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A Genome-Wide Screen for Copy Number Alterations in Aicardi Syndrome

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Abstract

Aicardi syndrome is a severe neurodevelopmental disorder that affects females or rarely males with a 47,XXY karyotype. Therefore it is thought to be caused by heterozygous defects in an essential X-linked gene or by defects in an autosomal gene with sex-limited expression. Because all reported cases are sporadic with one exception, traditional linkage analysis to identify the mutant gene is not possible, and the *de novo* mutation rate must be high. As an alternative approach to localize the mutant gene, we screened the DNA of 38 girls with Aicardi syndrome by high-resolution, genome-wide array comparative genomic hybridization for copy number gains and losses. We found 110 copy number variants (CNVs), 97 of which are known, presumably polymorphic, CNVs; eight have been seen before in unrelated studies in unaffected individuals. Four previously unseen CNVs on autosomes were each inherited from a healthy parent. One subject with Aicardi syndrome had a *de novo* loss of X-linked copy number in a region without known genes. Detailed analysis of this and flanking regions did not reveal CNVs or mutations in annotated genes in other affected subjects. We conclude that, in this study population of 38 subjects, Aicardi syndrome is not caused by CNVs detectable with the high-resolution array platform that was used.

Keywords

Aicardi syndrome; X-linked inheritance; array comparative genomic; hybridization; de novo mutation

INTRODUCTION

Aicardi syndrome (OMIM 304050) is characterized by developmental abnormalities that primarily affect the eyes and the brain and is associated with early-onset and often intractable seizures [Aicardi, 1999; Aicardi, 2005; Aicardi et al., 1965; Donnenfeld et al.,

1989]. Partial or complete agenesis of the corpus callosum, cerebral heterotopia, intracranial cysts and gyral abnormalities are present in many affected individuals [Aicardi 2005; Hopkins et al., 2008; Taggard and Menezes 2000]. Eye findings include multiple typical retinal lacunae, developmental anomalies of the optic nerves, microphthalmia and anophthalmia [Carney et al., 1993; Del Pero et al., 1986; Donnemfeld et al., 1989; Menezes et al., 1996]. In addition, costovertebral defects, skin abnormalities and characteristic facial features occur in some patients [Aicardi, 2005; Donnemfeld et al., 1989; Menezes et al., 1994; Sutton et al., 2005].

Although first described in 1961, the cause of Aicardi syndrome has remained elusive. This severe neurodevelopmental disorder affects almost exclusively females, and all reported cases are sporadic, except for one pair of affected sisters [Molina et al., 1989] and one pair of affected homozygous twins [Pons and Garcia, 2008]. Reported males with confirmed Aicardi syndrome all have a 47,XXY karyotype [Glasmacher et al., 2007; Hopkins et al., 1979]; male cases with a 46,XY karyotype have not been confirmed [Aicardi, 1980; Chappelw et al., 2008; Curatolo et al., 1980; Hunter, 1980]. This strongly suggests that Aicardi syndrome is caused by male-lethal or male-sparing mutations in an X-linked gene in females [Van den Veyver 2002], which is further supported by evidence of excess skewing of X-inactivation in females with Aicardi syndrome [Eble et al., 2008]. Alternatively, but less likely, would be a causal mutation in an autosomal gene with a sex-limited effect, that causes Aicardi syndrome in females but causes lethality in males, a different phenotype in males or spares males. Because no familial cases of Aicardi syndrome are available for study, traditional genetic linkage methods cannot be used to localize the mutated gene.

For many genetic disorders, a subset of affected individuals have copy number loss or gain, while others have point mutations of a particular causal gene, nonetheless each resulting in the same clinical phenotype [Lee et al., 2007; Stankiewicz and Beaudet, 2007]. Recent studies in such conditions, for example CHARGE syndrome [Vissers et al., 2004] and Goltz syndrome [Wang et al., 2007], have clearly demonstrated the benefit of array-based comparative genomic hybridization (aCGH) to find genes associated with selected syndromes. Therefore we hypothesized that in Aicardi syndrome, a region of copy number loss or gain found in one or several affected individuals might contain a gene that carries point mutations in other affected individuals. To evaluate this hypothesis, we used a genome-wide high-resolution aCGH platform to screen the DNA of girls with Aicardi syndrome for copy number changes.

