UNIQUE DELETIONS IN PRADER-WILLI SYNDROME

By

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By

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Prader-Willi syndrome (PWS) is a rare genetic disorder caused by an absence of paternally expressed genes within the 15q11-q13 region via one of the three genetic mechanisms: deletion of paternally inherited genes, maternal uniparental disomy (UPD) and imprinting defect (ID). Deletion is often subdivided into Type 1 and Type 2, while UPD is subdivided into heterodisomy and isodisomy. Despite PWS being a well-characterized genetic disorder, specific genetic factors contributing to specific PWS phenotypes are not fully understood.

The methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) is a newly developed technique that detects copy number changes and aberrant DNA methylation. In this study, we applied the MS-MLPA to further elucidate the genetic subtypes of deletion in 74 PWS subjects with deletion. Sixty-seven of 74 subjects had either Type 1 (n=25) or Type 2 (n=42) deletions. The remaining seven subjects had unique deletions other than Type 1 or Type 2. Subsequently, we verified the unique breakpoints with the microarray-based comparative genome hybridization (CGH).

Additionally, we reviewed the available medical records to examine the clinical characteristics of these seven subjects with unique deletions.

In conclusion, we identified unique deletions from approximately 9.5% of PWS subjects with deletion in our laboratory using the MS-MLPA, which was verified by the CGH. Case studies of the subjects with unique deletions may help identify the specific genetic factors contributing to the specific PWS phenotypes.

CHAPTER 1
INTRODUCTION

Prader–Willi syndrome (PWS) is the most frequently diagnosed genetic cause of obesity, characterized by pronounced hyperphagia (often described as “voracious appetite”) and early-onset morbid obesity (Cassidy and Driscoll 2009). Other most consistent major manifestations include hypotonia with poor suck and poor weight gain in infancy, mild to moderate mental retardation, hypogonadism, growth hormone insufficiency causing short stature, characteristic facial appearance, and behavioral and sometimes psychiatric disturbance (Cassidy 1997; Holm et al. 1993; State and Dykens 2000). Furthermore, the majority of PWS individuals suffer from various forms of abnormal restricted repetitive behavior, such as stereotypies, repetitive forms of self-injurious behavior, obsession, compulsion, ritualistic behavior, sameness behavior and restricted interests. For example, 69 to 100% of individuals with PWS showed skin-picking (Dykens et al. 1999; Thompson and Gray 1994; Torrado et al. 2006; Veltman et al. 2004; Webb et al. 2002; Whitman and Accardo 1987) and 37 to 58% of individuals with PWS manifested prominent obsessive compulsive symptoms, such as hoarding, ordering/arranging, concerns with symmetry/exactness, rewriting, need to tell/know/ask (Dykens et al. 1996).

PWS is a highly variable genetic disorder affecting multiple body systems with an estimated incidence rate of 1 in 15,000 (Butler 1990). PWS is caused by an absence of paternal contribution in the chromosome 15 q11-q13 region via three distinct genetic mechanisms including deletion of paternally inherited genes, maternal uniparental disomy (inheritance of two copies of maternal chromosomes) and imprinting defect (genetic mutation leading to abnormal silencing of gene expression from the paternally

inherited chromosome) (Cassidy and Driscoll 2009; Glenn et al. 1997; Nicholls and Knepper 2001). Deletion is responsible for approximately 70% of PWS cases, while maternal uniparental disomy (UPD) causes about 25% of PWS cases. Less than 5% of PWS cases are caused by imprinting defect (ID). Deletion is typically subdivided into two subgroups. Type 1 deletion encompasses a larger portion of the 15q11-q13 region between common breakpoints BP1 and BP3, whereas Type 2 deletion involves a shorter distance between BP2 and BP3 (Bittel and Butler 2005; Butler et al. 2004; Milner et al. 2005). Studies found at least two more rare breakpoints (BP4 and BP5) distal to BP3 (Sahoo et al. 2005; Sharp et al. 2006; Wang et al. 2004). In addition, the 15q11-q13 region may be deleted as a result of an unbalanced translocation in a few patients (Buiting and Horsthemke 2006).

Despite PWS being a well-characterized genetic disorder, specific genetic factors contributing to specific PWS phenotypes are not yet clearly understood (State and Dykens 2000). Interestingly, several studies have investigated the phenotypic characteristics across these genetic subtypes and reported that individuals with Type 1 deletion have more typical PWS phenotypes including higher levels of restricted repetitive behavior (RRB) than those with Type 2 deletion or UPD/ID (Bittel et al. 2006; Butler et al. 2004; Dykens et al. 1999; Torrado et al. 2006). Contrary to these reports, Milner et al. (2005) did not find differences in RRB scores across the genetic subtypes (Milner et al. 2005). This conflicting result may have been due to genetic heterogeneity. For example, patients with unique deletions other than Type 1 or Type 2 deletions may have been categorized as either subgroup.

The methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) is a relatively new technique to detect changes of copy number and DNA methylation (Bittel et al. 2007; Nygren et al. 2005; Procter et al. 2006). Compared to the traditional diagnostic methods, such as chromosomal analysis or Fluorescent *in situ* hybridization (FISH), the MS-MLPA provides more detailed information on the size of deletion as well as methylation status. The MS-MLPA is a labor and cost-effective alternative to the array based comparative genome hybridization (aCGH).

In the present study, we utilized the MS-MLPA to elucidate the size of deletions in our PWS subjects. Once we identified several subjects with unique deletions, we reviewed the available medical records to examine the genotype-phenotype relationships. Additionally, we applied aCGH to the subset of our subjects including those with unique deletions to verify the MS-MLPA findings.

CHAPTER 2
MATERIALS AND METHODS

Samples

We obtained DNA samples from 74 PWS individuals (Table 2-1) with deletion whose subtypes were unknown. In addition, we examined additional 128 DNA samples to compare the methylation patterns across the diagnostic categories. These include 14 PWS subjects with maternal UPD, 4 PWS subjects with ID, 8 Angelman syndrome (AS) subjects with deletion, 1 AS subjects with paternal UPD, 2 AS subjects with ID, 4 family members of the known maternal interstitial duplication of the 15q11-q13, 1 subject with maternal isodicentric duplication of the chromosome 15, 90 subjects with a history of early-onset morbid obesity (EMO) and 4 normal control subjects. The research protocol and informed consents were approved by the University of Florida Institutional Review Board (IRB).

