Published in final edited form as:
Am J Med Genet B Neuropsychiatr Genet. 2010 June 5; 153B(4): 960–966. doi:10.1002/ajmg.b.31055.

A Co-segregating Microduplication of Chromosome 15q11.2 Pinpoints Two Risk Genes for Autism Spectrum Disorder

Bert van der Zwaag[1], **Wouter G Staal**[2], **Ron Hochstenbach**[3], **Martin Poot**[3], **Henk A Spierenburg**[1], **Maretha V de Jonge**[2], **Nienke E Verbeek**[3], **R. van 't Slot**[4], **Michael A van Es**[5], **Frank J Staal**[6], **Christine M Freitag**[7], **Jacobine E Buizer-Voskamp**[4,8], **Marcel R Nelen**[3], **Leonard H van den Berg**[5], **Hans K Ploos van Amstel**[3], **Herman van Engeland**[2], and **J Peter H Burbach**[1]

[1] Rudolf Magnus Institute of Neuroscience, Department of Neuroscience and Pharmacology, UMC Utrecht, Utrecht, the Netherlands [2] Rudolf Magnus Institute of Neuroscience, Department of Child and Adolescent Psychiatry, UMC Utrecht, Utrecht, the Netherlands [3] Department of Medical Genetics, UMC Utrecht, the Netherlands [4] Complex Genetics Section, Department of Biomedical Genetics, UMC Utrecht, Utrecht, the Netherlands [5] Rudolf Magnus Institute of Neuroscience, Department of Neurology, UMC Utrecht, Utrecht, the Netherlands [6] Department of Immunology, Erasmus MC, Rotterdam, the Netherlands [7] Department of Child and Adolescent Psychiatry, Saarland University Hospital, Homburg, Germany [8] Department of Psychiatry, Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht, Utrecht, the Netherlands

Abstract

High resolution genomic copy-number analysis has shown that inherited and *de novo* copy-number variations contribute significantly to autism pathology, and that identification of small chromosomal aberrations related to autism will expedite the discovery of risk genes involved. Here, we report a microduplication of chromosome 15q11.2, spanning only four genes, co-segregating with autism in a Dutch pedigree, identified by SNP microarray analysis, and independently confirmed by FISH and MLPA analysis. Quantitative RT-PCR analysis revealed over 70 % increase in peripheral blood mRNA levels for the four genes present in the duplicated region in patients, and RNA *in situ* hybridization on mouse embryonic and adult brain sections revealed that two of the four genes, *CYFIP1* and *NIPA1*, were highly expressed in the developing mouse brain. These findings point towards a contribution of microduplications at chromosome 15q11.2 to autism, and highlight *CYFIP1* and *NIPA1* as autism risk genes functioning in axonogenesis and synaptogenesis. Thereby, these findings further implicate defects in dosage-sensitive molecular control of neuronal connectivity in autism. However, the prevalence of this microduplication in patient samples was statistically not significantly different from control samples (0.94% in patients vs 0.42% controls, p=0.247), which suggests that our findings should be interpreted with caution and indicates the need for studies that include large numbers of control subjects to ascertain the impact of these changes on a population scale.

Keywords

autism spectrum disorder; genetics; copy-number; gene-dosage; gene expression

Corresponding author: J. Peter H. Burbach, Universiteitsweg 100, 3584 CG, Utrecht, The Netherlands. Tel: +31-(0)88-7568810, Fax: +31-(0)88-7569032, J.P.H.Burbach@UMCUtrecht.nl.

