

Analysis of candidate imprinted genes in PWS subjects with atypical genetics: a possible inactivating mutation in the *SNURF/SNRPN* minimal promoter

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Abstract Prader-Willi syndrome (PWS) is a neurodevelopmental disorder associated with abnormalities of chromosome 15q11q13. The majority of cases result either from a deletion approximately 4 Mb in size, affecting chromosome 15 of paternal origin or from UPD(15)mat; these account for ~70 and ~20–25% of PWS cases, respectively. In the remaining 3–5% of PWS cases where neither the deletion nor UPD is detectable, PWS is thought to be caused either by a defect in the imprinting centre resulting in a failure to reset the paternally inherited chromosome 15 derived from the paternal grandmother or, very occasionally, from a balanced translocation involving a breakpoint in 15q11q13. Nine probands with a firm clinical diagnosis of PWS but who had neither a typical deletion in the PWS region nor UPD(15)mat were investigated for inactivating mutations in 11 genes located in the PWS

region, including *SNURF* and *SNRPN*, which are associated with the imprinting centre. Other genes studied for mutations included *MKRN3*, *NDN*, *IPW*, *HBII-85*, *HBII-13*, *HBII-436*, *HBII-438a*, *PARI* and *PAR5*. A possibly inactivating mutation in the *SNRPN* minimal promoter region was identified. No other inactivating mutations were found in the remainder of our panel of PWS subjects with atypical genetics. Expression levels of several of the candidate genes for PWS were also investigated in this series of probands. The results indicate that PWS may result from a stochastic partial inactivation of important genes.

Keywords PWS · Deletion · UPD · *SNRPN* minimal promoter mutation · Gene expression

Introduction

Prader-Willi syndrome (PWS) (MIM176270) is a complex neurodevelopmental disorder affecting ~1/22,000 live births but with a population prevalence of ~1/54,000 (Whittington et al. 2001). Clinically, PWS is characterised by two phases. In infancy there is a failure to thrive, with muscle hypotonia, genital hypoplasia, respiratory problems and feeding difficulties (Holm et al. 1993; Cassidy 1997). From as early as 2 years of age, an altered phenotype becomes apparent with evidence of mild developmental delay and learning disabilities and the onset of severe over-eating behaviour resulting from an abnormal satiety response to food intake (Holland et al. 1995). This can lead to severe obesity if not controlled. Other later-phase phenotypic characteristics include short stature, small hands and feet and significant behavioural problems. A

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points system of eight major criteria (1 point each) and 11 minor criteria (1/2 point each) has been devised to aid in the diagnosis of PWS. A firm clinical diagnosis of PWS must reach a score of at least 8 points including at least four of the major and several of the minor criteria as addressed by Holm et al. (1993).

In a population-based study of PWS (Whittington et al. 2001), it was found that the presence of five 'core' clinical criteria (floppy baby, weak cry or inactivity, poor suck, feeding difficulties, and hypogonadism) was necessary, but not sufficient, for a positive genetic diagnosis (Whittington et al. 2002). In this study, two people were identified who fulfilled the clinical criteria (Holm et al. 1993) for PWS but genetically showed a normal methylation pattern at the *SNURF/SNRPN* exon 1 region, with concomitant expression of *SNRPN*. One of these individuals was heterozygous at microsatellite loci spanning the whole PWS region, while the second person was found, by two independent genetics laboratories, to have a small paternally derived deletion in q12, encompassing D15S9 (*MKRN3*) and D15S543 but not extending as far as D15S541.

In a further, UK-wide study of psychosis in PWS (Soni 2006), another person was found with the five 'core' clinical criteria, but with normal genetics. Could these atypical people possibly have a mutation in, or a deletion of, a single maternally imprinted gene that would cause PWS? A hypothetical model of a single gene cause of PWS has been put forward (Holland et al. 2003) although it is contrary to mainstream opinion. However, the region thought to contain the 'PWS genes' has been shrinking over the past decade. If this single gene hypothesis were true, a logical starting point for the 'PWS gene' would be within the small deletion in the second case referred to above.

PWS is caused by the absence of expression of a cluster of paternally expressed genes located at 15q11q13, usually achieved by an interstitial paternal chromosomal deletion, UPD(15)mat or defects in the imprinting centre (IC) (Nicholls and Knepper 2001). The ~4-Mb common deletion that accounts for 70% of PWS cases (Nicholls and Knepper 2001; Christian et al. 1995; Amos-Landgraf et al. 1999) spans the 2-Mb imprinted domain and also several non-imprinted genes. To date, there are at least 11 known paternally expressed genes in the region with potential involvement in the etiology of PWS. These include *MAGEL2*, a related *MAGE* family member coding for the protein MAGE-like 2, of unknown function but with numerous potential nuclear localisation signals that is highly expressed in the hypothalamus and other regions of the brain (Boccaccio et al. 1999; Lee et al. 2000) and *NDN* encoding NECDIN, a MAGE protein family member

proposed to act as a neuronal growth suppressor and anti-apoptotic protein in post-mitotic neurons (Macdonald and Wevrick 1997; Jay et al. 1997; Pagliardini et al. 2005).