The Methylation-Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA)

A commercial MS-MLPA kit (ME028-A1) for Prader-Willi/Angelman syndrome (MRC-Holland, Amsterdam, The Netherlands) was utilized. This kit contained 25 probes specific for sequence in the 15q11-q13 region to detect copy number changes of the major genes. Among these 25 probes, five also contained methylation sensitive restriction enzyme HhaI recognition site, thereby amplifying only methylated sequence after digestion process (Procter et al. 2006) (Table 2-2). The manufacturer's protocol was followed for the DNA preparation, ligation, enzyme digestion and multiplex PCR reaction (www.mrc-holland.com). After the final PCR step, the Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, USA) and Peak Scanner™ Software (version 1.0) (Applied Biosystems) were used for capillary electrophoresis and

fragment analysis. The peak heights of the 15q11-q13 specific probe-amplified products were normalized using the averaged peak heights of neighboring control probe-amplified products. By comparing normalized peak heights of specific probe-amplified products with those of control subjects, we were able to identify copy number changes (e.g., deletion or duplication). To detect aberrant methylation, peak heights of the 15q11-q13 specific probes before and after digestion were normalized using global normalization process suggested by the manufacturer. Comparison of normalized peak heights before digestion with those after digestion provided information on quantity of methylation (Table 2-3). All MS-MLPA results were reviewed independently by three investigators. Whenever the results were inconclusive, the MS-MLPA reaction was repeated.

The Array-based Comparative Genome Hybridization (aCGH):

The MS-MLPA results from a subset of 24 PWS subjects were verified by an enhanced version of chromosome 15-specific genomic microarray, which included a total of 175 genomic bacterial artificial chromosome (BAC) clones across the 15q, with the higher density of clones spanning approximately 10 Mb of the 15q11-q14 interval in addition to 160 clones specific for the subtelomeric regions of all other chromosomes at the Baylor College of Medicine (Sahoo et al. 2007).

Clinical Evaluation

Clinical evaluations for 7 unique subjects were done as part of typical clinic visits and/or during clinical research encounters.

Table 2-1. Demographic information of the 74 PWS subjects with deletion

		Type 1	Type 2	Unique
Number of subjects		25	40 (+2-brain sample)	7
Gender (M:F)		16:9	22:18	4:3
	White	24	35	5
	Black	1	3	1
Race/ethnicity	Hispanic	0	0	1
	Asian	0	1	
	Other[1]	0	1	0

1. Other includes the subjects who did not report their ethnicity or are biracial.

Table 2-2. The MS-MLPA probes

Methylation[1]	Probe	Gene	Position[2]	Distance[3]
	1	TUBGCP5	20,398,305	122,299
	2	CYFIP1	20,520,604	841,839
100%	3	MKRN3	21,362,443	376
	4	MKRN3	21,362,819	78,685
	5	MAGEL2	21,441,504	966
100%	6	MAGEL2	21,442,470	40,021
	7	NDN	21,482,491	929
50%	8	NDN	21,483,420	1,136,387
50%	9	SNRPN U1B exon	22,619,807	96,908
	10	SNRPN U3 exon	22,716,715	34,499
50%	11	SNRPN alt exon 1	22,751,214	269
50%	12	SNRPN (CpG)	22,751,483	290
50%	13	SNRPN (CpG)	22,751,773	12,482
	14	SNRPN exon 3	22,764,255	372,148
	15	UBE3A, exon 15	23,136,403	31,346
	16	UBE3A, exon 9	23,167,749	67,104
0%	17	UBE3A, exon 1	23,234,853	332
0%	18	UBE3A, exon 1	23,235,185	342,162
	19	ATP10A, exon 2	23,577,347	82,014
0%	20	ATP10A, exon 1	23,659,361	684,889
	21	GABRB3, exon 9	24,344,250	224,854
0%	22	GABRB3, exon 1	24,569,104	1,201,013
	23	OCA2, exon 22	25,770,117	247823
0%	24	OCA2, exon 1	26,017,940	1,178,810
	25	APBA2	27,196,750	

1. Percentage of methylated sequence in blood-derived DNA
2. Base-pair position on March 2006 assembly on the UCSD genome browser (http://genome.ucsc.edu/)
3. Distance to the next probe in base pairs

Table 2-3. DNA methylation analyses from 5 methylation sensitive probes. Expected functional dosing for controls/EMO (Early-onset Morbid Obesity) would be 0.50; PWS 1.00; AS would be 0.00; maternal interstitial duplication 0.67 (2:1); and maternal isodicentric duplication 0.75 (3:1).

	NDN	SNRPN	SNRPN	SNRPN	SNRPN
Control (n=4)	0.38 ± 0.08	0.49 ± 0.07	0.55 ± 0.07	0.58 ± 0.08	0.54 ± 0.06
EMO (n=90)	0.39 ± 0.05	0.42 ± 0.05	0.54 ± 0.06	0.52 ± 0.05	0.49± 0.05
PWS (deletion) (n=74)	0.79 ± 0.17	0.77 ± 0.10	0.96 ± 0.11	0.99 ± 0.13	0.93 ± 0.15
PWS (UPD) (n=14)	0.79 ± 0.13	0.80 ± 0.11	0.93 ± 0.08	0.99 ± 0.09	0.91 ± 0.09
PWS (ID) (n=4)	0.81± 0.13	0.82 ± 0.09	0.97 ± 0.11	1.04 ± 0.12	0.93 ± 0.03
AS (deletion) (n=8)	0.04 ± 0.03	0.03 ± 0.02	0.06 ± 0.05	0.03 ± 0.02	0.02 ± 0.02
AS (UPD) (n=1)	0.00	0.00	0.02	0.00	0.00
AS (ID) (n=2)	0.07 ± 0.02	0.03 ± 0.01	0.05 ± 0.04	0.03 ± 0.04	0.02 ± 0.03
15q Mat Dup (n=4)	0.51 ± 0.05	0.57 ± 0.07	0.74 ±0.08	0.72 ±0.08	0.69±0.08
Mat isodicentric dup (n=1)	0.64	0.63	0.81	0.85	0.76

CHAPTER 3
RESULTS

Copy Number Analyses

Among 74 PWS subjects with deletion, we identified 25 subjects with Type 1 deletion (33.78%) and 42 subjects with Type 2 deletion (56.76%). The remaining seven PWS subjects (9.46%) had unique deletions other than Type 1 or Type 2 (Figure 3-1). In eight AS subjects with deletion, five subjects (62.5%) had Type 1 deletion and three subjects (37.5%) had Type 2 deletion. We also observed microdeletions within SNRPN in one of four PWS subjects with ID and two of two AS subjects with ID. We incidentally identified two unique deletions among 90 individuals with EMO. One subject with EMO had a deletion of the 15q26 region and the other subject with EMO had deletion between BP1 and BP2. The four subjects with maternal interstitial duplication of the 15q11-q13 as well as the subject with maternal isodicentric duplication of the chromosome 15 showed increased amplification pattern consistent with their molecular diagnoses.

