Human Molecular Genetics, 2003, Vol. 12, No. 20 **2723–2732**
DOI: 10.1093/hmg/ddg291

Somatic mosaicism for maternal uniparental disomy 15 in a girl with Prader–Willi syndrome: confirmation by cell cloning and identification of candidate downstream genes

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Received July 8, 2003; Revised and Accepted August 15, 2003

Although uniparental disomy often results from the postzygotic rescue of a meiotic non-disjunction event, mosaicism is usually confined to the placenta. We describe a girl with Prader–Willi syndrome (PWS) who is mosaic for normal cells and cells with maternal uniparental disomy 15 [upd(15)mat] in blood and skin. Somatic mosaicism was confirmed by cloning and genotyping of skin fibroblasts. X inactivation studies indicated that upd occurred prior to X inactivation. RNA samples from the cloned cells were used in DNA microarray experiments to study the effect of upd(15)mat on the gene expression pattern of fibroblasts. Proof of principle was obtained by detecting several chromosome 15 genes known to be imprinted. We did not obtain any evidence for novel 15q genes showing imprinted expression in fibroblasts. Differentially expressed genes on other chromosomes are candidates for downstream genes regulated by an imprinted gene and may play a role in the pathogenesis of PWS. The finding of strongly reduced mRNA levels in upd(15)mat cells of the gene encoding secretogranin II (*SCG2*), which is a precursor of the dopamine releasing factor secretoneurin, raises the question whether hyperphagia in patients with PWS might be due to a defect in dopamine-modulated food reward circuits.

INTRODUCTION

Maternal uniparental disomy for chromosome 15 [upd(15)mat] is the second most common finding in patients with Prader–Willi syndrome (PWS; reviewed in 1). PWS is characterized by neonatal hypotonia, hypogonadism, hyperphagia leading to obesity, short stature, small hands and feet, behavioral problems and mental retardation. Patients with upd(15)mat have two copies of all genes on chromosome 15, but they lack the activity of imprinted genes expressed from the paternal chromosome only (*MKRN3*, *MAGEL2*, *NDN*, *SNURF-SNRPN* and several snoRNA genes) and have a double dose of imprinted genes expressed from the maternal copy only (*UBE3A* and *ATP10C*), although the expression levels of the latter two genes has not been determined in these patients. The imprinted gene cluster within 15q11–q13 is under the coordinated control of an imprinting center (2). Apart from psychosis (3,4), there is no significant clinical difference between patients with upd(15)mat and patients with a paternal deletion of 15q11–q13 (5,6). Therefore it is generally assumed that it is the loss of function of one or more paternally expressed genes rather than increased expression of a maternally expressed gene that is responsible for the major clinical findings. It is unclear, however, which of the paternally expressed genes are relevant for PWS and which pathways are affected. A good candidate for the 'PWS gene(s)' are the HBII-85 snoRNA genes, which are located within the *SNURF-SNRPN* transcription unit (7–9). These genes are expressed

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predominantly in brain, but in other tissues as well. It has been suggested that the HBII-85 snoRNAs are involved in alternative splicing or mRNA editing (7), but the target RNAs have remained elusive.

Maternal uniparental disomy most often results from a combination of meiotic and mitotic errors (10). Non-disjunction of the homologous chromosomes 15 during female meiosis I or non-disjunction of the two sister chromatids during female meiosis II results in an oocyte with two chromosomes 15. Fertiliziation of such an oocyte by a sperm with one chromosome 15 will result in a zygote which is trisomic for chromosome 15. This condition is not compatible with normal development, but can be rescued by loss of one chromosome 15 (trisomy rescue). In two-thirds of cases, one of the two maternal chromosomes will be lost from the trisomic cell. This will result in a normal set of chromosomes. If , however, the paternal chromosome is lost, the cell is left with two maternal chromosomes 15. The trisomic cell line is often found to be confined to the placenta, but has occasionally been observed in the fetus also (11,12). In women with upd, X inactivation is often skewed (13), indicating that trisomy rescue occurred after X inactivation. Paternal uniparental disomy [upd(15)pat] results from the fertilization of an oocyte that is nullisomic for chromosome 15 and the postzygotic duplication of the paternal chromosome. Upd(15)pat is associated with the loss of function of the maternally expressed *UBE3A* gene, which leads to Angelman syndrome (AS).

Here we report on a patient with PWS, who is mosaic for a normal cell line and a cell line with upd(15)mat. We have used fibroblasts from this unique patient to study the effect of uniparental disomy on the global gene expression and to identify candidate downstream genes affected in PWS. By comparing cell lines from the same individual we have circumvented the problem of interindividual variation in gene expression. It is being realized that there is a substantial degree of natural variation in human gene expression (14,15), which is likely to confound global gene expression analysis. Even age- and sex-matched inbred mice differ in the activity of a significant fraction of genes (16). Nevertheless, by microarray analysis of tissues from mice with uniparental duplications of chromosomes 7 and 11, which include the orthologous PWS/AS region in 7B5, Choi *et al.* (17) detected four of five imprinted genes represented on their chips and identified two novel imprinted genes as well as a potential downstream target. This work established the use of expression profiling to detect genes affected directly or indirectly by imprinting.

RESULTS

In the course of routine diagnostic testing for PWS by a methylation-specific PCR assay (MS-PCR) we found that patient E.K. had a methylated and an unmethylated copy of *SNURF-SNRPN* in peripheral blood cells, but the methylated band was stronger than the unmethylated band (Fig. 1). Chromosome analysis of phytohemagglutin-stimulated lymphocytes revealed a normal karyotype (46, XX,9qh+,15ps+) in each of 62 metaphases analyzed. The parents also had a normal karyotype (father, 46,XY,9qh+; mother, 46,XX, 15ps+). By fluorescence *in situ* hybridization with a probe for *SNURF-SNRPN* we observed one signal on each of the two homologs in each of 50 metaphases studied. These results made it unlikely that a numerical or structural chromosome aberration accounted for the skewed signal ratio in the MS-PCR test.

