

Research Article

Investigation of Prader-Willi-like Phenotype using a Whole Genome Array

Esther N. Maina ^{a,b,*}, Tessa Webb ^b, John R. Arrand ^b, Joyce Whittington ^c, Sarita Soni ^c, Harm Boer ^d, David Clarke ^e, and Anthony J. Holland ^c

^a Department of Biochemistry, School of Medicine, University of Nairobi, Kenya

^b Medical School, University of Birmingham, UK.

^c Section of Developmental Psychiatry, Department of Psychiatry, University of Cambridge, UK

^d Janet Shaw Clinic, Coventry and Warwickshire Partnership NHS Trust, Birmingham, UK

^e Robertson Centre, Worcestershire Mental Health Partnership Trust, Kidderminster Hospital, UK

* **Corresponding author:** Department of Biochemistry, School of Medicine, University of Nairobi, P.O Box 30197-00100, Kenya; **Tel:** +254-718-733987; **Email:** njokimaina@uonbi.ac.ke

Background: Prader-Willi syndrome (PWS) is a complex human genetic disease that arises from lack of expression of paternally inherited imprinted genes on chromosome 15q11-q13. Prader-Willi syndrome.

Objective: To use whole genome array to investigate observed Prader-Willi phenotype by assaying differential gene expression patterns in Prader-Willi like subjects.

Methodology: Combined clinical and laboratory study. Three people who participated in a large study of Prader-Willi syndrome (PWS) were found to satisfy the criteria for a firm clinical diagnosis of the syndrome using the accepted consensus scores. A score of 8 is considered to be diagnostic for PWS but despite all three scoring >8, they were genetically negative for PWS. By using Affymetrix Cytogenetics Whole-Genome 2.7M arrays which identify both loss and gain in genomic DNA and also report loss of heterozygosity regions in which all three participants showed the same genomic abnormality were determined. By comparing these regions with the UCSC human genome database, a list of potential candidate genes was compiled in which the participants had all shown the same change. Confirmation of altered gene expression was sought using qPCR to study transcription levels in each of the genes identified.

Results: Three people who participated in the study showed both maternal and paternal bands after methylation-specific PCR and they all expressed *SNRPN*. Increase in copy number with concomitant elevated transcription levels were found in *SGSM2* which had previously been associated with severe obesity and in the protein-folding gene *PPIF*.

Conclusion: Over-expression caused by duplication may be a contributing factor to the PWS-like phenotype in these people.

Key words: PWS phenotype, microarray, copy-number, transcription

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1. Introduction

Prader-Willi syndrome (PWS) is characterised by obesity, short stature, small hands and feet, neonatal hypotonia with difficulty in feeding at birth, hypogonadism and eye problems. At about two years of age the feeding difficulties with poor suck are gradually

replaced by hyperphagia and obsession with food, leading to the obesity. In addition to developmental delay which is manifested by short stature, small hands and feet, growth hormone deficiency and hypogonadism/hypogonadism, there are also behavioural characteristics including learning disabilities, temper tantrums, aggression, repetitive

speech, obsessive compulsive behaviour, sleep disorder and skin picking (Cassidy and Driscoll, 2009). This disparate collection of symptoms led Holm et al (1993) to define the major and minor characteristics which allowed a clinical diagnosis of this the most common genetic form of obesity. Consensus diagnostic criteria were defined and weighted scores in which the major criteria were awarded one point and the minor criteria half a point calculated. A score of 8 or more is clinically diagnostic for PWS.

The majority of people with PWS have a paternally derived deletion of approximately 5-7Mb in 15q11-q13, others have maternal disomy of chromosome 15 (UPD15mat) and a minority have a defect of the imprinting centre located in exon 1 of the *SNRPN* gene which leads to a maternal imprint on the paternally derived chromosome. Any of these abnormalities will result in loss of the paternal contribution to the Prader-Willi syndrome critical region (PWSCR), demonstrated by loss of a paternally derived unmethylated band at the imprinting centre and a lack of expression of the *SNRPN* gene. Although these do not differentiate between the different genetic types of PWS they are diagnostic for the syndrome (Cassidy and Driscoll, 2009; Ramsden et al, 2010; Zeschgnig et al, 1997).