Autism spectrum disorders (ASD) are common neurodevelopmental disorders characterized by three core symptoms: impairment in reciprocal social interactions, communicative deficits, and repetitive and restricted patterns of behavior and interests. Relatively mild ASD cases exist, but most patients display severe symptomatology with a high prevalence of mental retardation and epilepsy. Whereas familial and twin studies have indicated a major role for genetics in the etiology of ASD only a few causal factors have been identified [Persico and Bourgeron, 2006]. Recently, a relatively large proportion of ASD cases were shown to carry genomic aberrations, and attention has focused on these copy-number variations (CNVs) as risk factors for developing ASD [Marshall et al., 2008; Morrow et al., 2008; Sebat et al., 2007]. The single most consistently and frequently reported genomic aberration associated with ASD is duplication at chromosome 15q11-q13 [Vorstman et al., 2006]. This region harbors a number of low-copy repeats serving as genomic breakpoints, making it prone to chromosomal rearrangement [Pujana et al., 2002]. Interestingly, the ASD-associated 15q11-q13 duplications largely overlap with deletions that cause Prader-Willi/Angelman syndrome (PWS/AS), in which ASD are often a co-morbidity [Veltman et al., 2005]. These data suggest that one or several genes from this region are involved in dosage-sensitive pathways that, when unbalanced, can lead to ASD. Recently, several submicroscopic aberrations in this genomic region on chromosome 15 have been associated with ASD or clinical features related to ASD. Murthy et al. reported a familial deletion at chr15q11.2 associated with neurological disorder and speech impairment [Murthy et al., 2007]. Miller et al. associated recurrent microdeletions and duplications of chromosome 15q13.2-q13.3 with a clinical picture including features of ASD and other neuropsychiatric disorders [Miller et al., 2008], and multiple cases with microdeletions of 15q11.2 with varying behavioral problems were described by Doornbos et al. [Doornbos et al., 2009]. Finally, a recent paper shows that relatively large genomic gains are significantly more prevalent in patients with disorders such as autism and intellectual disability than in control subjects. (Itsara et al., 2009)

Here we report the identification of a co-segregating submicroscopic chromosome 15q11.2 duplication in a Dutch pedigree with ASD, and show by expression analysis of the genes contained in the duplicated region that *CYFIP1 may be a* candidate for involvement in the ASD phenotype in this family.

The female proband (Figure 1a, subject 3) met the criteria for an ASD diagnosis, and was initially diagnosed with PDD-NOS. Her combined IQ was 83 (verbal IQ = 89, performance IQ = 81) on the Wechsler Intelligence Scale for Children-Revised (WISC-R) [Wechsler, 1974]. Her younger twin siblings did not meet the criteria for an ASD. Her mother did not have a psychiatric disorder, her father was diagnosed with Asperger syndrome and he also scored above the cut-off score on the Autism spectrum Quotient (AQ) [Baron-Cohen et al., 2001]. Two additional children of the father, both from a prior relationship, were also included (Figure 1a). Subject 7 was highly suspect for ASD, but a formal diagnosis of ASD could not be ascertained in this subject, as she has deceased since the start of this study, and the full clinical tests were not yet completed. Access to a report generated previously by a neurologist was provided, stating that at the age of nine years old she displayed general developmental delay, limited speech production, clumsiness, and a tendency to taste and lick many different objects. Apart from psychiatric screening, all cases were screened for associated medical conditions (such as fragile X syndrome, chromosomal abnormalities on karyogram, serious illnesses or severe perinatal events) and neurological conditions (such as seizures). No medical or neurological abnormalities were detected in any of the subjects, neither were dysmorphologies detected. Informed consent was obtained from all subjects.

We assessed segmental aneuploidies, including CNVs, in the ASD pedigree and to control for copy-number polymorphisms in 267 ethnically matched, adult Dutch (at least in second