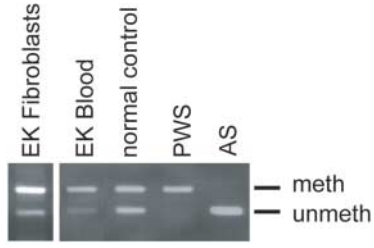

Figure 1. Analysis of the *SNURF-SNRPN* methylation pattern. The upper band (313 bp) is derived from the methylated allele and the lower band (221 bp) from the unmethylated allele (33). In contrast to a typical patient with PWS, EK does not lack the lower band, but has a lower band of reduced intensity both in blood and skin fibroblasts.

Table 1. Microsatellite analysis. Alleles are numbered according to increasing size. Blood and fibroblasts gave concordant results

Marker	Father	Mother	Patient
D15S817	2, 4	1, 3	1, 2, 3
C15orf2	2, 2	1, 2	1, 2
D15S128	1, 3	1, 2	1, 2, 3
AY065650	2, 3	1, 2	1, 2, 3
D15S122	2, 3	1, 1	1, 3
D15S1234	1, 3	2, 2	2, 3
D15S822	3, 4	1, 2	1, 2, 4
CYP19	1, 3	1, 2	1, 2, 3
FES	1, 2	1, 2	1, 2

By microsatellite analysis we found three alleles at five of nine loci tested (*D15S817*, *D15S128*, *AY065650*, *D15S822* and *CYP19* (Table 1; for a typical result see Fig. 2). The additional alleles are of maternal origin. At the other four loci the patient had two alleles, but in some cases the signal intensity appeared to be skewed (data not shown). The analysis of microsatellite loci on chromosomes 1, 2, 8, 13 and 14 revealed normal biparental inheritance (not shown) and made chimerism unlikely. As there was no cytogenetic indication for trisomy 15, the results suggested that the patient might be mosaic for a normal cell line and a cell line with maternal uniparental disomy 15. As upd most often results from trisomy rescue, we investigated fibroblasts for the presence of trisomic cells. As in blood, there was no indication for trisomy 15 in 46 metaphases analyzed (karyotype: 46, XX,9qh+,15ps+). MS-PCR and microsatellite analysis of the fibroblast DNA gave similar results compared to those obtained in blood (Table 1; for a typical result see Fig. 2), although the skewing of the signal intensities was slightly different. X inactivation in blood was random (0.47), but skewed in fibroblasts (0.77, preferential inactivation of the maternal chromosome; Fig. 3).

To confirm somatic mosaicism in the patient and to obtain cells for expression profiling (see below), we decided to separate the cells by cloning. To reduce the effect of interstrain variation in gene expression, which may result from somatic mutations or epigenetic changes in a single cell and confound

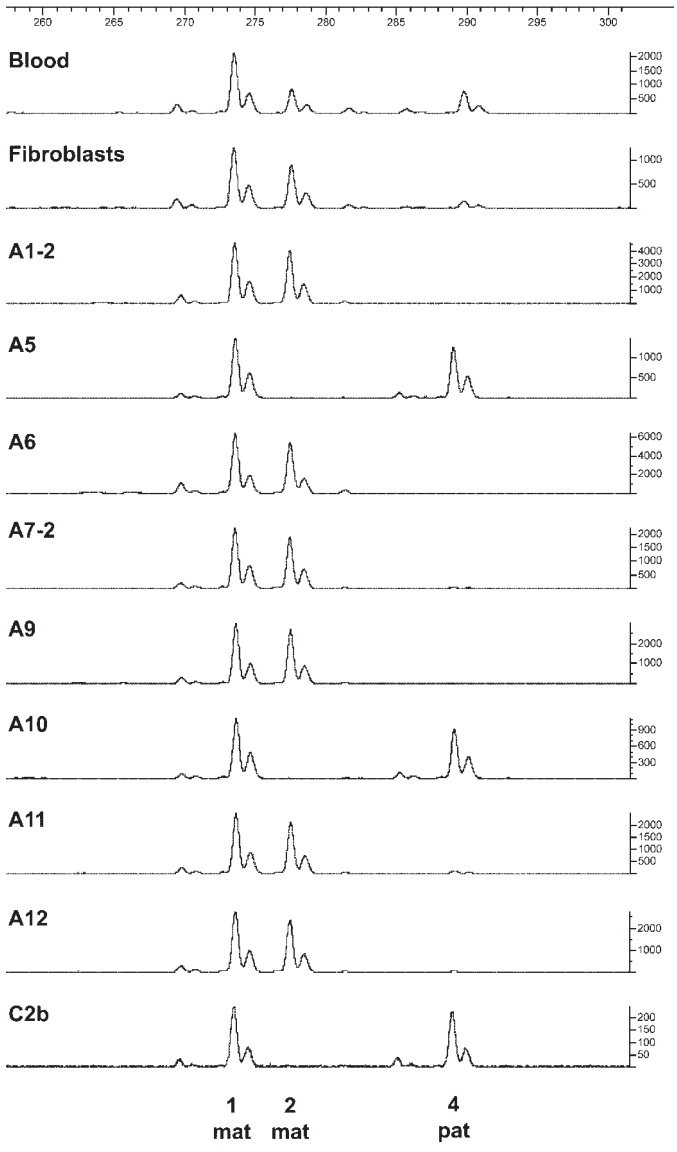

Figure 2. *D15S822* alleles in blood, fibroblasts and cell lines. Note that there are two maternal alleles and one paternal allele in blood and fibroblasts. The cell lines have either two maternal alleles [upd(15)mat] or one maternal and one paternal allele (normal chromosome 15 status). mat, maternal; pat, paternal.

genome-wide expression profiling studies (18), we did not perform single cell cloning, but picked three neighboring cells each. Of 22 cultures, nine cell lines grew and could be used for DNA and RNA preparation. Analysis of the microsatellite locus *D15S822* revealed that three cell lines had biparental chromosomes 15 and six cell lines hat upd(15)mat (Fig. 2). The finding of cells with normal chromosomes and cells with upd(15)mat confirmed that the patient has a somatic mosaic.

X inactivation was studied in eight of nine cell lines (Fig. 3). A12, which has upd(15)mat, could not be studied because we ran out of DNA. In all but one of the other cell lines (A11), X inactivation was non-random. This indicates that at least A11, which is upd, is not a clonal cell line. In the other four upd cell lines, either the paternal X chromosome (two cell lines) or the maternal X chromosome (two cell lines) was inactivated. In each of the three cell lines derived from normal cells the maternal chromosome was inactive.