Another known gene, *MKRN3*, encodes a putative ribonucleoprotein, the makorin ring finger protein 3 (Jong et al. 1999). The complex IC-*SNURF/SNRPN*-snoRNA polycistronic locus encodes several functions including a *cis*-acting regulatory region, two nuclear proteins (*SNURF* and *SNRPN*) (Glenn et al. 1996; Gray et al. 1999), five classes of box C/D small nucleolar RNAs (snoRNAs) (Cavaille et al. 2000; de los Santos et al. 2000; Runte et al. 2001) and a *UBE3A-AS* antisense transcript (Rougeulle et al. 1998; Runte et al. 2001; Landers et al. 2004). *SNURF/SNRPN* encodes an arginine-rich polypeptide, small ribonucleoprotein N, whose putative function includes RNA binding (Gray et al. 1999). The snoRNAs in the PWS region lack the usual rRNA complementarity and may target cellular mRNAs for methylation or alternative splicing (Cavaille et al. 2000; de los Santos et al. 2000; Runte et al. 2001). *HBII-85*, a member of the snoRNA family, has been shown to be highly conserved in rodents, and Gallagher et al. (2002) showed that a 121-kb region thought to be critical in PWS contained both *HBII-85* and *HBII-438*, suggesting that these genes may play a major role in the PWS phenotype.

Angelman syndrome (AS) is clinically distinct from PWS, although it also results from abnormalities in the 15q11q13 region, but here the deletion is maternally inherited and the UPD is paternally derived (Nicholls and Knepper 2001). AS therefore results from the inactivity of maternally, rather than paternally, inherited genes in 15q11q13. About 20% of the people with AS carry a causative mutation in the *UBE3A* gene, located in the maternally active but paternally imprinted part of the bipartite PWS/AS region. The *UBE3A* gene was not originally considered to be a candidate gene for AS, as it is not imprinted in blood lymphocytes. Only when the gene was found to be paternally imprinted in certain regions of the brain were mutations searched for and identified as associated with AS (Kishino et al. 1997; Rougeulle et al. 1997). To date, unlike AS, no single gene mutation has been implicated in the etiology of PWS although candidate genes have been proposed.

The human *SNRPN* minimal promoter region (SMP) upstream of the *SNURF* transcript has been hypothesized as a likely candidate where a mutation could inactivate the *SNRPN* gene and also affect subsequent downstream expression. The SMP region includes 71 bp of upstream sequence and the first 51 bp of *SNURF/SNRPN* exon 1 (Finberg et al. 2003).

This work describes a search for mutations in imprinted genes in the PWS region of chromosome 15 that might have a causative role in the syndrome combined with a study of expression levels in some of these candidate genes. Starting with the three individuals described above, the cohort was expanded to include further individuals with unusual or possibly informative genotypes.

Materials and methods

Participants

The probands included in this study were a subset from the large extended population study involving approximately a third of the people with PWS resident in the UK (Whittington et al. 2001; Soni 2006). On entry into the study, the participants were clinically and psychologically assessed, and blood samples were taken and sent to the genetics laboratory where both RNA and DNA were extracted by routine methods.

Molecular diagnosis of PWS

The absence of a paternally inherited non-methylated band at the *SNURF/SNRPN* locus after treatment of DNA samples with bisulphite followed by methylation-specific PCR was taken as laboratory confirmation of the diagnosis of PWS (Kubota et al. 1997; Zeschnigk et al. 1997). Confirmation of the molecular diagnosis was also obtained by a lack of expression of *SNRPN* (Wevrick and Francke 1996).

Microsatellite analysis

Differentiation between participants who carried a deletion in 15q11q13 and those with UPD(15)mat was made by detection of alleles at a combination of 14 microsatellite loci lying within the common deletion and two lying distal to the common breakpoint BP3 within the *HERC2* gene (Chai et al. 2003). Some loci were studied by incorporation of ^{32}P -[dCTP] followed by autoradiography, and others by the use of fluorescence-tagged primers and Genescan software (Applied Biosciences). When more densely packed markers were needed, the tool “repeat master” (ensembl.org) was used to identify further polymorphic CA repeat sequences. The microsatellite markers so obtained are identified by the contig from which they were derived.

Where possible, confirmation of the genetic subtype was obtained from parental DNA. All of the partici-

pants were studied at D15S128, which lies within the *SNRPN* IC region.

PCR amplification and sequencing analysis

The ExonPrimer program was used to design PCR primers flanking each exon of the 11 candidate genes. Sequences were obtained from the chromosome 15q11q13 draft human genome sequence (primer sequences are available on request). Direct sequencing was performed using the dideoxy chain termination method (Applied Biosciences, ABI BigDye 3.0 system) on an ABI 3730 DNA Sequencer, and sequences were analysed using Chromas v2.0 software. All base variations were verified bidirectionally.