METHODS

Subjects and DNA preparation

Girls with clinically confirmed Aicardi syndrome and their parents were enrolled in this study under a protocol approved by Baylor College of Medicine Institutional Review Board for Human Subject Research. Clinical information and results of laboratory testing and imaging studies pertinent to the diagnosis of Aicardi syndrome were collected and stored in a secure database. Venous blood samples were collected for DNA extraction from peripheral blood leukocytes (PBL) and for establishment of permanent lymphoblastoid cell lines (LCL). Total genomic DNA was extracted with the Puregene DNA extraction kit (Gentra Systems, Inc. Minneapolis, MN) according to the manufacturer's protocol. Reference human female or male DNA samples were prepared from de-identified single human placentas.

aCGH hybridization and data analysis

Three micrograms of genomic DNA extracted from PBL or LCL of female or male subjects and from gender-matched reference DNA were differentially labeled and co-hybridized to

Agilent human genome 244K DNA 60-mer arrays (Agilent Technologies, Inc, Santa Clara, CA) as described previously [Wang et al., 2007]. Briefly, DNA was first digested with *RsaI* and *AclI* for 2 hours at 37°C, followed by heat inactivation at 65°C for 20 minutes. The Agilent Genomic DNA Labeling Kit PLUS with random primers and the exo-Klenow fragment was used to differentially label experimental DNA with Cyanine-5 dUTP and reference DNA with Cyanine-3 dUTP fluorescently labeled nucleotides for two hours at 37°C. After heat inactivation and sample clean-up, equal amounts of experimental and gender-matched reference DNA were mixed, heat-denatured and blocked for 30 minutes at 37°C with Cot-1 DNA in blocking buffer. The mixture was applied to the whole genome 244K DNA array and hybridized in a rotating oven (20 rpm) at 65°C for 40 hours. Slides were washed and dried with Agilent's oligo-array wash buffers 1 and 2, followed by one acetonitrile wash, drying and stabilization wash. Slides were scanned on an Agilent DNA microarray scanner model G2565BA, and data extraction was performed with Agilent G2567AA Feature Extraction 9.1 software with the CGH014693 design file as the template for automated gridding and with the CGH-v4_91 protocol to assign spot-intensity values and ratios to each extraction set. Data were visualized and analyzed with Agilent's CGHanalytics software package at the following settings: 1-fold cut-off, ADM-2 aberration algorithm with threshold 10.0 and a 2 Mb moving-average window. Data were displayed as log2 ratios. Within the 2 MB moving average windows, only significant changes with 2 or more adjacent probes were called as deletion or duplication. All the coordinates for identified unreported CNVs in this study are according to UCSC hg17 (NCBI build 35), as annotated in the Agilent CGHanalytics package used for analysis of the data. For the unreported CNV found on the X chromosome, a blat alignment of the most telomeric and most centromeric deleted Agilent probes onto UCSC hg18 was done to define the deleted region in the most recent annotated version of the human genome (NCBI build 36) at the time of data analysis.

Sequence analysis

Based on the analysis of the CGH data, several regions were selected for detailed analysis. Primers were designed for amplification of a predicted putative small RNA region, all three predicted coding exons of NT_011786.159, all eight predicted exons of flanking gene NT_011786.158 and the two predicted exons of flanking gene NT_011786.160. Primer sequences, product sizes and PCR amplification conditions are listed in Supplemental Table I (online). PCR amplification was performed for 35 cycles from 50 nanograms (ng) of total genomic DNA extracted from PBL or LCL. All PCR products were purified and sequenced directly with the forward or reverse primers used for amplification. All identified sequence variants were verified with repeat sequencing in both orientations. Sequence files were compared to the reference genomic sequence derived from Mar. 2006 hg18 assembly of the UCSC human genome browser (<http://genome.ucsc.edu>) and sequencing profiles were inspected visually to detect heterozygous or mosaic changes. Identified sequence variants were named according to the Human Genome Variation Society recommendations (www.hgvs.org/mutnomen/) as described [Wang et al., 2007].