To study the effect of upd(15)mat on gene expression, we determined the expression profile of three normal cell lines and three upd cell lines. For unknown reasons, two upd cell lines (A1-2 and A6) and one normal cell line (C2b) proliferated faster than the other upd cell line (A7-2) and the other two normal cell lines (A5 and A10). The fast and slowly proliferating cell lines reached mid-confluency after 2–5 days and 14–22 days, respectively. Total RNA was extracted, amplified and hybridized onto Affymetrix DNA-chips HG-U133A and HG-U133B containing approximately almost 45 000 probe sets representing more than 39 000 transcripts derived from ~33 000 well-substantiated human genes. The expression values are available as supplementary material from the author's homepage (www.uni-essen.de/humangenetik/expressionswerte/)

Scatter plot analyses (not shown) suggested that the global expression profiles of the cells were highly similar. By hierarchical cluster analysis, two clusters containing the fast and slowly proliferating cell lines, respectively, were obtained (data not shown). These results showed that the chromosome 15 status, as expected, did not have a major effect on the global gene expression pattern in fibroblasts. In order to identify genes differentially expressed between normal and upd(15)mat cells, we determined the fold-change of the weighted geometrical mean of the expression values between the two classes of cell lines (see Methods). The weighted mean was used in order to reduce the confounding effect of the proliferation rate on gene expression.

First, we had a specific look at the expression level of 15q11–q13 genes known to be imprinted. As shown in Table 2, *MKRN3* and *MAGEL2* were not found to be expressed in any of our cell lines. As expected, the paternally active *SNURF–SNRPN–IPW* (detected by multiple probe sets querying different parts of this transcript) and *NDN* genes were expressed in the normal cell lines only, although one normal cell line did not express *NDN*. The fold-changes were between 3.65 and 18.52. *UBE3A* was expressed in all cell lines at a similar level (0.96-fold change). This result is in agreement with previous findings showing that imprinting and maternal expression of *UBE3A* do not occur in all tissues. *ATP10C*, which is expressed from the maternal chromosome only, was represented twice on the chips. The two probe sets query regions flanking an alternative poly(A)site. *ATP10C* was scored 'absent' in all cell lines with the 3′ probe set, but scored 'present' in two upd cell lines with the more 5′ probe set. The fold-change of the short transcript is 0.30. This value is in agreement with maternal-only expression of *ATP10C*, although it is somewhat lower than expected (0.5).

To identify novel imprinted genes on 15q and candidate downstream genes, we generated, for each type of chip, lists of probe sets/genes where the fold change is above 3.3 (Tables 3 and 4) or below 0.3 (Tables 5 and 6). The first two lists contain genes with a higher steady-state mRNA level in normal cells and the latter two lists contain genes with a higher steady-state mRNA level in upd cells. The thresholds are rather stringent, but we reasoned that the complete loss of expression of a paternally expressed 15q gene in a upd(15)mat cell should result in a high fold change of this

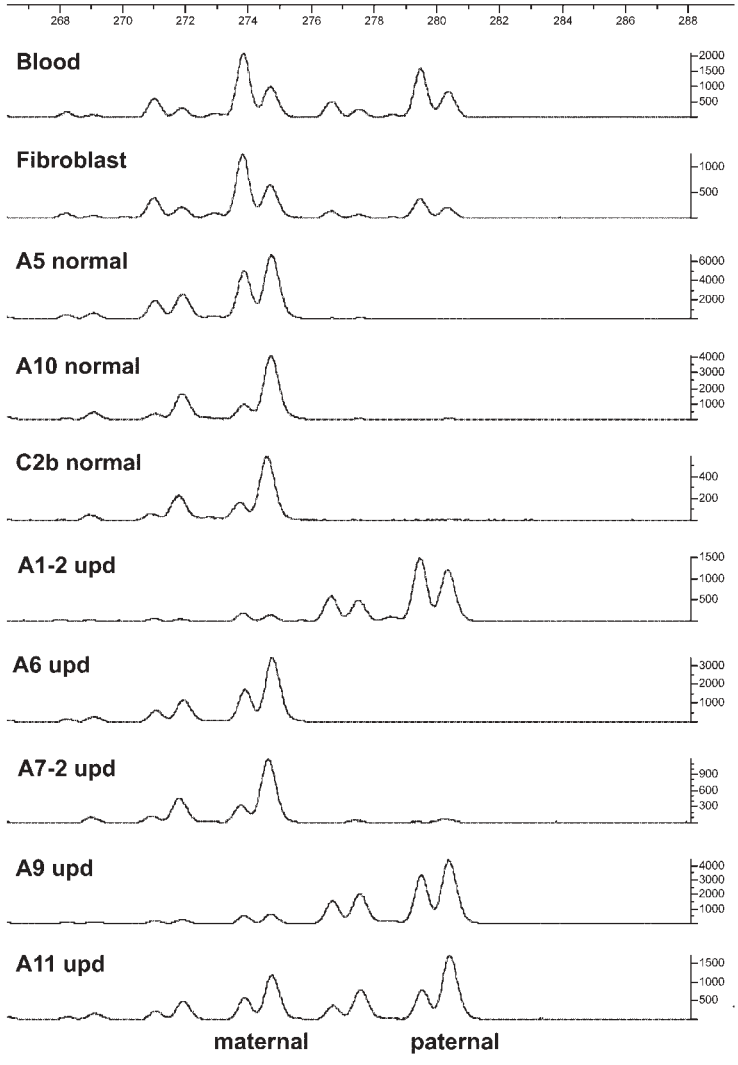

Figure 3. X inactivation. Undigested DNA samples and DNA samples digested with the methylation-sensitive restriction enzyme *Hpa*II were amplified with fluorescence-tagged PCR primers flanking the CAG repeat in the first exon of the androgen receptor gene and analyzed by capillary gel electrophoresis. For clarity, only the results of the *Hpa*II digest are shown. A peak indicates that this allele is methylated as the result of X inactivation.

gene and have a strong effect on the expression level of downstream genes.

