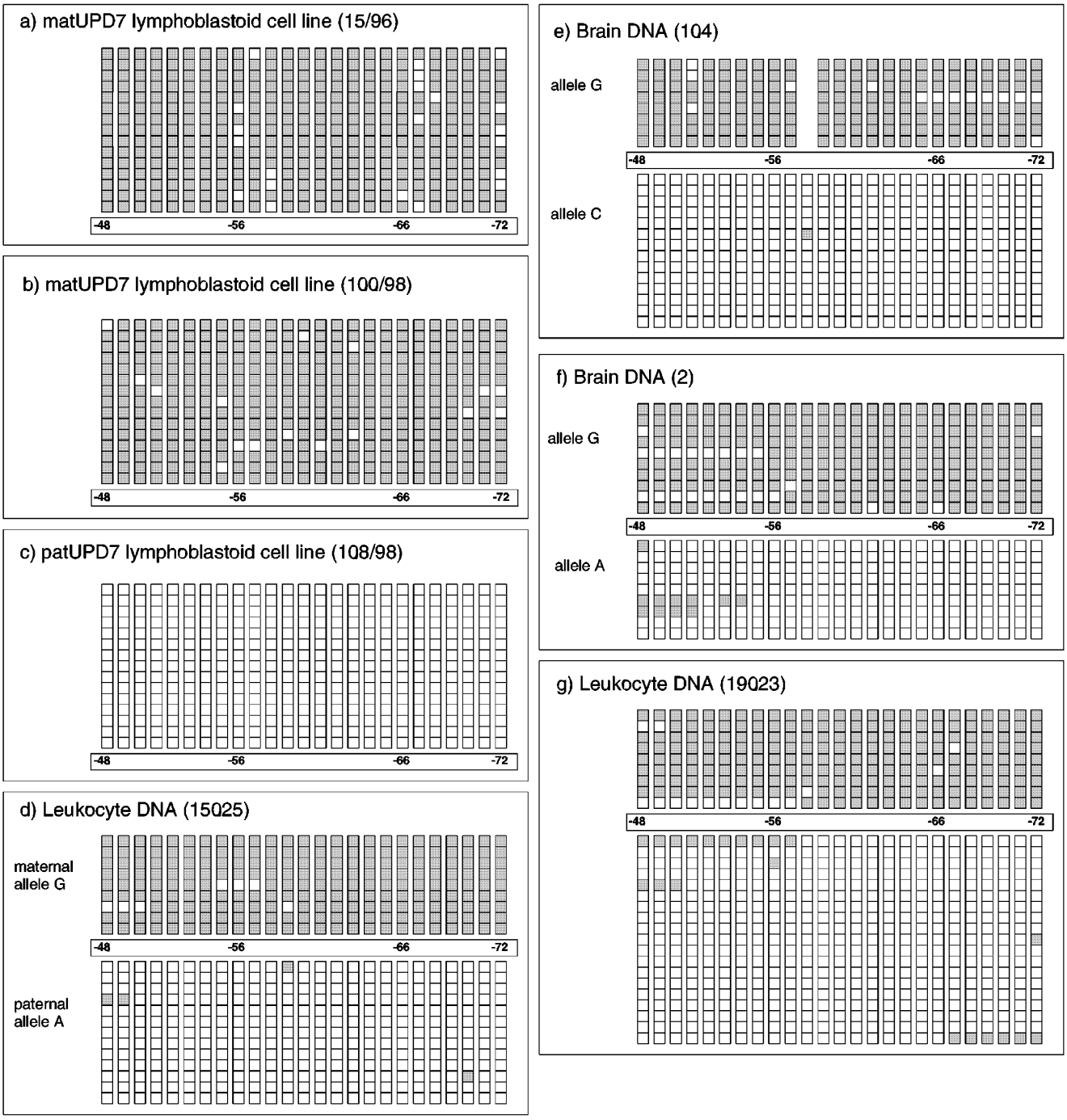

Figure 2 Distribution of 5-methylcytosines within the putative *SGCE* promoter region in genomic DNA isolated from different tissues. Bisulphite treated DNA was PCR-amplified and cloned. For each sample, at least 15 clones were sequenced. Each horizontal set of squares represents the methylation pattern of a single clone. Every column represents the position of a CpG dinucleotide. The position of the 5-mC residues are numbered relative to the start codon of the *SGCE* gene. Filled squares indicate methylated, open squares unmethylated CpG dinucleotides. In (**e**), the CpG dinucleotide at position −58 is disrupted by the polymorphism (−911C→G) in one of the alleles.

