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Replication of the Association between Copy Number Variation on 8p23.1 and Autism by Using ASD-specific BAC Array

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Abstract

To discover genetic markers for autism spectrum disorder (ASD), we previously applied genome-wide BAC array comparative genomic hybridization (array-CGH) to 28 autistic patients and 62 normal controls in Korean population, and identified that chromosomal losses on 8p23.1 and on 17p11.2 are significantly associated with autism. In this study, we developed an 8.5K ASD-specific BAC array covering 27 previously reported ASD-associated CNV loci including ours and examined whether the associations would be replicated in 8 ASD patient cell lines of four different ethnic groups and 10 Korean normal controls. As a result, a CNV-loss on 8p23.1 was found to be significantly more frequent in patients regardless of ethnicity ($p < 0.0001$). This CNV region contains two coding genes, *DEFA1* and *DEFA3*, which are members of *DEFENSIN* gene family. Two other CNVs on 17p11.2 and Xp22.31 were also distributed differently between ASDs and controls, but not significant (p=0.069 and 0.092, respectively). All the other loci did not show significant association. When these evidences are considered, the association between ASD and CNV of *DEFENSIN* gene seems worthy of further exploration to elucidate the pathogenesis of ASD. Validation studies with a larger sample size will be required to verify its biological implication.

Keywords: 8p23.1, array-CGH, autism, copy number variation, defensin

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Introduction

Autism spectrum disorder (ASD) is neurodevelopmental syndrome with a broad spectrum of phenotypes including profound deficits in social relatedness and communication, repetitive behaviors, and restricted interests (Rutter, 2005). There is accumulating evidence which suggests substantial genetic contribution to ASD development (Folstein and Rosen-Sheidley, 2001; Veenstra-VanderWeele and Cook, 2004). For example, the concordance rate of autism was significantly higher in monozygotic twins than in dizygotic twins (Folstein and Rosen-Sheidley, 2001; Veenstra-VanderWeele and Cook, 2004). Although previous studies including linkage analyses have revealed a number of potential ASD-associated loci, genetic and phenotypic heterogeneity of the disease has made it difficult to replicate the results as well as to identify ASD-associated genetic mechanisms (Folstein and Rosen-Sheidley, 2001; Klauck, 2006; Veenstra-VanderWeele and Cook, 2004).

Recently a new type of human genetic variation named copy number variant (CNV) has been suggested as one of the major sources of genomic diversity ranging from normal phenotypic heterogeneity to disease susceptibility (Freeman *et al*., 2006; Iafrate *et al*., 2004; Sebat *et al*., 2004). Indeed, lines of evidence have been reported which demonstrated the association between CNV and ASD (Cho *et al*., 2009; Cook and Scherer, 2008; Glessner *et al*., 2009; Marshall *et al*., 2008; Sebat *et al*., 2007). In our previous study, we identified 38 copy number variable regions in ASD patients, two of which (CNVs on 8p23.1 and 17p11.2) were found to be significantly associated with ASD (Cho *et al*., 2009).

Using 27 CNV loci previously reported to be associated with ASD including ours, we developed an 8.5K ASD-specific BAC array and screened whether the association between those CNVs would be replicated in patient cell lines of four different ethnic groups.

Methods

Study samples

We used DNA extracted from B-lymphocyte cell lines of 8 ASD patients of four different ethnicities; Caucasian, Chinese, Asiatic Indian, and Hispanic (Coriell Institute for Medical Research, Camden, NJ, USA). General characteristics of the 8 ASD patients are shown in Table 1. As

normal control subjects, we used 10 normal Korean adults without any sign of ASD and other genetic disorders. Using the D5500A PUREGENE DNA Isolation kit (QIAGEN, Valencia, CA, USA), we extracted genomic DNA from the blood of controls according to manufacturer' s instructions. DNA quantity and quality were checked by a NanoDrop assay and 1% agarose gel electrophoresis. This study was performed under the approval of the Institutional Review Boards of The Catholic University of Korea (CUMC07U025).