degree) healthy individuals using the HumanHap300 Genotyping Beadchip and Beadstudio V2.3.41 software (Illumina Inc., USA) as described [Peiffer et al., 2006]. In the female proband a total of six chromosomal gains or losses were identified (Table I), of which only a duplication at 15q11.2, spanning approx. 471kb, was not observed in the 267 controls. Routine FISH analysis in the proband using a region specific probe (RP11-80H14) confirmed the duplication in the proband (Figure 1b). The identical duplication was also found in the affected father and the ASD-suspect half-sister; none of the other family members had this specific chromosomal gain (Table I). Multiplex ligation-dependent probe amplification (MLPA) analysis of the 15q11-q13 region, using the Salsa MS-MLPA kit ME028 (MRC Holland, the Netherlands) and Genemarker software (SoftGenetics, USA), independently confirmed the presence of the duplication in the three affected subjects, and did not detect a difference in methylation status between duplication and non-duplication carriers (Figure 1, and data not shown). Analysis of 678 additional, psychiatrically unaffected, healthy adult Dutch controls, genotyped on the same platform, identified 4 identical duplications of 15q11.2. As such the prevalence in the total control group (n=945) is 0.42% versus 0.95% in our patient sample (1 duplication in 105 total ASD patients screened). In the Autism Genetic Resource Exchange (AGRE) Consortium dataset, recently made available to the research community, 7 out of 744 ASD patients with duplication of the 15q11.2 region were identified, yielding a prevalence of 0.94%, comparable to our primary finding. The difference in prevalence of totals did not reach statistical significance (p=0.247, 8 out of 849 in ASD patients versus 4 out of 945 in controls; Fischer's exact test, two tailed). These data suggest non-penetrance of the 15q11.2 duplication in some individuals, which has previously also been observed for large 15q11-q13 duplications (Bolton et al., 2001; Itsara et al., 2009). However, the study by Itsara et al. on more than 5000 subjects showed that the large 15q11-q13 duplication occurred significantly more frequent in patients when compared to healthy controls.

To see whether transcript levels of the four RefSeq genes contained in the duplicated region (*TUBGCP5*, *CYFIP1*, *NIPA1*, and *NIPA2*) (Figure 1c) were altered in the affected subjects of our pedigree, we performed real-time quantitative RT-PCR. For this we used whole blood derived total RNA isolated with the PAXgene blood RNA system (PreAnalytix, Switzerland)) and the QuantiTect SYBR Green RT-PCR Kit and Primer Assays (Qiagen Inc., USA) in a LightCycler 2.0 Real-Time PCR System (Roche Diagnostics, Switzerland). *GAPDH* and *β-ACTIN* were used as reference genes for the calculation of relative transcript quantities using qBASE analyzer [Hellemans et al., 2007]. Significantly elevated transcript levels (> 70 % increase) were present in duplication carrying subjects for all four genes (Table II). These findings are in line with the reported strong correlation between reduced transcript levels of these genes and ASD-related endophenotypes in distinct PWS/AS cases [Bittel et al., 2006]

In order to further dissect the genes in the duplication that may operate during brain development we performed transcript-specific RNA *in situ* hybridization on serially cut embryonic and adult mouse brain sections. Transcript specific Digoxigenin-labeled cRNA probes were generated (probe nucleotide positions available on request), hybridized to the sections, and images were recorded as described before [van der Zwaag et al., 2005]. From embryonic day (E) 12.5 onwards, both *Cyfip1* and *Nipa1* were broadly expressed in the brain (Figure 2). At later developmental stages and in the adult brain expression became restricted to distinct but still overlapping patterns. Overlapping expression domains were identified in the cerebral cortex, hippocampus, and cerebellum. Transcripts of *Nipa2* and *Tubgcp5* were not detectable in embryonic or adult brain (two different probes used per gene, data not shown).

There are arguments of gene function in favor of *CYFIP1* and *NIPA1* being autism candidate genes. Both *CYFIP1* and *NIPA1* are implicated in axonal growth, neuronal connectivity, and neuronal morphology [Schenck et al., 2003; Wang et al., 2007]. CYFIP1 directly interacts with FMRP, the protein encoded by *FMR1*, which is involved in dendritic mRNA transport and translational regulation [Schenck et al., 2004; Zalfa et al., 2006]. Repeat expansions in the *FMR1* gene preventing appropriate FMRP synthesis, have been shown to lead to alterations in the expression levels of *CYFIP1*, which may explain the ASD phenotype often observed in Fragile-X syndrome (FXR) patients [Nowicki et al., 2007]. Genome-wide expression profiling in ASD patients further strengthens the link between *FMR1*/*CYFIP1* and ASD, as changes in shared *FMR1* or *CYFIP1* downstream pathways were identified in both FXR patients with ASD and ASD patients with 15q11-q13 duplications [Nishimura et al., 2007]. In addition, RNAi knockdown experiments have shown that CYFIP1 plays an important role in control of neuronal connectivity through actin remodeling mediated by the WAVE/SCAR complex, downstream of Rac1 [Kunda et al., 2003]. Missense mutations in *NIPA1* may cause autosomal dominant spastic paraplegia (MIM600363) [Rainier et al., 2003], a syndrome in which the peripheral nervous system is affected. ASD is not a co-morbid feature in this syndrome; and neither do any of the patients in our pedigree show clinical symptoms resembling spastic paraplegia. Thus gene-dosage alterations of *NIPA1* are less likely to contribute to ASD, but cannot be completely excluded as yet.

