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A family with autism and rare copy number variants disrupting the Duchenne/Becker muscular dystrophy gene *DMD* and *TRPM3*

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Abstract Autism spectrum disorder is a genetically complex and clinically heterogeneous neurodevelopmental disorder. A recent study by the Autism Genome Project (AGP) used 1M single-nucleotide polymorphism arrays to show that rare genic copy number variants (CNVs), possibly acting in tandem, play a significant role in the genetic aetiology of this condition. In this study, we describe the phenotypic and genomic characterisation of a multiplex autism family from the AGP study that was found to harbour a duplication of exons 31–44 of the Duchenne/Becker muscular dystrophy gene *DMD* and also a rare deletion involving exons 1–9 of *TRPM3*. Further characterisation of these extremely rare CNVs was carried out using quantitative PCR, fluorescent in situ hybridisation, long-range PCR amplification and sequencing of junction fragments. The maternal chrX:32,097,213-32,321,945 tandem duplication and paternal chr9:72,480,413-73,064,196 deletion (NCBI build 36 coordinates) were transmitted to both affected boys, potentially signifying a multi-hit mechanism. The *DMD* reading frame rule predicts a Becker phenotype, characterised by later onset and milder symptoms. When last evaluated, neither child had developed signs of muscular dystrophy. These data are consistent with a degree of comorbidity between autism and muscular dystrophy and suggest that genomic background as well as the position of the mutation within the *DMD* gene may impact on the neurological correlates of Duchenne/Becker muscular dystrophy. Finally, communicating unexpected findings such as these back to families raises a number of ethical questions, which are discussed.

Keywords Autism · Becker · Duchenne · CNV · Comorbid · DMD

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Introduction

Autism is a highly heritable and heterogeneous neurodevelopmental condition affecting approximately 0.2% of the general population (Fombonne 2009). Whilst a number of rare, highly penetrant genetic variants have been reported, a large proportion of the condition's heritability remains unaccounted for (Abrahams and Geschwind 2008). A recent high-resolution genome scan carried out by the Autism Genome Project consortium (AGP) comprising almost 1,000 individuals with autism spectrum disorder (ASD) suggests that rare genic copy number variants (CNVs) play a significant role in the aetiology of this

condition (Pinto et al. 2010). Interestingly, more than 0.6% of affected individuals had two or more de novo CNVs (Pinto et al. 2010), a figure consistent with an earlier single-nucleotide polymorphism (SNP) array study comprising 427 ASD cases (Marshall et al. 2008). Another recent study detected an increased frequency of "second-hit" CNVs in individuals with developmental delay and deletion of 16p12.1 (Girirajan et al. 2009). A third study found two rare CNVs involving *CNTNAP5* and *DOCK4* (inherited from the father and mother, respectively), each transmitted to both members of an autistic sibling pair (Pagnamenta et al. 2010). These studies highlight the emerging evidence for multiple rare genomic variants combining in the same individual resulting in a particular phenotype (Cook and Scherer 2008).

Recent studies show that the incidence of autistic traits in both Duchenne (DMD) and Becker muscular dystrophy (BMD) cohorts is higher than observed in the general population. In the largest study, 11 of 351 (3.1%) boys with DMD were reported to have ASD, according to parental questionnaires (Hendriksen and Vles 2008). In another study, direct screening of 85 boys with DMD or BMD revealed 16 (19%) meeting the criteria for ASD (Hinton et al. 2009). In a smaller study focussing on the milder BMD phenotype, 2 of 24 (8.3%) were described as having autism (Young et al. 2008). About 20% of patients with DMD have intellectual disability, whereas the frequency is lower (~10%) in those with BMD (Young et al. 2008; Emery and Muntoni 2003). Data suggest that the location of the mutation within the dystrophin gene (*DMD*) may impact on the risk of cognitive impairment. One such analysis showed that for individuals with mutations in exons 46–79, the IQ was an average of 17 points lower compared to individuals with mutations proximal to exon 45 (Taylor et al. 2010). Thus, mutations at the 3′ end of *DMD*, which disrupt all dystrophin products, including the shorter brain-expressed isoforms Dp140 and Dp71, are more likely to result in cognitive impairment (Taylor et al. 2010; Daoud et al. 2009). To better determine the nature of this comorbidity and understand why only a proportion of cases have social and/or cognitive impairments, it is important to describe additional autism/learning disability pedigrees harbouring *DMD* mutations and provide full details not only of the *DMD* genotype and phenotype but also of the genomic background and potential environmental risk factors. The difficulty of establishing a true increase in the risk for neurological outcomes when studying a group of children with identifying developmental syndromes is also worth noting, given the inherent challenges in blinding and the use of standardised diagnostic instruments which still contain some variability in their use between sites and assessors.

A recent genome-wide scan CNVs carried out by the AGP (Pinto et al. 2010) found that three individuals from 996 ASD families harboured exonic CNVs in *DMD* compared to a general population incidence of DMD and BMD in males of 1 in 3,800 and 1 in 18,000, respectively (Mostacciuolo et al. 1987). In addition to an ~225-kb duplication within *DMD*, one of these individuals and his affected brother carried a second rare deletion of ~580 kb on chromosome 9q21, potentially contributing to their autism phenotype. In this study, this family is described in more detail, with a combination of molecular methods being used to characterise both of these CNVs at base pair resolution.