Within 15q11-q13 the complex imprinted *SNURF/SNRPN* gene hosts several untranslated snoRNA genes located within intronic sequences. The finding of a microdeletion involving *SNORD116* in a boy with PWS led to the identification of this snoRNA as the candidate gene for the syndrome (Sahoo et al, 2008).

In the course of a large study of PWS in the UK (Whittington et al, 2001; Soni et al, 2007) three people were identified who fulfilled the criteria for a clinical diagnosis of the syndrome but not the genetic laboratory diagnostic criteria.

The Affymetrix Cytogenetics Whole-Genome 2.7M array while providing high resolution whole genome coverage reliably detects changes in copy number. Deletions and/or duplications present in all three participants if involved in annotated genes could potentially contribute to the Prader-Willi-like phenotype. Candidate genes can subsequently be evaluated to estimate their transcription levels and compared with those shown by people with PWS and with unaffected individuals.

2. Methods

2.1 Study subjects

The three subjects in this study had all been participants in a population study of PWS carried out in the UK (Whittington et al, 2001; Whittington et al, 2002; Soni et al, 2007). Each was assessed by the clinical criteria of Holm et al (1993). Genetic testing was carried out blind to the results. Despite the fulfilment of the clinical criteria with all three scoring >8 (**Table 1**), genetic testing proved negative. Participant 2 (also case 2 in Whittington et al, 2002) had a clinical diagnostic score of >15.

Table 1: Clinical data for three people with characteristics of Prader-Willi syndrome

| Criterion | Participant | | |
|----------------------------|-------------|-----|-----|
| | 1 | 2 | 3 |
| Floppy at birth | yes | yes | yes |
| Poor suck | yes | yes | yes |
| Feeding problems | yes | yes | yes |
| Obesity if no intervention | yes | yes | yes |
| Hypogonadism | yes | yes | ? |
| Developmental delay/ID | yes | yes | yes |
| Food foraging or obsession | yes | yes | yes |
| Behavioural problems | yes | yes | yes |
| Sleep disturbance | ? | yes | no |
| Short height for family | yes | yes | ? |
| Skin picking | yes | yes | yes |
| Scoliosis | yes | no | no |
| Bruises easily | yes | ? | yes |

2.2 Molecular Studies

Nucleic acids were extracted from peripheral blood lymphocytes according to manufacturers' protocols and stored at -80°C. RNA was isolated using TRI-reagent (Sigma, UK) and DNA using Puregene methodology (Qiagen, UK). Quality and concentration were estimated using Nanodrop ND-1000 UV-Vis spectrophotometry.

Genetic analysis for PWS was carried out both by bisulphite treatment of genomic DNA followed by methylation-specific PCR (MS-PCR) at the *SNRPN* (exon 1) locus and by expression of the *SNRPN* gene (Zeschgnig et al, 1997; Kubota et al, 1997; Ramsden et al, 2010). Microsatellite analysis was used to determine zygosity within 15q11-q13.

For expression studies, RNA (1-5µg) was treated with DNase1 prior to cDNA synthesis using a high capacity reverse transcription kit (Applied Biosystems) with random hexamers as primers.

The integrity of the PWSCR was investigated by sequencing genomic DNA using primers from genes located within 15q11-q13. Individual primers from all 29 copies of *SNORD116* were designed within flanking sequences unique to each member of the cluster, ensuring distinction between individual copies and where possible including SNPs. Primer sequences were derived using Primer 3 (<http://frodo.wi.mit.edu>) and sequencing was carried out using BigDye Terminator technology (Applied Biosystems). Copy number at the *IPW* locus was determined by real time qPCR of genomic DNA using *GUSB* as the reference gene and expression levels at a series of genes from 15q11-q13

were obtained by qPCR using cDNA and SYBR Green technology (Applied Biosystems). Reference genes were

GAPDH and *ACTB*. Quantitative PCR was carried out according to MIQE guidelines (Bustin et al, 2010).

