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ARTICLE

Maternal repression of the human *GRB10* gene in the developing central nervous system; evaluation of the role for *GRB10* in Silver-Russell syndrome

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The *GRB10* gene encodes a growth suppressor and maps to human chromosome 7p11.2-p13. Maternal duplication (matdup) of this region has recently been associated with Silver-Russell syndrome (SRS), which is characterised by pre- and postnatal growth restriction, craniofacial dysmorphism and lateral asymmetry. Maternal uniparental disomy for chromosome 7 (mUPD7) occurs in approximately 7% of SRS patients. Exposure of a recessive allele due to isodisomy has been ruled out in five mUPD7 cases, suggesting genomic imprinting as the basis for disease. Assuming SRS patients with matdup of 7p11.2-p13 and mUPD7 share a common aetiology, this would implicate a maternally expressed gene from this interval, which is involved in growth inhibition. Murine *Grb10* was identified as a maternally expressed gene by subtractive hybridisation using normal and androgenetic mouse embryos. *Grb10* maps to the homologous region of proximal mouse chromosome 11, for which mUPD incurs reduced birthweight. A role for *GRB10* in SRS was evaluated by determining its imprinting status in multiple human foetal tissues using expressed polymorphisms, and by screening the coding region for mutations in 18 classic non-mUPD7 SRS patients. Maternal repression of *GRB10* was observed specifically in the developing central nervous system including brain and spinal cord, with biallelic expression in peripheral tissues. This is in contrast to mouse *Grb10*, and represents the first example of opposite imprinting in human and mouse homologues. While a role for *GRB10* in mUPD7 SRS cases can not be ruled out on the basis of imprinting status, no mutations were identified in the patients screened. *European Journal of Human Genetics* (2001) **9**, 82–90.

Keywords: *GRB10*; SRS; tissue-specific; genomic imprinting

Introduction

Silver-Russell syndrome (SRS) [MIM 180860] is primarily a growth disorder, characterised by pre- and postnatal growth restriction, relative sparing of cranial growth, triangular facies, lateral asymmetry, fifth finger clinodactyly and mild cognitive deficit in some cases.[1–3] The genetic aetiology of SRS is heterogeneous, but maternal uniparental disomy for chromosome 7 (mUPD7) accounts for approximately 7% of patients.[4–6] Lack of consistent isodisomy for 40 loci distributed over the full-length chromosome 7 has been demonstrated in a group of five mUPD7 cases, indicating that SRS is due to an imprinting effect, as opposed to unmasking of a recessive allele.[7]

Recently, two patients with classical features of SRS have been described, each with a maternal duplication of 7p11.2-

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p13.[8,9] Assuming the duplicated regions in these two patients are functionally active, this suggests that the aetiological basis for SRS in mUPD7 cases may be over-expression of a maternally expressed gene involved in growth suppression, rather than lack of a paternally-derived growth promoter. The duplications, which contain the insulin-like growth factor binding protein 1 and 3 genes (*IGFBP1* and *3*) and *GRB10*, define a candidate region for SRS on the short arm of chromosome 7. The *IGFBP1* and *3* genes are not imprinted in adult and foetal tissues[10,11] and no mutations were detected in either gene in a cohort of non-mUPD7 SRS patients,[12] making these unlikely candidates for this disorder. *GRB10* has previously been proposed as a candidate for the SRS phenotype on the basis of its protein function as a growth suppressor, its chromosomal location and imprinting status in the mouse.[13]

GRB10 contains a central pleckstrin homology (PH) domain and a carboxy-terminal Src homology 2 (SH2) domain. The human and mouse orthologues are highly conserved, with 88 and 99% amino acid similarity in the PH and SH2 domains respectively, and 70–99% similarity in the remaining segments of the two proteins.[14–16] Four GRB10 isoforms have been identified in humans, which differ at the amino-terminus and the PH domain due to alternative splicing events (Figure 1); GRB10α (previously referred to as GRB-IR),[14] GRB10β (or GRB-IRSV1), which is the most widely and abundantly expressed isoform,[15,16] GRB10γ,[17] and *GRB10*δ, which has an extended 3′ untranslated region.