Quantitative real-time PCR

Twenty ng of genomic DNA was amplified with PCR primers designed with Primer Express software (Applied Biosystems, Foster City, CA) and are listed as Supplemental Table II (online). All reactions were done in triplicate. Each target region was amplified with the Biorad iQ5 optical system (Biorad Laboratories, Inc., Hercules, CA) for quantitative real-time PCR (qPCR) with SYBR-Green chemistry. Data were normalized to an endogenous reference gene (GAPDH) and a melting curve analysis was performed to verify PCR product specificity. Relative gene copy number was determined by the comparative threshold cycle

method ($\Delta\Delta C_t$) after standard curves with serial dilutions were obtained for each amplification reaction as described [del Gaudio et al., 2006; Wang et al., 2007].

To obtain copy number ratios and to generate copy number graphs, we calculated the ΔC_t of threshold cycles of normal female DNA [$\Delta C_t(HF)$] minus the ΔC_t of threshold cycles of the tested DNA sample from each patient (or parent), either $\Delta C_t(Pt)$, $\Delta C_t(Mo)$, or $\Delta C_t(Fa)$, and the ΔC_t of threshold cycles of normal male DNA [$\Delta C_t(HM)$] minus the ΔC_t of threshold cycle of the tested patient's (or parent's) DNA sample, either $\Delta C_t(Pt)$, $\Delta C_t(Mo)$ or $\Delta C_t(Fa)$. As controls, we also calculated the ΔC_t of threshold cycles of normal female DNA [$\Delta C_t(HF)$] minus the ΔC_t of threshold cycles of normal male DNA [$\Delta C_t(HM)$]. Ratios for each amplification product and comparisons were then calculated as $2^{\Delta\Delta C_t}$.

RESULTS

Identified copy number variants

We studied DNA samples from 38 well-characterized patients with Aicardi syndrome [Hopkins et al., 2008; Sutton et al., 2005]. A summary of the physical features of these subjects, relevant to the diagnosis of Aicardi syndrome are shown in Table I. Total genomic DNA extracted from either LCL or PBL (if available) was co-hybridized with reference DNA to human whole-genome Agilent 244K oligo arrays. We found a total of 110 CNVs among all samples (Supplemental Table I online). Ninety-seven of these were known, likely polymorphic, CNVs already annotated in the database of genomic variants (<http://projects.tcag.ca/variation>); eight had been found previously by us in control DNA samples or in unrelated aCGH experiments with the same 244K array platform. Five CNVs, four on autosomes and one on the X chromosome had never been observed before and were analyzed in more detail. A list of all known genes located in those five identified CNV regions appears as Table II.

Detected autosomal CNVs inherited from unaffected parents

We validated all the copy number changes found in this study by qPCR. Among four new CNV regions identified on autosomes, three overlapped with multiple genes and one did not overlap with any known or predicted genes (Table II; Fig. 1). For the CNV region on chromosome 7 in subject AIC38, we did not choose the *TAS2R39* gene for qPCR validation because it belongs to the taste receptor 2 family, an unlikely candidate gene for Aicardi syndrome. However, we confirmed by qPCR that there was a loss in the same CNV region of one copy of *PIP*, encoding prolactin-induced protein, and found it to be inherited from her unaffected mother (Fig. 1A). We also validated the copy number losses in affected individual AIC4 of the *OR3A3* and *OR1E2* genes (Fig. 1B) and in individual AIC2 of a deleted region without annotated known genes on chromosome 17 (Fig. 1C), each of which was inherited from an unaffected father. The region with evidence for copy number gain on chromosome 12 in AIC22 involved the genes *ACAD10*, *ALDH2* and *MAPKAPK5*. The copy number gain of these three genes could not be confirmed by qPCR (Figure 1D). The $2^{\Delta\Delta C_t}$ ratio of subject AIC22/HM was 1.60, while the $2^{\Delta\Delta C_t}$ ratio of AIC22/HF was 1.0, which is inconsistent with a heterozygous deletion. The intermediate values on the AIC22/HM qPCR, as well as the low level gain seen on aCGH, suggest that this is a polymorphic CNV region.