Taken together, we have characterized a submicroscopic genomic aberration of chromosome 15q11.2, that may co-segregate with ASD in a Dutch pedigree. *CYFIP1* is the best candidate gene in this region for a causal role in ASD as it has previously been shown to act in a dosage-sensitive molecular pathway controlling neuronal network formation and maintenance, its expression pattern suggests involvement in establishing and maintaining brain structures necessary for cognitive processing, and a robust dosage effect in gene expression was observed in patients. Together with other genes that have been linked to more severe forms of ASD with mental retardation, particularly *SHANK3*, *FMR1*, and *NLNG3/NLNG4* [Abrahams and Geschwind D H, 2008], and recent evidence for the involvement of neuronal activity responsive genes and glycosylation enzymes in ASD [Morrow et al., 2008; van der Zwaag et al., 2009], our data suggest that axonogenesis and synaptogenesis are main biological pathways affected in ASD. Furthermore, in a recent large study in schizophrenia patients deletions of the exact same region on chromosome 15 were found to be significantly associated with schizophrenia [Stefansson et al., 2008]. Although the direct genetic changes are opposite, the functional studies mentioned above have shown that lower as well as higher levels of *CYFIP1* expression result in a similar phenotypical outcome. Thereby we can add yet another genetic locus to the growing number of overlapping susceptibility regions between ASD and schizophrenia, suggesting that these disorders may be caused by genetic alterations in shared biological mechanisms and pathways [Burbach and van der Zwaag, 2008].

However, our data should be interpreted with caution. In the study reported here, the difference in prevalence of the 15q11.2 microduplication between patients and controls did not reach statistical significance ($p = 0.247$). As such it remains under debate to what extent the observed 15q11.2 microduplication is a risk factor for autism, and if other confounding factors are involved. Clearly, investigation of much larger cohorts of ASD patients and control subjects will be required to address this issue.

Acknowledgments

We thank the ASD family and healthy control individuals for their participation in this study. B. van der Zwaag was supported by the ‘Breedtestrategie’ program and the Prestigous Master Neuroscience and Cognition of the Utrecht University. Part of this research was funded by the Hersenstichting (Netherlands Foundation for Brain Research), grant #2008(1).43 to M. Poot. We gratefully acknowledge the resources provided by the Autism Genetic Resource

Exchange (AGRE) Consortium and the participating AGRE families. The Autism Genetic Resource Exchange is a program of Autism Speaks and is supported, in part, by grant 1U24MH081810 from the National Institute of Mental Health to Clara M. Lajonchere (PI).”

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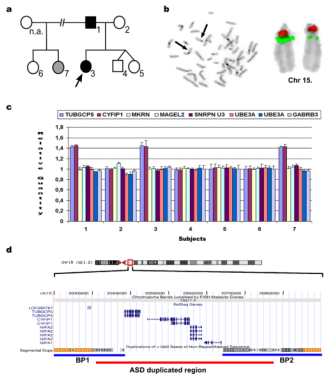

Figure 1.

