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Array Comparative Genomic Hybridization (aCGH) Analysis in Prader–Willi Syndrome

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Abstract

Prader–Willi syndrome (PWS) is due to loss of paternally expressed genes in the 15q11–q13 region generally from a paternal 15q11–q13 deletion. The proximal deletion breakpoint in the 15q11–q13 region occurs at one of two sites located within either of two large duplicons allowing for identification of two typical deletion subgroups. The larger type I (TI) deletion involving breakpoint 1 (BP1) is nearer to the centromere and located proximal to the microsatellite marker D15S1035, while the smaller type II (TII) deletion involves breakpoint 2 (BP2) and distal to D15S1035. Breakpoint 3 (BP3) is located at the distal end of the 15q11–q13 region and common to both typical deletion subgroups. Using high resolution aCGH, BP1 spanned a region from 18.683 to 20.220 Mb, BP2 from 20.812 to 21.357 Mb and BP3 from 25.941 to 27.286 Mb. The TI deletion ranged in size from 5.721 to 8.147 Mb (mean 6.583) and the type II deletion from 4.770 to 6.435 Mb (mean 5.330). A subset of the TI subjects showed larger deletions including the loss of at least three genes/transcripts (i.e., *LOC283755*, *POTE5*, *OR4N4*) in addition to the four genes between BP1 and BP2 (i.e., *GCP5*, *CYFIP1*, *NIPA1*, *NIPA2*). Interestingly, four PWS subjects had duplications of the 15q11 region in addition to the typical deletion. Furthermore, most PWS subjects had copy number variation (CNV) of 50 kb or larger in other chromosome regions; most common were deletions and duplications of 8p and 3q, previously recognized sites of CNV in the human genome.

Keywords

Prader–Willi syndrome (PWS); type I and II deletions; array comparative genomic hybridization (aCGH); chromosomal breakpoint; copy number variation (CNV)

INTRODUCTION

Prader–Willi syndrome (PWS) is a neurodevelopmental disorder resulting from loss of function of paternally expressed genes from the 15q11–q13 region and characterized by severe hypotonia, feeding difficulties, hypogonadism, small hands and feet, developmental delay, behavior problems and genital hypoplasia. However, children with PWS between 2 and 4 years of age develop an insatiable appetite and severe obesity if food intake is not

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strictly controlled [Cassidy et al., 1997; Butler and Thompson, 2000; Bittel and Butler, 2005].

A paternal 15q11–q13 deletion is found in about 70% of PWS subjects, approximately 25% have uniparental maternal disomy 15 (UPD), and the remaining 2–5% have imprinting defects or translocations [Nicholls and Knepper, 2001; Bittel and Butler, 2005]. The proximal deletion breakpoint in the 15q11–q13 region is reported to occur at one of two sites located within either of two large duplicons centromeric to locus ZNF127 [Pujana et al., 2001; Chai et al., 2003] which allows for the identification of two typical deletion subgroups [Knoll et al., 1990; Amos-Landgraf et al., 1999; Butler et al., 2004; Bittel and Butler, 2005]. The larger type I (TI) deletion involves breakpoint 1 (BP1), which is close to the centromere and proximal to the microsatellite marker D15S1035, while the smaller type II (TII) deletion involves breakpoint 2 (BP2) located closeby but distal to D15S1035. Breakpoint 3 (BP3) is located at the telomeric end of the 15q11–q13 region, distal to *OCA2* and common to both deletion subgroups.