DNA Methylation Analyses

DNA methylation analyses for NDN and SNRPN confirmed characteristic methylation patterns for PWS, AS and normal control subjects, respectively (Table 2-3). However, we noted individual variations in some methylation-sensitive probes (Table 2-3).

Agreement Rate Between the MS-MLPA and aCGH:

We conducted both MS-MLPA and aCGH in a subset of our PWS subjects (n=24). Among these 24 subjects, seven subjects had unique deletions identified by MS-MLPA. The remaining 17 subjects were the study participants of the Rare Disease Clinical

Research Network (RDCRN). Not surprisingly, the agreement rate between MS-MLPA and aCGH was 100%. However; the aCGH revealed more precise location of breakpoints.

Case Studies

We reviewed the available medical records of the seven subjects with unique deletion (Figure 3-1 and Table 3-1).

PW235P: The patient was a 21 year-old white male who was last seen just prior to his accidental death. He was born at 43 weeks gestation with a birth weight of 3.73Kg. He was noted to be very hypotonic and have a poor sucking as a neonate. He was fed via nasogastric tube for the first three months of life and thereafter by a bottle with a widened nipple. He fed slowly and poorly for the first nine months. However, by 18 months his weight started going up quickly and by 24 months he was obese with a body mass index (BMI) standard deviation score (SDS) = 2.05. His developmental history indicated that he sat at 10 months, was late to walk at 2 ½ years, and his first words were at 2 ½ years.

He was first seen by the genetics service at 4 11/12 years of age to evaluate for PWS. His BMI SDS was +4.12 and hand length was at approximately 65th percentile (%ile). High resolution chromosomal analysis at that time was reportedly normal. In his follow up at 5 11/12 years, his geneticist noted that he had “some but not all of the features of PWS.” The reluctance to label him as PWS at that time was partly due to his macrocephaly (95th %ile) in relation to his younger brother’s (60th %ile) and mother’s (60th %ile) head sizes. In addition, his birth weight (3.73Kg) and his height (30th %ile) were higher than expected, and he lacked a voracious appetite. He did, however, have

obesity and hypogonadism. At age 10 ½ years, he had been started on growth hormone (GH) therapy by an endocrinologist to improve his muscle mass. His height was noted at the 40th %ile prior to starting GH.

He was seen again by the genetics service at 11 ½ years of age to re-evaluate for PWS. At that time, his height was at the 75th %ile and head circumference was >95th %ile, with a BMI SDS = 2.38. His voracious appetite had begun at 8 years of age but he did not develop food stealing problems yet. Skin picking was also noted at that time. Academically, he was in regular classes and achieving good grades, but had speech difficulties. On exam, he did have almond shaped eyes and bitemporal narrowing. His saliva was viscous. He had hypogonadism. He was not hypopigmented relative to his family. He had large hands (85th %ile) and feet (85th %ile). This time, the SNRPN FISH analysis and DNA methylation analysis using the SNRPN probe (Glenn et al., 1996) revealed a deletion and aberrant methylation compatible with a diagnosis of PWS.

By 14 years of age, he started having severe behavioral problems and he had physically threatened family members several times. He was arrested for breaking into an occupied home and stealing food. He was subsequently placed into a psychiatric facility for his out of control behavior. His GH was stopped at this time and not restarted again till 5 years later at 19 years. He was last seen at 21 years on our inpatient clinical research unit. At that time he was noted to have mild bitemporal narrowing but otherwise he lacked the facial gestalt of PWS. His height was at the 25th %ile, head circumference was 98th %ile, and BMI SDS was 2.65. Cognitive testing revealed an IQ=63 on the Woodcock-Johnson III and a total achievement score of 78.

Unique features not typical of PWS include macrocephaly, large hands and feet, tall stature for PWS (before the start of GH), higher than average birth weight, normal skin pigmentation, and lower than average pain tolerance. The MS-MLPA showed a deletion of MKRN3 to intron 2 of ATP10A, sparing exons 1 and 2 of ATP10A. Array CGH showed a deletion of 2.46 Mb.

HBTB_113: The subject was a 42 year-old deceased white male who came to our attention after his brain was donated to the Human Brain Tissue Bank (HBTB) program at the University of Florida. Therefore, we obtained minimal records on him which included his autopsy and his hospital records prior to his death. At autopsy he was found to have chronic right ventricle hypertrophy (RVH), right sided heart failure, pulmonary hypertension, chronic gastritis and acute renal tubular necrosis. The pathologist attributed the cause of his death as "pulmonary hypertension leading to RVH and right sided heart failure resulting in hypotensive injury to the liver and to the kidneys. A probable contributing factor was stress secondary to his recent surgery." Of note, he had undergone a full dental extraction for multiple abscessed carries. He went into acute renal failure for unclear reasons 48 hours after his dental surgery, and died 3 days later. He was not tested for central adrenal insufficiency (de Lind van Wijngaarden et al. 2009), but his adrenal glands were described as within normal limits for size and sectioning showed normal architecture.

On physical exam he was 149.9cm in height (SDS = -4.21) and weighed 88.5 Kg (SDS = 4.54). He was short and obese (BMI SDS = 3.41) with small hands and feet with a small phallus and testes in the inguinal canal. Spermatogenesis was not present. Preoperative notes revealed that he had "profound mental retardation." We have no

information regarding behavior, psychiatric or pigmentation status. He was on no medications prior to his dental surgery.

The MS-MLPA found a deletion between BP1 and distal to ATP10A sparing GABRB3 and the more distal genes. This was confirmed by array CGH that showed a deletion size of 3.60 Mb.

PW173P: The patient is a 49 year-old white male, who was first seen in our clinic at 39 years. He was 3.18Kg, full term produced to a 37 year-old G3P2 mother by spontaneous vaginal delivery. Early feedings were problematic, and he needed to be fed via an eyedropper. He was noted to have poor muscle tone. He was discharged from the hospital at a few months of age. The family history revealed that he was one of five children born from the union of his parents, and the only one with a birth defect. He was diagnosed at 22 years with PWS after a chromosome report in 1982 was interpreted as “abnormal and consistent with PWS.” We have no further information on that report. At 24 years the Wechsler Adult Intelligence Scale-Revised (WAIS-R) revealed a full scale IQ of 62 (verbal IQ=63 and performance IQ=62).