Expression analysis

Prior to the isolation of cDNA, the RNA samples were treated with DNase1 to ensure that they did not contain any contaminating genomic DNA. cDNA was then isolated by standard methods using a Taqman reverse transcription kit (Applied Biosciences, Warrington, UK). Expression of candidate genes was then measured by quantitative methodology using SYBR green as the detector. As SYBR green will only fluoresce when it is bound to double-stranded DNA, the point at which the concentration of PCR product is above a set background threshold will be related to the amount of cDNA template in the PCR mix. The actual product concentration is estimated by constructing a standard curve produced by serial dilutions of cDNA from a control individual. To allow for differences in cDNA concentration, these values are normalised against those from a housekeeping gene such as *β -actin* (*BA*) or *GAPDH*. Thus the level of expression of any sample can be calculated for different types of proband, relative to controls. Expression analysis was performed using an Applied Biosystems ABI prism 7900HT sequence detection system. Primers were derived using the programme Primer 3. Where possible, primer pairs crossing an intron were selected. Those used for *SNRPN* crossed intron 6.

Results

A total of nine participants from the Cambridge, UK, PWS studies (Whittington et al. 2001; Soni 2006) were recruited into the subset. These participants all had a confirmed clinical and behavioural diagnosis of PWS but presented with unusual genetics.

Clinical description and genetic studies

The clinical details for all participants are shown in Table 1. All were examined by at least two of the authors and were found to fulfil the criteria for a clinical diagnosis of PWS as described by Holm et al. (1993).

Participant id015

This person (case 1 in Whittington et al. 2002) demonstrated both a maternally and a paternally derived band at the *SNURF/SNRPN* exon 1 region by methylation-specific PCR (MS-PCR) and also expressed the *SNRPN* gene when studied by reverse transcriptase (RT-PCR) (Fig. 1; Table 2). However, a series of microsatellite markers across the 15q11q13 region revealed a small paternally derived deletion located proximal to *SNRPN*. It included D15S543, D15S18 and D15S9 (*MKRN3*) but neither D15S541 nor D15S11. Thus the maximum size of the deletion was 1.8 Mb (Fig. 2). Microsatellite analysis at a series of loci extending throughout the remainder of the PWS/AS region demonstrated both maternal and paternal al-

les (Tables 3, 4), although he was uninformative at D15S128. Expression studies carried out on a series of candidate genes showed that this proband expressed *HBII-85* and *PAR5* at levels comparable to those shown by control subjects (Table 2), while sequencing studies showed SNPs close to exons 2, 7 (rs705) and 12 of the *SNRPN* gene but no inactivating mutation in any candidate gene located in the PWS region (Table 5).

Participant id027

This person also presented with classical PWS. She fulfilled the genetic diagnostic criteria as she did not express *SNRPN* and had only a maternally derived non-methylated band after MS-PCR at *SNURF/SNRPN* exon 1. A paternal sample was unavailable, but while the proband was mono-allelic across a region that included D15S11, D15S817, and D15S1021, she carried a non-maternally derived allele at other loci including D15S128, D15S1513 and *GABRB3* that lie within the PWS region (Tables 3, 4). These data suggest that either id027 has a smaller than usual deletion in 15q11q13 (Fig. 2) or the proband has an IC

Table 1 Clinical assessment of people in the study

	id015	id026	id027	id153	id081	id090	id094	id096	id119
Floppy at birth	+	++	++	++	++	++	++	++	++
Poor suck/difficulty feeding at birth	++	++	++	++	++	++	++	++	++
Childhood obesity	++	++	++	++	++	++	++	++	++
Age of onset of obesity	2	8	2	3	N/K	2	4	3	N/K
Abnormal/delayed sexual features	++	++	N/A	++	++	N/A	++	++	++
Undescended testes	-	N/A	N/A	++	++	N/A	++	++	++
Learning difficulties	++	+	++	++	++	++	++	++	++
Overeating/food obsession	++	+	++	++	++	++	++	++	++
Age of onset of overeating	2	8	2	2	N/K	3	4	3	N/K
Decreased foetal movement	++	++	++	++	N/K	++	++	-	N/K
Weak cry/inactivity	++	++	++	++	N/K	++	++	++	N/K
Disturbed sleep	++	-	++	++	N/K	-	N/K	-	N/K
Short height	++	++	++	++	+	++	-	-	+
Thick saliva	++	-	-	++	N/K	-	++	-	N/K
Small hands and feet	++	+	++	++	N/K	++	++	++	N/K
Articulation difficulty	++	++	++	++	N/K	-	++	-	N/K
Skin-picking*	++	+	+++	++	+	+	+++	+	+
Temper tantrums ever*	+	+	+++	++	+	+	++	+	+
Obsessional behavior*	+	-	+++	+++	+	+++	++	+++	-
Violent ever*	-	-	+++	++	++	-	+++	++	-
Mood swings*	-	++	+	+++	+	-	++	-	-
Argumentative ever*	-	+	+	+++	+	++	+++	+++	-
Lying ever*	+++	-	-	+	N/K	-	+++	-	N/K
Stealing ever*	+	-	-	+++	+	-	+++	++	-
Stubborn*	+++	+++	+++	+++	N/K	+++	+++	+++	N/K
High pain threshold	++	+	+	++	N/K	+	++	++	N/K
Jigsaw skills	++	++	-	-	N/K	-	-	++	N/K
Scoliosis	-	-	-	-	+	+	-	++	-