GRB10 has been shown to interact with several tyrosine kinase growth factor receptors including the insulin[14–16] and insulin-like growth factor-I (IGF-I)[15,18] receptors, and the platelet-derived growth factor (PDGF) receptor,[16] and so may serve a broad role in growth suppression through various receptor pathways. Over-expression of the murine Grb10α has been shown to inhibit the tyrosine phosphorylation of two endogenous insulin receptor substrates upon insulin stimulation.[14] An excess of this protein was also shown to cause suppression of IGF-I mediated cell proliferation in mouse fibroblasts.[19] The SH2 domain of human GRB10 has been shown to inhibit both insulin and IGF-I stimulated mitogenesis in fibroblast cell lines.[15,17] GRB10 also interacts with the non-tyrosine kinase growth hormone (GH) receptor, causing down regulation of the GH signalling pathway.[20]

The region of human chromosome 7p11.2-p13 containing *GRB10*,[21] is homologous to an imprinted region on mouse proximal chromosome 11, and mice with a maternal duplication/paternal deficiency of this region are growth restricted.[22] Mouse *Grb10* was identified in a systematic screen for maternally expressed genes by subtractive hybridisation, and is a candidate for this growth phenotype.[13] Asynchronous replication has been demonstrated at the human *GRB10* locus in normal lymphocytes and lymphoblasts, giving a preliminary indication that the human homologue is also imprinted.[9]

To determine if *GRB10* contributes to the SRS phenotype, firstly the imprinting status of the gene during human foetal development was investigated by direct analysis of allelic transcription using intragenic polymorphisms. We demonstrate repression of expression from the maternal allele for human *GRB10* specifically in the foetal central nervous system (CNS), accompanied by biallelic expression in all other major foetal tissues. This is in contrast to the maternally expressed mode of imprinting of the mouse homologue.[13] Secondly, a group

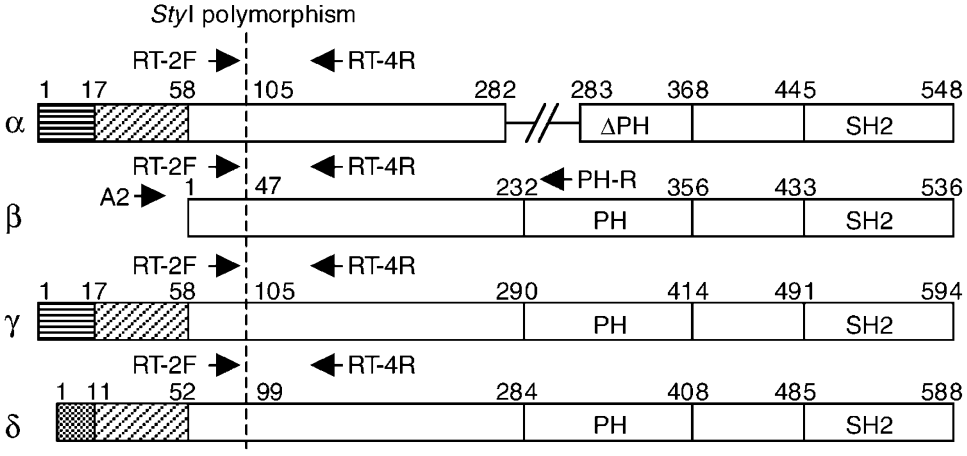

Figure 1 GRB10 protein isoforms. Schematic of the four known GRB10 isoforms aligned according to regions of similarity. GRB10α (GRB-IR) has an incomplete PH domain with a 46-amino acid deletion due to skipping of exon 7A. GRB10β has a short amino terminus and complete PH domain. GRB10γ has the same amino terminus as GRB10α, but with a full-length PH domain. The *GRB10*δ cDNA encodes a unique amino terminus and full PH domain. Regions common to all isoforms are represented by white boxes. Patterned boxes denote the different amino termini. Amino acid residues are numbered. A polymorphic *Sty*I site in exon 3, used for imprinting analysis, is indicated by a vertical dashed line. The positions of RT–PCR primers with respect to the protein isoforms are indicated by arrows.

of 18 classical SRS patients in whom mUPD7 and cytogenetic abnormalities had been ruled out, were analysed for mutations of the coding region of *GRB10*. No mutations were detected in any of the patients, but two novel polymorphisms were identified. Thus, while an imprinting effect of *GRB10* on SRS patients can not be ruled out, the lack of any mutations in these patients suggests that this gene is neither likely to contribute to SRS in a significant number of patients, nor be responsible for the full disease spectrum.

Materials and methods

Tissue samples for *GRB10* imprinting analysis

Foetal tissues were obtained from terminated pregnancies at Queen Charlotte's and Chelsea Hospital (QCCH). Samples were washed in sterile PBS prior to freezing in liquid nitrogen. Corresponding maternal peripheral blood was also acquired at QCCH. Local ethics approval for obtaining foetal and maternal samples was granted by the Research Ethics Committee of the Royal Postgraduate Medical School (96/4955), and informed consent was obtained for each sample pair. Normal fibroblasts were obtained from anonymous volunteers by 3-mm punch skin biopsy.