He moved to a PWS group home at 39 years and was seen shortly after in our clinic. At his first clinic visit with us, his height was <5th %ile, weight was >95th %ile, head circumference was <3rd %ile with a BMI SDS = 4.11. He had a pleasant personality with a mild PWS facial gestalt, but not classical. For example, he did not have striking bitemporal narrowing. He did have esotropia, dry and viscous saliva and a hypernasal voice. He was very obese with decreased muscle mass and hanging skin. He had hypogonadism as well as small hands and feet measuring at <5th %ile. His skin pigmentation was darker than his younger brother and his mother (his father is

deceased). He had no acute skin lesion and no evidence of post skin picking (i.e., scarring). The PWS group home staff, his mother and his brother all stated that he was "good natured" and pleasant. The PWS group home staff further stated that he was a "model client." They rarely have any difficulties with him. However, he shows rigid behavior and does not tolerate changes well. He has no psychiatric history and he has never done any active skin picking. Since being in the group home, he has lost weight and his last BMI was 26.2 (BMI SDS = 1.00) at 49 years.

Chromosomal and FISH analyses done by our service revealed a chromosomal translocation involving chromosomes 6 and 15 with a karyotype that was interpreted as the following: 45,XY,der(6)t(6;15)(p25;q12),-15. FISH analysis confirmed a 15q11.2 deletion with the SNRPN probe. The MS-MLPA revealed a deletion of gene interval extending to the probe for GABRB3, sparing OCA2 (there were no MS-MLPA probes for GARBA5 and GABRG3). The research array CGH confirmed this finding and further delineated the location of distal BP, which was within the intron 3 of GABRG3, sparing exons and genes telomeric to intron 3 of GABRG3 including OCA2. Furthermore, our assumption of proximal BP being further upstream than BP1 in light of the chromosomal analysis finding of translocation was confirmed by the array CGH with a deletion of 5.09 Mb. Of note, aCGH demonstrated no deletion of unique 6p material. Unique features not typical of PWS include normal pigmentation, pleasant and mild mannered behavior, microcephaly, not argumentative, no history of skin picking, and chromosomal translocation.

PW246P: The patient is now 17 year-old white female who was first seen in our genetics clinic at 12 years. She was the 2.1Kg full-term product to a 31 year G3P2 who

was delivered by a C-section due to fetal distress. She was in the neonatal intensive care unit for 3 weeks. It is unknown if she received assisted feeding. She was reportedly seen by a geneticist in infancy and diagnosed with PWS; however it is unknown if genetic testing was ever done at that time.

Her weight began to climb significantly at 4 years and by 5 ½ years she was obese (BMI>97th %ile). Her weight continued to increase abnormally and at 11 ½ years, the child protection team recommended that she be removed from the mother's custody and be placed in a medical foster home. At 12 years she entered a PWS group home weighing 136Kg (SDS = 5.07) and was shortly thereafter seen in our genetics clinic. On examination she was morbidly obese, but did not have the typical PWS facial gestalt. Her height was 151.7cm (SDS = 0.27) with a BMI 59.1 (SDS = 4.68). Her head circumference was at the 2nd %ile. She had mild bitemporal narrowing, dry and viscous saliva, esotropia, a hypernasal voice, and a mild pectus excavatum. She did not have heart murmur. Breast and pubic hair were at Tanner stage II. She had an abdominal obesity and lacked appropriate tapering at the ankles and wrists. There were old healed skin excoriations, but no new, open lesions. Hand length was 16cm and foot length was 21.5cm. She did not appear hypopigmented, but there were no family member available for comparison. The karyotype and FISH analysis were reported as chromosomal translocation involving the chromosomes 14 and 15 with a karyotype of 45,XX,der(14),t(14;15)(p11.1;q13).

She was started on growth hormone therapy at 15 years. She lost a great deal of weight in the group home and at her last visit at 17 years her BMI was 21.2 (SDS = 0.131). She occasionally has inappropriate verbal behavior and bed wetting. She was

reported by the group home staff as “higher functioning” than many other PWS clients in the group home. She rarely engaged in skin picking. She does not have any specific psychiatric issues but has mild “autistic-like” behaviors including some self-stimulatory behavior and keeping to herself. Unique features, not typical of PWS, include the autistic-like behavior, microcephaly, smaller than average birth weight for PWS and chromosomal translocation.

The MS-MLPA revealed a deletion of all the proximal loci extending to GABRB3. The research aCGH revealed a deletion extending from the 15 centromeric region to intron 5 of GABRG3. The size of the deletion was 5.58 Mb. The aCGH did not demonstrate any loss of unique 14q material.

PW133P: This is now a 47-old white female living in a PWS group home. She was the 2.4Kg, 42 weeks gestational product to a 26 year old G3P2 who was delivered vaginally. Hypotonia and feeding difficulties were noted at birth. She was fed through an nasogastric tube (NG-tube) for the first 4 months of life. She was obese by 3 years. She was seen by a geneticist at 13 years when the possible diagnosis of PWS was raised; however, the chromosomal analysis at that time was inconclusive.

She was first seen by our genetics service at 30 years of age. She was accompanied by her parents to the research study, but she had been living for many years in a group home specifically designed for individuals with PWS. She was weighed daily in the group home and her diet was well controlled. She had oligomenorrhea. She had no specific psychiatric illnesses. Previous IQ testing was reported to be 55-60, but we do not have a copy of the report.

On physical examination at 30 years, she was a pleasant, smiling adult female with a typical PWS facial gestalt. Length was 150.1cm (SDS = -2.28); weight 56.0Kg (SDS = -0.29); BMI=24.9 (SDS = 0.82) and head circumference at just <2nd %ile (versus the father who was ~75th %ile and the mother at ~40th %ile). She had bitemporal narrowing and dry, sticky saliva. She had mild, moderate skin picking and her skin pigmentation was lighter than either parent. Her hands and feet were small at <3rd %ile. Genetic testing done as part of our research study at 30 years showed that her DNA methylation was positive for PWS at the DN34 locus (Driscoll et al., 1992) and a deletion was detected by dosing for the 189-1, 34, 3-21, and GABRB3 loci but intact at IR10-1 and biparental at the CMW-1 loci (Robinson et al. 1993). She was subsequently shown several years later in the lab to also be PWS positive at the PW71 and SNRPN methylation sensitive loci (Glenn et al., 1997). Unique feature, not typical of PWS, was the lower than average birth weight for PWS (Butler et al. 2009).