Characteristics starred (*) are on a 4-point scale (- absent, +++ severe). All other characteristics are on a 3-point scale

N/A Not applicable, N/K not known

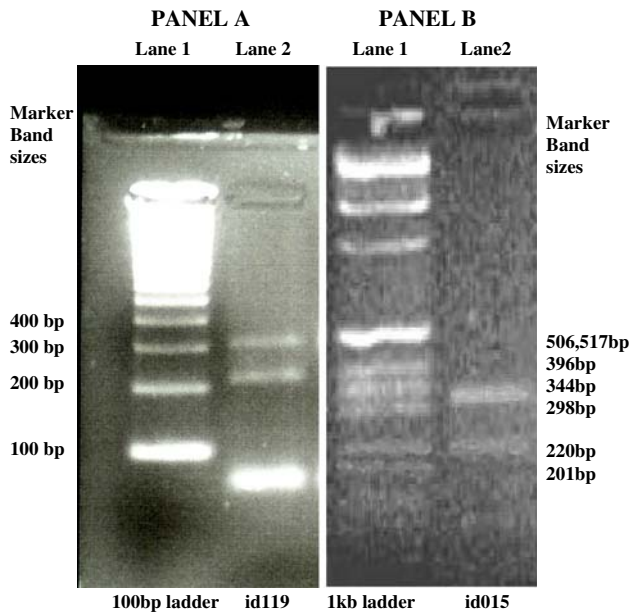


Fig. 1 Methylation-specific PCR of the *SNURF/SNRPN* exon 1 region. *Panel A* Lane 1 100-bp ladder, lane 2 id119. *Panel B* Lane 1 1-kb ladder, lane 2 id 015. Maternally derived methylated band is at 313 bp, paternally derived non-methylated band is at 221 bp, both are present in id119 and id015

epimutation. She carried variants near *SNRPN* exon 12 and *IPW* exon 3 (Table 5).

Participants id026, id090, id094 and id096

These four people fulfilled the criteria for a clinical diagnosis of PWS, but each was found to carry an IC mutation. All four have only the maternal methylated band at *SNURF/SNRPN* exon 1, and none of them expresses *SNRPN*. Parental samples were obtained in each case, and microsatellite analysis showed bi-allelic

Locus	Mb	Participant Identification								
		015	026	027	090	094	096	081	119	153
D15S541	20.46									
D15S543	21.18									
D15S11	21.64									
D15S817	22.15									
D15S1021	22.6									
D15S128	22.7									
D15S122	23.2									
D15S210	23.3									
D15S1513	23.6									
NT026446	23.7									
D15S1002	23.9									
GABRB3	24.3									
NT010280	25.5									
↑PWSCR↑										
D15S165	29.9									
D15S130	92.5									

Fig. 2 Microsatellite map of the nine probands showing the size and the relative positions of the deletions. These are maximum deletions in that if the locus is mono-allelic it has been included even if it is uninformative. Distances are given in megabases from the centromere, and the *hatched region* defines the limit of the deletion

inheritance throughout most of the 15q11q13 region (Tables 3, 4). Participants id026 (case 4 in Whittington et al. 2002) and id096 had only a maternally inherited copy of D15S128, but id090 and id094, although mono-allelic, were uninformative at this locus.

All four demonstrated a paternal allele at informative loci throughout the region (Tables 3, 4). The maximum extent of the deletion carried by each of them is shown in Fig. 2. Subject id026 had only the C allele at rs220030 lying within the *SNURF/SNRPN* minimal promoter region and participants id090, id094 and id096 only the T allele.

None of these four people had an inactivating mutation in any of the candidate genes studied (Table 5).

Table 2 Expression values for PWS subjects compared to controls at genes within the PWS/AS region

Subject	<i>MKRN3</i>	<i>SNRPN</i>	<i>HBII-85</i>	<i>PAR 5</i>	<i>UBE3A</i>	<i>ATP10C</i>
id015	ND	1.0	1.05	1.0	2.3	4.3
id026		ND	ND	ND	1.0	1.6
id027		ND	ND	ND	1.5	1.4
id090		ND	ND	ND	3.6	3.3
id094		ND	ND	ND	1.2	2.5
id096		ND	NR	NR	NR	NR
id81	0.5	0.5	0.5	0.6	1.6	1.6
id119		ND	ND	ND	1.5	1.3
id153	0.9	0.4	0.7	0.4	1.9	1.6
UPD(15)mat		ND (n = 10)	ND (n = 5)		2.5 ± 1.3 (n = 8)	2.6 ± 1.4 (n = 10)
del15q11q13	<0.03 (n = 10)	ND (n = 30)	ND (n = 5)		1.0 ± 0.6 (n = 25)	1.1 ± 0.9 (n = 30)
Normal subjects	1.1 ± 1.0 (n = 12)	1.1 ± 0.4 (n = 7)	1.5 ± 0.4 (n = 17)	1.3 ± 0.6 (n = 18)	1.2 ± 0.8 (n = 20)	1.1 ± 0.7 (n = 30)