Genetic analysis of ASD pedigree with submicroscopic duplication of chromosome 15q11.2. (a) ASD pedigree. The arrow indicates the proband, affected subjects are indicated in black. Subject 1.7 was highly suspect for ASD, full clinical diagnosis was not possible (gray circle, see text). n.a.: no DNA available. (b) FISH results on metaphase spread of subject 3 (arrows indicate the separate chromosomes 15), magnification shows a clearly more intense signal on one of the two chromosomes 15 for subject 3 (red = centromere probe D15Z1, green = RP11-80H14). (c) MLPA analysis results using a 15q11-q13 region specific MS-MLPA kit. Subjects 1, 3, and 7 show a marked increase in the probe signals for TUBGCP and CYFIP1, indicative of a duplication of these probes. (d) RefSeq gene content of the 15q11.2 region (20.20–20.90 Mb, UCSC Genome Browser, May 2006 freeze). Approximate positions of flanking low-copy repeat regions are indicated by a blue bar, segmental duplications in the breakpoint regions are shown above the blue lines. The region duplicated in the ASD pedigree is indicated by a red bar. BP: breakpoint region.

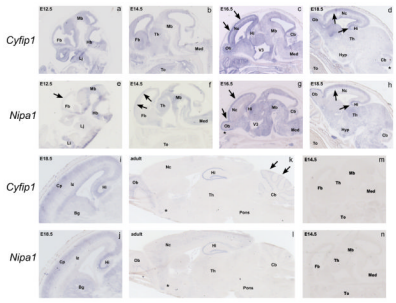

Figure 2.

RNA *in situ* hybridization of *Cyfip1* and *Nipa1* probes on adjacent embryonic and adult mouse brain sections. (a-d, i, k): *Cyfip1* antisense probe, (e-h, j, l): *Nipa1* antisense probe. (a-c) *Cyfip1* expression was detected throughout the brain during early brain development. From E16.5 onwards, the cortical plate (including the hippocampus) showed a more intense staining compared to surrounding tissues (arrows in c). (d) At E18.5, *Cyfip1* expression has become restricted to the forebrain, particularly to the cortical plate of the neocortex and hippocampus (arrows). A low level of expression was also observed in the external granular layer of the cerebellum (asterix). (e) At E12.5, *Nipa1* was expressed throughout the embryonic brain, with exception of the forebrain (arrow). (f-h) Expression of *Nipa1* in the midbrain, and more posterior brain regions (including the spinal cord) was maintained throughout embryogenesis, and from E14.5 onwards, expression of *Nipa1* was also observed in the outer cell layer of the olfactory bulb and the cortical plate of the neocortex (arrows in f, g, h). (i,j) Adjacent E18.5 sections of the forebrain show overlapping expression domains for *Cyfip1* and *Nipa1* in the cortical plate of the neocortex and in the hippocampus. (k) In adult brain, *Cyfip1* was expressed in the external plexiform layer of the olfactory bulb, and in the neuronal layers of the hippocampus, and the cerebellum (arrows). A small subset of neurons in the piriform cortex also shows expression of *Cyfip1* (asterix). (l) *Nipa1* is widely expressed in the adult brain, expression was observed in the olfactory bulb, the cerebral cortex (including the piriform cortex, asterix), the neuronal cell layer of the hippocampus, and several other brain nuclei (not shown). (m,n). Sense probed sections for *Cyfip1* and *Nipa1* did not show significant staining. Magnification: a-h, k-n: 12.5 X and i,j: 25 X. Abbreviations: Bg: basal ganglia, Cb: cerebellum, Cp: cortical plate, Fb: forebrain, Hb: hindbrain, Hi: hippocampus, Hyp: hypothalamus, Iz: internediate zone, Li: liver, Lj: lower jaw, Mb: midbrain, Med: medulla oblongata, Nc: neocortex, Ob: olfactory bulb, Th: thalamus, To: tongue, V3: third ventricle.

Table I

CNVs in the ASD pedigree

Begin and end base pair (bp) numbers represent genomic positions of the first and last SNP deviating from the normal (SNP position according to NCBI build35). Family subjects numbered as in Figure 1, affected subjects in bold. The final column lists the number of overlapping CNVs observed in 267 ethnically matched healthy controls. Het.: heterozygous.