Although the approximate location of the breakpoints involved in the typical PWS deletion was reported in the early 1990s, newer technology allows for a more precise localization of the breakpoints. Commercially available whole genome microarrays for array comparative genomic hybridization (aCGH) have resolution better than 10 kb allowing for a much higher resolution for localizing breakpoints. In addition, recent interest in copy number variation (CNV) in the human genome has indicated a greater than expected level of variation in copy number with implications for genetic diversity and human disease [Barrett et al., 2004; Conrad et al., 2006; Fiegler et al., 2006; Khaja et al., 2006; Komura et al., 2006; Perry et al., 2006; Redon et al., 2006; Sebat et al., 2007; Stranger et al., 2007]. Therefore, we have undertaken an evaluation of 25 subjects with PWS due to typical deletions using high resolution aCGH focusing on a more precise location of breakpoints in the 15q11–q13 region and examining CNVs in the remaining genome.

SUBJECTS AND METHODS

Subjects

Our study included 12 TI PWS subjects (5 male, 7 female; mean age 24.7 ± 9.5 years) and 13 TII PWS subjects (4 male, 9 female; mean age 18.6 ± 6.1 years). PWS diagnosis was confirmed by DNA methylation testing and deletion status by cytogenetic analysis including fluorescence in situ hybridization (FISH) with the *SNRPN* probe and by genotyping informative microsatellites (e.g., D15S1035) between breakpoints BP1 and BP2. The paternal deletion in this region was confirmed and deletion subtype established as previously described [Butler et al., 2004].

Methods

DNA was isolated from peripheral blood using a Gentra (Madison, WI) DNA isolation kit following the manufacturer's protocol. All microarrays used were Agilent 244K whole human genome microarrays with an average distance of 6.4 kb between each probe (Agilent Technologies, Santa Clara, CA). Comparison genomic DNA was obtained commercially

(Promega, Madison, WI) and matched for sex. The methods for genomic DNA preparation, labeling, hybridization and scanning can be found at <http://www.home.agilent.com>. The hybridization and initial analysis was performed commercially at Cogenics Inc. (Morrisville, NC).

Copy number variants were identified with CGH Analytics software using the Aberration Detection Method 2 (ADM-2) analysis algorithm (Agilent Technologies) with a user specified statistical threshold. The ADM-2 quality-weighted interval score algorithm identifies aberrant intervals (e.g., CNVs) in a sample that have consistently high or low log ratios based on their statistical score. ADM-2 uses an iterative procedure to find all genomic intervals with a score above a user specified statistical threshold value (e.g., a minimum of 12 and the aberration filter set at 20). The score represents the deviation of the weighted average of the normalized log ratios from its expected value of zero and incorporates quality information about each probe measurement.

Fifteen of our PWS subjects were more extensively analyzed to determine CNVs outside the 15q11–q13 region. In our study, CNVs were included only if they met the same statistical threshold stringency. Several copy number variants outside the Prader–Willi chromosome region (e.g., 3q, 8p) were confirmed using quantitative PCR with primers specific to the region of interest (i.e., deleted or duplicated) and compared to CNVs reported in the human genome (see <http://projects.tcag.ca/variation/>). Equal quantities of genomic DNA from PWS subjects and chromosomally normal comparison subjects were added to a hot-start quantitative PCR mix including SYBR green (Invitrogen, Inc., Carlsbad, CA) and PCR performed in an ABI 7000 thermocycler according to the manufacturer's instructions as previously described [Bittel et al., 2006, 2007b]. Five replicates of each sample were run. A dissociation analysis and agarose gels were run on PCR reactions to verify that single fragments of the correct size were amplified. In addition, the initial PCR products were sequenced and the sequence blasted against the human genome to verify the correct product.

RESULTS

We analyzed 25 subjects with PWS (12 with a typical type I deletion and 13 with a typical type II deletion) by high resolution aCGH analysis using 244K microarrays. The spatial resolution of the probe set in the 15q11–q13 region was approximately 14.5 kb which allowed us to more accurately identify breakpoints than previous analyses involving typical deletions leading to PWS.