The MS-MLPA revealed a deletion of the gene interval from BP1 to within the OCA2 gene which was confirmed by the aCGH. Specifically the aCGH revealed the distal BP within intron 18 of OCA2, sparing exons (1 to 18 of OCA2) with a deletion size of 5.55 Mb. Interestingly, the aCGH also identified another small deletion of less than 300 Kb which was approximately 300 Kb, distal to BP5. At this time we do not know whether this deletion is on the same (paternally inherited) chromosome and/or whether this second deletion has any clinical implications in this patient. We are in the process of obtaining the parents' DNA to determine if this is a copy number variant. In addition, we plan to obtain the patient's blood to do FISH studies to determine if they are on the same chromosome.

PW231P: The patient is now a 7 2/12 year-old Hispanic female. She was born at 40 weeks gestation with a birth weight of 3.18 Kg. Her mother recalled decreased fetal movements during her pregnancy. Even though she was a poor breast feeder and therefore the mother needed to bottle feed with a widened nipple, she was in the nursery for only four days. She never needed a feeding tube placed. She was referred to a neurologist at 2 1/2 months of age because of the hypotonia. She was diagnosed with a 15q11.2-q13 deletion and PWS at 3 months of age by FISH and DNA methylation analyses. By 5 months of age, she no longer had difficulty feeding. She was first seen by our service at 9 months of age with a length of 68 cm (25th %ile), weight 8.26Kg (46th %ile) and a head circumference 45.0cm (80th %ile) and weight/length at the 75th %ile.

Developmentally she sat at 8 1/2 months, first words at 12 months and walked independently at 20 months. At 7 2/12 years she underwent cognitive testing using the Woodcock-Johnson III tests of cognitive and achievement. Her general IQ was 89 and her achievement IQ was 109.

She started growth hormone treatment at 19 months. Her BMI exceeded the 97th %ile at 3 years of age, but her appetite did not become greater than average until 3 3/12 years. By 6 years she started exhibiting a very aggressive appetite typical of PWS. By 7 2/12 years her head circumference was 98th %ile, weight was 40.7 Kg (SDS=+2.68), and height was 126.2 cm (SDS=0.35) with a BMI SDS=+2.48.

On physical examination, she lacks the PWS typical gestalt. She has frontal bossing, almond shaped eyes, esotropia and a hypernasal voice. She has inverted nipples, bilateral transverse palmar creases, genu valgum and pes plantar. She has

mild hypoplasia of her labia major and minor and clitoris. She has the typical weight distribution of PWS at the buttocks, hips, thighs and legs. Her skin pigmentation is appropriate for her family background, but her irises are lighter than her two sibs and both parents. Of note, no one in her family or school noted any seizure activities. However, given the recent report of 15q13 microdeletion syndrome and epilepsy (Sharp et al. 2008), we administered an EEG during her most recent visit at our inpatient clinical research unit. During the EEG session, she was noted to have absence seizure that corresponded to the abnormal EEG recording.

Unique features not typical of PWS include the frontal bossing, macrocephaly, inverted nipples, bilateral transverse palmar creases large hands and feet, higher than typical IQ, and abnormal EEG with subclinical seizure activities. The MS-MLPA revealed a deletion from MKRN3 to APBA2. Array CGH demonstrated a 9.06 Mb deletion extending from MKRN3 to CHRNA7.

PW259P: The patient is now a 4 11/12 year-old black male. He was born at 40 weeks gestation and weighed 2.36 Kg at birth. He was very hypotonic which prompted his neonatologist to suspect PWS. This was confirmed by chromosomal, FISH and DNA methylation analysis at 6 weeks of age. In addition, a maternally inherited paracentric inversion of chromosome 12 was noted with a karyotype of 46,XY,inv(12)(q13.1q22),del(15)(q11.2q13).

He spent 2 months in the NICU. Initially he was fed by an NG-tube and then received a gastric feeding tube (G-tube) at 1 ½ months of age. He was first seen by our genetics service at 11 months of age with severe failure-to-thrive despite continuous feeding through the G-tube. His weight was 4.83 Kg (SDS = -6.64), length was 64.0 cm

(SDS = -4.20), head circumference was 41.5 cm (much less than 3rd %ile) and weight/length was also much less than 3rd %ile. Growth hormone treatment was begun at 13 months of age and was discontinued at 39 months of age due to insurance issues. He received a Nissen fundoplication at 14 months of age for gatroesophageal reflux disease (GERD) and was fed through his G-tube until 2.5 years of age. He appeared well-nourished at 24 months of age with a weight for length at the tenth percentile.

Developmentally he did not sit unsupported until 18 months and did not begin saying his first words until 3 years. At 4 11/12 years, he was still not walking independently and was speaking in three word sentences. At 7 months of age, an EEG was done and showed high amplitude background rhythm, but no specific epileptiform activities. An EEG repeated at 4 11/12 years revealed runs of medium-high amplitude delta activities over the left parietal region. While the findings were non-specific it raised the concern for potential focal lower seizure threshold over the left parietal region, although to date he has had no frank seizures.

His appetite began to abnormally increase at 3 9/12 years and by 4 11/12 years his BMI SDS was +3.48. His height was 101 cm (SDS = -1.78), weight was 24.3 Kg (SDS = 2.12) and head circumference SDS was -2.0. On physical examination, he is microcephalic and lacked the PWS facial gestalt with no bitemporal narrowing. He is obese. He has a round face and dry and sticky saliva. He has estropia bilaterally and a very hypoplastic scrotal sac with undescended testes on his most recent exam. He does have self-stimulatory behavior and moderate hypotonia. Unique features not typical of PWS include greater than expected severity of developmental delay, the microcephaly, atypical facial features, increased hypotonia and the prolonged failure-to-thrive which

lasted until 24 months of age. The MS-MLPA detected a deletion from MRKN3 to APBA2. Array CGH demonstrated a 9.31 Mb deletion extending from MKRN3 through CHRNA7.