Table 2: Laboratory criteria for three people with a firm clinical diagnosis of PWS

| | Subject 1 | Subject 2 | Subject 3 | Controls |
|------------------------------------|---------------------|---------------------|---------------------|---------------|
| Karyotype | 46XY | 46XY | 46XX | |
| MS-PCR bands | Maternal & Paternal | Maternal & Paternal | Maternal & Paternal | |
| <i>SNRPN</i> expression | 0.7 | 0.6 | 4.0 | 1.0±0.1, n=9 |
| <i>PAR5</i> expression | 1.1 | 0.5 | 2.0 | 1.0±0.1, n=10 |
| <i>SNORD116</i> expression | 0.5 | 0.7 | 1.0 | 1.0±0.3, n=14 |
| <i>IPW</i> expression | 2.3 | 1.7 | 3.3 | 1.0±0.4, n=5 |
| <i>UBE3A</i> expression | 1.6 | 0.9 | ND | 1.0±0.1, n=14 |
| <i>ATP10A</i> expression | 1.4 | 1.0 | ND | 1.0±0.1, n=30 |
| <i>GABRB3</i> expression | 1.5 | 1.4 | 1.0 | 1.0±0.4, n=16 |
| Alleles at <i>D15S128</i> | 203, 206 | 203, 206 | 206, 209 | |
| Alleles at <i>D15S822 (GABRG3)</i> | 289, 296 | 289, 296 | 260, 270 | |
| Copies of <i>IPW</i> | 1.6 | 1.9 | 2.2 | |
| Conclusion | Not PWS | Not PWS | Not PWS | |

ND = not done; n=number of individuals

Note: To normalise the Ct values standard curves for the target gene and the reference gene were plotted using the same control and relative expression calculated from the standard equations. The ΔCt method was then used to determine the relative expression values for the participants and a series of control individuals averaged as one. Expression values were calculated for a series of at least 4 separate control and PWS individuals

Table 3: Informative SNPs either inside or close to genes within the PWSCR

| Gene | SNP id | SNP | Subject 1 | Subject 2 |
|---|------------|--------|-----------|-----------|
| <i>MAKORIN3</i> | rs2239669 | C/T | C/T | C |
| <i>NDN</i> | rs2192206 | C/T | C/T | C/T |
| <i>SMP (SNRPN minimal promoter)</i> | rs220030 | A/G | T/C | T |
| <i>SNRPN Exon 5 (first coding exon)</i> | rs705 | C/T | C/T | C/T |
| <i>SNORD116-15</i> | rs1972694 | A/T | A/T | A/T |
| <i>SNORD116-21</i> | rs3863396 | C/T | C/T | C |
| <i>IPW Exon 1</i> | rs28481747 | C/T | C | C/T |
| <i>IPW Exon 3</i> | rs13526 | A/C | A/C | A |
| <i>IPW Exon 3</i> | rs16416 | -/ACAG | -/ACAG | - |
| <i>PAR 1</i> | rs2249378 | C/T | A/G | G |

2.3 Array analysis

DNA samples from each participant were processed according to the Affymetrix Cytogenetics Array protocol (<http://media.affymetrix.com/support/downloads/manuals/cyto assay usermanual.pdf>) then hybridised to Affymetrix Cytogenetics Whole-Genome 2.7M arrays and the results analysed using the Affymetrix

Chromosome Analysis Suite (ChAS). These results were evaluated by identifying overlapping areas of genomic change shown by all three of the study participants. The location and size of each shared alteration obtained from the analysis identified potential candidate genes and their expression levels were ascertained by real-time qPCR.

The mRNA sequence for each gene was obtained from the UCSC or NCBI databases (<http://genome.ucsc.edu/>; <http://www.ncbi.nlm.nih.gov>). Where possible primer pairs spanning an intron/exon boundary were chosen

and each pair was first studied by *in vitro* PCR to ensure that any product was unique and that different isoforms of the same gene would be detected.