Values are shown as an arbitrary scale compared to controls, which are set at 1

ND None detected, NR no result

Table 3 Microsatellite analysis at selected loci within 15q11q13 detected by autoradiography

Subject	Methylation band present	D15S543 20.46 (Mb)	D15S541 21.18 (Mb)	D15S11 21.64 (Mb)	D15S817 22.15 (Mb)	D15S128 22.68 (Mb)	D15S122 23.23 (Mb)	D15S210 23.3 (Mb)	GABRB3 24.34 (Mb)
id015	Mat and pat	2, 2	2, 3	1, 2	1, 2	1, 1	1, 2	1, 2	3, 3
	Father	1, 1	1, 2	1, 2	2, 2	1, 2			2, 3
	Mother	2, 2	2, 3	1, 2	1, 1	1, 1			1, 3
id026	Mat	1, 2	1, 2	1, 1	2, 2	1, 1 (205)	1, 1	1, 3	2, 3
	Father	1, 2	1, 2	1, 2	2, 3	2, 3 (207, 213)	1, 1	1, 3	1, 2
	Mother	1, 2	1, 2	1, 1	1, 2	1, 1 (205, 205)	1, 1	1, 2	2, 3
id027	Mat	ND	ND	1, 1	1, 1	1, 3	1, 2	1, 2	1, 2
	Mother	ND	ND	ND	1, 3	1, 2			1, 3
id090	Mat	2, 2	ND	1, 2	1, 1	2, 2 (207)	1, 3	2, 3	2, 3
	Father	2, 2	ND	1, 2	1, 2	1, 2 (205, 207)	3, 4	2, 3	2, 3
	Mother	1, 2	ND	2, 3	1, 1	2, 2 (207, 207)	1, 2	1, 2	1, 3
id094	Mat	ND	ND	2, 3	2, 3	1, 1 (203)	1, 2	1, 2	1, 3
	Father	ND	ND	2, 3	2, 3	1, 2 (203, 207)		1, 2	1, 3
	Mother	ND	ND	1, 3	1, 3	1, 2 (203, 207)		2, 2	2, 3
id096	Mat	ND	ND	1, 2	1, 2	4, 4 (209)			1, 2
	Father	ND	ND		2, 2	1, 2 (201, 205)			2, 3
	Mother	ND	ND		1, 1	3, 4 (207, 209)			1, 4
id81	Mat and pat	ND	ND	1, 2	1, 2	1, 2			1, 2
id119	Mat and pat	ND	ND	1, 1	2, 2	1, 1			1, 1
	Father			1, 2	1, 2	2, 2			1, 2
	Mother			1, 3	2, 3	1, 1			1, 2
id153	Mat and pat	ND	ND	1, 2	1, 2	1, 2	1, 2	1, 2	1, 2

Distances from the centromere of chromosome 15 are given in megabases (Mb). The numbers *in brackets* for D15S128 are allele sizes from gene scanning

mat Maternal, *pat* paternal, *ND* not done

Participant id81

This person entered the study during its second phase (Soni 2006). Both a paternally derived and a mater-

nally derived band could be detected after MS-PCR at *SNURF/SNRPN* exon 1. Parental samples were not available, but this person was also bi-allelic at microsatellite loci throughout the PWS/AS region including

Table 4 Microsatellite alleles at selected loci within chromosome 15q11q13 using Genescan analysis

Subject	D15S817 (22.15 Mb)	D15S1021 (22.6 Mb)	D15S1513 (23.6 Mb)	NT026446 (23.7 Mb)	D15S1002 (23.9 Mb)	NT010280 (25.5 Mb)	D15S130 (92.5 Mb)
id015	153, 157	231, 236	225	226, 229	109, 121	335	ND
	Father						
	Mother						
id026	157	220	231	239, 242	109, 120	319, 334	289, 296
	Father	144, 157	220	227, 231	120	319, 334	
	Mother	157, 165	217, 220	231, 231	109, 120	319, 334	
id027	157	220	226, 231	ND	1,2	318, 335	217, 220
	Mother	220	226, 234		1,2	319, 335	
id090	153	231	230	242	122, 124	321, 337	289
	Father	153	217, 231	230	109, 122		
	Mother	153	217, 231	230	122, 124		
id094	149, 153	219	231	225, 228	120, 122	319, 336	289, 296
	Father	153	219, 220	207, 231		319, 336	
	Mother	149	219, 232	231		319, 336	
id096	148, 155	218	231, 235	228	109,123	321, 337	289, 296
	Father	155	235	231	109,120	321, 337	
	Mother	148	218, 235	231, 235	123		
id81	149, 157	220, 236	221	228	111,122	330, 337	289, 296
id153	161, 168	219, 230	231	228	107,123	321, 325	288, 296
id119	157	220	235	228	109	336	287, 289
	Father	149, 157	220, 232	227, 230	228, 230	111,120	321
	Mother	157, 164	220, 231	231, 235	228	109,126	320, 336