SRS patient and normal control samples

Eighteen classical SRS patients (demonstrating at least three of the following characteristics; low birthweight (at least 2SD below the mean), short stature (at least −2SD), lateral asymmetry and characteristic craniofacial dysmorphism) were screened for mutations of *GRB10*. These patients were a subset of 53 previously described.[7] Major structural abnormalities including duplications, inversions and translocations had previously been excluded by cytogenetic analysis. Trisomy mosaicism and mUPD for chromosome 7 had also been ruled out by microsatellite repeat analysis of the nuclear pedigrees. Peripheral blood samples for DNA analysis were obtained with the patients' informed consent. This study was approved by the Joint Research Ethics Committee of the Great Ormond Street Hospital and the Institute of Child Health (1278). Genomic DNA from peripheral blood of 10 anonymous normal individuals served as controls.

PCR amplification of genomic DNA

Genomic DNA was extracted from placenta and peripheral blood using standard phenol-chloroform separation.[23] For PCR analysis, 200 ng genomic DNA was amplified in 50-μl reactions containing $1\times NH_4$ reaction buffer, 50 ng each oligonucleotide primer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$ and 2 U DNA *Taq* DNA Polymerase (Bioline). PCR was performed for 35 cycles of denaturation at 94°C for 60 s, followed by 45 s annealing (temperature as stipulated for each primer pair), and extension at 72°C for 60 s.

Analysis of expression by reverse–transcription (RT)–PCR

Total RNA was isolated from foetal tissues and normal adult fibroblasts using the guanidium isothiocyanate extraction technique.[24] For RT–PCR, 2 μg denatured total RNA from each sample was used to synthesise cDNA in 20-μl reactions with 1 × RT buffer, 40 U M-MLV reverse transcriptase (Promega), 1 unit RNase inhibitor (Promega), 1 mM each dNTP, and 0.2 μg primer at 37°C. Poly-dT primed cDNA was used for study of the *GRB10* 3′ UTR, and random hexamers were used to produce cDNA for analysis of the *GRB10* coding region. A duplicate set of samples was processed, with RT omitted to detect any genomic DNA contamination of the RNA. One tenth of the resulting cDNA and RT samples were subjected to PCR amplification, as above. The number of cycles varied such that PCR reactions were terminated in the log-linear phase (to minimise any allelic bias in amplification). RT–PCR primers were designed to amplify between exons, across at least one intron, to detect amplification of genomic contamination or antisense RNA on the basis of product size.

Sequencing

PCR and RT–PCR samples were purified for sequencing using MicroSpin S-300 HR columns (Pharmacia). Sequencing was performed using the BigDye Terminator ready reaction kit on an automated ABI PRISM 377 DNA sequencer (PE Biosystems).

Analysis of *GRB10* imprinting

A *Sty*I polymorphism within *GRB10* exon 3 was used to study the allelic origin of expression of *GRB10*, and specifically the *GRB10β* isoform (GenBank: U69276). To identify informative foetal-maternal pairs, genomic DNA extracted from placenta and maternal blood was PCR amplified using intronic primers 3F and 3R (Table 1) and analysed by restriction digestion with *Sty*I. Digestion reactions were performed using 5 μl PCR product with 1 × Buffer F and 10 U *Sty*I (Promega) in 20-μl volumes at 37°C for 3 h. For RT–PCR analysis of all *GRB10* isoforms concomitantly, a forward primer from within exon 2 (RT-2F: AGTCTGACCGACTTGCGAAT) and reverse primer from exon 4 (RT-4R: CCACAGAGTTCAGGAAAAGG), were used to amplify cDNAs from heterozygous foetal tissues with annealing at 60°C. To study the expression of the *GRB10β* isoform individually, *GRB10β*-specific forward primer A2 (AGACCTAAGCCTGTTTGCTC)[16] and reverse primer PH-R (CAGGACAACTACTGGAGTTC) were used with annealing at 56°C. Between 6 and 15 μl RT–PCR product was digested with *Sty*I to determine imprinting status.

Imprinting of the *GRB10δ* (GenBank: D86962) mRNA was analysed using a novel G→A polymorphism present in the extended 3′ UTR of *GRB10δ*. Genomic DNA was amplified using primers from within the extended exon 15 sequence, which flanked the polymorphism (15-3′F: GTGATAAAGACAAGGAAACG and 15-3′R: AAACAACTAGAGTGTGGTCT),

Table 1 Primers used for PCR amplification and sequencing of *GRB10* from genomic DNA