Table 4: Expression levels of annotated candidate genes in which all three participants demonstrated the same change in copy number

| Gene | Position | Copy number | Control average | Subject 1 | Subject 2 | Subject 3 | PWS mUPD | PWS 15q11-q13del |
|----------------|----------|-------------|-----------------|-----------|-----------|-----------|---------------|------------------|
| <i>ACY</i> | 3p21.1 | 3 | 1.0±0.2, n=4 | 0.8 | NR | 0.5 | 0.4±0.04, n=4 | 0.9±0.2, n=4 |
| <i>RPL29</i> | 3p21.1 | 3 | 1.0±0.2, n=4 | 0.9 | 6.3 | 1.9 | 1.2±0.2, n=4 | 3.5±1.4, n=4 |
| <i>SERF1B</i> | 5q13.2 | 1 | 1.0 | NR | NR | 0.5 | 2.0 | 2.1 |
| <i>SMN2</i> | 5q13.2 | 1 | 1.0 | 2.8 | 5.9 | 0.3 | 1.5 | 0.3 |
| <i>OCN</i> | 5q13.2 | 1 | NR | NR | NR | NR | NR | NR |
| <i>NAIP</i> | 5q13.2 | 1 | 1.0±0.6, n=6 | 1.0 | NR | 1.0 | 0.8±0.6, n=6 | 1.3±0.6, n=5 |
| <i>KIF25</i> | 6q27 | 1 | 1.0 | NR | NR | NR | 1.0 | 0.8 |
| <i>POLR2J</i> | 7q22.1 | 1 | 1.0 | 0.4 | 3.8 | 0.1 | 1.5 | 0.3 |
| <i>RASA4</i> | 7q22.1 | 1 | NR | NR | NR | NR | NR | NR |
| <i>CNTNAP3</i> | 9p13.1 | 1 | 1.0±0.3, n=13 | 1.8 | 2.3 | 1.8 | 0.8±0.4, n=9 | 1.0±0.3, n=9 |
| <i>PARG</i> | 10q11. | 1 | 1.0 | NR | NR | 1.0 | 1.8 | 1.7 |
| <i>ZMIZ1</i> | 10q22 | 3 | 1.0±0.1, n=8 | 0.8 | 0.7 | 1.3 | 1.4±0.1, n=8 | 0.9±0.2, n=6 |
| <i>PPIF</i> | 10q22 | 3 | 1.0±0.4, n=12 | 2.1 | 4.0 | 2.2 | 2.2±0.9, n=12 | 2.5±0.7, n=11 |
| <i>GPHN</i> | 14q23 | 1 | 1.0 | 4.3 | 3.1 | 0.6 | 0.8 | 1.5 |
| <i>APBA2</i> | 15q13 | 3 | 1.0±0.1, n=5 | 1.7 | 1.4 | 1.0 | 1.0±0.2, n=9 | 0.9±0.2, n=6 |
| <i>NDNL2</i> | 15q13 | 1 | 1.0±0.2 | 0.6 | 1.3 | 1.0 | 1.6±0.5 | 1.3±0.4 |
| <i>TJP1</i> | 15q13 | 1 | NR | NR | NR | NR | NR | NR |
| <i>CHRNA7</i> | 15q13 | 1 | 1.0 | 2.8 | 2.3 | 0.1 | 1.2 | 1.3 |
| <i>NOMO2</i> | 16p12 | 1 | NR | NR | NR | NR | NR | NR |
| <i>EIF3C</i> | 16p11 | 1 | 1.0 | 0.8 | 2.7 | 0.1 | 1.3 | 0.2 |
| <i>SGSM2</i> | 17p13 | 3 | 1.0±0.2, n=17 | 2.2 | 3.1 | 1.6 | 1.3±0.2, n=12 | 1.2±0.4, n=8 |
| <i>MNT</i> | 17p13 | 3 | 1.0±0.2, n=10 | 2.0 | NR | 1.1 | 1.0±0.4, n=12 | 0.9±0.5, n=8 |
| <i>FMNL1</i> | 17q21 | 3 | 1.0±0.2 | 1.1 | 0.5 | 0.8 | 0.9±0.2 | 0.6±0.2 |
| <i>TCEB3C</i> | 18q21 | 1 | 1.0 | 1.6 | 2.1 | 0.1 | 1.1 | 2.5 |
| <i>GGT1</i> | 22q11 | 1 | 1.0 | 1.0 | 0.8 | 0.5 | 0.7 | 0.3 |
| <i>PCDH11</i> | Xq21 | gain | 1.0 | NR | NR | NR | 1.1 | 1.4 |