Among the HG-U133A and HG-U133B probe sets detecting transcripts present at a higher level in normal cells, *SNURF–SNRPN–IPW* gene had the highest fold change. Apart from various parts of *SNURF–SNRPN–IPW* and *NDN* gene, we did not detect any other 15q gene with a fold change >3.3. Four other genes (*CD36*, *CLCA2*, *RNF29* and *HOXB6*) were represented by two different probe sets each, which gave concordant results. In total, only 46 genes/ESTs were above the threshold of 3.3. Among the probe sets detecting transcripts present at a higher level in upd cells (Tables 5 and 6), two sets detected 15q genes/sequences that had a fold change <0.3 (*ATP10C* and *KIAA1199*). In total, only 33 genes/ESTs were below this threshold. The identification of *SNURF–SNRPN–IPW*, *NDN* and *ATP10C* demonstrates that our algorithm is capable of detecting genes expressed differentially between normal and upd(15)mat cells.

KIAA1199 does not map to the Prader–Willi/Angelman syndrome critical region. Although there is no other region on 15q known to be imprinted, the expression data suggested that this EST might be imprinted and expressed predominantly from the maternal chromosome. To test this hypothesis we performed RT–PCR on somatic cell hybrids containing either the maternal or the paternal chromosome 15 on a rodent background. As the EST was found to be expressed in both cell lines (data not shown), it is likely to be expressed biallecically.

Several non-imprinted genes on other chromosomes have a very high fold change because of complete loss or strong reduction of expression in upd cells. *DUSP4A*, *RNF29*, *HOXB6*, *ARL5* and several ESTs are expressed in the normal cell lines, but not expressed in the upd cell lines. *CD36* and *SCG2* have strongly reduced expression levels in the upd cell lines. These genes have a fold change that is higher than that of *NDN* and most parts of *SNURF–SNRPN–IPW*, and the expression differences are apparent in both fast and slowly

Table 2. Expression values of 15q genes known to be imprinted

Probe set ID	upd(15)mat			Normal			Accession no. (gene)	Fold change
	F A1-2	F A6	S A7-2	S A10	S A5	F C2b		
206585_at	72 A	20 A	24 A	111 A	52 A	10 A	NM_005664 (*MKRN3*)	n.d.
219894_at	9 A	14 A	11 A	316 A	98 A	19 A	NM_019066 (*MAGEL2*)	n.d.
209550_at	48 A	21 A	150 A	4986 P	6751 P	18 A	NM_002487 (*NECDIN*)	4.82
206042_x_at	40 A	103 A	83 A	1079 P	1210 P	825 P	NM_022804 (*SNURF-SNRPN-IPW*)	3.88
201522_x_at	207 A	178 A	691 P	9846 P	8902 P	6330 P	NM_003097 (*SNURF–SNRPN–IPW*)	18.52
228370_at	70 A	418 A	459 A	4919 P	6941 P	4039 P	NM_005678 (*SNURF–SNRPN–IPW*)	12.61
213447_at	64 A	171 A	85 A	1290 P	1317 P	776 P	AI672541 (*SNURF–SNRPN–IPW*)	4.02
221974_at	3 A	2 A	67 A	1442 P	1635 P	544 P	AW770748 (*SNURF–SNRPN–IPW*)	3.65
211285_s_at	1413 P	1170 P	1417 P	1748 P	1538 P	1029 P	NM_130838 (*UBE3A*)	0.96
214255_at	194 A	526 P	1987 P	291 A	96 A	199 A	NM_024490 (*ATP10C*)	0.30
214256_at	125 A	33 A	29 A	46 A	29 A	47 A	AB011138.1 (*ATP10C*)	n.d.

F, fast growing cell line; S, slowly growing cell line; A, absent; P, present; n.d., not done. The genes are ordered according to their location on chromosome 15 from centromere to telomere.

Table 3. Expression values of genes on chip HG-U133A with a higher mRNA level in normal cells

Probe set ID	upd(15)mat			Normal			Accession no. (gene)	Chromosome	Fold change
	F A1-2	F A6	S A7-2	S A10	S A5	F C2b			
201522_x_at	207 A	178 A	691 P	9846 P	8902 P	6330 P	NM_003097 (*SNURF–SNRPN–IPW*)	15	18.52
209555_s_at	254 P	353 P	337 P	725 P	4951 P	4479 P	NM_000072 (*CD36*)	7	9.18
204035_at	1920 P	277 P	419 P	4705 P	4440 P	3531 P	NM_003469 (*SCG2*)	2	7.27
204014_at	439 P	19 A	245 A	3181 P	3440 P	1137 P	NM_001394 (*DUSP4*)	8	6.74
204580_at	519 P	8382 P	730 P	3558 P	5512 P	11257 P	NM_002426 (*MMP12*)	11	5.72
209550_at	48 A	21 A	150 A	4986 P	6751 P	18 A	NM_002487 (*NECDIN*)	15	4.82
206488_s_at	198 A	283 P	97 A	177 A	1392 P	2376 P	NM_000072 (*CD36*)	7	4.59
212192_at	1154 P	921 P	1781 P	5276 P	5077 P	7235 P	NM_138444 (EST)	13	4.52
205226_at	69 A	152 P	321 P	953 P	1322 P	1170 P	NM_006207 (*PDGFRL*)	8	4.04
213447_at	64 A	171 A	85 A	1290 P	1317 P	776 P	AI672541 (*SNURF–SNRPN–IPW*)	15	4.02
206042_x_at	40 A	103 A	83 A	1079 P	1210 P	825 P	NM_022804 (*SNURF–SNRPN–IPW*)	15	3.88
218559_s_at	40 A	88 A	1901 P	11433 P	5932 P	821 P	NM_005461 (*MAFB*)	20	3.77
218451_at	853 P	841 P	1451 P	9232 P	5468 P	2447 P	NM_022842 (*CDCP1*)	3	3.76
201286_at	819 P	411 P	559 P	2016 P	2234 P	2158 P	NM_002997 (*SDC1*)	2	3.76
204472_at	658 P	1084 P	539 P	1307 P	1588 P	4465 P	NM_005261 (*GEM*)	8	3.76
206165_s_at	82 A	109 P	738 P	6801 P	2432 P	619 P	NM_006536 (*CLCA2*)	1	3.70
221974_at	3 A	2 A	67 A	1442 P	1635 P	544 P	AW770748 (*SNURF–SNRPN–IPW*)	15	3.65
205630_at	915 P	125 A	362 P	2500 P	1535 P	1144 P	NM_000756 (*CRH*)	8	3.60
217528_at	40 A	286 P	1003 P	7961 P	1983 P	856 P	NM_006536 (*CLCA2*)	1	3.56
209687_at	13500 P	3243 P	1018 P	6440 P	4975 P	13923 P	NM_000609 (*CXCL12*)	10	3.42
213110_s_at	406 P	290 P	305 P	2232 P	919 P	840 P	NM_000495 (*COL4A5*)	X	3.39
218899_s_at	889 P	1228 P	329 A	2600 P	2167 P	1666 P	NM_024812 (*BAALC*)	8	3.39
204726_at	809 P	157 P	274 P	2108 P	1717 P	740 P	NM_001257 (*CDH13*)	16	3.38
211990_at	114 P	52 A	167 P	2802 P	2840 P	194 P	NM_033554 (*HLA-DPA1*)	6	3.36
209732_at	1588 P	7912 P	673 P	6867 P	6959 P	3877 P	NM_005127 (*CLECSF2*)	12	3.35
209160_at	2579 P	267 P	717 P	1743 P	2681 P	3024 P	NM_003739 (*AKR1C3*)	10	3.32