Preparation of autism-specific BAC array prototype

In total, 846 BAC clones representing the 27 candidate CNV regions were selected from the DNA BAC library (Macrogen, Seoul, Korea) and the BAC clone DNA was extracted as described elsewhere (Cho *et al.*, 2009; Chung *et al.*, 2004). In brief, each BAC clone was isolated, grown in 500ml of culture media, pelleted, from which DNA was extracted using an alkaline lysis method. All selected clones were bi-directionally sequenced using the ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA), and their sequences were blasted and mapped according to their linear positions. Extracted BAC DNA was dissolved in 50% DMSO with a concentration of 400~500ng/ul and spotted in triplicate onto the glass by the Genemachines OmniGrid 100 arrayer (Digilab Genomic Solutions, Holliston, MA, USA). We used Corning UltraGAPS amine coating slides (Corning, Acton, MA, USA) and Telecam SMP4 pins (Arrayit Corporation, Sunnyvale, CA, USA) for DNA spotting. We followed processes for a general contact type spotter.

Array-CGH experiment

Array-CGH was performed as described elsewhere using the MAUI hybridization station (BioMicro Systems, Salt Lake City, Utah, USA) (Joo *et al.*, 2008). In one tube, Cy3-labeled sample and Cy5-labeled reference DNAs were mixed together and 50 μg human Cot I DNA (HybMasker, ConnectaGen, Seoul, Korea), 20 μl of 3 M sodium acetate and 600 μl of cold 100% ethanol were added to precipitate DNA. The obtained pellet was re-suspended in 40 μl of hybridization solution containing 50% formamide, 10% dextran sulfate, 2 SSC, 4% SDS and 200 ug of yeast tRNA. Hybridization was performed in slide chambers for 48 hr at 37C. After the hybridization, slides were washed serially at room temperature in solution 1 (2X saline-sodium citrate (SSC), 0.1% SDS) for 10 min (1X), in solution 2 (0.1X SSC, 0.1% SDS) for 10 min (2X) and in solution 3 (0.1X SSC) for 1 min (3X) followed by rinsing in distilled water for 10 sec. Finally, the slides were spin-dried for 2 min at 1000 rpm. All the experiments were duplicated and dye-swapping was done for more reliable interpretation.

Copy number analysis

Arrays were scanned and analyzed using the GenePix 4200A two-color fluorescent scanner (MDS Analytical Technologies, Sunnyvale, CA, USA) and the images were processed using the MacViewer 1.6 imaging software (Macrogen, Seoul, Korea). Signal intensity ratios (test/reference) were measured and converted to log2 scale. Background corrected signal intensity ratios were normalized using Lowess normalization. For defining CNVs, we set the cut-off values of signal intensity ratios at above 0.25 (log2 scale) for copy number gain and at below −0.25 for copy number loss according to our previous study (Cho *et al.*, 2009; Jeong *et al.*, 2008). We also analyzed dye-swap experiment results to get more reliable CNV call. If a CNV call is true, its test/reference intensity ratio value must be inverted by switching the dyes. Only the original ratios and inverted dye swap signals were observed, the according CNV was called as a true one.

Table 1. General characteristics of 8 ASD patients

Sample ID	Age (year)	Sex	Ethnicity	Remarks
AU10000	6	Male	Caucasian	Neurologically impaired; history of febrile seizure at age 10～14 months
AU10010	12	Female	Caucasian	-
AU10063	6	Male	Other*	Pervasive developmental disorder
AU10078	7	Female	Other*	-
AU10090	2	Male	Asiatic Indian (East Indian)	Pervasive developmental disorder
AU10114	5	Male	Asian (Chinese)	Pervasive developmental disorder
AU10115	4	Male	Asian (Chinese)	Pervasive developmental disorder
AU10184	10	Male	Hispanic/Latino	-

*Other indicates that the sample was not specified in sample overview of Coriell Cell Repositories.