Distances of loci from the centromere of chromosome 15 are given in megabases (Mb). Alleles are given as sizes in base pairs

Table 5 Mutation analysis at candidate genes within the PWS critical region

Gene	Exon/frag	SNP	PWS	Controls
<i>SMP</i>		rs220030	ids 026, 81	3/10
<i>SNRPN</i>	2	IVS2-3 + 25A → C	id015	0/10
<i>SNRPN</i>	7	rs705	ids 015, 026, 81, 153	
<i>SNRPN</i>	12	IVS12-13, +40A → C,+41C → A	ids 015, 027, 81, 153, 094	6/10
<i>HBII-85</i>	4	+94G → A	id094	2/60
<i>HBII-85</i>	7	+84A → G	All	All
<i>IPW</i>	3	rs1043164	All	6/13
		rs691	ids 026, 027	9/10
		rs13526	ids 015, 027, 81, 153, 094	9/10
<i>PARI</i>	1	+38G → A	None	2/10
<i>NECDIN</i>	1	+493T → A	None	2/10
<i>MKRN3</i>	1	rs2239669	id81	

No SNPs were detected in any of the other genes studied

D15S128 (Tables 3, 4). Quantitative RT-PCR detected expression of *SNRPN* at a reduced level (Table 2). Expression of *UBE3A* and *ATP10C*, which are located within the AS rather than the PWS region, were within the normal range indicating that the reduced expression levels were confined to the PWS part of the region. He was heterozygous C/T at SNP rs220030, which is the only one recorded as located within the *SNURF/SNRPN* minimal promoter region (Table 5).

Participant id119

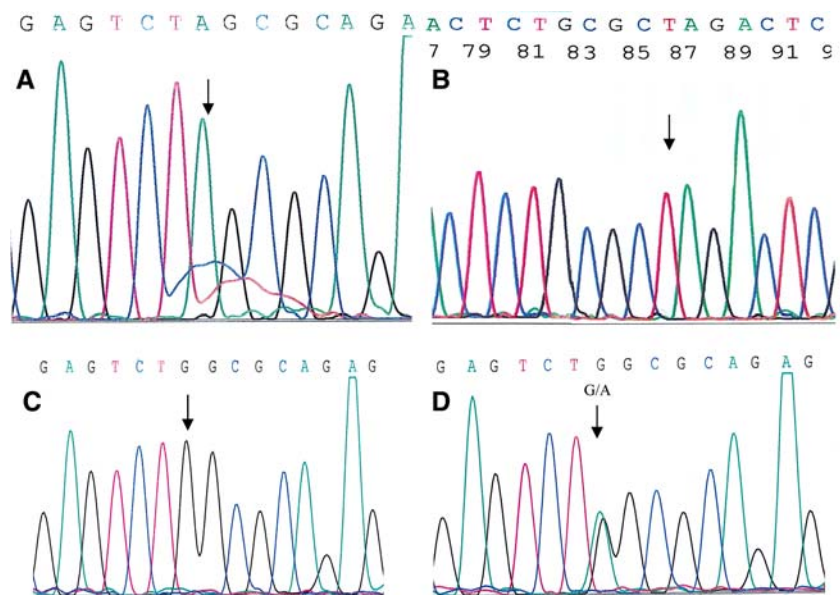
This participant is a member of a family in which an intrachromosomal inversion of 15p11q12 segregates (Webb et al. 1992; Clayton-Smith et al. 1993), and he has a cousin with AS. Microsatellite analysis of id119 and his parents showed that he carried a paternal deletion at several loci in the PWS/AS region including

D15S128, and he did not express *SNRPN* (Tables 2, 3, 4). However repeated MS-PCR of *SNURF/SNRPN* exon 1 always revealed the presence of both a methylated and a non-methylated band (Fig. 1), and sequencing of the *SNRPN* minimal promoter showed that id119 had only the T allele at rs220030 and a G → A transition at base 22,751,225 which is the fourth base prior to the start of transcription of the *SNURF/SNRPN* gene (Fig. 3). This change was confirmed by bi-directional sequencing. A paternal sample showed only the normal G allele, but the maternal sample showed that she was a heterozygous carrier G/A (Fig. 3).

Participant id153

Despite fulfilling the clinical criteria for a diagnosis of PWS (case 2 in Whittington et al. 2002), MS-PCR at

Fig. 3a–d Sequencing of part of the *SNURF/SNRPN* minimal promoter region in the family of id119. The change is indicated by arrows. **a** id119 forward. **b** id119 reverse. **c** Father of id119 forward. **d** Mother of id119 forward



SNURF/SNRPN exon 1 demonstrated both a paternally derived non-methylated band and a maternally derived methylated band, precluding a laboratory diagnosis of PWS. Although parental samples were not available, this person also demonstrated bi-allelic inheritance throughout the entire PWS/AS region including D15S128 (Tables 3, 4). Sequencing detected SNPs near to the *SNRPN* gene exons 7 (rs705) and 12, but no inactivating mutations were found in any candidate gene, including the *SNURF/SNRPN* minimal promoter. Despite this, quantitative RT-PCR demonstrated that, although expression of *SNRPN* was detected, it was at a reduced level. Low expression was also found at certain other loci within the PWS region including *PAR5* and *HBII-85* (Table 2).