For the abbreviations see legend to Table 2. The genes are ordered according to the fold changes in normal and upd cells (for details see Material and Methods).

growing cultures. The latter, for example, is not the case for *MMP12*, although the fold change is high (5.72).

DISCUSSION

To the best of our knowledge, this is the first patient described who is mosaic for a normal cell line and a cell line with upd(15)mat. Other PWS patients with mosaic upd(15)mat had a trisomic cell line (11,12) or a cell line with an inv dup(15) (19), but no normal cell line. We cannot exclude the presence of a trisomic cell line at a very low level in blood and fibroblasts or at an unknown level in other tissues. In view of the fact that the patient is severely retarded, especially as speech development is concerned, it is tempting to speculate that trisomic cells are present in the brain.

As judged from the MS–PCR results, microsatellite studies and cell cloning experiments, the upd cell line is somewhat more prominent than the normal cell line, at least in blood and fibroblasts. The upd cell line is heterozygous at many

Table 4. Expression values of genes on chip HG-U133B with a higher mRNA level in normal cells

Probe set ID	upd(15)mat			Normal			Accession no. (gene)	Chromosome	Fold change
	F A1-2	F A6	S A7-2	S A10	S A5	F C2b			
228370_at	70 A	418 A	459 A	4919 P	6941 P	4039 P	NM_005678 (*SNURF–SNRPN–IPW*)	15	12.61
236175_at	290 A	33 A	484 A	8784 P	1831 P	4202 P	NM_033058 (*RNF29*)	8	11.37
236892_s_at	827 P	95 A	19 A	3273 P	5875 P	3069 P	NM_018952 (*HOXB6*)	17	10.88
228766_at	660 P	790 P	351 P	755 P	5563 P	13846 P	AW299226 (EST)	7	10.59
239791_at	1134 A	134 A	183 A	2395 M	3937 P	3097 P	NM_018952 (*HOXB6*)	17	8.45
226034_at	1631 P	359 A	1142 P	12298 P	13507 P	4715 P	BE222344 (splicing factor)	8	8.34
238332_at	348 A	289 A	310 A	1906 P	1389 P	4091 P	NM_173505 (EST)	18	8.22
228155_at	505 P	755 M	416 P	9174 P	4791 P	2175 P	NM_032333 (EST)	10	7.49
229308_at	40 A	147 A	50 A	1280 A	483 P	3570 P	NM_012097 (*ARL5*)	2	6.70
236110_at	203 A	205 A	654 A	6822 P	2810 P	1245 P	BF968243 (EST)	18	5.78
226950_at	1333 A	1178 A	45 A	2447 M	1302 A	5840 P	T63524 (EST)	12	5.77
232721_at	364 A	88 A	225 A	5277 P	676 A	1051 P	NM_033058 (*RNF29*)	8	5.13
238408_at	106 A	197 A	429 A	5286 P	1016 P	1143 P	AW086258 (EST)	8	4.97
224506_s_at	57 A	66 A	130 A	529 A	1673 P	1520 P	NM_032728 (EST)	9	4.78
241726_at	3474 P	329 A	747 M	6733 P	7259 P	2292 P	NM_000411 (*HLCS*)	21	4.48
231807_at	1914 P	1319 P	1210 P	4966 P	6258 P	6633 P	NT_008705 (EST)	10	4.38
223395_at	165 A	381 A	196 A	176 A	1341 P	2458 P	NM_015429 (*TARSH*)	3	4.30
224964_s_at	3404 P	875 P	620 A	5118 P	5278 P	3633 P	AK026424 (*GNG2*)	14	4.20
229331_at	1877 P	875 P	2348 P	6932 P	3760 P	10257 P	NM_145263 (EST)	4	4.17
231725_at	52 A	237 A	913 P	3833 P	3939 P	973 P	NM_018936 (*PCDHB2*)	5	4.07
224435_at	212 A	43 A	92 A	2569 P	1243 P	553 M	NM_032333 (EST)	10	3.98
226064_s_at	319 A	1263 A	772 A	3921 P	3373 P	2025 P	NM_032564 (*DGAT2*)	11	3.88
235561_at	702 P	165 A	650 P	1351 P	2218 P	2202 P	T16544 (EST)	18	3.74
222484_s_at	84 A	32 A	624 A	7814 P	8111 P	58 A	NM_004887 (*CXCL14*)	5	3.57
227226_at	1269 A	124 A	534 A	1572 A	2817 P	1729 P	NM_138409 (EST)	6	3.48
232570_s_at	802 M	137 A	57 A	986 A	1569 A	1067 P	NM_022139 (*GFRA4*)	20	3.44
244117_at	79 A	539 A	510 P	2585 P	1584 P	1096 P	R49389 (EST)	6	3.44
224327_s_at	164 A	937 A	2311 P	6459 P	5504 P	2088 P	NM_032564 (EST)	11	3.34

M, marginal. For other abbreviations and details see legends to Tables 2 and 3.