Statistical Analysis

We used Stata version 10.0 (Stata Corporation, College Station, Tx) and performed the chi-square or Fisher's exact test to compare the distribution of CNV regions between cases and controls. False discovery rate (FDR) was used for multiple comparisons correction. p values less than 0.05 was considered statistically significant.

Results

Design of ASD-specific BAC array

We developed a ASD-specific BAC array named 'MacArray Karyo Autism Prototype' which covers a total of 27 CNV regions that have been reported to be associated with autism in previous studies including ours (Cho *et al*., 2009; Klauck, 2006; Schellenberg *et al*., 2006; Sultana *et al*., 2002; Vorstman *et al*., 2006). A total of 846 BAC clones representing the candidate 27 CNV regions were selected for designing ASD-specific BAC array (Fig. 1). Locus specificities of selected clones were improved by removing multiple loci-binding clones under standard fluorescence in situ hybridization (FISH). The information about those targeted 27 genomic regions and 846 BAC clones is available in Supplementary data 1.

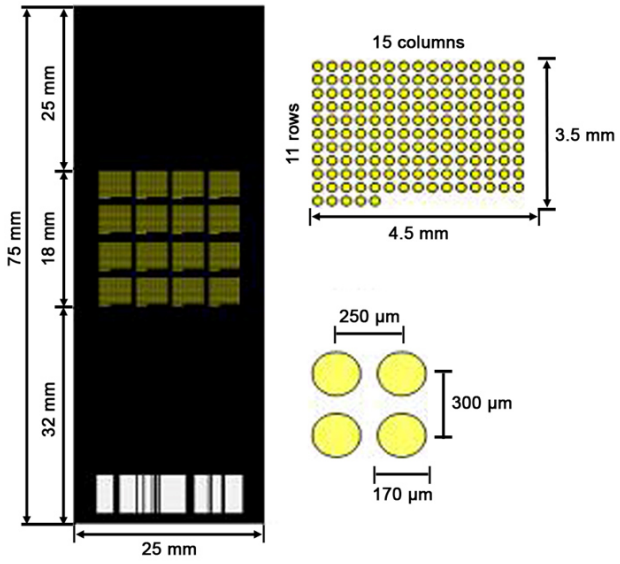

Fig. 1. MacArray Karyo Autism Prototype. We developed an ASD-specific BAC array named 'MacArray Karyo Autism Prototype' which would cover a total of 27 CNV regions that have been reported to be associated with autism in previous studies. The array is 75 by 25 mm which contains 846 spots with diameter of 0.17 mm and applied in triplicate.

CNVs distributed differently between ASDs and normal controls

Among the 846 BAC clones, 77 spots (15 loci) showed CNVs in at least 2 study participants. Details of the CNV profiles of the 8 ASDs and 10 normal controls for the 77 spots are available in supplementary data 2. Of the 77 spots, a CNV-loss on 8p23.1 (BAC168_A06) was found to be significantly more frequent in ASD patients (7 out of 8 ASD patients versus none in controls, $p < 0.0001$) (Table 2). Two other CNVs were distributed differently between patients and controls, but not significant. A CNV-loss on 17p11.2 (BAC130_G23) was found in 3 out of 8 ASD patients, but not in controls (p=0.069). A CNV-gain on Xp22.31 (BAC231_F19) was found in 4 out of 10 controls, but not in ASDs (p=0.092) (Table 2).

Validation of CNVs on 8p23.1 and 17p11.2 in ASD patients

The most significant CNV region (BAC168_A06, 8p23.1) contains two coding genes, *DEFA1* and *DEFA3*, which are members of *DEFENSIN* gene family. Interestingly, the copy number loss CNV on 8p23.1 was identified in all four different ethnic groups and also consistent in duplicated experiments. Fig. 2 shows the examples of CNVs on 8p23.1 and 17p11.2 in ASD patients, which are duplicated and dye swapped and no CNVs in normal control individuals.