3. Results

3.1 Molecular studies

The three study participants all had normal G-banded karyotypes, they demonstrated both maternal and paternal bands after MS-PCR and they all expressed *SNRPN* (Whittington et al, 2002). These data exclude a genetic diagnosis of PWS (Jin, 2011). Microsatellite analysis showed heterozygosity throughout 15q11-q13 including at the *SNRPN* locus, gene scanning with fluorescent PCR detected two copies of *GABRG3* and

genomic qPCR detected two copies of *IPW* in each participant. Genes *PAR5*, *SNORD116*, *IPW*, *UBE3A*, *ATP10A* and *GABRB3* were all expressed (Table 2).

Following the sequencing of participant DNA in all 29 copies of *SNORD116*, the current candidate gene for PWS, using primers distinguishing each individual copy, no point mutations or deletions were detected within the coding region of any copy of *SNORD116*.

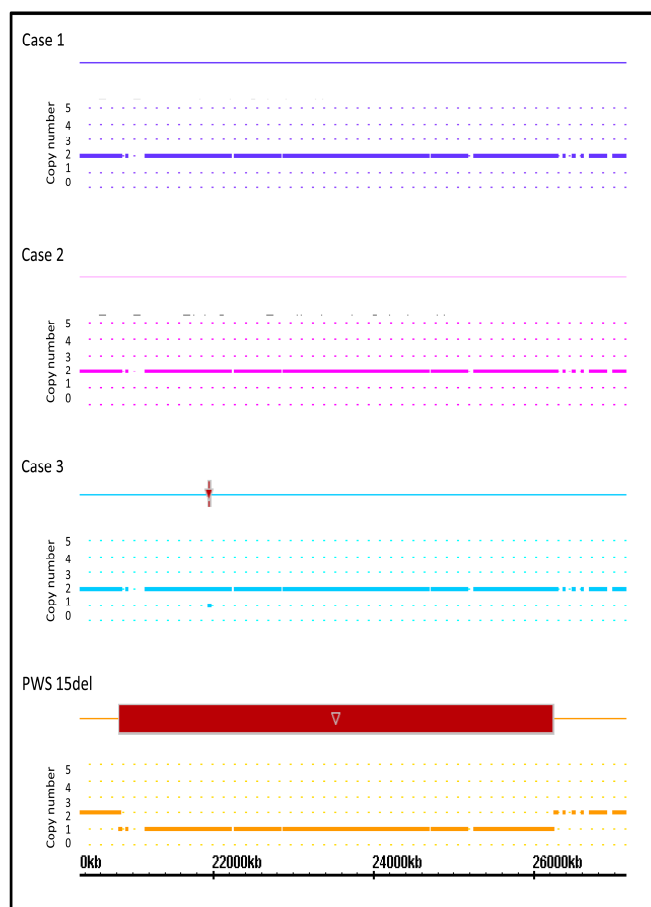
In subjects 1 and 2 sequencing of a series of other genes located within 15q11-q13 again did not detect

mutations in any coding region. These genes included *MAKORIN3*, *NDN*, *SMP* (*SNRPN* minimum promoter), *SNRPN/SNURF*, *SNORD107*, *PAR5*, *SNORD64*, *IPW* and *PARI*. Of 45 SNPs within the sequenced regions, subject 1 was informative at 9 ranging from 15:21,362,686 in *MAKORIN3* to 15:22,933,834 in *PARI* and subject 2 was informative from 15:21,482,600 in *NDN* to 15:22,912,959 in *IPW* (Table 3).

Expression studies showed all three of the participants to express genes within 15q11-q13, showing that PWSCR is intact in all three participants.