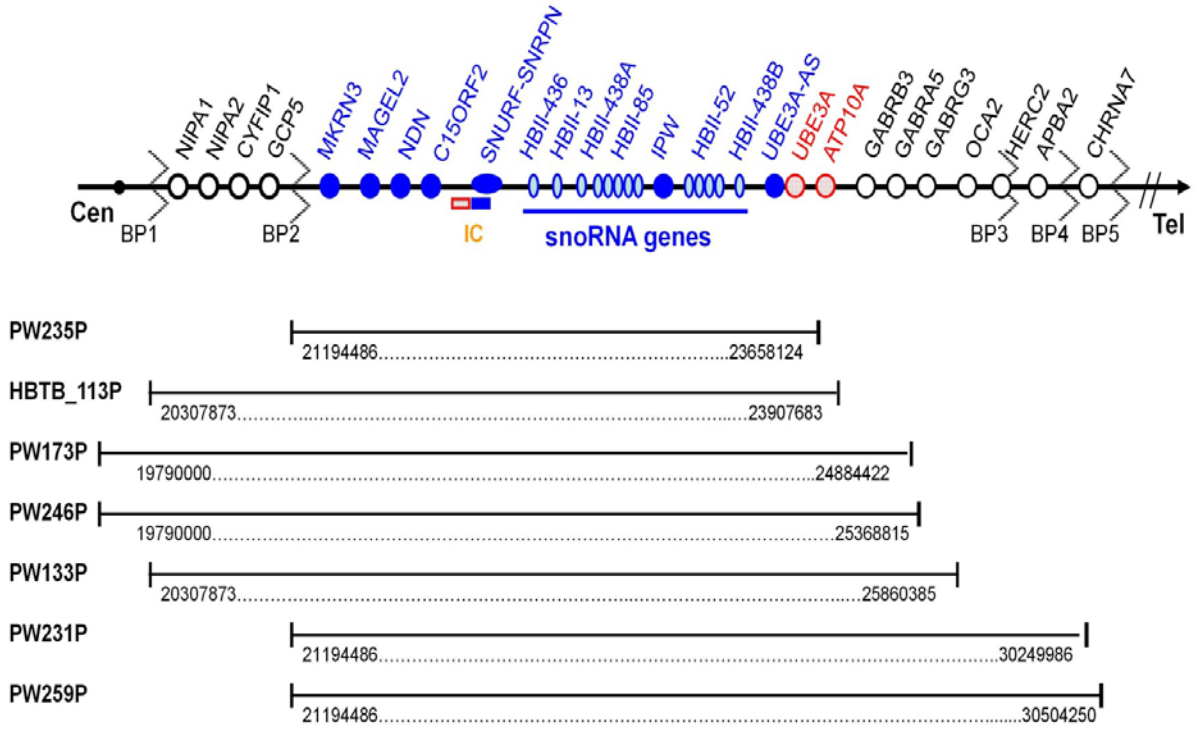

Figure 3-1. Seven unique PWS deletions. The position of genes and genetic markers (circles) in the chromosomal 15q11-q13 region are shown. In the PWS region (shown in blue), there are six paternal-only (PWS region) expressed unique copy genes (MKRN3, MAGEL2, NECDIN, C15ORF2 and SNURF- SNRPN and a family of 5 paternal-only expressed snoRNA genes). Only UBE3A and ATP10A (shown in red), related to Angelman syndrome (AS), have maternal-only expression in mouse and humans, and this imprinted expression is limited to certain tissue specific regions (i.e., mostly regions in the brain). The bipartite imprinting center (IC) lies proximal to SNURF-SNRPN and within the 3 Mb PWS/AS imprinted region. The cluster of GABA receptor genes (GABRB3, GABRA5 and GABRG3), OCA2 (Type 2 albinism) and HERC2 are not imprinted and have biparental expression (shown in open black circle). The jagged vertical lines denote the common PWS deletion breakpoints, which lie within the segmental duplications associated with BP1 to BP5. Type 1 deletions extend from BP1 to BP3 and type 2 deletions extend from BP2 to BP3. The 7 unique deletions (i.e., neither type 1 or 2) identified from this study are shown in solid lines with base pair positions of breakpoints confirmed by array CGH. These base pair positions are derived from the UCSC genome browser March 2006 (hg18) freeze (http://www.genome.ucsc.edu/). There is a lack of agreement in the literature regarding the order of the genes between BP1 and BP2 (Chai et al. 2003; Makoff and Flomen 2007). Note that there are more copies of the HBII-85 and HBII-52 snoRNA genes than are shown and map distances are not drawn exactly to scale.

Table 3-1. Subjects with unique deletions

	PW235P_DG	HBTB_113	PW173P_PB	PW246P_EM	PW133P_BL	PW231P_IB	PW259P_AH
Molecular genetics							
Age of PWS diagnosis	11 years	Unknown	22 years	"Infancy"	12 years	3 months	6 weeks
Base pair positions of breakpoints	21,194,486 to 23,658,124	20,307,873 to 23,907,683	19,790,000 to 24,884,422	19,790,000 to 25,368,815	20,307,873 to 25,860,385 (2^{nd} deletion 31.2Mb to 31.5Mb)	21,194,486 to 30,249,986	21,194,486 to 30,504,250
Deleted genes	MRKN3 to ATP10A	GCP5 to ATP10A	Centromeric to GABRG3	Centromeric to GABRG3	GCP5 to OCA2	MKRN3 to CHRNA7	MKRN3 to DKFZp434L187
Demographics							
Gender	Male	Male	Male	Female	Female	Female	Male
Race/ethnicity	White	White	White	White	White	Hispanic / White	Black
Age of subject for clinical features	Died at 21 years	Died at 42 years	39 years	12 years	30 years	7.2 years	4.9 years
Pregnancy/neonate							
Reduced fetal movement	Yes	Unknown	Unknown	Unknown	Yes	Yes	Yes
Gestational age	43 weeks	Unknown	Full term	Full term	42 weeks	40 weeks	40 weeks
Birth weight	3.73 kg	Unknown	3.18 kg	2.1 kg	2.4 kg	3.18 kg	2.36 kg
Hypotonia	Yes	Unknown	Yes	Yes	Yes	Yes - mild	Yes - severe
Feeding difficulty as a neonate	Yes	Unknown	Yes	Yes	Yes	Yes - mild	Yes - severe
Assisted feeding	Yes - NG tube	Unknown	Yes - eye dropper	Unknown	Yes - NG tube	No	Yes - G tube until 2.5 years
Age range of assisted feeding	0 to 3weeks	Unknown	Several months	Unknown	0 to 4 months	NA	0 to 2.5 years
Duration of poor feeding	0 to 9 months	Unknown	Unknown	Unknown	0 to 4 months	0 to 5 months	0 to 2.5 years

Table 3-1. Continued.