Table 2. CNV regions distributed differently between ASDs and normal controls

Location of CNV	BAC clone	ASD cases (n=8)			Normal control (n=10)		
		CNV		No CNV	CNV		No CNV
		Loss	Gain		Loss	Gain	
8p23.1	BAC168_A06	7	0	1	0	3	7
17p11.2	BAC130_G23	3	0	5	0	0	10
Xp22.31	BAC231_F19	0	0	8	0	4	6

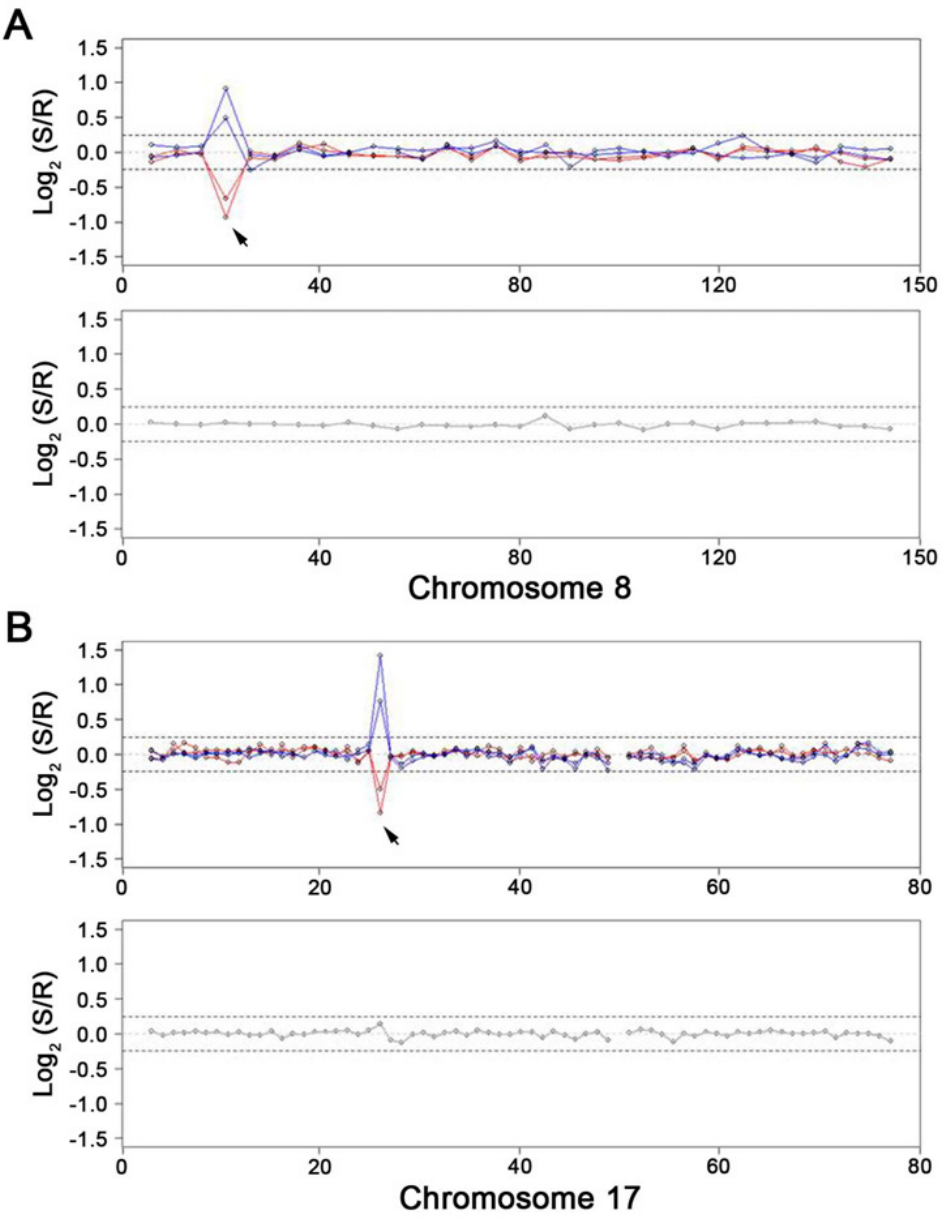

Fig. 2. Examples of copy number loss CNV on 8p23.1 and 17p11.2. (A) A copy number loss on 8q23.1 region from a patient, AU10000 (upper box) and from a normal control, LSG0001 (lower box). (B) A copy number loss on 17p11.2 region from a patient, AU10114 (upper box) and from a normal control, LSG0001 (lower box). Test and reference DNAs were labeled with Cy3 and Cy5, respectively, and cohybridized. Then, the same test and reference DNAs were labeled by switching the dyes. Red graph represents test/reference ratio, copy number loss. Blue graph represent dye swapped ratio. To get more reliable interpretation, the dye swap experiment was repeated two times. Dotted horizontal lines indicate cut-off values that determine copy number changes (0.25 for copy number gain and −0.25 for copy number loss). Arrow heads indicate the CNVs on 8p23.1 and 17p11.2.

Discussion

It has been widely accepted that ASD has a strong genetic background (Folstein and Rosen-Sheidley, 2001; Rutter, 2005; Veenstra-VanderWeele and Cook, 2004). Although genomic loci or genes reported to be associated with ASD were not easily replicated in subsequent studies for many reasons such as disease heterogeneity per se and relatively high noise levels of genomics technology used, some of the ASD-associated CNVs have been successfully replicated. For example, CNVs in *NRXN1*, *NLGN4* and *SHANK3* genes, which had been identified in target gene studies, were replicated in genome-wide CNV studies for ASD (Glessner *et al*., 2009; Marshall *et al*., 2008). Given that SNPs or other genetic markers were not very consistent in ASD, this suggests that structural variation could be a more consistent and important marker in ASD and facilitate the elucidation of genetic mechanisms behind the disease.