A 157-kilobase (kb) region of copy number loss located on Xq25 was detected in one individual with Aicardi syndrome (AIC11) (Fig. 2A, Pt). This deletion was not present by array CGH in unaffected parents (Fig. 2A, Mo, Fa) and has never been observed in 123 other unrelated array CGH hybridizations [Wang et al., 2007]. The deleted region contains

no known genes but contains one Genscan-Predicted Gene NT_011786.159 (Chr X: 126,367,719-126,582,412) (Fig. 2B). We also found a small RNA secondary structure (Chr X:126,417,442-126,417,665) predicted by the Evofold RNA secondary structure prediction algorithm [Pedersen et al., 2006] (Fig. 2B,C). The region containing this predicted small RNA was highly conserved, as demonstrated by vertebrate multiZ alignment (Fig. 2C). There were two known annotated CNVs within the 157-kb deleted region that do not overlap with the exons of the Genscan-Predicted Gene NT_011786.159 or with the predicted small RNA. The centromeric flanking region contains the Genscan-Predicted Gene NT_011786.158 and the telomeric flanking region contains the Genscan-Predicted Gene NT_011786.160 (Figure 2B). FISH with BAC RP11-81E3 located in the deleted region did not show a deletion either in AIC11 or in her parents (Fig. 2D).

To study the copy number loss further and to test efficiently additional subject and control DNAs for loss of a smaller segment containing the predicted conserved RNA, we performed qPCR with a set of primers complementary to this predicted RNA sequence. This confirmed the copy number loss in the patient's DNA. Interestingly, in contrast to the aCGH and FISH results, the qPCR analysis also showed copy number loss in the mother's DNA. The discrepant data between array, FISH and qPCR, as well as intermediate $2^{\Delta\Delta C_t}$ ratios of AIC11/HF (0.73), AIC11/HM (1.47), mother of AIC11/HF (0.68) and mother of AIC11/HM (1.37) (Fig. 2E) likely indicate that repeated sequences are present in this region.

Mutation analysis of predicted exons and small RNA in the deleted region in Xq25

We pursued an extensive analysis in and around the 157-kb deleted region in Xq25. We performed PCR amplification and direct sequencing of all three predicted coding exons of Genscan-Predicted Gene NT_011786.159 on genomic DNA of 30 girls with Aicardi syndrome who were not deleted for this region. Additional detailed bioinformatic analysis of the region revealed no other putative exons. We identified a *de novo* single nucleotide sequence (T) insertion in one affected individual (AIC22) in intron 2 of NT_011786.159, not present in her unaffected parents or in genomic DNAs from 83 unrelated male or female control individuals. Its significance is unknown but likely not related to Aicardi syndrome, as no other changes in this region were detected in other affected patients. Although the Evofold algorithm revealed over 48,000 putative functional RNAs through an exhaustive genome-wide search, it is possible that the stringent 8-genome alignment excluded a functional non-coding RNA that is largely conserved in mammals [Pedersen et al., 2006]. Furthermore, it is possible that small and regulatory non-coding RNAs that have structures other than those tested expressed from this region may play a role in this disease. We also sequenced this region on genomic DNA from 30 girls with Aicardi syndrome. We found two known single nucleotide polymorphisms (SNPs) in 8 patients, each of which was inherited from either the subject's mother or father; four of them carried both SNPs.

Because genomic deletions can disrupt distant regulatory elements and affect the expression of genes as far as 4–6 Mb from the deletion, we also amplified and sequenced the two coding exons of the flanking Genscan-Predicted Gene NT_011786.160 and all 8 coding exons of flanking Genscan-Predicted Gene NT_011786.158 on genomic DNA of 20 girls with Aicardi syndrome. We found three known SNPs in NT_011786.158 in one patient (AIC17). One was a C→T change in exon 1, which did not result in amino acid change. One was a known SNP with T→C change in intron 1 (rs.4289953). One was a known SNP with a C→G change in exon 6 (rs36076888), which result in amino acid change from *Ser*→*Cys*. Each was inherited from either the unaffected mother or father.

In conclusion, no disease-causing mutations were detected in at least 20 Aicardi syndrome subjects in all analyzed predicted genes and other sequences in and around the 157-kb deletion region on Xq25.