CNV type	Cytoband	Begin (bp)	End (bp)	Size (kb)	Subject	Controls
Het. Loss	3p12.3	75511365	75650909	139.5	**1**, **3**, 4, 5,	29
Het. Loss	5q14.3	91116560	91208460	91.9	2	0
Het. Loss	6p12.3	22371817	22378099	6.2	**7**	35
Het. Loss	6q12	67075448	67104015	28.6	**1**, **3**, 4, 5,	35
Het. Loss	6q13	75788804	75854022	65.2	2	0
Het. Gain	7q11.21	61487774	62143104	655.3	**1**, 4, 5	5
Het. Loss	7q21.2	91821291	91918187	96.9	2	0
Het. Loss	8q11.21	50317691	50428283	110.6	2	0
Het. Gain	9p24.1	5296824	5325470	28.6	2, **3**, 4, 5,	3
Het. Gain	10p13	16023051	16045847	22.8	2	0
Het. Gain	10q11.22	47013328	47173619	160.3	6	26
Het. Gain	10q26.3	135155270	135246120	90.9	**1**, **3**, 4, **7**	10
Het. Gain	12p13.31	7891603	8014573	123	**7**	17
Het. Gain	12p12.3	19360345	19420335	60.0	**1**, **3, 7**	10
Het. Gain	14q13.3	36515241	35544401	29.2	2, 4, 5	0
Het. Gain	15q11.2	20306549	20777695	471.1	**1**, **3, 7**	0
Het. Loss	16p13.11	15032942	15039475	6.5	2, 4, 5	1
Het. Gain	18q22.1	63805807	64547842	742.0	2, 4, 6	0
Het. Loss	22q11.23	24012122	24234365	222.2	**1**, 5	10
Het. Loss	Xp21.1	33659102	33780813	121.7	2, 4, 5	0

Table II

Real-time quantitative RT-PCR analysis of 15q11.2 candidate gene mRNA levels in subjects from the ASD pedigree

Individual results from qBASE analyzer were combined to form a copy-number neutral (Group N = 2, subjects S2, S4, S5, S6), and duplication carrier group (Group N = 3, subjects S1, S3, S7). *GAPDH* and *β-ACTIN* were used as reference genes, and showed no significant difference in relative transcript quantity between the two groups. Relative transcript quantities were calculated using the results of the non-affected mother for normalization. Errors indicated are measured as the standard error of the mean (SEM). The final column shows average percent change in transcript levels for duplication carriers compared to non-affected family members.

Gene	S1	S2	S3	S4	S5	S6	S7	Group N=2	Group N=3	% change
GAPDH	1.22 ± 0.04	1.00 ± 0.08	1.14 ± 0.05	1.64 ± 0.10	1.35 ± 0.07	1.20 ± 0.05	1.14 ± 0.10	1.29 ± 0.08	1.17± 0.07	− 9.7 %
β-ACTIN	0.82 ± 0.02	1.00 ± 0.06	0.88 ± 0.03	0.61 ± 0.07	0.76 ± 0.03	0.83 ± 0.02	0.88 ± 0.05	0.80 ± 0.05	0.86 ± 0.03	+ 7.5 %
TUBGCP5	3.13 ± 0.19	1.00 ± 0.04	1.74 ± 0.09	1.95 ± 0.18	1.59 ± 0.07	1.64 ± 0.05	3.51 ± 0.12	1.54 ± 0.09	2.79 ± 0.13	+ 81, 0 %
CYFIP1	2.23 ± 0.13	1.00 ± 0.08	1.60 ± 0.11	1.03 ± 0.13	1.35 ± 0.10	1.20 ± 0.05	2.03 ± 0.08	1.14 ± 0.09	1.95 ± 0.10	+ 70, 5 %
NIPA2	2.38 ± 0.19	1.00 ± 0.05	2.21 ± 0.10	1.39 ± 0.14	1.37 ± 0.13	1.41 ± 0.09	2.26 ± 0.11	1.29 ± 0.10	2.28 ± 0.13	+ 76,8 %
NIPA1	2.50 ± 0.32	1.00 ± 0.08	2.45 ± 0.10	1.54 ± 0.35	1.62 ± 0.34	1.57 ± 0.16	2.89 ± 0.11	1.43 ± 0.23	2.61 ± 0.18	+ 82, 1 %