In our previous study, we identified 38 CNV regions in 28 Korean ASD patients and two of them (CNVs on 8p23.1 and 17p11.2) were significantly associated with ASD. In this study, we designed and prepared an ASD-specific BAC array for CNV analysis and examined

whether the significant CNV markers would be replicated in independent ASD patients of diverse ethnic origins.

The ASD-specific BAC array contains the two significant CNV regions identified in our previous study and also 25 putative ASD-associated regions reported by previous linkage studies (Cho *et al*., 2009; Klauck, 2006; Rutter, 2005; Schellenberg *et al*., 2006; Sultana *et al*., 2002; Vorstman *et al*., 2006). However, all 25 candidate regions suggested by linkage analyses were not replicated in our study population. This data seems coherent with previous observations that positive findings from one linkage study often fail to replicate in the other observation (Glessner *et al*., 2009), but it could be due to small sample size of the study.

In this study, the association of copy number loss CNV on 8p23.1 with ASD was consistently replicated in diverse ethnic groups. 8p23.1 locus is known to be a frequent region of DNA structural variation in human (Hollox *et al*., 2008a). *DEFENSIN* gene family, which is a well-known component of innate immunity, is clustered in this region (Ganz, 1999; Hollox *et al*., 2008a). CNV of *DEFENSIN* family, especially beta-defensin, has been reported as a risk factor for different diseases such as psoriasis, Crohn's disease and cystic fibrosis (Hollox, 2008b; Hollox *et al*., 2008c), which suggests that CNV of *DEFENSIN* family is likely to be associated with autoimmune diseases. Immunological dysfunction has been suggested to be associated with autism (Folstein and Rosen-Sheidley, 2001; Rutter, 2005) and the copy number loss on 8p23.1 was reported to be associated with behavioral problems or mental slowness (Pettenati *et al*., 1992). When this evidence is considered, the association between ASD and CNV of *DEFENSIN* gene seems worthy of further exploration to elucidate the pathogenesis of ASD. Validation studies with a larger sample size will be required to verify its biological implication.