15q loci and has most likely arisen from a trisomic zygote containing two maternal chromosomes that were non-disjoined at meiosis I. We note in this context that the mother's age at birth was advanced (43 years). The finding of upd fibroblasts with either an inactive maternal or an inactive paternal X chromosome suggests that trisomy rescue occured prior to X inactivation, i.e. very early during embryonic development. To explain the presence of a normal cell line in the patient, it is necessary to invoke a second independent rescue event, by which one of the two maternal chromosomes was lost from a trisomic cell. Although each of the three normal fibroblast cell lines had an inactive maternal X chromosome, the number of cell lines is too small to determine whether this rescue event occurred after X inactivation. However, as we did not detect trisomic cells in blood and fibroblasts of the patient and both tissues contain normal cells and upd(15) cells, the two rescue events have probably occurred within a narrow time window during early embryogenesis. This raises the question whether there is an active mechanism surveilling chromosome number.

We have used fibroblasts from this unique patient to identify candidate genes involved in the etiology of PWS. This approach has one big advantage, but also drawbacks. The advantage is that the genetic background of the two cell types is identical, apart from chromosome 15. Therefore, a significant fraction of the differences in gene expression should be the consequence of the imprint differences (one paternal and one maternal versus two maternal imprints). A drawback of our approach is that the major symptoms in PWS are related to brain and not fibroblast dysfunction. We reasoned, however, that some of the affected genes and pathways might be expressed in fibroblasts also. Indeed, we identified several genes which are predominantly expressed in brain and pituitary (e.g. *SCG2*). Second, cloned fibroblasts show considerable interstrain variation (18). However, by pooling three fibroblasts and by using a weighted mean of the expression values to account for the obvious difference in the proliferative capacity of the cells we tried to minimize this problem.

There are two arguments for assuming that the expression profiles determined by our chip analysis are valid: (i) several genes (*SNURF–SNRPN–IPW*, *CD36*, *CLCA2*, *RNF29* and *HOXB6*) are represented by multiple probe sets, which gave highly concordant results; this suggests that the chip mesasurements are highly reproducible; and (ii) we have detected all 15q genes known to be expressed and imprinted in fibroblasts. *SNURF–SNRPN–IPW* and *NDN* are among the genes with the highest fold change as expected for imprinted genes expressed from the paternal chromosome only. The fold change of *ATP10C* (0.30) is in agreement with imprinted expression of this gene, although somewhat lower as expected for a gene expressed from the maternal chromosome only (expected fold change, 0.5). The lower than expected fold change is probably due to the low level of expression of this gene in

Table 5. Expression values of genes on chip HG-U133A with a higher mRNA level in upd cells

Probe set ID	upd(15)mat			Normal			Accession no. (gene)	Chromosome	Fold change
	F A1-2	F A6	S A7-2	S A10	S A5	F C2b			
212314_at	964 P	3569 P	3841 P	404 P	121 P	382 P	AB018289 (*KIAA0746*)	4	0.13
204614_at	3803 P	19819 P	1154 P	28 A	12 A	828 P	NM_002575 (*SERPINB2*)	18	0.14
222108_at	2499 P	3452 P	7815 P	676 P	1754 P	606 P	AC004010 (BAC clone GS1-99H8)	12	0.17
209596_at	9472 P	1286 P	7108 P	3057 P	2560 P	281 P	NM_015419 (DKFZP564I1922)	X	0.18
207316_at	25 A	7524 P	1356 P	61 A	33 A	166 A	NM_001523 (*HAS1*)	19	0.18
204748_at	14619 P	8344 P	4902 P	1015 P	1183 P	1668 P	NM_000963 (*PTGS2*)	1	0.18
205476_at	102 A	4767 P	1284 P	29 A	12 A	30 A	NM_004591 (*CCL20*)	2	0.21
212942_s_at	12736 P	8202 P	1598 P	90 A	1211 P	1371 P	AB033025 (*KIAA1199*)	15	0.21
205066_s_at	3976 P	1799 P	6592 P	716 P	951 P	1328 P	NM_006208 (*ENPP1*)	6	0.25
218332_at	1568 P	11850 P	8534 P	2692 P	2482 P	1012 P	NM_018476 (*HBEX2*)	X	0.27
201843_s_at	80 P	975 P	4751 P	206 P	1795 P	228 P	NM_004105 (*EFEMP1*)	2	0.27
203440_at	2714 P	8597 P	4607 P	642 M	7326 P	753 P	NM_001792 (*CDH2*)	18	0.27
212950_at	82 A	59 A	3323 P	151 A	50 A	63 P	NM_015234 (*GPR116*)	6	0.27
215034_s_at	3008 P	14133 P	118 P	44 A	295 P	460 P	NM_014220 (*TM4SF1*)	3	0.28
207030_s_at	1792 P	4121 P	7813 P	3026 P	2171 P	649 P	NM_001321 (*CSRP2*)	12	0.28
203184_at	8004 P	6123 P	3379 P	440 P	307 P	5180 P	NM_001999 (*FBN2*)	5	0.28
205207_at	21497 P	23604 P	2984 P	374 M	987 P	9191 P	NM_000600 (*IL6*)	7	0.29
207426_s_at	1114 P	184 P	1810 P	270 P	290 P	288 P	NM_003326 (*TNFSF4*)	1	0.29
212488_at	4012 P	9382 P	13607 P	2704 P	3601 P	2297 P	NM_000093 (*COL5A1*)	9	0.29
205047_s_at	414 P	1132 P	2413 P	375 P	522 P	329 P	NM_001673 (*ASNS*)	7	0.30
214255_at	194 A	526 P	1987 P	291 A	96 A	199 A	NM_024490 (*ATP10C*)	15	0.30

For abbreviations and details see legend to Tables 2 and 3.