Exon number	*Primer sequences (5′ to 3′). Forward (F), Reverse (R)*	*Tm °C*	*Product size (bp)*	*GenBank number*
1	1F: GGCTTGGCTTCTCACAGTCTG[a]	58	213	AF073363
	1R: ATCTATGGCTGGTGGCGACAT[a]			
2	2F: TTCTGCTGCGGTCCTGTTTTT[a]	55	159	AF073364
	2R: CAAGCTAGAACTGGGAGTGT[a]			
3	3F: CCTGATCACCAAGATGTACA[a]	58	321	AF073365
	3R: GAGGCCTGGACCTACCTG[a]			
4	4F: GTGTAAGGCTGGGTCAT[a]	58	298	AF073366
	4R: ACTTCCCGCCCTTCTTCC[a]			
5	5F: TTTCTTGAAAGCCCGAAGTT[a]	62	242	AF073367
	5R: AGGTGCCAATCCTGTTCTGA[a]			
6	6F: TACCATGAATTTCCCACCTGT[a]	53	248	AF073368
	6R: TGATACTATGAAAACCCAAGT[a]			
7	7F: CTTTGAAGCTAACCTTTTACG[a]	51	172	AF073369
	7R: CAAGAGGATTTCTATTCTGAA[a]			
7A	7AF: GCTGATAACATGTCTGCTTA[a]	57	242	AF073370
	7AR: ATTAGGGCTGGTGGTGGTAGC[a]			
8	8F: TGCTCTTTGCTGTGCTTGAG	59	213	AF073371
	8R: AGTCTCCTGTGGGCTGCTGAG[a]			
9	9F: GTTTCTGACTCCCTGTGTAACAC	59	232	AF073372
	9R: CTGCCAGAATAGACATCAAGT			
10	10F: TGCTGTGGCGTTTGTCAC[a]	56	198	AF073373
	10R: TGAAGCTGAAAAGGCACT[a]			
11	11F: CTGCGGCCTTTCCTTTTC[a]	61	188	AF073374
	11R: GGCTACCACCTTGAGGGT[a]			
12	12F: GTCTGCTGTCCTCGGTGCTAA[a]	58	164	AF073375
	12R: GGGGTGCTGTTTGATTTTCTT[a]			
13	13F1: CTCTGAGTTGATCAGAGTTG	54	216	AF073376
	13R1: TTAGCAGATGACAGCTCTTA			
14	14F1: CAGAATTACCGTCTTGGTGC	55	416	AF073377
	14R1: CTGTCTGATACTTACCGGTC			
15	15F1: CTGCCTCTAACCTGGCAAGTG	60	428	AF073378
	15R1: CTGGATCTTCCATGCCCTCC			

[a]Previously published primer sequences.[25] All primers are intronic. Tm = annealing temperature.

at an annealing temperature of 52°C. The PCR products were sequenced across the polymorphic site with 15-3′R. For RT–PCR analysis, a 1.932-kb cDNA product was amplified using a forward primer from coding exon 14 (RT-14F: CAGAGTAATCCAAAGGCATT) and reverse primer 15-3′R, with annealing at 53°C and 1.75 mM $MgCl_2$. RT–PCR products were sequenced with primers 15-3′F and 15-3′R to determine the allelic origin of expression.

Screen for *GRB10* mutations in SRS patients

GRB10 exons 1–15[25] were PCR amplified from genomic DNA of 18 SRS patients and 10 anonymous normal controls using primers listed in Table 1. The PCR products were directly sequenced. SRS patient sequences were analysed for mutations and polymorphisms using the Sequence Navigator program, and through direct comparison to the published *GRB10* exon sequences (Table 1) by BLAST analysis.

Results

Tissue-specific imprinting of *GRB10*

The imprinting status of *GRB10* was studied during the first and second trimesters of foetal development and in adult fibroblasts by exploiting expressed sequence polymorphisms to distinguish the parental alleles. The allelic origin of expression was traced in the cDNA of heterozygous foetal tissue samples for which the corresponding maternal allele was known.

Initially, the imprinting status of the undifferentiated *GRB10* transcripts was studied by RT–PCR using primers capable of co-amplifying each splice variant across a previously described *Sty*I polymorphism within exon 3,[25] that is present in all four known isoforms (Figure 1). Imprinting of *GRB10* was thus studied by restriction digestion of the amplified cDNAs. In total, 68 foetuses were screened for heterozygosity, as detected by presence of both the 319 bp undigested *Sty*I− PCR product and the *Sty*I+ digested fragments of 257 and 62 bp (Figure 2A). *GRB10* imprinting was studied in 19 heterozygous foetuses, which ranged from 7–15 weeks gestation, for which a variety of tissues were available. Nine of the sample sets were informative with respect to parental origin of the alleles, with four maternal samples homozygous for the *Sty*I− allele and five homozygous for the *Sty*I+ allele.

