Published in final edited form as:

Hum Genet. 2015 June ; 134(6): 569–575. doi:10.1007/s00439-015-1537-6.

Replicated linear association between DUF1220 copy number and severity of social impairment in autism

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Abstract

Sequences encoding DUF1220 protein domains exhibit an exceptional human-specific increase in copy number and have been associated with several phenotypes related to brain size. Autism is a highly heritable and heterogeneous condition characterized behaviorally by social and communicative impairments, and increased repetitive and stereotyped behavior. Given the accelerated brain growth pattern observed in many individuals with autism, and the association between DUF1220 subtype CON1 copy number and brain size, we previously investigated associations between CON1 copy number and autism-related symptoms. We determined that CON1 copy number increase is associated with increasing severity of all three behavioral features of autism. The present study sought to replicate these findings in an independent population (N = 144). Our results demonstrate a replication of the linear relationship between CON1 copy number and the severity of social impairment in individuals with autism as measured by Autism Diagnostic Interview – Revised Social Diagnostic Score, such that with each additional copy of CON1 Social Diagnostic Score increased 0.24 points (se = 0.11, p = 0.036). We also identified an analogous trend between CON1 copy number and Communicative Diagnostic Score, but did not replicate the relationship between CON1 copy number and Repetitive Behavior Diagnostic Score. Interestingly, these associations appear to be most pronounced in multiplex children. These results, representing the first replication of a gene dosage relationship with the severity of a primary symptom of autism, lend further support to the possibility that human brain evolution and autism severity may involve the same protein domain family.

Introduction

Autism Spectrum Disorder as classified by the DSM-5 represents a group of developmental disorders featuring behavioral deficits in social and communicative measures, and increased repetitive behaviors and stereotyped interests. Children and adults with autism display a broad heterogeneity of symptom severity and manifestation (Levy et al. 2009) which,

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considering the strong genetic component of the condition, suggests a complex and varied etiologic mechanism (Folstein and Rosen-Sheidley 2001; Geschwind 2011).

A subset of children with autism exhibit more rapid brain growth, a larger head circumference, and greater neuron number than their typically developing peers (Courchesne et al. 2003; Courchesne et al. 2005; Courchesne et al. 2011). These findings suggest that some cases of autism involve atypical brain growth trajectories that may result from mechanisms leading to excessively dense neuronal structures. It has been suggested that the macrocephalic and behavioral phenotypes are a direct result of this overly dense pattern of neurons which may bias connective architecture to favor short-distance and impair long-distance connectivity, a critical mix for social functioning (Courchesne and Pierce 2005; Just et al. 2012).

Head circumference has similarly been implicated in degree of impairment in affected individuals, such that increased head circumference is associated with increasing symptom severity (Deutsch and Joseph 2003; Lainhart et al. 2006; Davis et al. 2013; Chaste et al. 2013). However, the opposite association has also been found (Dementieva et al. 2005), which suggests a varying etiologic relationship with brain growth. Considering the vast clinical heterogeneity of autism and that much of the phenotype is based on clinical review and parent report, it is not surprising that association studies of biological indicators, such as head circumference or various genetic markers, may identify different associations among different autistic populations.

Copy number of sequences encoding DUF1220 domains has previously been implicated in brain size, exhibiting a dosage-dependent positive relationship (Dumas et al. 2012). Given the association between excessive brain growth and autism, and between brain size and symptom severity, it is therefore plausible that DUF1220 domain copy number variation may play a role in the symptoms of autism. Further, sequences encoding DUF1220 domains are almost exclusively embedded in the *NBPF* gene family in the 1q21.1 region of the genome (Vandepoele et al 2005; Popesco et al 2006; O'Bleness et al 2012a; O'Bleness et al 2012b). Large scale duplications in this region that contain DUF1220 domains have been repeatedly connected to autism, macrocephaly and other neurodevelopmental phenotypes (Mefford et al. 2008; Brunetti-Pierri et al. 2008; Dumas and Sikela 2009; Crespi and Crofts 2012; Dumas et al. 2012; O'Bleness et al. 2014; Keeney et al. 2014b). Genome wide association studies, meanwhile, have implicated the 1q21 region in autism yet have not been able to identify a causative gene (Ylisaukko-oja et al. 2004; Goodbourn et al. 2014).