Table 6. Expression values of genes on chip HG-U133B with a higher mRNA level in upd cells

Probe set ID	upd(15)mat			Normal			Accession no. (gene)	Chromosome	Fold change
	F A1-2	F A6	S A7-2	S A10	S A5	F C2b			
228253_at	6309 P	14252 P	6559 P	685 A	240 A	3964 P	NM_013247 (*PRSS25*)	2	0.16
228360_at	4379 P	836 A	7521 P	806 A	534 A	908 A	NM_177964 (EST)	2	0.20
241763_s_at	362 A	1112 P	4662 P	52 A	847 P	270 A	BF244402 (EST)	8	0.20
231559_at	1366 M	604 A	1387 P	205 A	185 A	167 A	NM_006169 (*NNMT*)	11	0.22
229088_at	4768 P	2130 P	4548 P	684 A	638 P	1094 P	BF591996 (EST)	6	0.22
230135_at	481 A	5683 P	7612 P	1238 P	903 P	751 P	AI822137 (EST)	4	0.25
229493_at	1192 P	844 P	1284 P	142 A	453 A	193 A	BF315468 (EST)	2	0.26
230291_s_at	14 A	1557 P	1368 P	108 A	59 A	18 A	T90642 (EST)	9	0.27
235086_at	29600 P	13767 P	10696 P	2212 M	4801 P	5065 P	AW956580 (EST)	?	0.28
230793_at	698 P	1310 P	1402 P	297 A	578 P	192 A	NM_017640 (EST)	6	0.28
222668_at	2012 P	2158 P	3648 P	1848 P	1441 P	398 A	NM_024076 (EST)	19	0.29
240382_at	243 A	1041 P	1673 P	119 A	342 P	29 A	AW444944 (EST)	6	0.29

For abbreviations and details see legend to Tables 2–4.

normal fibroblasts; as shown in Table 2, *ATP10C* mRNA levels in the normal cell lines are below the threshold of detection.

We did not obtain any evidence for novel 15q genes showing imprinted expression in fibroblasts. In addition to *SNURF–SNRPN–IPW*, *NDN* and *ATP10C*, only one other chromosome 15 sequence (*KIAA1199*) appears on our lists. Although the fold change (0.21) was much lower than expected for a maternally expressed gene, we examined *KIAA1199* expression in somatic cell hybrid studies. As we observed biallelic expression of this gene, it may be indirectly affected by upd(15)mat. It should be noted that our study is not well suited to discovering maternally expressed genes on chromosome 15, because the expected fold change is only 0.5. In contrast, loss of expression of a paternally expressed 15q gene in upd(15)mat cell should lead to a high fold change, unless expression in fibroblasts is weak. As we did not detect any 15q gene, apart from *SNURF–SNRPN–IPW* and *NDN*, with a fold change >3.3, there is probably no other gene which is represented on the HG-U133 chips, expressed in fibroblasts at reasonable levels, and active on the paternal chromosome only.

Approximately 50% of the genes listed in Tables 3–6 encode hypothetical proteins of unknown nature. The other genes encode membrane-bound proteins including receptors and ion channels, transcription factors, signalling proteins, enzymes and structural proteins. The non-imprinted genes identified by

us are potential candidates for downstream genes regulated by an imprinted 15q gene, although it is likely that the lists contain also genes which differ in their expression values between the three biparental and the three upd cell lines just by chance. On the other hand, weakly affected genes with a small fold-change will go undetected.

Several non-imprinted genes have a very high fold-change because of complete loss or strong reduction of expression in the upd(15)mat cells: *DUSP4A* (dual specificity phosphatase 4), *RNF29* (ring finger protein 29), *HOXB6* (homeo box B6 protein), *ARL5* (ADP-ribosylation factor-like 5), *CD36* (CD36 antigen), *SCG2* (secretogranin II) as well as several ESTs. These genes are good canidates for downstream genes, because they have a fold change that is higher than that of *NDN* and most parts of *SNURF–SNRPN–IPW* and not affected by the proliferation rate of the cell lines. A role of these genes in PWS is not immediately obvious, apart from *SCG2*, which is third on list 3 (fold change, 7.27). Secretogranin II is mainly found in the core of catecholamine-storage vesicles within cells of the neuroendocrine system (reviewed in 20). It is a precursor of secretoneurin, which induces dopamine release (21). In the context of the present study on Prader–Willi syndrome it is of interest to note that dopamine agonists are anorexigenic and that dopamine antagonists are orexigenic. Dopamine seems to regulate food intake by modulating food reward via the mesolimbic circuitry of the brain (22). Recent imaging studies in humans (23–25) have linked dopamine with eating behavior and obesity, and Wang *et al.* (23) have suggested that obese individuals may perpetuate pathological eating as a means to compensate for a decreased reward. Akefeldt *et al.* (26) have found that the concentrations of dopamine and serotonin metabolites were increased in the cerebrospinal fluid of patients with PWS and conclude that their findings implicate dysfunction of the serotonergic system and the dopamine system in PWS.

Previous studies on adiposity signals (leptin and insulin) and their neuropeptide effectors [e.g. neuropeptide Y (NPY), agouti-related protein and melanocortins] have not provided any clue to the cause of dysregulated energy homeostasis in PWS. Goldstone *et al.* (27) concluded that the pathogenesis of obesity may lie in downstream or separate circuits, although studies in a transgenic mouse model by Ge *et al.* (28) suggest that melanocortin signaling may be augmented in PWS. Recently it has been found that serum levels of the orexigenic gut–brain peptide ghrelin are reduced in obese individuals, but elevated in patients with PWS (29,30). However, as ghrelin signals through NPY neurons (31) and NPY responses appear to be normal in the hypothalamus of patients with PWS (27), the role of ghrelin in the pathogenesis of this syndrome remains to be elucidated. The gene was not expressed in our fibroblast cell lines.

We are well aware that the *SCG2* mRNA levels in fibroblasts may not reflect *SCG2* mRNA levels in neuroendocrine cells and that decreased mRNA levels do not necessarily mean decreased protein levels and impaired function. Also, it will be necessary to obtain independent confirmation of altered *SCG2* mRNA levels in other patients with PWS, but this is not possible at present, because the gene is not expressed in blood, and skin biopsies from a large series of PWS patients are not available. However, intrigued by our findings on *SCG2*, the role of SCG2/secretoneurin in dopamine release, and the association of dopamine with eating behaviour, we propose that in addition to adiposity and satiety signalling pathways, dopamine-modulated food reward circuits should be studied in patients with PWS. It is tempting to speculate that a defect in these circuits may contribute to the pathogenesis of hyperphagia.