using primers specific for unmethylated DNA providing evidence that all sequences are methylated (data not shown).

Furthermore, we sequenced 15 clones of a paternal UPD7 lymphoblastoid cell line (108/98). Sequences of all clones showed completely unmethylated CpG dinucleotides

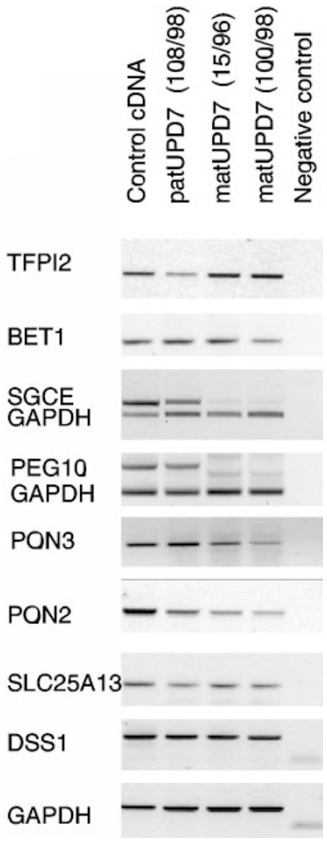

Figure 3 RT–PCR analysis of *SGCE*, *PEG10* and adjacent genes in UPD7 lymphoblastoid cell lines. RNA was isolated from UPD7 and one control lymphoblastoid cell line. RNA was treated with DNase I and reverse transcribed to cDNA. In case of *SGCE* and *PEG10*, multiplex PCR was performed with gene-specific primers and primers for *GAPDH*. As a control, the same amounts of cDNA were used as template for *GADPH* RT–PCR. RT–PCR products were separated by agarose gel electrophoresis and visualised by ethidium bromide staining.

(Figure 2c). In this case, it was possible to amplify a product with primers specific for methylated DNA, indicating that the CpG dinucleotides of a small proportion of the sequences may be methylated (data not shown).

Leukocyte and brain DNA

We further investigated two brain and one leukocyte DNA sample. To be able to distinguish the maternal from the paternal allele, we searched for polymorphisms in 45 leukocyte DNA samples and 33 brain DNA samples by sequencing part of the CpG island. We identified a polymorphism in a single leukocyte DNA (DNA 15025, −1062A/G). The allele containing the guanine was inherited from the mother. Furthermore, we found polymorphisms in two brain DNA samples (DNA 2, −1067A/G; DNA 104, −911C/G). DNA of the parents was not available to determine the origin of the alleles.