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Supplementary data 1. Twenty-seven targeted regions of MacArray Karyo Autism Prototype

Cytoband	Start (bp)	End(bp)	# of BAC clones for detection
1q23.2	157300000	158800000	9
2q31.1	169500000	177700000	50
3p25.2	11500000	12400000	6
4q23	99100000	102500000	15
5p15.33	1	4400000	27
6q22.1	113900000	117100000	17
7q22.1	97900000	104400000	47
7q36.1	147500000	152200000	36
7q36.2	152200000	154700000	16
8p23.1	6200000	12700000	31
9p24.1	4600000	9000000	22
11p13	31000000	36400000	33
11q25	130300000	134452384	37
15q23	65300000	70400000	39
15q24	70400000	76100000	39
15q25	76100000	86900000	56
16p13.13	10300000	12500000	13
16q22.3	69800000	73300000	29
17p11.2	15900000	22100000	23
17q11.2	23200000	28800000	47
18q21.1	41800000	46400000	35
18q23	71300000	76117153	38
19p13.12	13800000	16100000	11
22q11.2	16300000	24300000	52
22q13.3	42600000	49691432	54
Xp22.3	1	9500000	33
Xp22.2	9500000	17100000	31
Total			846

Supplementary data 2. Copy number variations identified in ASD cases and normal controls

Chr	Clone ID	Cytoband	start (Mb)	end (Mb)	ASD (n=8)		Control (n=10)	
					Gain	Loss	Gain	Loss
7	BAC127_M11	7q22.1	98.53	98.63	0	0	0	2
7	BAC247_E05	7q22.1	100.41	100.48	0	0	0	2
7	BAC165_L12	7q22.1	101.38	101.46	0	0	0	2
8	BAC168_A06	8p23.1	6.8	6.87	0	7	3	0
8	BAC135_H20	8p23.1	7.33	7.42	1	2	1	1
8	BAC194_P23	8p23.1	8.6	8.7	0	0	3	0
8	BAC64_D05	8p23.1	11.65	11.77	0	0	3	0
11	BAC127_F16	11p13	31.37	31.45	0	0	2	0
11	BAC209_B17	11p13	32.37	32.48	0	0	3	0
11	BAC149_C22	11q25	132.76	132.84	0	0	2	0
11	BAC134_M14	11q25	133.52	133.62	0	0	3	0
15	BAC194_M03	15q24.1	70.75	70.85	0	0	0	2
17	BAC130_G23	17p11.2	21.83	21.85	0	3	0	0
17	BAC208_O06	17q11.2	23.59	23.71	0	0	0	3
18	BAC56_O16	18q23	75.84	76.01	0	0	3	0
19	BAC132_O15	19p13.12	15.97	16.1	0	0	0	2
22	BAC185_K22	22q11.21	17.65	17.73	0	0	2	0
22	BAC17_E13	22q11.22	21.33	21.44	0	2	0	0
22	BAC172_P19	22q13.31	43.97	44.05	0	0	0	3
X	BAC195_D03	Xp22.33	2.78	2.88	1	0	1	0
X	BAC32_O13	Xp22.33	2.78	2.89	1	0	2	0
X	BAC55_C05	Xp22.33	2.8	2.96	0	0	2	0
X	BAC170_E06	Xp22.33	2.89	2.98	1	0	2	0
X	BAC119_C10	Xp22.33	3.26	3.35	1	0	2	0
X	BAC172_C15	Xp22.33	4.02	4.11	0	0	2	0
X	BAC38_L03	Xp22.32	4.41	4.55	2	0	2	0
X	BAC14_K21	Xp22.32	4.61	4.71	1	0	3	0
X	BAC226_K11	Xp22.32	4.83	4.93	1	0	3	0
X	BAC165_G03	Xp22.32	5.03	5.13	0	0	2	0
X	BAC204_C10	Xp22.32	5.12	5.22	1	0	2	0
X	BAC247_D06	Xp22.32	5.63	5.72	1	0	2	0
X	BAC28_F18	Xp22.32	5.8	6.02	1	0	2	0
X	BAC37_K21	Xp22.31	6.09	6.19	1	0	3	0
X	BAC243_C15	Xp22.31	6.6	6.68	1	0	4	0
X	BAC221_A12	Xp22.31	7.09	7.19	1	0	4	0
X	BAC191_E24	Xp22.31	7.4	7.5	1	0	4	0
X	BAC231_F19	Xp22.31	7.55	7.64	0	0	4	0
X	BAC31_E12	Xp22.31	7.61	7.73	2	0	5	0
X	BAC26_J10	Xp22.31	7.86	7.96	1	0	3	0
X	BAC177_O15	Xp22.31	8.24	8.35	2	0	3	0
X	BAC185_C21	Xp22.31	8.55	8.66	1	0	2	0
X	BAC178_N11	Xp22.31	8.57	8.66	0	0	2	0
X	BAC170_I18	Xp22.31	8.58	8.66	0	0	3	0
X	BAC58_F06	Xp22.31	8.58	8.68	2	0	2	0
X	BAC175_B18	Xp22.31	8.64	8.74	1	0	2	0
X	BAC135_L05	Xp22.31	8.65	8.73	1	0	3	0
X	BAC201_I22	Xp22.31	8.94	9.02	0	0	2	1
X	BAC180_P24	Xp22.31	9.1	9.23	2	0	3	0
X	BAC252_L05	Xp22.2	9.52	9.65	1	0	3	0
X	BAC153_J20	Xp22.2	9.78	9.81	1	0	1	0
X	BAC224_E17	Xp22.2	9.8	9.88	2	0	3	0
X	BAC143_P20	Xp22.2	10.21	10.3	1	0	2	0
X	BAC165_B04	Xp22.2	10.43	10.53	2	0	2	0
X	BAC181_H09	Xp22.2	10.65	10.75	1	0	3	0