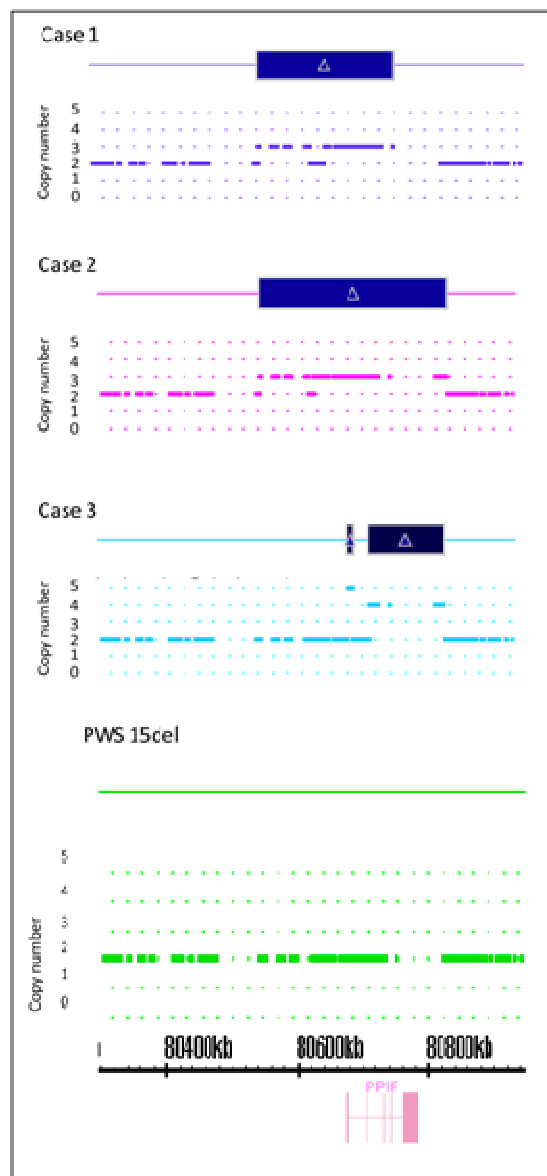
3.2 Array Results

Genomic DNA from the three participants was studied alongside a sample from a PWS patient with del15q11-q13pat. The array confirmed the presence of the deletion which was shown to be 5,957 kbp in size and also showed that it was not present in any of the three participants (Figure 1). Subject 3 did have a small deletion of 21kbp in a repetitive region near the *PWRN2* gene.



The copy number throughout the region is shown on the Y axis by the coloured lines. The large PWS deletion and the small 21kb deletion in subject 3 are shown as solid red bands. The numbers along the X-axis at the base of the figure show genomic position on chromosome 15 in kilobases (*hg18*).

Figure 1: Array copy number analysis of 15q11-q13 for the three participants (cases 1-3) and a person with PWS due to a paternal deletion (PWS 15q11-q13del).



The copy number throughout the region is shown on the Y axis by the coloured lines. The duplicated regions in the three subjects are shown as solid blue bands. The numbers along the X-axis indicate genomic position on chromosome 10 in kilobases (*hg18*) and the position of *PPIF* is shown below.

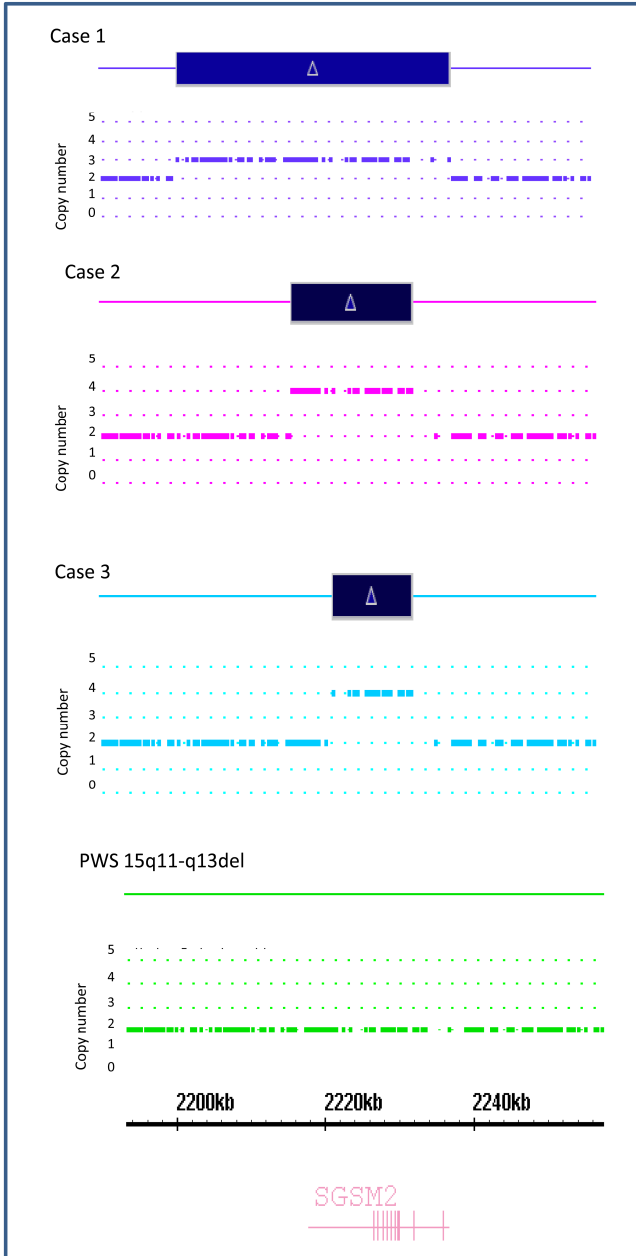
Figure 2: Array copy number analysis of 10q22.3 for the three participants (cases 1-3) and a person with PWS due to a paternal deletion (PWS 15del).