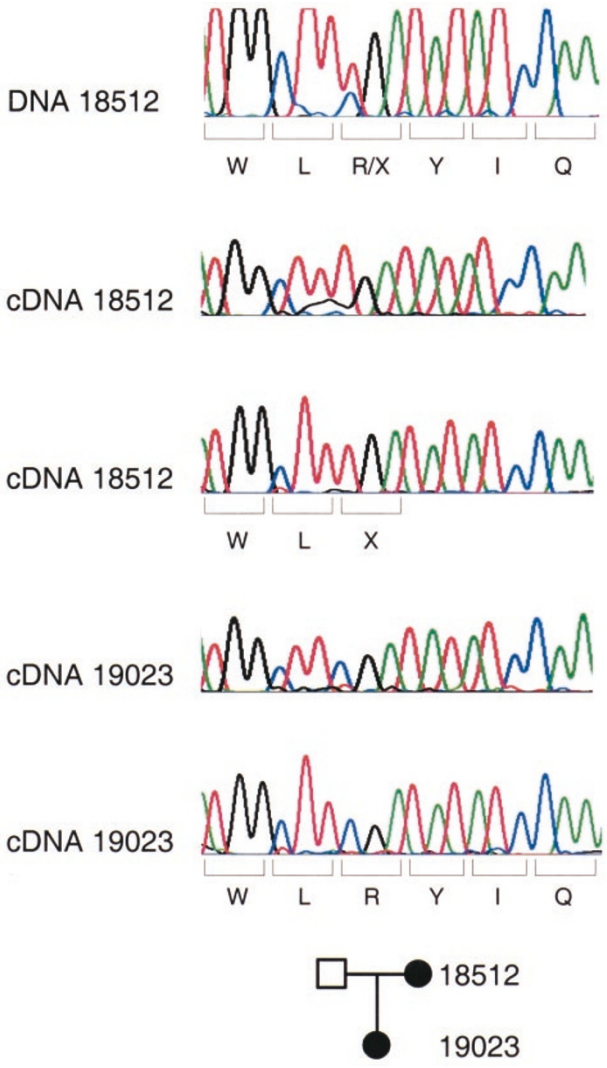

Figure 4 RT–PCR analysis of a patient which is inconsistent with a maternal imprinting mechanism. MDS pedigrees are in accordance with a maternal imprinting mechanism. As an exception, we observed a few affected individuals, who inherited a *SGCE* mutation from their mother. Sequencing of a *SGCE* RT–PCR product from peripheral blood leukocytes in an affected mother (18512) and daughter (19023) showed, that the mother expressed the mutant allele (forward and reverse strand in lines 2 and 3, respectively) whereas the daughter expressed the paternal wildtype allele (forward and reverse strand in lines 5 and 6, respectively). The first line of the figure shows the genomic DNA of the mother containing both alleles.

We sequenced 22 clones of the leukocyte DNA 15025. The CpG dinucleotides of the sequences containing the paternally derived allele were almost completely unmethylated (99%), whereas the CpG dinucleotides within the maternal allele were mainly methylated (97%) (Figure 2d).

The same results were obtained for the brain DNAs. In one brain DNA (#104), all sequences containing a G at posi-

tion −911 were mainly methylated (92%), whereas the unmethylated sequences contained the allele C at position −911 (Figure 2e). In the other brain DNA (# 2), the mainly unmethylated sequences contained an A, the methylated sequences contained a G at position −1067 (Figure 2f).

Expression analysis

We confirmed that *SGCE* is paternally expressed by RT–PCR using UPD7 lymphoblastoid cell lines as template. *SGCE* is clearly expressed in paternal UPD7 but only weakly expressed in maternal UPD7 cell lines.

Imprinted genes are often organised in clusters. We therefore analysed the expression of adjacent genes within a region starting 700 kb upstream and ending 2 Mb downstream of SGCE. The investigated genes map in the following order to 7q21.3: pter – *TFPI2* – *GNGT1* – *GNG11* – *BET1* – *COL1A2* – *C7ORF12* – *SGCE* – *PEG10* – *PPP1R9A* – *PON1* – *PON3* – *PON2* – *ASB4* – *PDK4* – *DNCI1*– *SLC25A13* –*DSS1*– qter. Six genes from this list (*TFPI2, BET1, PON3, PON2, SLC25A13,* and *DSS1*) could be consistently amplified with one round of RT–PCR in lymphoblastoid cell lines. They did not show differential expression (Figure 3). We could also confirm the paternal expression of *PEG10*[10] which lies 7.2 kb q-terminal to *SGCE*.