DISCUSSION

We performed a high-resolution genome-wide aCGH-based screen for copy number changes in DNA from 38 patients with well-characterized Aicardi syndrome in an attempt to identify a region with altered copy number that might harbor the gene mutated in this condition, but we failed to find such a region in the studied DNA samples. The search for the Aicardi syndrome gene has been complicated by the fact that the condition is rare and mostly sporadic, precluding classic methods of linkage analysis [Van den Veyver, 2002]. Aicardi syndrome is detected primarily in younger girls [Aicardi, 2005] who are typically severely affected (and none are known to have reproduced), or in males with a 47,XXY karyotype [Hopkins et al., 1979]. This suggests strongly that the mutated gene is on the X chromosome and, prior to this study, all efforts to identify the gene have focused predominantly on the X chromosome. We initially followed a candidate gene approach, based on phenotypic overlap with other conditions [Prakash et al., 1999; Schaefer et al., 1996; Schaefer et al., 1997; Van den Veyver et al., 1998] for which mapping information is known, or based on known or putative function of selected candidate genes [Van den Veyver et al., 2004]. Yilmaz and colleagues performed in-depth aCGH copy number approaches focused on the X chromosome with a X-chromosome specific BAC array, which also did not identify a candidate for the mutated gene [Yilmaz et al., 2007].

While the unique presentation of Aicardi syndrome in females or rare 47,XXY males is highly indicative for the presence of an X-linked genetic defect expressed in females and causing male lethality, we did not want to exclude the possibility that it is caused by an autosomal mutation with sex-limited expression and thus elected a genome-wide search for copy number variants by aCGH. Several arguments justify such an approach. First, although not always present in X-linked disorders [Amir and Zoghbi, 2000; Van den Veyver, 2001], especially when caused by *de novo* dominant mutations, skewing of X inactivation, although increased, is not universally present in Aicardi syndrome [Eble et al., 2008]. Indeed, previous smaller studies reached disparate conclusions, one showing increased skewing [Neidich et al., 1990], while another did not [Hoag et al., 1997]. Second, the technology for genome-wide aCGH-based screening has become both feasible and affordable. Third, such approaches have yielded identification of the mutated gene for other mostly sporadic genetic conditions [Vissers et al., 2004; Wang et al., 2007]. For example, in Goltz syndrome (Focal Dermal Hypoplasia), a numerable proportion of affected patients have a deletion, and the chance of finding the gene by this approach is high [Grzeschik et al., 2007; Wang et al., 2007]. However, for other disorders such as CHARGE syndrome, only a rare affected patient has a genomic rearrangement, and thus the chance of finding the gene by this method is relatively low [Vissers et al., 2005]. Nonetheless, the CHARGE syndrome experience exemplifies that it is feasible and that the DNA of only one or a few individuals may yield the critical information. In addition, copy number variation has been recognized recently as an important mutational mechanism for both *de novo* mutation in sporadic genetic disorders [Lupski, 2007] and for inherited disease [Estivill and Armengol, 2007; Stankiewicz and Beaudet, 2007].

Thus, we used a high-resolution genome-wide screen for copy number alterations, but the technologies for copy number detection continue to evolve, as more sensitive denser higher-resolution arrays are generated. Hence, although our study on 38 subjects yielded negative results, they do not exclude the presence of copy-number alterations in Aicardi syndrome. With the development of higher-density arrays, additional patients must be studied by this approach, as there are currently few alternative strategies to find the mutated genetic material that causes this syndrome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. autosomal copy number variants

Log2 ratio display of aCGH data on subjects indicating copy number loss (A, B and C) or gain (D) is shown on the left. Bar graphs show the $2^{\Delta\Delta CT}$ ratios of qPCR amplicons for subjects compared with normal female (HF) and normal male (HM). A $2^{\Delta\Delta CT}$ ratio of 0.5:1 for subject/HF or subject/HM, indicates the presence of only one copy in subjects (gray bars), while a $2^{\Delta\Delta CT}$ ratio of 1:1 for subject/HF or subject/HM (white bar), indicates the presence of two copies in subjects.