The *GRB10* cDNAs from heterozygous tissue samples were RT–PCR amplified using primers flanking the polymorphism and introns 2 and 3, and digested with *Sty*I (Figure 2B). In the majority of foetal tissues, including vertebral column, eye,

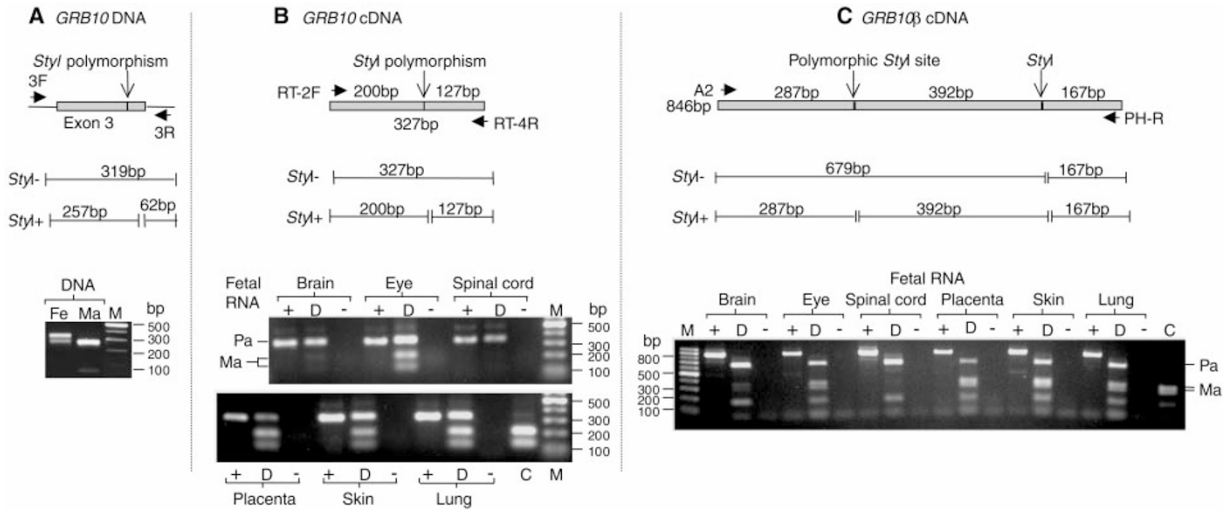

Figure 2 Imprinting analysis of *GRB10* and the *GRB10β* isoform in human foetal tissues. (**A**) Informative foetal-maternal pair for the *GRB10* exon 3 *Sty*I polymorphism. Top: schematic of exon 3 amplification product from genomic DNA using intronic primers 3F and 3R, and the position of the *Sty*I polymorphism. Fragment sizes of the *Sty*I+ and *Sty*I− alleles are shown beneath. Bottom: Electrophoresed *Sty*I digested PCR products of heterozygous 11-week foetal DNA (Fe) and the corresponding maternal DNA (Ma), which is homozygous for the *Sty*I+ allele. (**B**) RT–PCR imprinting assay of undifferentiated *GRB10* cDNA in tissues from foetus in **A**. Top: schematic of undifferentiated *GRB10* cDNA amplified with primers RT-2F and RT-4R across the *Sty*I polymorphism, and its restriction fragment sizes for the *Sty*I+ and *Sty*I− alleles. Bottom: Gel containing *Sty*I digested RT–PCR products of *GRB10* cDNA. The paternally derived (Pa) *Sty*I− transcripts and maternal *Sty*I+ bands are indicated. The paternal allele is predominant in brain and the only allele visualised in spinal cord. Both parental alleles were detected in the RNA of all other tissues. A homozygous *Sty*I+ control to demonstrate complete *Sty*I digestion (C) was included. (**C**) Imprinting assay for *GRB10β* isoform in same foetal samples. Top: *Sty*I restriction map of the *GRB10β* RT–PCR product, showing the positions of the polymorphic and non-polymorphic *Sty*I sites, and the restriction fragments generated for the *Sty*I+ and *Sty*I− alleles. Bottom: electrophoresed *Sty*I digested *GRB10β* RT–PCR products. The 679 bp *Sty*I− paternal allele only is detected in spinal cord and is predominant in brain. The same *Sty*I digestion control, (C) was used. (D) amplification products digested with *Sty*I. (+) reactions containing RT. (−) RT negative reactions. (M) 100 bp size marker.

tongue, heart, lung, kidney, stomach, intestine, pancreas, adrenals, skin, limb, placenta, umbilical cord and yolk sac, expression was detected from both parental alleles (Figure 2B and Table 2). *GRB10* is widely expressed, but is most abundant in insulin responsive tissues including the pancreas, skeletal muscle and adipocytes.[14,16] Biallelic expression was clearly observed in the pancreas. Although *GRB10* expression in the limb and skin was also biallelic, these tissues are relatively heterogeneous, so that skeletal muscle and adipocytes were not examined separately. Both parental alleles were similarly observed in adult fibroblasts (Table 2). In eight foetal brain and three spinal cord informative samples, a very different pattern of *GRB10* expression was observed, with distinct repression of the maternal allele (Figure 2B, Table 2). In spinal cord of 8–12 weeks post-conception, expression was detected exclusively from the paternal allele. In foetal brain, expression occurred predominantly or monoallelically from the paternal allele. Since the RT–PCR assay was not quantitative, the levels of maternally derived transcripts could not be accurately assessed. This imprinted pattern of expression in the CNS was observed irrespective of whether the expressed allele was the *Sty*I+ or *Sty*I− genotype.