DUF1220 domains are uniquely and exceptionally expanded in the human lineage such that copy number in the genome increases with increasing phylogenetic proximity to humans (Popesco et al. 2006; O'Bleness et al. 2012a). The domains can be grouped into 6 different subtypes based on sequence similarity: conserved (CON) clades 1, 2 and 3 and human lineage specific (HLS) clades 1, 2, and 3 (O'Bleness et al. 2012a). Unlike many copy number variable regions in the genome, DUF1220 domains are coding regions that display a Gaussian distribution in the human population, which suggests they may confer a broad and continuous range of variation within the phenotype(s) they affect (Davis et al. 2014a). Increased DUF1220 CON1 copy number has been associated with increased gray matter

volume in healthy individuals (Dumas et al. 2012), incrementally increased CON2 copy number has been associated with improved cognition (Davis et al. 2014b), and increased DUF1220 expression has been implicated in promoting neuronal stem cell proliferation (Keeney et al. 2014a).

Given these findings, we hypothesized in previous work that CON1 would be associated with symptoms of autism and would exhibit a similar dosage effect. We found that increased copy number of CON1 had a linear dose-response relationship with the symptoms of autism, such that with increased copy number of the CON1 subtype of DUF1220, social, communicative and repetitive behavior phenotypes each became incrementally more severe (Davis et al. 2014a). This study sought to replicate those findings in an independent sample and explore associations between HLS1 and autism-related characteristics.

Results

In the population of 166 non-Hispanic white individuals with autism, CON1 copy number ranged from 54 to 78 diploid copies, and HLS1 copy number from 125 to 257 diploid copies. As in the previous study, copy number of each clade followed a Gaussian distribution (Table 1). CON1 copy number also exhibited a similar range to that found in the previous population (56 to 88 copies, (Davis et al. 2014a). In this population ADI-R scores are correlated with each other such that Social and Communication Scores ($r=0.65$), Social and Repetitive Behavior Scores ($r=0.36$) and Communication and Repetitive Behavior Scores ($r=0.39$) co-vary. Copy numbers of CON1 and HLS 1 are also mildly correlated ($r=0.24$).

We identified an association between increased copies of CON1 and increased Autism Diagnostic Interview – Revised (ADI-R) Social Diagnostic Score ($p=0.036$), much as we found in the original investigation (Table 2). With each additional copy of CON1, Social Diagnostic Score increased 0.24 points ($SE = 0.11$), becoming progressively more severe. We also identified trends between increased CON1 copies and both increased ADI-R Verbal Communicative Diagnostic Score and decreased Vineland Adaptive Behavior Scales (VABS) Social Adaptive Score, results similar to the initial investigation (Table 2). The association originally identified between CON1 and repetitive behaviors in the previous study was not replicated here.

We also investigated potential associations between HLS1 copy number and autism-related symptoms. HLS domains represent an attractive candidate for influencing neurologic symptoms as they account for much of the unique and unparalleled expansion of DUF1220 copy number between humans and non-human primates. Approximately 149 copies of HLS-type DUF1220 domain sequences have been added to the human genome since divergence from chimpanzee (O’Bleness et al. 2014). In the autistic population examined we identified protective associations between increased HLS1 copy number and decreased severity of autism-like symptoms. Increased copy number of HLS1 was associated with improved Social Adaptive Score as measured from the VABS ($p=0.02$), improved ADI-R Verbal Communicative Diagnostic Score ($p=0.046$), and a progressively increased IQ in children younger than 10.6 ($n=80$) as measured by the Raven Matrices ($p=0.02$)(Table 2). These associations were not identified in the original population, which may be due to

characteristics of this study's group that are subtly different than those found in the original study population (discussed further below).