	PW235P_DG	HBTB_113	PW173P_PB	PW246P_EM	PW133P_BL	PW231P_IB	PW259P_AH
Developmental milestones							
Sitting	10 months	Unknown	Unknown	Unknown	11 months	8 months	18 months
Walking	30 months	Unknown	36 months	24 months	30 months	20 months	Not walking at 59 months
First words	30 months	Unknown	Unknown	Unknown	Unknown	12 months	36 months
Intellectual disability	WJ-III at 19 years; GIA = 63, TIA=78	Described as "very mentally retarded"	WAIS-R at age 31 years; full scale = 62, verbal = 63	Yes	IQ 55-60 per parents	WJ-III at 7.2 years, GIA = 89, TIA = 109	Moderate/severe MR
Growth							
Growth hormone treatment	10 to 14 and 19 to 21 years	NA	NA	15 years to present	30's to 40's	19 months to present	13 to 39 months
Weight (SDS)	+ 2.65	+ 1.28	+ 2.07	+ 3.75	- 0.24	+ 2.47	+ 1.88
Height (SDS)	- 0.76	- 3.72	- 3.72	+ 0.07	- 2.04	+ 0.65	- 1.59
BMI (SDS)	+ 2.65	+ 2.53	+ 2.93	+ 3.08	+ 0.76	+ 2.48	+ 3.51
Head circumference in percentile	98%	Unknown	<2%	<2%	<2%	98%	2%
Small hands and feet before GH treatment	No	Yes	Yes	Yes	Yes	No	Yes
Obesity and appetite							
Childhood obesity	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Subject became obese (BMI >97%)	2 years	Unknown	Unknown	4.5 years	3 years	3 years	3.9 years
Voracious appetite began	8 years	Unknown	Unknown	Unknown	3 years	6 years	4.9 years

Table 3-1. Continued.

	PW235P_DG	HBTB_113	PW173P_PB	PW246P_EM	PW133P_BL	PW231P_IB	PW259P_AH
Other clinical issues							
Facial gestalt suggestive of PWS	Yes at 5 years before GH, no at 21 years	Unknown	Mild	No	Yes	No	No
Dry/sticky saliva	Yes	Unknown	Yes	Yes	Yes	No	Yes
Hypo-pigmentation	No	Unknown	No	No	Yes	Only in iridies	No
Skin picking	Moderate	Unknown	No	Very mild	Mild to moderate	No	No
Scoliosis	Moderate	Unknown	No	No	Mild	Mild	No
Hypogonadism	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Menstruation	NA	NA	NA	No	Rarely	NA	NA
Seizure activity	No	No	No	No	No	Yes	No clinical seizures, but abnormal EEG
Other medical problems	Hypothyroidism	Right ventricular hypertrophy & chronic gastritis	Hypertension, Inguinal hernia	Enuresis	NA	Hypothyroidism, Premature adrenarche Hip dysplasia	Reflux Hip dysplasia
Psychiatric & behavioral problems	Destructive behavior, Rigid thinking	Unknown	Resistance to change	Imaginary friends still at 14 years	No issues now	Nail biting, resistance to change, stubborn, labile	Plays with feces, Self stimulatory behavior
Pain threshold	Lower than average	Unknown	Higher than average	Unknown	Higher than average	Higher than average	Higher than average
Vomiting	Much lower than average	Unknown	Much lower than average	Much lower than average	Never vomited	Much lower than average	NA due to Nissen fundoplication

SDS: Standard deviation score
WJ-III: Woodcock-Johnson Tests of Cognitive Abilities and Achievement, Third Edition
WAIS-R: Wechsler Adult Intelligence Scale-Revised
NA: not applicable
Center for Disease Control curves (2000) used for weight, height and BMI SDS
Nellhaus (1968) used for head circumference percentages

CHAPTER 4
DISCUSSION

PWS is the most frequently diagnosed genetic cause of obesity, and also an example of genomic imprinting disorders. It is a contiguous genomic disorder most frequently caused by deletion of the 15q11-q13 region. This 15q11-q13 region is highly vulnerable to structural rearrangements due to repeated sequences.

Several efforts to identify phenotypic characteristics across the genetic subtypes of PWS (Type 1 vs. Type 2 vs. UPD/ID) were not conclusive, although some studies provided evidence for more severe and typical PWS phenotypes in Type 1 deletion compared to Type 2 deletion or UPD/ID. The lack of consistent findings across genotype-phenotype studies may have arisen from heterogeneous sample set, for example, patients with unique deletions may have been categorized as either Type 1 or Type 2, skewing the data one way or another.

The primary focus of this study was to examine the copy number changes and aberrant methylation patterns in our PWS subjects using the MS-MLPA method. Among 74 PWS subjects with deletion, we found 25 Type 1, 42 Type 2 and 7 unique deletions. The prevalence of unique deletions (~9.5%) was higher than our expectation, which may provide an explanation as to conflicting results across the genotype-phenotype studies. During the course of the study, we also applied this method to additional study participants to compare the results across the diagnostic categories. Interestingly, we incidentally identified unique deletions in 2 EMO subjects, whose clinical implications would require further investigation.

To find clues for underlying genetic factors for specific PWS phenotypes, we reviewed the available medical records of these seven PWS subjects with unique

deletions. Because of the nature of retrospective chart review, our description of clinical phenotypes was not comprehensive. Despite this limitation, we were able to confirm that intact OCA2 was associated with absence of hypopigmentation, consistent with the function of OCA2 in skin pigmentation (cases of PW235P, PW173P and PW246P). From the case study of PW133P, we also learned that even with majority of exons of OCA2 (exons 1 to 18) spared, disruption of distal part of this gene still could cause hypopigmentation. In addition, we learned that smaller sizes of deletion (PW235P) may have resulted in more atypical features of PWS, thereby, contributing to the delay of diagnosis. For example, this subject had above average birth weight, macrocephaly, normal-sized hands and feet, normal stature and delayed onset of voracious appetite and food-stealing behavior. The atypical features may be explained by the sparing of genes distal to ATP10A, which include the GABAA receptor subunit gene clusters and OCA2. However, the prominent skin-picking behavior in this subject was rather puzzling. Given the clinical efficacy of Topiramate on skin-picking behavior supposedly mediated via GABA pathway (Shapira et al. 2004), we anticipated that sparing the GABAA receptor subunit gene clusters may lessen this behavior. The likely explanation for this phenomenon may involve the positional effects of proximal deletion acting epigenetically preventing proper expression of the genes distal to the deletion. Unfortunately, detailed behavioral phenotypic information on HBTB_113 was not available, therefore, we could not make further hypothesis regarding behavioral effects of sparing the GABAA receptor subunit gene clusters at this time.