Individuals with a type I deletion have breakpoints near the centromere. Seven individuals with a type I deletion had a breakpoint at BP1 of 20.220 Mb (measured from the p terminus); one at 20.080 Mb; two at 19.806 Mb; one at 18.722 Mb and one at 18.683 Mb. The 244K CGH microarray did not contain any probes nearer to the centromere than 18.683 Mb and no probes were available on the short arm of chromosome 15. Therefore, 18.683 Mb may be a minimal proximal boundary for BP1. The most distal breakpoint for BP1 was 20.220 Mb. The overall span for breakpoints in the BP1 region in the PWS subjects with type I deletions was 1.537 Mb. Furthermore, the largest deletion seen in our subjects with

type I deletions was 8.147 Mb and the smallest was 5.721 Mb with an average of 6.583 Mb (Fig. 1).

Individuals with a type II deletion have breakpoints distal to marker D15S1035 beginning at nucleotide number 20,459,962. Two individuals with a type II deletion had a breakpoint at BP2 of 21.357 Mb; one at 21.320 Mb; two at 21.236 Mb; four at 21.219 Mb; one at 21.208 Mb; one at 21.195 Mb; one at 20.851 Mb and one at 20.812 Mb. The most proximal breakpoint in subjects classified as having a type II deletion was 20.812 and the most distal breakpoint was at 21.357 Mb. The overall span of breakpoints in the BP2 region from PWS subjects with type II deletions was therefore 0.545 Mb. Furthermore, the largest deletion seen in our subjects with type II deletion was 6.435 Mb and the smallest was 4.770 Mb with an average size of 5.330 Mb (Fig. 1).

The third breakpoint (BP3) was common to PWS subjects with either the type I or type II deletion (Fig. 2). In the individuals with type I deletions, BP3 was located at 25.941, 25.979, 26.194 Mb (three individuals), 26.209, 26.365, 26.830 Mb (three individuals), 27.015 and 27.041 Mb (Fig. 3). In the individuals with type II deletions, BP3 was located at 26.127, 26.182, 26.209 Mb (six individuals), 26.830 Mb (two individuals), 27.112 Mb (two individuals) and 27.286 Mb. The region on chromosome 15 between 26.233 and 26.739 Mb has only a single probe located at 26.365 Mb in the Agilent 244 K CGH microarray. Thus, there is a 0.5 Mb gap in the array that makes the determination of BP3 less precise. However, there appears to be two localized regions involved in the break site at BP3, one located at approximately 26.2 Mb (at the proximal side of the gap) and the other is larger at about 26.8 Mb (at the distal side of the gap). There does not seem to be an association with the deletion type as to where the specific site of BP3 occurs. The most proximal breakpoint for BP3 in subjects with either typical deletion was 25.941 Mb and the most distal breakpoint was at 27.286 Mb. The overall span of the breakpoints in the type II subjects was therefore 1.889 Mb (Fig. 1).

Interestingly, our aCGH analysis identified four individuals (out of 25) with PWS with a duplication in the 15q11 band at approximately 18.5 Mb and ending at approximately 20 Mb (Fig. 3), one of whom we reported previously [Butler et al., 2002]. The breakpoint for the duplicated region appears to be near the centromere but does not include the most proximal five or six array probes. This also appears to be the case for the largest type I deletion (data not shown) suggesting a possible additional BP site (?BP0) potentially resulting from a duplicon-like sequence near the centromere as previously suggested [Ji et al., 2000]. In addition, there appears to be an area which was not deleted and not duplicated between the duplicated and deleted regions of approximately 0.5–1 Mb in size in two of the four subjects which closely corresponds to the area between BP1 and BP2 breakpoints (see Fig. 3A).