Discussion

DUF1220 CON1 Subtype and Autism Symptoms

This report presents a replication of our previous study demonstrating a linear association between CON1 copy number and progressively more impaired Social Diagnostic Score as measured by the ADI-R. This association, implicating DUF1220 dosage in the severity of a primary autism phenotype, has now been validated in two independent populations of individuals with autism... It is particularly important to note that the magnitudes of the effects (beta values) of CON1 are remarkably similar across social and communicative metrics despite the exceptional heterogeneity of the condition. Even though CON1 approached but did not reach statistical significance in the communication analysis or the VABS-based social analysis, the fact that the beta values were highly similar, with similar standard errors (Table 2), suggests that with a larger study these associations may also be significant. Given these highly similar effects the presented replicated findings lend credibility to the hypothesis that DUF1220 CON1 copy number may modulate social symptoms in autistic individuals. Additionally, the social ability measured in these studies is heavily reliant on parent report. Although the ADI-R is a clinical gold standard, continued improvement of social metrics will refine the assessment of the condition and precise associations between social functioning and CON1 copy number may be further clarified. Future investigations of CON1 in exceptionally well-phenotyped populations with additional social measures such as the Social Response Score or a latent social phenotype may also further elucidate the relationship between CON1 and autism-related social symptoms. The association previously reported between CON1 copy number and severity of repetitive behaviors, however, did not replicate. This may indicate a spurious result in the initial analysis or may indicate that there are important differences, perhaps unmeasured, between these populations that affect the association between CON1 and repetitive behavior symptom severity.

Autism is remarkably heterogeneous and different covariates were important in these investigations with CON1. This may suggest that these populations are subtly different. Importantly, after adjustment the effect of CON1 is independent of other predictors of symptoms of the condition and further implicates CON1 as having a specific role in autism-related social ability. Further, our previous investigation suggested that CON1 effects are most important in multiplex individuals, and this investigation demonstrated the same association. These findings suggest that the role of CON1 may be most important in relatively higher functioning individuals with autism. Many previous CNV-related genomic studies, meanwhile, have focused on rare, larger scale events in individuals with simplex autism (Sebat et al. 2007; O'Roak et al. 2012; Girirajan et al. 2013). In these simplex populations as many as 10% of cases can be explained by rare CNVs (Sebat et al. 2007). Simplex individuals, however, may not be exclusively representative of the autistic population, as they frequently exhibit greater prevalence of intellectual disability, dimorphism (Miles et al. 2005) and cognitive impairment relative to multiplex individuals

(Davis et al. 2013). Our findings suggest a possible role for common variation of CON1 copy number in multiplex children and, as such, point to a promising novel direction in autism research. Expanding genomic efforts to investigate multiplex children and their families, as unaffected family members have shared subclinical traits (Virkud et al. 2009; Constantino et al. 2010), could identify important associations between CON1 and social phenotypes. Expanding genomic research beyond simplex-based investigations is also important given that a large portion of children with autism have IQs above 70, a feature not as common in simplex children, (Charman et al. 2011) and sibling recurrence could be more common than previously reported (Constantino et al. 2010).

DUF1220 HLS1 Subtype and Autism Symptoms

Interestingly, we identified a protective association between HLS1 copy number and autism symptoms, such that increased copies of HLS1 were associated with improved symptoms. These findings were not seen in the original population and remain to be replicated. The lack of replication could be due to different population characteristics between the studies and the sample size available. The original work identified the most pronounced CON1 social effect in multiplex individuals. Stratification in that analysis reduced the effective sample size by half giving 85 multiplex individuals for multiplex-specific analyses. This would have decreased power to detect lesser effects of HLS1 that may be most pronounced in multiplex individuals as well. This study aimed to replicate our original findings by examining a larger sample of mostly (96%) multiplex children, and found a protective relationship between HLS1 copy number increase and both autism communication symptoms and Raven's Progressive Matrices (RPM) IQ. The cognitive findings are consistent with previous analyses of DUF1220 in typically developing youths, which demonstrated that increases in copy number of CON2 were significantly associated with increased Wechsler Intelligence Scale for Children (WISC) IQ in males under 11 (Davis et al. 2014a). Future investigations with larger samples of multiplex children should be informative in testing the replicability of our findings regarding HLS1 copy number and autism-related symptoms. Such investigations may also elucidate the nature of the protective associations with HLS1, as these findings suggest an association with IQ may play a mediating role. As IQ adjustments did not change the beta observed with the HLS1 VABS Social Standard Score analysis, there appears to be a complex relationship in which HLS1 and IQ are associated but also independently affect autism related symptoms. It should also be noted that this sample of individuals had IQ measured from the RPM. Future research in this area should strongly consider the RPM for cognitive research as IQ in autistic children may have been historically underestimated (Dawson et al. 2007) and precise phenotypic measures should improve our understanding of the role HLS1 and IQ may play in other autism related symptoms.