Supplementary data 2. Contined

Chr	Clone ID	Cytoband	start (Mb)	end (Mb)	ASD (n=8)		Control (n=10)	
					Gain	Loss	Gain	Loss
X	BAC121_N22	Xp22.2	11.13	11.21	2	0	2	0
X	BAC166_K19	Xp22.2	11.21	11.3	1	0	2	0
X	BAC247_L21	Xp22.2	11.4	11.47	1	0	2	0
X	BAC151_D12	Xp22.2	11.54	11.62	1	0	3	0
X	BAC51_J21	Xp22.2	11.85	11.99	1	0	2	0
X	BAC31_M18	Xp22.2	12.17	12.25	2	0	3	0
X	BAC81_D07	Xp22.2	12.43	12.53	1	0	2	0
X	BAC194_L05	Xp22.2	12.63	12.73	2	0	2	0
X	BAC162_J15	Xp22.2	12.81	12.93	1	0	2	0
X	BAC72_G18	Xp22.2	12.87	12.95	1	0	2	0
X	BAC172_E18	Xp22.2	13.32	13.42	2	0	3	0
X	BAC161_F20	Xp22.2	13.46	13.57	1	0	2	0
X	BAC19_P08	Xp22.2	13.56	13.66	0	0	3	0
X	BAC236_M15	Xp22.2	14.43	14.52	1	0	3	0
X	BAC33_D22	Xp22.2	14.76	14.85	1	0	3	0
X	BAC107_A08	Xp22.2	15.39	15.54	2	0	3	0
X	BAC39_M09	Xp22.2	15.43	15.52	2	0	3	0
X	BAC122_M06	Xp22.2	15.59	15.68	1	0	3	0
X	BAC244_J12	Xp22.2	15.66	15.73	1	0	2	0
X	BAC184_J09	Xp22.2	16.29	16.37	1	0	3	0
X	BAC31_O22	Xp22.2	16.56	16.65	1	0	2	0
X	BAC230_P12	Xp22.2	16.89	17	1	0	2	0
X	BAC155_G06	Xp22.2	17	17.09	1	0	2	0