Analysis of imprinting in individual *GRB10* isoforms

Isoform-specific imprinting, through the utilisation of different promoters, has been demonstrated in the *IGF2*[26] and *MEST*[27] genes. In the case of the paternally expressed *MEST* gene, biallelic expression was observed specifically in lymphocytes.[27] Recently the existence of a second non-imprinted isoform in lymphocytes was reported, which had previously masked the imprinted expression of the original isoform.[28] The possibility of isoform-restricted imprinting of *GRB10* was investigated using isoform-specific RT–PCR.

To examine the allelic expression of the ubiquitous *GRB10β* isoform (GenBank: U69276) individually, RT–PCR was performed using forward primer A2 from the 5′ UTR unique to *GRB10β*[16] and reverse primer from exon 7A (Figure 1). The amplified cDNA included the exon 3 polymorphic *Sty*I site, but also incorporated a second non-polymorphic *Sty*I site (Figure 2C). Imprinting analysis of *GRB10β* was performed using the same foetal-maternal sample pairs and adult fibroblasts used for analysis of the undifferentiated *GRB10* transcripts. *GRB10β* was also expressed exclusively from the paternal allele in spinal cord and predominantly or mono-allelically from the paternal allele in the brain (Figure 2C, Table 2). Samples demonstrating preferential expression were

Table 2 Allelic origin of expression of *GRB10*, and individual *GRB10* isoforms β and δ, in foetal tissues and adult cells

Tissue	*Allelic expression of* GRB10 *using* Sty*I polymorphism*	*Allelic expression of* GRB10β *using* Sty*I polymorphism*	*Allelic expression of* GRB10δ *studied with 3′ UTR polymorphism*
Brain	6/8 preferential paternal 2/8 paternal only 1 uninformative monoallelic	6/8 preferential paternal 2/8 paternal only 1 uninformative monoallelic	4/5 preferential paternal 1/5 paternal only 1 uninformative monoallelic
Spinal cord	3/3 paternal only	3/3 paternal only	–
Vertebral column	3/3 biallelic	3/3 biallelic	–
Eye	6/6 biallelic	6/6 biallelic	8/8 biallelic
Tongue	2/2 biallelic	2/2 biallelic	–
Heart	5/5 biallelic	5/5 biallelic	6/6 biallelic
Lung	5/5 biallelic	5/5 biallelic	4/5 biallelic
Liver	–	–	3/3 biallelic
Kidney	3/3 biallelic	3/3 biallelic	2/2 biallelic
Stomach	2/2 biallelic	2/2 biallelic	2/2 biallelic
Intestine	6/6 biallelic	6/6 biallelic	6/6 biallelic
Pancreas	5/5 biallelic	5/5 biallelic	–
Adrenals	5/5 biallelic	5/5 biallelic	–
Skin	5/5 biallelic	5/5 biallelic	7/7 biallelic
Limb	2/2 biallelic	2/2 biallelic	4/4 biallelic
Placenta	9/9 biallelic	9/9 biallelic	7/7 biallelic
Umbilicus	3/3 biallelic	3/3 biallelic	1/1 biallelic
Yolk sac	4/4 biallelic	4/4 biallelic	–
Adult fibroblasts	2/2 biallelic	2/2 biallelic	1/1 biallelic

Note: all tissues are foetal, except where stated.

concordant for the β splice form and the mixed *GRB10* isoforms. Biallelic expression was observed in all other foetal tissues tested (Figure 2D) and adult fibroblasts (Table 2).

Imprinting of the *GRB10δ* isoform (GenBank: D86962) was analysed using a novel G→A polymorphism at nucleotide 4001 of the *GRB10δ* cDNA (Figure 3). This occurs in the elongated 3′ UTR of the δ cDNA, which is contiguous with the previously reported exon 15 sequence in genomic DNA (data not shown). Although this polymorphism occurs beyond the extent of the other reported *GRB10* isoforms through alternative polyadenylation signalling, it may not be unique to *GRB10δ*, but could be present in other as yet unidentified splice variants. The frequency of the 'A' allele was 21% in 52 foetuses screened for informative heterozygotes. In total, tissue samples from 13 heterozygous fetuses were analysed for *GRB10δ* imprinting, of which seven were informative. The maternal DNA in four sample pairs was homozygous for the 'G' allele and in three cases, homozygous for the 'A' allele. Three of the foetuses were also heterozygous for the exon 3 *Sty*I polymorphism and had previously been used to analyse the undifferentiated *GRB10* and *GRB10β* imprinting status. The gestational ages of the foetuses analysed were between 7 and 18 weeks. RT–PCR of *GRB10δ* was performed from exon 14 within the coding region, across the G/A polymorphism, to the extended 3′ UTR. Allelic origin of expression was examined by automated sequencing of the RT–PCR products across the polymorphic site. As was the case for *GRB10* and *GRB10β*, the *GRB10δ* transcript was expressed predominantly or specifically from the paternal allele in 7–18-week foetal brain (Figure 3, Table 2). This pattern of expression was consistently observed in the sequences in both the forward and reverse-complement directions, whether the paternal allele was a 'G' or an 'A'. All other foetal tissues tested demonstrated biallelic expression (Table 2), with equal heights of both the G and A peaks in the sequence electropherograms (Figure 3). Biallelic expression was also detected in the adult fibroblasts from a single individual. Thus the imprinting profiles observed for the *GRB10β* and *GRB10δ* isoforms were similar to those detected in the undifferentiated *GRB10* cDNAs (Table 2) suggesting that imprinting of *GRB10* is co-ordinately controlled in neural tissues.