Loss of both alleles was not detected at any autosomal locus. So each deleted region was one of potential haploinsufficiency. When qRT-PCR was performed on seventeen genes to determine whether loss of a single copy resulted in a detectable deficiency of expression in

dosage sensitive genes, no correlations were found between values obtained from the three subjects and people with PWS due either to matUPD or a 15q11-q13 deletion or from control individuals (Table 4).

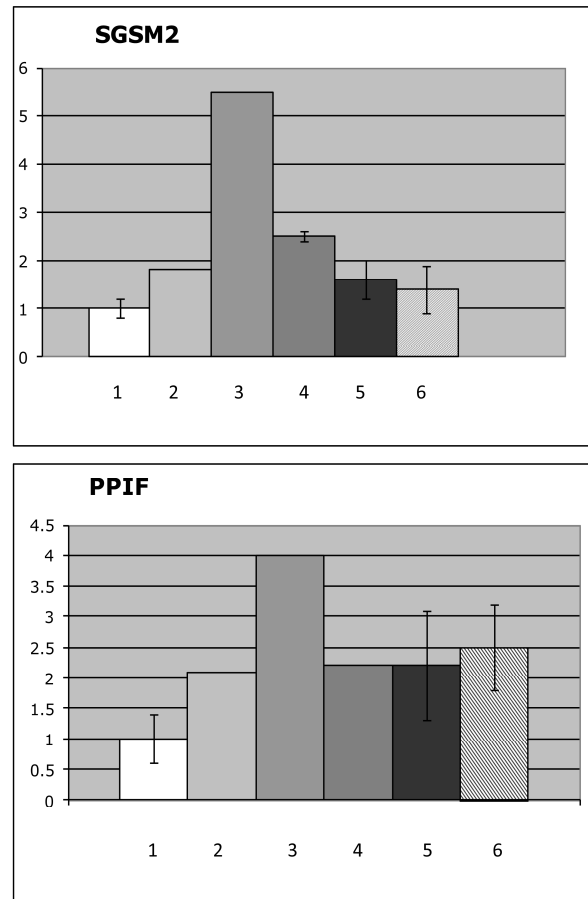
Nine genes; *ACY*, *RPL29*, *ZMIZ1*, *PPIF*, *APBA2*, *SGSM2*, *MNT*, *FMNL1* and *PCDH11* showed a gain in copy number for all three subjects. Two of these genes (*SGSM2* and *PPIF*) which are located on 10q22.3 and 17p13.3 respectively showed a copy number increase, (Figure 2 and 3) and (also had elevated transcription

levels when compared to controls. Normal transcription of *SGSM2* was found in people with either of the common genetic types of PWS but levels of *PPIF* were raised in people with PWS due to either maternal UPD or del15q11-q13 pat. (Table 4, Figure 4). Comparison with either genetic type of PWS using the Mann-Whitney test showed that for *PPIF*, PWS due to either a deletion or maternal UPD differed from controls with $p < 0.05$.