Maternal transmission of MDS in two cases

Our results showed that the *SGCE* gene is maternally imprinted and that the analysed CpG dinucleotides in the putative promoter region are mainly methylated on the maternal allele and unmethylated on the paternal allele. In general, this imprinting mechanism is in accordance with the inheritance pattern present within MDS pedigrees: RT–PCR in one unaffected individual that inherited the mutated allele from his mother (MD8, del488−497) revealed that only the paternal wild type allele is expressed in leukocytes. Furthermore, in an affected MDS patient that inherited the mutated allele from her father (MD6, R97X), only the mutated paternal allele was expressed in leukocytes (data not shown). In contrast, two out of 29 individuals, who inherited a *SGCE* mutation from their mother, were affected. We investigated leukocyte DNA by bisulphite genomic sequencing from one of these patients (pedigree MD7, DNA 19023) with a *SGCE* stop mutation (R102X). No polymorphism was present within the CpG island and we were therefore unable to distinguish the parental alleles. Nineteen of 28 sequenced clones were mainly unmethylated (96%), whereas nine were mainly methylated (93%) (Figure 2g). Taking into account that the unmethylated DNA is often amplified preferentially after bisulphite treatment,[11] these results provide no evidence for an irregular imprinting pattern. We then performed RT–PCR using leukocyte cDNA from the same patient as template. The primers were designed to include the maternal mutation in order to distinguish the parental alleles. Sequencing showed only the normal paternal allele, thus excluding that the mutant maternal allele is expressed to a larger extent in peripheral blood leukocytes (Figure 4).

Discussion

Over 50 genes have been reported to be imprinted in man and mouse (http://www.mgu.har.mrc.ac.uk). Several of them are involved in human diseases, such as Prader-Willi syndrome, Angelman syndrome, Beckwith-Wiedemann syndrome, pseudohypoparathyroidism 1, and transient neonatal diabetes mellitus. In man, four genes have been described to be imprinted on chromosome 7: (i) *MEST* (*PEG1*);[12,13] (ii) *COPG2*;[14] (iii) *GRB10*;[9,15] and (iv) *PEG10*.[10] Imprinting has further been described in three mouse orthologues of human chromosome 7 genes: (i) *Copg2as2*;[16] (ii) *Asb4*;[17] and (iii) *Sgce*.[2]

We showed that also the human *SGCE* gene is maternally imprinted in matUPD7 lymphoblastoid cell lines and in leukocytes of MDS patients. We further confirmed the maternal imprinting of PEG10. *SGCE* maps adjacent to PEG10 in 7q21.3. Maternal UPD in the homologous mouse region has been shown to be associated with embryonic lethality.[18] In humans, imprinting in this region has been excluded to be responsible for the Silver-Russell syndrome as a case with a partial matUPD7 has narrowed the responsible interval to 7q31-qter.[19] We investigated additional genes in 7q21.3 but could not find them imprinted. ASB4, which has been shown to be paternally imprinted in mice,[17] could not be amplified from lymphoblastoid cell lines with one round of RT–PCR.

The most likely epigenetic mechanism causing imprinting is allele-specific methylation, which is established during male and female gametogenesis.[20] We showed that the examined part of the CpG island around the first exon of *SGCE* is methylated on the maternal allele of lymphoblastoid cell lines, leukocytes and, most likely, in brain tissue whereas the paternal allele is unmethylated.

Our results provide strong evidence for the maternal imprinting of the *SGCE* gene. Such a mechanism is in accordance with the inheritance pattern within MDS families. However, there are two cases in the families studied that do not fit into this pattern. We investigated one of these cases where a female patient inherited the disease from the affected mother. Surprisingly, we only found the normal paternal allele expressed in peripheral blood leukocytes. Furthermore, we observed methylated and unmethylated clones by bisulphite genomic sequencing. These results argue for a complete maternal imprinting in peripheral blood leukocytes in this patient. The irregular imprinting pattern suggested by the disease phenotype could be explained by metastable epialleles resulting in phenotypic mosaicism between cells and also between individuals.[21] Imprinting of the mouse *Sgce* gene has been reported to be incomplete in brain tissue[2] although bisulphite sequencing in the two human brain samples we investigated showed complete differential

methylation. That the maternal allele can escape imprinting under certain conditions is also suggested by the fact that Silver-Russell syndrome patients with a matUPD7 do not show MDS. The mechanism responsible for a reduced expression of the paternal allele present in the described MDS patient remains elusive.

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