Mutation screening of *GRB10* in SRS cases with unknown genetic aetiology

Eighteen classic SRS patients, in whom cytogenetically distinguishable abnormalities and mUPD7 had been excluded, were analysed for disease-causing mutations of all 16 exons[25] of the *GRB10* gene by sequencing. No mutations were detected in any of the 18 SRS patients screened, when compared to the previously published *GRB10* exon sequences (Table 1). However, two novel polymorphisms were identified in *GRB10* which were also identified amongst 10 normal controls (Table 3). These polymorphisms were predicted to have no effect on splicing events and did not change any amino acid.

Discussion

We have consistently demonstrated paternal expression of *GRB10* in the developing CNS, accompanied by biallelic transcription in a wide range of other organs and peripheral tissues. In the foetal spinal cord, expression was clearly monoallelic from the paternal allele, but in the majority of

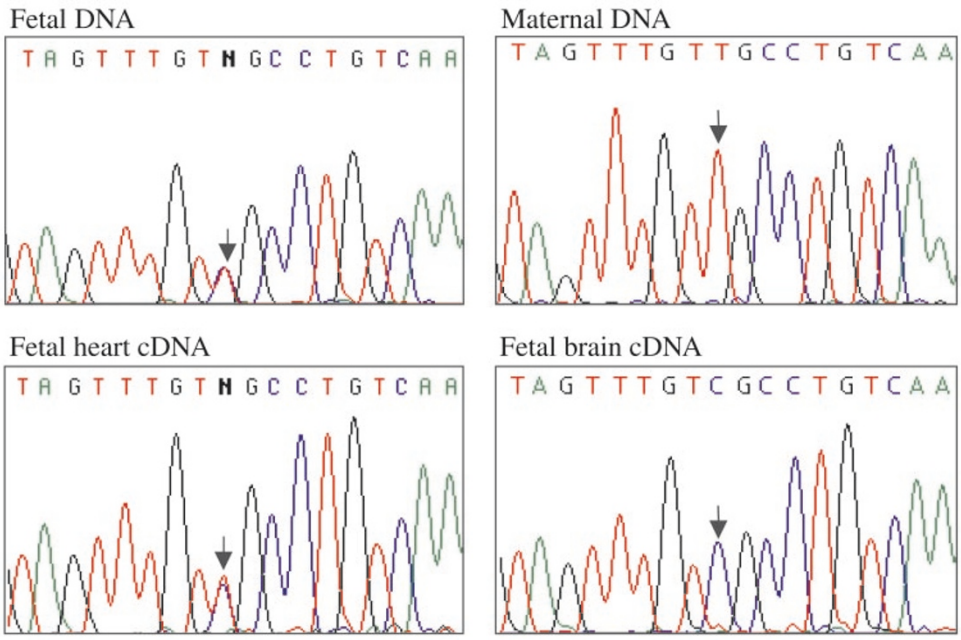

Figure 3 Imprinting analysis of the *GRB10δ* isoform. Sequence electropherograms are shown across nucleotides 4392–4409 of *GRB10δ* (GenBank: D86962) for PCR and RT–PCR products from a single informative foetal-maternal pair. Sequences were acquired with primer 15-3′R and are in the reverse-complement direction. Arrows indicate the position of the newly identified G→A polymorphism at nucleotide 4001. The maternal allele of the heterozygous foetus is a 'T'. The brain RNA was monoallelic for the paternally derived 'C' allele. Both parental alleles were detected equally in the heart cDNA, illustrating the biallelic expression pattern observed in all other tissues tested.