We also performed more extensive analysis in 15 of our PWS subjects to identify CNVs in chromosomes other than chromosome 15 and compared the CNVs to those reported in the human genome literature [Barrett et al., 2004; Sebat et al., 2004; Conrad et al., 2006; Khaja et al., 2006; Komura et al., 2006; Redon et al., 2006; Scherer et al., 2007]. We observed a total of 47 CNVs ranging in size from 50 kb to 1.6 Mb in our PWS subjects. Thirty CNVs (17 deletions and 13 duplications) ranged in size between 50 and 300 kb (Fig. 4) while 17

CNVs (11 deletions and 6 duplications) were greater than 300 kb. The smallest CNV that met our criteria for statistical significance was 60 kb and the largest was 1.6 Mb. The average size of the 47 CNVs was 384 kb with an average of 3.1 CNVs per individual and only one CNV per individual had an average size greater than 300 kb. The most common CNV occurred on chromosome 3 at approximately 164 Mb from the p terminus (CNV_locus 0383—annotations from Agilent Technologies, CGH Analytics software) and chromosome 8 at approximately 39 Mb from the p terminus (CNV_locus 1769; see Fig. 4). The CNVs on chromosomes 3 and 8 were verified by quantitative PCR (data not shown). These regions have been reported previously to contain CNV in non-PWS individuals [Perry et al., 2006; Redon et al., 2006] (for summary, see <http://projects.tcag.ca/variation/>).

We observed the largest CNV of 1.6 Mb in other genomic regions (e.g., a deletion at Xp22) in a PWS male which included the steroid sulfatase gene (*STS*) and also a previously reported CNV; CNV_locus 0334. We previously examined gene expression in this region in the PWS male by whole genome microarray analysis [Bittel et al., 2007b] and detected no expression of *STS* in RNA isolated from lymphoblastoid cells. However, *STS* was easily detectable in other males with no X deletion which is consistent with the gene deletion observed in the aCGH analysis.

DISCUSSION

Previous estimates of deletion size were approximately 4 Mb for the large type I deletion and approximately 3.5 Mb for the smaller type II deletion. However, our analysis using high resolution aCGH suggests that the deletion sizes can be significantly larger (e.g., 8.147 Mb) with the most common size of approximately 6.6 Mb in subjects with a type I deletion. The subjects with the largest TI deletions (e.g., breakpoint proximal to 20 Mb) included the loss of at least three genes/transcripts (i.e., *LOC283755*, *POTE5*, *OR4N4*) in addition to the four genes previously recognized between BP1 and BP2 (i.e., *GCP5*, *CYFIP1*, *NIPA1*, *NIPA2*). The possible impact (if any) of the loss of these additional genes on clinical outcome is unknown. However, studies are underway at our center to address genotype/phenotype relationships with those subjects with PWS having varying deletion size and potential altered gene expression in the 15q11–q13 region. The largest deletion in the type II subjects was 6.435 Mb and the smallest being 4.770 Mb. The BP1 breakpoint occurred at 20.220 Mb in seven of 12 subjects suggesting the presence of a sequence at this site that is prone to possible misalignment and breakage. The BP2 break site seemed to be localized at approximately 21.22 Mb but was less well defined by the aCGH analysis, perhaps suggesting a wider region of instability or fewer repetitive sequences. This is in agreement with previous observations of the duplicated structure of repeats in this region [Christian et al., 1999]. The large span seen in the location of BP3 perhaps reflects the larger span of duplcon repeats seen at the BP3 locus [Christian et al., 1999]. We observed a possible bimodal distribution of BP3 breakpoints with breakpoints centered around 26.2 and 27 Mb which is in agreement with previous observations of two breakpoints, BP3A and BP3B, at the distal breakpoint BP3 region [Wang et al., 2004]. Additionally, in a recent analysis of PWS genetic subtypes using Methylation-Specific Multiplex Ligation-Dependent Probe Amplification (MLPA), we observed the BP3 site to occur either proximal to or distal to the

APBA2 gene which further supports a bimodal distribution of BP3 in subjects with PWS having typical deletions [Bittel et al., 2007a].