PATIENTS AND METHODS

Patient

The proposita is the third child of a non-consanguineous German couple. The older sister died at the age of 21 years during sleep. No diagnosis was made. There was no history of illness. The older brother is healthy. Caesarean section was performed after 39 weeks of gestation. Anthropometric measurements were decreased [weight 2220 g (−2.3 SD), length 44 cm (−7 SD), head circumference 31 cm (−2 SD)]. The mother's age at birth was 43 years. The first days of life were complicated by muscular hypotonia and feeding problems. However, no tube feeding was necessary. In addition, recurrent episodes of apnea and bradycardia were present.

The girl showed delayed developmental milestones. Walking without support was possible at the age of 26 months, first words were present at about the same time. She had severe articulation problems. Massive hypertrophy of the adenoids caused obstructive sleep apnea. Therefore supported ventilation with CPAP was necessary during night for about 1 year. After tonsillectomy oxygenation was much better and supported ventilation could be stopped. Obesity started at about 18 months. At the age of 2 years 7 months weight was 23 kg (+3 SD), and height at that time was 93 cm (mean). The last clinical examination was done at the age of 4 years 6 months. Her height was normal (106 cm, mean) while she was still overweight (24.6 kg, +2 SD) corresponding to a BMI of 21.5 kg/m^2, which is way beyond the 97th percentile (18.8 kg/m^2) of girls this age. She is globally retarded. Speech development, especially is retarded and corresponds to a 2-year-old girl. The study was approved by the local ethics committee.

Chromosome analysis

Cytogenetic and molecular cytogenetic studies were performed on chromosomes derived from peripheral blood and fibroblasts. Chromosome preparations and GTG banding were performed according to standard techniques (32). Fluorescence *in situ* hybridization with a probe for *SNRPN* was performed according to the manufacturer's protocol (Vysis Inc., Downers Grove, IL, USA).

Cloning of fibroblasts

Skin fibroblasts were cultured in Petri-dishes containing AmnioMax-Medium (Gibco Invitrogen, Karlsruhe, Germany). Under an inverted microscope (Zeiss), the cells were detached with Trypsin/EDTA and transferred to a 96-well dish with the help of an extended Pasteur pipette. Each well received three neigboring cells. Growing cultures were expanded in separate culture flasks.

Genomic DNA analysis

DNA methylation at the *SNURF–SNRPN* locus was determined by bisulfite treatment of genomic DNA and methylation-specific PCR as described by Zeschnigk *et al.* (33). For microsatellite analysis, fluorescence-tagged PCR products were analyzed using an Abi 3100 automatic capillary genetic analyzer and GeneScan and Genotyper software (Applied Biosystems, Foster City, CA, USA). The inactivation status of the X chromosome was assessed by studying DNA methylation at the androgen receptor locus (34). Undigested DNA samples and DNA samples digested with *Hpa*II were amplified with fluorescence-tagged PCR primers flanking the CAG repeat in the first exon of the gene. DNA samples from the parents were used as controls and to determine the parental origin of the two X chromosomes in the patient. PCR products were analyzed on a capillary genetic analyzer as described above. The degree of X inactivation was calculated as (pd1/pu1)/(pd1/pu1+pd2/pu2), where pd1 and pd2 represent the peak integrals of the stronger and weaker *Hpa*II-digested allele, respectively, and pu1 and pu2 are the corresponding peak integrals from the undigested samples (13).

Expression profiling

Fibroblasts were grown in Chang medium to mid-confluency. Total RNA was extracted according to the QIAamp RNA preparation protocol (QIAgen, Hilden, Germany). To remove residual traces of genomic DNA, RNA was treated with DNase. To prove the integrity of the RNA, RT–PCR was carried out using primers for the β-actin locus (35). A PCR product of 496 bp was obtained in each RNA sample tested. As RNA yields from the slowly growing cell lines were rather low, biotinylated cRNA targets from all cell lines were prepared by two rounds of amplification with 250 ng of total RNA as starting material in round 1 as described (36) with minor modifications (L. Klein-Hitpass, to be described elsewhere). Fragmented cRNA samples were hybridized to HG-U133A and HG-U133B oligonucleotide arrays. Hybridization, washing, staining and scanning was performed following standard Affymetrix protocols (Technical Manual).

Expression values were obtained using Affymetrix™ Microarray Suite™ Version 5.0. This includes the background correction of the average of the lowest two percentiles of intensities on a 4-by-4 grid on the chip, the introduction of an 'ideal mismatch' forced to be lower than the corresponding perfect match, and the usage of Tukey's biweight to elicit an expression value out of single probe intensity pairs. Annotation of the probe sets was taken from the Bioconductor packages hgu133a, version 1.1.1 and hgu133b, version 1.1.1.

Expression values, after setting all values <500 to 500, were log-transformed before being contrasted. Because different characteristic lines of expression values were detected, the chip types HG-U133A and HGU-133B were not normalized to fit each other, but instead analyzed separately.

Contrasts between uniparental and biparental expressions were calculated as differences between the transformed values. As replicate values were available for uniparental/fast and biparental/slow, the log expression values of these were averaged before the contrasts were formed. Lists of genes were generated where the fold-change was above 3.3 or below 0.3. Genes called 'present' in only one cell line were not considered.

Reverse transcription PCR

To check the imprinting status of *KIAA1199*, reverse transcription PCR (GeneAmp RNA-PCR kit, Perkin Elmer) was performed on RNA from the somatic-cell hybrids A9+15 and t75-2maz-34-4a, which contain a maternal or a paternal human chromosome 15, respectively (37). We used the primers KIAA1199_F, 5′-GAAGCATATGGGACAGCAG-3′ and KIAA1199_R, 5′-GGAGGGTTCCAGACTTGACA-3′. The annealing temperature was 58°C. PCR products were verified by sequencing using fluorescence-tagged dideoxynucleotides and the *Taq* cycling procedure (ABI). Sequences were analyzed on an ABI 3100 automatic capillary genetic analyzer (Applied Biosystems, Foster City, CA, USA).

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft. We thank Drs G. Kistner, P. Pawlowski and J. Karsten-Schäfer for clinical data and patient material, Dr R.D. Nicholls for somatic cell hybrid RNA, and Drs M. Zeschnigk, K. Bauer and J. Hebebrand for helpful discussions.

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