Conclusion

Unraveling the exact nature of these relationships will require both more precise phenotyping and more thorough investigations of the nature and localization of DUF1220 copy number change within the genome. ddPCR, the assay tool utilized in this study, produces a global genomic estimate of copy number but is unable to localize copy number changes to specific *NBPF* genes. This is especially important given that CON1 and HLS1

subtypes are found on numerous NBPF genes that are interspersed among many non-NBPF genes in the 1q21 region. It is plausible that copy number alteration of certain CON1 and HLS1 copies may be contributing to autism etiology, while changes in other CON1 and HLS1 copies do not. Knowledge of such localizations may inform our understanding of DUF1220 associations with autism etiology, particularly in light of the fact that there are no apparent differences in global DUF1220 copy number between individuals with autism and those typically developing (Davis et al. 2014a). Further, the fact that the relationship between CON1 and communication score was nearly significant after adjusting for HLS1 suggests a complex relationship between DUF1220 domain subtype dosage variations and autism related symptoms, and perhaps etiology. Additionally, although copy number differences were not detected between individuals with autism and unrelated peers (Davis et al. 2014a), this does not preclude possible differences that may exist within affected families. As technologies improve, the identification of gene-specific and domain-specific variations could shed light on any causal mechanisms DUF1220 domains may have on autism.

The findings presented demonstrate a significant association between DUF1220 subtype copy number and autism severity. Specifically, HLS1 copy number appears to exhibit a protective association with Communication Diagnostic Score, VABS Social Score, and Raven Matrices IQ. CON1 copy number increase, meanwhile, exhibits a significant linear association with increased severity of Social Diagnostic Score in autism, and a trending association with increased severity of Communication Diagnostic Score. To our knowledge, this represents the first replication of a gene dosage relationship with the severity of a primary symptom of autism. This is an important and unique effect given the broad range of severity observed in individuals with autism and reinforces our suggestion that copy number polymorphic gene sequences such as DUF1220 may be important and underexamined contributors to complex genetic conditions (Davis et al. 2014a). This is also the second of only two reports exploring the relationship between the dosage of a highly copy number polymorphic sequence with the heterogeneity of the autism condition. In genetic studies of autism the condition is frequently classified dichotomously (ie; autism or unaffected) when identifying risk factors, even though the variation of the phenotype within autism is substantial and well documented. This classification may be an oversimplification and studies directly addressing the heterogeneity of the condition are warranted. Despite the limitations of imperfect measurements common in complex conditions, the findings presented here provide additional incentive, and novel direction, to further explore the role of DUF1220 domains in the severity and etiology of autism.

Methods

166 non-Hispanic white, primarily multiplex individuals with autism or autism spectrum as classified by the Autism Diagnostic Observations Schedule (ADOS), and with Vineland Adaptive Behavior Scales (VABS) social standardized scores above 20 were selected for replication studies. 144 of these individuals had IQ scores available. The Autism Diagnostic Observation Schedule was used only as an enrollment mechanism due to nontrivial severity measures based from the ADOS. Children diagnosed with autism or autism spectrum disorder were included. Multiplex individuals were preferentially selected due to previous

evidence suggesting the most pronounced effects of DUF1220 CON1 copy number would be identifiable in this group (Davis et al. 2014a). Non-Hispanic white individuals were selected to control for population stratification.

Droplet digital PCR (ddPCR) a third generation PCR technique was used to assay CON1 and HLS1 copy number as described in (O'Bleness et al. 2014). Primer sequences were as follows: CON1 Left 'AATGTGCCATCACTTGTTC AAATAG', Right – 'GACTTTGTCTTCCTCAAATGTGATTTT', Hyb – 'CATGGCCCTTATGACTCCAACCAGCC'; HLS1 Left – 'GCTGTTCAAGACAAGTGAAGGA', Right - 'GGGAGCTGCTGGAGGTAGT', Hyb – 'AGAGCCTGAAGTCTTGCAGGACTCAC'; reference RPP30 Left – 'GATTTGGACCTGCGAGCG', Right – 'GCGGCTGTCTCCACAAGT', Hyb – 'TTCTGACCTGAAGGCTCTGCGC'. Standard deviation of measurements of CON1 varied from 0.57 copies to 3.4 copies with one sample at 7.6 per triplicate. HLS1 standard deviation ranged from 3.1 to 28. Importantly, repeated measurements were conducted in triplicate and the mean value was utilized to maximize accuracy as discussed in exposure measurement approaches (White et al. 2008).