Expression analysis

None of the people who demonstrated a single maternally derived methylated band at the *SNRPN* locus expressed *SNRPN*, but the person with a very small deletion, id015, did so with a value within the normal range. The two probands who had a firm clinical, but not a genetic, diagnosis of PWS also expressed this transcript but at a reduced level. The average expression level for control participants was 1.1 ± 0.4 , whereas for id153, the average value obtained over five separate measurements, each performed in triplicate, was 0.4, and for id81 it was 0.5 over four separate measurements, each performed in triplicate. These values were significantly different from those obtained for a series of unaffected control participants when subjected to the student's *t* test. Also the average control value for the relative expression of *HBII-85* was 1.5 ± 1.4 , for id153 it was 0.7, and for id81 it was 0.5. The wider range of values obtained for *HBII-85* reflects the difficulties in detecting expression quantitatively in the more distal part of the PWS region. In the AS region, however, neither participant showed reduced values for *UBE3A* or *ATPI0C* (Table 2).

Discussion

Mutation analysis has been carried out on 11 imprinted genes located within 15q11q13 using DNA from nine participants who clinically have PWS but who do not fulfil the genetic diagnostic criteria. If the proband is >3 years of age, a clinical diagnosis of PWS will include a score of 8 points, of which 4 should be from the eight major diagnostic criteria (Holm et al. 1993; Cassidy 1997). Two of the authors, who examined all of the participants who entered the Cambridge study, were

convinced that id81 and id153 were clinically indistinguishable from those with a confirmed laboratory diagnosis of PWS (both were >40 years of age). Mosaicism was not detected. They not only expressed *SNRPN* and *HBII-85* (Ding et al. 2005), although possibly at a reduced level, as shown by quantitative real-time PCR, but were also heterozygous for loci within the deletion region. While the latter does not eliminate an imprinting defect, expression of *SNRPN* usually does. These results suggested that the signs and symptoms of PWS in these participants could be caused by another genetic abnormality, such as a mutation in the *SNRPN* gene, particularly in the minimal promoter region (SMP/exon1) containing the putative imprinting control centre, or possibly in any other paternally expressed gene including *SNURF*, *HBII-85*, *HBII-13*, *HBII-436*, *HBII-438a*, *IPW*, *PAR1*, *PAR5*, *NECDIN* and *MKRN3*.

As no DNA mutation was found in any of these people, and naturally occurring SNP polymorphisms do not seem to be associated with clinical symptoms (Oeffner et al. 2001), their PWS phenotype could be attributed to a phenocopy, a gene located elsewhere in the genome (D'Angelo et al. 2006), or to partial expression of the epimutation error described by El-Maarri et al. (2001) in people with IC mutations but without a microdeletion, or it may be that both probands lie at the low expressing end of a normal distribution. If as El-Maarri et al. (2001) have suggested, the majority of people with an IC fail to reset the parental imprint correctly after fertilisation rather than in the gamete, or in other cases fail to erase the maternal imprint during spermatogenesis (Buiting et al. 2003), either explanation could account for the reduced expression of *SNRPN* in these two people.

The loss in id015 of a small upstream, paternally derived part of the PWSCR with apparently no involvement of the IC, but accompanied by clinical manifestation of PWS, would suggest that despite the genetic evidence, any disruption of the imprinted region may result in the syndrome. Even a maternal deletion encompassing only the *ATPI0C* gene has been associated with PWS-like symptoms (Ninomiya et al. 2005). In contrast, id015 was found to over-express both *UBE3A* and *ATPI0C*, the two paternally imprinted genes associated with AS. As this tendency was also detected in some other participants with unusual genetics (Table 2) and *UBE3A* is associated with a paternally expressed antisense transcript, it is tempting to suggest that the over-expression is related to the PWS phenotype.

In one proband (id119), sequencing of the SMP/exon1 region of the *SNRPN* gene revealed a point

mutation (G → A) at position –4 from the start of transcription of *SNRPN*. He also has a paternally inherited deletion that extends throughout the entire PWS/AS region, providing an explanation for his clinical diagnosis of PWS. As he has only the A allele, his father only the G and his mother is heterozygous A/G, then the change in sequence must be maternally inherited. In addition, id119 also consistently demonstrated a non-methylated band at the *SNRPN* promoter region when investigated by MS-PCR and was considered clinically to be relatively mildly affected by PWS. These three factors are likely to be linked. The mutation is not in one of the important 7-bp elements (SBE) described by Ohta et al. (1999) but is closer to the start of transcription than any of them. It is likely not only that the non-methylated band is maternally derived but also that the G → A mutation is responsible for a failure to reset the imprinting pattern completely. Such “leaky” imprinting could also account for the milder than expected clinical picture presented by id119.