The most interesting finding from this study was the similarity and differences between two subjects, PW231P and PW259P, who had similar pattern of deletion. Both

subjects had a large deletion spanning over 9Mb between MKRN3 and CHRNA7, but PW259P had additional 250 Kb deletion telomeric to CHRNA7 involving DKFZp434L187. Interestingly, CHRNA7 encodes the alpha-7 subunit of neuronal nicotinic acetylcholine receptor (Agulhon et al. 1999). Recently several groups have implicated CHRNA7 as a candidate gene for the 15q13.3 microdeletion syndrome whose clinical manifestations include mental retardation (MR), seizures, facial and digital dysmorphology, expressive language deficit, various neuropsychiatric disorders, such as schizophrenia or autism (Agulhon et al. 1999; Dibbens et al. 2009; Erdogan et al. 2007; Freedman et al. 2001; Helbig et al. 2009; Martin et al. 2007; Miller et al. 2009; Sharp et al. 2008; Stefansson et al. 2008; Xu et al. 2001).

In line with these reports, during the most recent visit, we noted that PW231P had absence seizures documented on her EEG. No one noted any clinical seizure activities from PW259P, but his EEG revealed abnormal waves, suggesting the possibility of unrecognized seizure disorder. However, other than the fact both subjects had abnormal EEG recordings, the clinical presentation of these two subjects appeared widely different. For example, PW231P had much milder postnatal course, less severe developmental delay, higher intellectual and academic functioning, whereas PW259P had much more severe failure to thrive requiring feeding tube over 9 months, markedly delayed gross and fine motor as well as speech development.

This wide difference in their clinical features despite similar genetic findings including deletion of CHRNA7 led us consider the following possibilities: (1) It is plausible that genetic variation in the 15q13-q14 on the maternal chromosome is “unmasked” by the paternally-derived deletion, which may have contributed to more

severer clinical phenotype in PW259P. (2) Although it is less likely, the gene(s) downstream to CHRNA7, such as DKFZp434L187 may require further evaluation for its potential implication in developmental delay and failure to thrive, given much severer clinical course of PW259P who has additional deletion beyond CHRNA7. (3) Genetic variation elsewhere in the genome may have contributed to the composite clinical features in PW259P. As described earlier, PW259P has another chromosomal rearrangement (12q inversion inherited from his mother) that may have influenced on his phenotype. However, given the normal phenotype of his mother, it is less likely that this 12q inversion resulted in disruption of essential genes. (4) In addition to genetic abnormality, modifying environmental factors, such as the level of prenatal care, maternal health, and family support system may also have contributed to the clinical course, although their effect sizes are probably modest at most.

We also identified several strengths and weaknesses of the MS-MLPA methods. The MS-MLPA reliably found the presence of deletion in our subject (100% agreement between our MS-MLPA results and aCGH). It also provided more detailed information than fluorescent *in situ* hybridization (FISH), as it has 25 probes across the 15q11-q13 region, thereby allowing us to make educated guesses in regards to the position of breakpoints. Compared to the aCGH, the MS-MLPA was much more labor and cost-effective, although the aCGH provides much more detailed information regarding extent of deletion than the MS-MLPA. In addition, the methylation analysis component of the MS-MLPA allows us to differentiate PWS vs. AS (and vs. normal control). Therefore, this method can be a good screening tool when a patient has clinical features of PWS, especially because it also detects aberrant methylation status. The main weaknesses

include lack of probes proximal to BP1 and distal to APBA2 and relative paucity of probes between GABRB3 and OCA2, thereby, making it difficult to identify more accurate position of proximal or distal BP.

Additionally, we have learned several lessons through this study. First, we should not categorize all deletions to either Type 1 or Type 2 for genotype-phenotype studies, as a small but significant percentage of deletions (~9.5% in present study) may have unique breakpoints. Genotype-phenotype studies on individuals with unique deletions would be very important in further elucidating specific genetic factors for specific PWS associated phenotypes. Second, mechanisms for unique deletions need further clarification, as it does not appear at the typical low copy repeat (LCR) regions. Third, we need to continuously monitor clinical courses of subjects with larger deletion extending to the 15q13.3 region, as the 15q13.3 microdeletion syndrome has been implicated in several neuropsychiatric disorders including schizophrenia and epilepsy (Sharp et al. 2008; Stefansson et al. 2008). Fourth, at this point, it may not be necessary to distinguish subtypes of deletion in all cases until we know what Type 1 vs. Type 2 deletion means. However, it may be worthwhile clinically to investigate the types of deletions if phenotype is milder or severer than expected.

In our future studies, we plan to obtain more detailed phenotypic information using specific rating scales and tests, such as cognitive profile, behavioral profile, comorbid psychiatric illnesses, and response to certain mediation, to examine genotype-phenotype relationship in these subjects. We believe further elucidation of genetic subtypes beyond the conventional classification system of Type 1 or Type 2 would be necessary for successful genotype-phenotype studies.

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BIOGRAPHICAL SKETCH

Soo-Jeong Kim, MD is an assistant professor in the departments of psychiatry and pediatrics. She is board certified in general psychiatry and in child and adolescent psychiatry. She currently serves as a director of the autism clinic at the Shands Medical Plaza.

Dr. Soo-Jeong Kim obtained her M.D. degree at Seoul National University in South Korea where she also received general psychiatry residency training (1993-1998). After completing residency training in Korea, Dr. Kim studied the molecular genetics of childhood-onset psychiatric disorders in Dr. Ed Cook's laboratory at the University of Chicago (1998-2001). To pursue further clinical training in both psychiatry and child psychiatry, Dr. Kim completed a three-year general psychiatry residency and the first year of Child Psychiatry fellowship at the University of Chicago (2001-2005). She finished her second year of Child Psychiatry fellowship at the University of Illinois at Chicago (2005-2006). During the clinical training period, Dr. Kim remained engaged in Dr. Cook's laboratory working on several interesting projects and publications.

Upon completion of clinical training in June 2006, Dr. Kim joined the University of Florida faculty as a tenure-track assistant professor (2006-present). At this time, Dr. Kim is most interested in studying the RRB genetics in two clinical populations, autism spectrum disorders and Prader-Willi syndrome. To support her research programs, Dr. Kim has attained external funding, including the NARSAD Young Investigator Award (2007), PWSA (USA) Research Award (2008), NIMH R03 Award (2008) and NIMH K23 Award (2009), as well as support from the University of Florida's NIH K30 program.