Table 3 Polymorphisms identified in *GRB10* in SRS and normal individuals

Site	*Nucleotide* (n) *change*	*Amino acid change*	*Enzyme change*	*Allele frequency*	*Number of alleles tested*
Exon 3[a]	428G→A	P47P	*Sty*I	62%	36
Intron 4	IVS+35G→A	–	–	23%	66
Exon 11	1460G→A	A391A	–	19%	54

[a]Previously published.[25] Exonic polymorphism sites are given with respect to the *GRB10β* cDNA or protein isoform (GenBank U69276).

foetal brain samples analysed, there was incomplete repression of the maternal allele. Although the RT–PCR assays were not strand-specific, such that amplification of a putative antisense transcript was not precluded, the amplification products crossed at least one intron and were of the correct cDNA size. Antisense RNAs have now been described for several imprinted genes,[29–31] but in each case these have contained intronic sequences not detected in our RT–PCR assays, making it unlikely that we were measuring antisense expression.

The pattern of tissue-specific imprinting of *GRB10* strongly resembles that of the Angelman syndrome gene *UBE3A*, except the latter is expressed predominantly from the maternal chromosome in brain.[32,33] *In situ* hybridisation studies in mice with reciprocal disomies for the region containing *Ube3a* showed that monoallelic maternal expression occurred in specific regions of the brain, including hippocampus and Purkinje cells, whereas other regions demonstrated preferential maternal or biallelic *Ube3a* expression.[34] A similar situation may occur for *GRB10* to give an overall pattern of predominant paternal expression in brain. Incomplete silencing of the maternal *GRB10* allele could also account for the trace of maternal transcripts detected. The $p57^{KIP2}$ gene demonstrates preferential expression from the maternal allele in several foetal tissues.[35] Alternatively, specific *GRB10* isoforms may be biallelically expressed in brain. However, two of the four known isoforms, *GRB10β* and *δ*, similarly demonstrated monoallelic or predominant expression from the paternal allele in foetal brain. This suggests that regulation of *GRB10* imprinting in the CNS is not isoform-specific.

The imprinting status of the human *GRB10* gene dramatically contrasts with the maternal-specific expression of mouse *Grb10*.[13] The *Igf2r* gene is expressed monoallelically

from the maternal allele in the mouse, but in a polymorphic manner in humans, indicating a partial loss of imprinting during evolution.[36] However, *GRB10* is the first gene to be identified for which contrasting imprinting between the two species has been described. This confounds the arguments surrounding the evolutionary significance of genomic imprinting.

Paternal-specific expression in the foetal brain has also recently been reported by Blagitko *et al*[37] corroborating our data: In their study, similar RT–PCR analyses of expressed polymorphisms in foetal tissues for which parental DNA was available were performed. However, a conflicting report published very recently by Yoshihashi *et al*[38] states that monoallelic expression of *GRB10* is likely to be of maternal origin in the brain. In their study, *GRB10* expression was found to be monoallelic in brain, but no corresponding parental DNA samples were available to determine the allelic origin of transcription in this tissue. Instead, *in vitro* RT–PCR assays were performed in somatic cell hybrids derived from human lymphoblasts and Chinese Hamster ovary cells, containing either a maternal or a paternal human chromosome 7, to determine which parental allele was active. *GRB10* expression was detected solely in the cell line with the maternal human 7, so paternal repression in brain was inferred.[38] While *GRB10* may feasibly be maternally expressed in the somatic cell hybrid system, we and Blagitko *et al*[37] have clearly demonstrated that expression is paternally derived in brain through direct analysis of allelic transcription.

The role *GRB10* might play in the aetiology of SRS involving chromosome 7 is difficult to predict in view of the imprinting profile of this gene. Absence of an active paternal allele in the developing CNS may contribute to certain aspects of the clinical phenotype, specifically in mUPD7 patients. However, this would not be the case for patients with matdup 7p11.2-p13, as a normal paternal homologue is present. A novel maternally expressed splice variant, *GRB10*γ-1, has very recently been identified in foetal skeletal muscle.[37] Over-expression of this isoform in this insulin-responsive tissue could more plausibly contribute to the growth restriction and lateral asymmetry phenotypes of SRS.

No sequence mutations were identified in the *GRB10* coding region in 18 SRS cases screened. The possibility that epigenetic mutations of *GRB10* are associated with SRS was not investigated. Recently, a nucleotide substitution resulting in a P95S change in GRB10 was identified in two unrelated Japanese SRS patients, but not in 100 normal controls. Functional studies will aid in determining the effect this substitution has on GRB10 activity, and whether it is a disease-causing mutation in these patients.[38] The variability in the clinical presentation of SRS no doubt reflects the genetic heterogeneity of this disorder. However, in cases where the genetic aetiology involves a chromosome 7 disruption, more than one gene may contribute to different aspects of the syndrome. *GRB10* is the first gene within the SRS candidate region of 7p11.2-p13 to be found to be imprinted, but sequence mutations appear to be exceedingly rare. Possible disease-causing mutations of *GRB10* have only been identified in two of a total of 136 SRS patients screened, including the 18 cases in this report. This region of chromosome 7 is currently under investigation for other imprinted genes that may contribute to SRS.

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