Although there are many differences between the mouse and human *SNRPN* genes, the striking similarities validate the use of mouse models. In the mouse, the methylation pattern of the maternally inherited homologue is erased at an early stage of gametogenesis and established either during oogenesis (Shemer et al. 1997) or, as has been shown in cases of IC deletion, after fertilisation (El-Maari et al. 2001).

Ohta et al. (1999), while studying three patients with an imprinting mutation but with no detectable mutation, identified the SMP/exon1 region as the PWS-IC. However Shemer et al. (2000), using mouse models, found that the SMP/exon1 alone was insufficient but needed the *cis*-acting AS-SRO both for the removal of the maternal methylation imprint and for promoter expression. By using a transgene consisting of human AS-SRO and mouse SMP/exon1, they were able to establish that the maternal allele was methylated and not expressed while the paternal allele was unmethylated and expressed. A series of papers then dissected the transgene using the CAT expression vector to determine expression and methylation-sensitive enzymes to determine the extent of methylation.

Ohta et al. (1999), Hershko et al. (2001), and Finberg et al. (2003), using AS-SRO-mouse SMP mini-transgenes to establish and maintain the imprinted state, found an SBE (7-bp element) within the SMP to be an absolute requirement for promoter activity. Differences between mouse and human imprinting mechanisms were revealed when the orthologous human SMP, which has sequence-identical but not positional-identical SBEs, failed to methylate the maternal

allele. The mouse SBE, when unmethylated, bound a specific protein required for promoter activity. Mutation of the SBE in the mouse did not completely abolish methylation of the maternal allele indicating that sequences outside the SBE are also required. AS-SRO-human-SMP/exon1 did not induce methylation of the promoter upon maternal transmission, and AS-SRO-human SMP/mouse exon1 had neither promoter activity nor methylated state on female transmission.

The same group (Kantor et al. 2004), using five different mini-transgenes, isolated five *cis* elements involved in different steps of the imprinting process. The original SMP consisted of –84 bp upstream/exon1. If base pairs –84 to –42 were removed then the maternal allele did not become methylated during oogenesis. If bp +1 to +40 were removed again, loss of maternal methylation resulted. So *de novo* methylation signals map both to upstream sequences of the SMP and to exon1 of the gene itself, and both are required.

Buiting et al. (2003) studied 51 patients with PWS and an IC mutation. Sequencing of the SMP/exon1 in 32 patients without an IC deletion revealed no point mutations, but 7/32 had a C → G change at bp–83. All were heterozygous, and the change was found in 7/68 controls. The G → A change at bp–4 was also detected in one PWS patient. Because it was also found in the mother and in a single proband with AS without a deletion (although not in a series of controls), it was considered to be a neutral variant. However, this patient differed from id119 in that he/she did not have a paternally transmitted deletion, which accounted for the clinical picture of PWS, but all paternal sequences were apparently intact.

The integrity of the SMP/exon1 region is vital for establishing and maintaining the imprinted state in both mice and humans, and upstream sequences are critical for the correct function of either mechanism. Changes in the non-coding regions can cause genetic disease by altering gene expression (Buckland 2004). We suggest that in the case of id119, the change from G → A at bp–4 alters the binding of a factor, such that methylation of the maternal allele is not fully established, and results in a non-methylated band during MS-PCR, together with partial expression of SMP/exon1. This may explain the relatively mild clinical picture presented by this person.

A similar situation has recently been described by Wu et al. (2006). They showed that mutations upstream of *Snrpn*-exon1 caused lack of methylation in the maternal *Snrpn* promoter and activation of maternally expressed genes including a rescue from the lethality and growth retardation normally shown by the PWS mouse.

DNA sequence variants such as the one described here would affect the binding of factors that are involved in establishing the correct epigenetic state (Lee and Wevrick 2000). Genetic factors have been found to be responsible for an increased risk of an imprinting defect in AS (Buiting et al. 2001) and are considered to be responsible for the majority of individual, parentally dependent variability in epigenetic modification in other regions of the genome (Sandovici et al. 2003). In a recent study of childhood absence epilepsy, Urak et al. (2006) were able to demonstrate that certain promoter haplotypes present in the *GABRB3* gene were not only associated more strongly with the disease but at the same time lowered the transcriptional activity of the putative disease gene.

In summary, a study of nine probands who clinically had PWS but with atypical genetics has suggested that changes in the maternal SMP may alleviate some of the more severe manifestations of the disease, that PWS can result from a stochastic partial change in gene expression, and that even minor disruption of the PWSCR outside the SRO can still cause PWS.

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URLS and genome browsers used in this study

- http://www.ensembl.org/Homo_sapiens/index.html
- <http://www.genome.ucsc.edu/index.html>
- <http://www.ncbi.nlm.nih.gov/>
- <http://www.gdb.org>
- <http://www.frodo.wi.mit.edu>

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