Figure 2. Characterization of the Xq25 deletion

(A) aCGH data. Log₂ ratio display of results on patient sample (Pt) showing a *de novo* 157-kb region of copy number loss in Xq25, not seen in the maternal (Mo) or paternal (Fa) DNA. (B) Map of deletion region in Xq25, showing the position of Genscan-predicted genes NT_011786.159, its flanking genes NT_011786.158 and NT_011786.160 (green), the Evofold predicted RNA (red) and the BAC clone RP11-81E3 used for FISH (blue). (C) Evofold-predicted RNA in the deleted region which is highly conserved. (D) Lymphocyte metaphase spreads hybridized with BAC clone RP11-81E3 (red) and a pericentromeric X chromosome control probe (green) in the affected individual (Pt), her mother (Mo) and her father (Fa). (E) Bar graphs showing the $2^{-\Delta\Delta CT}$ ratios of qPCR amplicons for patient (Pt),

her mother (Mo) and father (Fa) compared to normal female control DNA (HF) and normal male control DNA (HM). qPCR results on subjects showing the copy number loss in patient (Pt) and her mother (Mo), but not in her father (Fa).

Summary of main clinical features of research subjects

Table 1

Subject	Agensis of the Corpus Callosum	Heterotopias	Polymicrogyria	Chorioretinal Lacunae	Seizures
Aic 1	yes	present	N/A	Present	yes
Aic 2	yes	present	present	Present	yes
Aic 3	yes	absent	absent	N/A	yes
Aic 4	yes	present	present	Present	yes
Aic 5	yes	present	present	Present	yes
Aic 6	yes	present	N/A	Present	yes
Aic 7	yes	N/A	N/A	Present	yes
Aic 8	yes	present	present	Present	no
Aic 9	yes	present	present	Present	yes
Aic 10	yes	N/A	N/A	Present	yes
Aic 11	yes	present	absent	Present	yes
Aic 12	yes	absent	absent	Present	yes
Aic 13	yes	present	present	Present	yes
Aic 14	yes	present	present	Present	yes
Aic 15	yes	N/A	N/A	Present	yes
Aic 16	yes	N/A	N/A	Present	yes
Aic 17	yes	N/A	N/A	Present	yes
Aic 18	yes	N/A	N/A	Present	yes
Aic 19	yes	N/A	N/A	Present	yes
Aic 20	yes	present	N/A	Present	yes
Aic 21	yes	present	present	Present	yes
Aic 22	yes	present	absent	Present	no
Aic 23	yes	present	present	Present	yes
Aic 24	yes	absent	present	Absent	yes
Aic 25	yes	N/A	N/A	Present	yes
Aic 26	yes	present	present	Present	yes
Aic 27	yes	N/A	N/A	Present	yes
Aic 28	yes	absent	absent	Present	yes
Aic 29	yes	present	present	Present	yes

Subject	Agenesis of the Corpus Callosum	Heterotopias	Polymicrogyria	Chorioretinal Lacunae	Seizures
Aic 30	yes	present	N/A	Present	yes
Aic 31	yes	N/A	N/A	present	yes
Aic 32	yes	N/A	N/A	present	yes
Aic 33	yes	N/A	N/A	present	yes
Aic 34	yes	N/A	N/A	present	yes
Aic 35	yes	present	present	present	yes
Aic 36	yes	N/A	N/A	present	yes
Aic 37	yes	N/A	N/A	present	yes
Aic 38	yes	N/A	N/A	present	yes

Notes: N/A=information not available about this feature; Numbering of cases has been altered compared to tables in our previous publications to improve deidentification of published data on research subjects.

Table II

Detected unreported copy number changes.

Subject	Chr.	Start ^{\$}	End ^{\$}	Length (bp)	change	Annotated known genes
Aic38 [*]	7	142342680	142407505	64,825	Loss	<i>PIP, TASS2R39</i>
Aic22 [#]	12	110646841	110771649	124,808	Gain	<i>ACAD10, ALDH2, MAPKAPK5</i>
Aic2 [*]	17	73744539	73781100	36,561	Loss	No known genes annotated
Aic4 [*]	17	3252323	3282949	30,626	Loss	<i>ORIE1, OR3A3</i>
Aic11 [*]	X	126276056	126433060	157,004	Loss	No known genes annotated

^{\$}Coordinates are according to UCSC hg17 (NCBI Build35) as annotated in the Agilent CGHanalytics tool.

^{*}Classic triad of seizures, chorioretinal lacunae and agenesis of the corpus callosum;

[#]Chorioretinal lacunae, agenesis of the corpus callosum and heterotopia;