The advent of new technology for analyzing CNV has led to the discovery of a surprising amount of deleted and duplicated genetic material in the human genome [Conrad et al., 2006; Khaja et al., 2006; Komura et al., 2006; Redon et al., 2006; Stranger et al., 2007]. The estimated number of CNVs in a single genome ranges from a few to as many as 100, depending on size, but the phenotypic consequences of CNV are not yet clear. We observed an average of three CNVs per individual with PWS. However, most CNVs (i.e., 40 of 47 CNVs) occurred in areas containing previously identified copy number variants without known clinical significance. Furthermore, seven CNVs in our PWS subjects occurred in chromosome regions located within or close to gene families with multiple copies of related gene sequences (e.g., HLA genes on 6p; olfactory receptor genes on 1q, 14q and 15q) which are consistent with regions containing repeated sequences being less stable and prone to rearrangement [Giglio et al., 2001]. The average size of the CNVs in our PWS subjects was 384 kb with a range of 60 kb–1.6 Mb which compares with 25 kb–3 Mb reported previously with oligonucleotide arrays [Sebat et al., 2004; Scherer et al., 2007]. In addition, a cluster of *ADAM* genes were seen at a CNV site (i.e., CNV_locus 1769) frequently observed on chromosome arm 8p.

Our interest in genotype–phenotype relationships in Prader–Willi syndrome prompted us to examine genomic CNV in a group of PWS subjects with typical deletions using high resolution CGH microarrays. We found that PWS subjects showed CNVs similar to those seen in the general population (e.g., 8p deletion/duplication). Interestingly, we observed a previously unreported deletion within 15q14 and four of our subjects had duplications of approximately 1 Mb within 15q11 in addition to the typical deletion. This duplication within 15q11 was previously reported in one of our four PWS subjects and was also found in unaffected family members (e.g., father and uncle) [Butler et al., 2002]. Segmental duplication involving 15q11 has been reported as a CNV in the general population in the Human Database of Genomic Variants (<http://projects.tcag.ca/variation/?source=hg18>). Another subject had a chromosomal deletion involving Xp22 which involved the *STS* and CNV_locus 0334. No message from the *STS* gene could be detected by whole genome expression microarray analysis of this subject but *STS* was easily detected in other subjects [Bittel et al., 2007b]. This observation underscores the potential importance of CNVs and gene expression levels. Because limited differences in genome structure have been reported in PWS, the presence of anomalies within the genome need to be considered when studying genotype/phenotype relationships as well as the size of the type I versus type II deletion among subjects with typical deletions.

We previously reported the first association of larger deletion size in PWS and poorer clinical outcomes [Butler et al., 2004] and described an association between expression levels of the four reported genes between BP1 and BP2 and aberrant behavior [Bittel et al., 2006]. Our aCGH analysis also shows larger deletions than previously identified with more variation in size. The larger deletion will obviously involve a greater number of potential genes/transcripts than will shorter deletions in the 15q11–q13 region. Therefore, a re-

examination of genotype/phenotype correlations in our PWS subject population is warranted based on the findings from our aCGH study.

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Chromosome 15q11-q13 breakpoint assignment

Fig. 1.
Typical type I and type II 15q deletion sizes and breakpoint assignments.

Fig. 2.
Representative examples of chromosome 15 comparative genomic hybridization (CGH) of individuals with Prader-Willi syndrome and type I or type II deletions using the Agilent 244 K CGH microarrays.

Fig. 3. Comparative genomic hybridization (CGH) of individuals with Prader–Willi syndrome and 15q11 duplications and typical 15q deletions identified using Agilent 244K CGH microarrays showing areas with normal copy number, deletions and duplications.

Fig. 4.

Human chromosome ideograms showing regions of copy number variation (deletion and/or duplication) observed in high resolution (244K) whole genome hybridization (aCGH) analysis of subjects with Prader-Willi syndrome (PWS). Deletions are shown on the left side of the chromosome ideogram and duplications on the right. Numbers indicate the frequency of copy number variants (CNVs) observed in PWS subjects studied. * Duplication of 15q11 observed in four PWS subjects in addition to the typical deletion.