The copy number throughout the region is shown on the Y axis for each subject by the coloured lines. The duplicated regions in the three subjects are shown by solid blue bands. The numbers along the X-axis indicate genomic position on chromosome 17 in kilobases (hg18) and the location of *SGSM2* is shown below

Figure 3 Array copy number analysis of 17p13.3 for the three participants (cases 1-3) and a person with PWS due to a paternal deletion (PWS15q11-q13del).



Column 1 - control subjects; column 2 - participant 1; column 3 - participant 2; column 4 - participant 3; column 5 - PWS due to UPD(15)mat; column 6 - PWS with del15q11-q13pat.

Figure 4: Expression of *SGSM2* and *PPIF*.

4. Discussion

This study has identified two genomic regions, 10q22.3 and 17p13.3, common to three people in which duplications with resulting over expression of genes, may contribute to a PWS phenotype.

Since PWS is a neurodevelopmental disorder, tissue specificity of gene expression could mean that genes highly expressed in brain may not be so in lymphocytes and vice versa. Despite this, some authors have employed both with no apparent problem [Hong et al., 2008] and others have used lymphoblastoid lines in behavioural studies (Bittel et al, 2006; Nishimura et al, 2007).

Microdeletions in PWS subjects with negative molecular diagnostic tests have established snoRNA *SNORD116* as a strong candidate gene (Sahoo et al, 2008; de Smith et al, 2009; Duker et al, 2010). In each of these studies, array genotyping led to the identification of a microdeletion (of ~200kbp) encompassing the entire snoRNA cluster. although expression of *SNRPN* remained. The Affymetrix Cytogenetics Whole Genome array should not have missed a deletion of this size and

did detect a much smaller microdeletion of 21kbp at a more proximal location within the PWSCR (**Figure 1**).

In the present study each of three subjects also demonstrated a functional imprinting centre showing both methylated and unmethylated bands on MS-PCR. PWS could still have resulted from abnormalities such as a small deletion or a point mutation elsewhere in the PWSCR but sequencing did not detect a mutation in any 15q11-q13 gene including *SMP* the *SNRPN* minimal promoter and *SNORD116*. Sequencing of *MAKORIN3*, *SNRPN* and *IPW* indicated two intact copies in both subjects 1 and 2 as they contained informative SNPs and all three participants had two alleles at *D15S128* and *D15S822*. All three expressed candidate genes including *SNORD116*, *SNRPN*, *IPW* and also *UBE3A*, *ATP10C* and *GABRB3*. Taken together these data suggest that they all had two copies of the PWSCR, that genes within it were being normally expressed and that the PWS phenocopies were the result of downstream changes elsewhere in the genome. Substrates for *SNORD116* are still not identified and its functional role is as yet unclear.

Studies on candidate genes identified by array technology demonstrating loss or gain in copy number showed that no loss of an allele had led to a marked reduction of transcription in any of those identified but elevated transcription levels were observed in two putative obesity-related genes *PPIF* and *SGSM2* where there was a gain in copy number. An increase in transcription levels of *PPIF* was also found in individuals with PWS due to either a 15q11-q13 deletion or 15(UPD)mat, suggesting its possible involvement as a downstream gene contributing to the etiology of PWS.

SGSM2 (OMIM 611418) which is upregulated in all three participants, is located within the small 17p13.3 gain described previously by Bochukova et al [2010] in a study of obesity. *PPIF* (OMIM 604486) or cyclophilin F potentiates neuronal and synaptic stress [Du et al 2008]. Mice lacking the *Ppif* gene demonstrated improvement in both learning and memory when compared with controls and were also subject to adult-onset obesity (Luvisetto et al, 2008).

A bioinformatics screen (Bazeley et al, 2008) searching for guide sites for orphan C/D box snoRNAs located 21 possible targets for *SNORD116* within protein coding genes. One of the 21 purported targets for *SNORD116* is *PPIL2* which belongs to the same family and performs a similar function to *PPIF* while another, *RASAL2* was hemizygous in participant 1.

Conflict of Interest declaration

The authors declare no conflict of interest

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