Autism symptom scores were taken from the Autism Diagnostic Interview – Revised (ADI-R), the VABS, and the Raven Progressive Matrixes (RPM). All DNA was obtained from cell lines from the Rutgers branch of the Autism Genetic Resource Exchange (Lajonchere 2010). All DNA assays were conducted in a blinded randomized manner to guard against differential misclassification. Randomization leading to non-differential misclassification of exposure is a highly important step in this work, and suggests that results presented are conservative estimates (White et al. 2008).

Statistical analysis

Assays were conducted on 96 well plates and despite high reproducibility ($r > 0.9$) variability from plate to plate exists, such that subtle correlations exist within plates and variability exists between plates. To accommodate this and to more precisely calculate standard errors and beta estimates, we utilized mixed models with maximum likelihood estimation with a random intercept for plate similar to previous work (Yu et al. 2008; Zhang et al. 2013). Sample was nested within plate to account for the experimental design in all regression analyses. Akaike Information Criterion suggested a marked improved model fit in HLS1 cognitive analyses and was uninformative in CON1 analyses. Covariates sex, age, head circumference, HLS1, and RPM IQ were explored in all models. Reduced models, including CON1, were developed through backwards selection. Covariates that remained in the ADI-R Social Score analysis were CON1 ($p=0.036$) and IQ ($p=0.02$). CON1 ($p=0.07$), and HLS1 ($p=0.046$) remained in the ADI-R communication analysis. The VABS analysis included CON1 ($p=0.11$), HLS1 ($p=0.02$), IQ ($p=0.005$), and age ($p<0.001$). Even though CON1 did not universally meet a strict alpha of less than 0.05 it was left in models to explore trending effects (Table 2). IQ investigations explored effects stratified by mean age group (<10.6) due to previous findings that cognitive effects of DUF1220 may be most pronounced in males younger than 11 (Davis et al. 2014b). HLS1 was the only significant covariate in the IQ analyses. As this is an independent replication study and the outcomes are correlated with

one another ($r=0.36 - 0.65$), corrections that assume independence are excessively conservative. As such we did not make corrections for multiple comparisons in the analyses conducted. R version 3.0.2 (<http://cran.r-project.org/>) with the nlme package (Bates et al. 2011) was used for analyses.

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Table 1

Full Population Characteristics (n=166)

Characteristic	Proportion of Total	1 st quartile	mean	median	3 rd quartile
Sex (Male)	79.8%				
Multiplex	96.4%				
Age		7.8	10.6	9.9	12.5
ADOS Classification (Autism, Autism Spectrum)	69.1%, 31.0%				
ADI-R Social Diagnostic Score		16.0	20.2	21.0	25.0
ADI-R Verbal Communication Diagnostic Score		13.0	16.4	17.0	20.0
ADI-R Repetitive Behaviors Diagnostic Score		4.0	6.1	6.0	8.0
VABS Social Standard Score		53.0	64.1	64.0	74.0
Raven Matrices IQ (n=144)		93.2	100	100	110
CON1 Copy Number		63	66	66	69
HLS1 Copy Number		185	198	196	209

Table 2

Adjusted CON1 Social Diagnostic Score Replication

CON1 associations	beta	SE	p-value
ADI-R Social Diagnostic Score, Study1 *	0.25	0.11	0.021
ADI-R Social Diagnostic Score, Study2	0.24	0.11	0.036
ADI-R Verbal Communication Diagnostic Score, Study 1 *	0.18	0.08	0.030
ADI-R Verbal Communication Diagnostic Score, Study 2	0.16	0.09	0.072
ADI-R Repetitive Behavior Diagnostic Score, Study 1 *	0.10	0.05	0.047
ADI-R Repetitive Behavior Diagnostic Score, Study 2	0.00	0.05	1.0
VABS Social Standard Score, Study 1 *	−0.43	0.23	0.056
VABS Social Standard Score, Study 2	−0.38	0.26	0.143

*
(Davis et al 2014a)

Table 3

Adjusted HLS1 Associations from the Current Study

HLS1 associations	beta	SE	p-value
ADI-R Verbal Communication Diagnostic Score	−0.04	0.02	0.046
VABS Social Standard Score	0.14	0.06	0.023
Raven Matrices IQ in children younger than 10.6 years	0.19	0.08	0.025