Materials and methods

Family details

The family is from the IMGSAC cohort described previously (IMGSAC 2001) and consists of father (3019.1), mother (3019.2), two affected brothers (3019.3 and 3019.5) and an unaffected sister (3019.4). Both affected individuals were classified with narrowly defined autism using the Autism Diagnostic Interview-Revised and Autism Diagnostic Observation Schedule (ADOS) instruments (Rutter et al. 2003; Lord et al. 2001). Karyotype and *FMR1* testing gave negative results. Genomic DNA obtained from blood was available for all members of the pedigree. The family provided informed consent and the study approved by the Oxfordshire Psychiatric Research Ethics Committee A and locally by the Institute of Psychiatry Ethical Research Committee.

Clinical details for 3019.3 (proband)

Participant 3019.3 was born by spontaneous vaginal delivery after a normal pregnancy, weighing 3,140 g and with head circumference in the 10–25 centile. No abnormalities were seen at birth and he had an Apgar score of 9. Language development was delayed, with first words at 30 months and short phrases at 42 months. He sat unaided at 9 months, walked at 13 months and achieved toilet training after age 4.5. However, his medical notes show that there was a developmental disorder of language, with a diagnosis of articulation dyspraxia. He was noted to be clumsy and have poor coordination (although his handwriting appears to be fine), with odd head movements and frequently getting his body into strange contortions. A school report (age 5.5) noted that the proband's gross motor control was very poor and that his legs did not bend. His walking and running abilities were akin to those of a much younger child.

Obsessional interests were noted from age 2, and he did not play normally with toys or with other children. At

5 years of age, he responded well to speech therapy in all areas except articulation. He was an active child liking a lot of stimulation, but was afraid of crowds, preferring to remain indoors with his mother, and had temper tantrums daily. Autistic features were described as being clearest until the age of 4; however, he still met criteria for autism when assessed with the ADOS, aged 20 years and 10 months. His verbal and performance IQ, measured with the Wechsler Preschool and Primary Scale of Intelligence at age 5 years and 8 months, was 87 and 76, respectively. However, his verbal IQ was measured again using the British Picture Vocabulary Scale (BPVS) at age 22 years and 7 months and was 130.

Clinical details for 3019.5

Participant 3019.5 (younger brother of 3019.3) was born by spontaneous vaginal delivery after a normal pregnancy, weighing 3,380 g and with head circumference in the 10–25 centile. No abnormalities were seen at birth. First words were at 12 months, with first phrases delayed at 60 months. He sat unaided at 8 months and walked at 17 months; toilet training was not fully achieved at age 8 when he was seen for diagnostic assessment. Clinical notes describe a general developmental delay, and as an adult, he has moderate learning difficulties. His verbal IQ was measured using the BPVS aged 11 years and 2 months and was 62. Our existing medical notes do not indicate any specific problems with his motor development.

Quantitative PCR

Quantitative PCR (qPCR) experiments were performed using iQ™ SYBR® Green Supermix (BioRad) following the manufacturer's protocol. qPCR primers were designed using Primer3 (Electronic supplementary material (ESM) Table 1 and Fig. 1a). Thermocycling conditions are given in the Electronic supplementary material. Relative copy number was determined using the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001).

Long-range PCR and sequencing of junction fragments

Long-range PCR was performed for the *DMD* and *TRPM3* CNVs using the BIO-X-ACT long DNA polymerase (Bioline) and the SequalPrepTm Long PCR kits (Invitrogen), respectively. Reactions were performed according to the manufacturers' instructions, with thermocycling conditions listed in the Electronic supplementary material. Primers for the *DMD* duplication used combinations of qPCR primers. Those for *TRPM3* were designed using Primer3 (ESM Table 1 and Fig. 1). PCR products were purified using Exonuclease I (NEB, Ipswich, MA) and shrimp alkaline phosphatase (USB, Cleveland, OH). Sanger sequencing was by BigDye v3.1 chemistry (Applied Biosystems, Foster City, CA).

Sequencing *TRPM3* and microRNA MIR204

Primer sequences (ESM Table 2) and PCR conditions are listed in the Electronic supplementary material. PCR purification and sequencing were performed as described above.

Fluorescence in situ hybridisation

Fluorescence in situ hybridisation (FISH) was performed on chromosomes obtained from EBV-transformed B-lymphoblastoid cell lines. For the *DMD* duplication, a BAC (RP11-168F15) and a fosmid (G248P86682a6) that map within the CNV were chosen as probes (ESM Fig. 1a). For the *TRPM3* deletion, two BAC clones were used: RP11-89K20 within the CNV and RP11-240L5 outside it (ESM Fig. 1b). More details are available in the Electronic supplementary material.

Results

Exonic CNVs in *DMD* from a recent AGP study

In a recent genome-wide scan for CNVs performed by the AGP using Illumina 1M SNP arrays, three male probands from 996 ASD families harboured exonic CNVs involving *DMD* (Pinto et al. 2010). In one male proband, a 29.4-kb deletion removed exon 48 of *DMD*. This leaves the reading frame intact, but is predicted to remove 62 amino acids. This variant would thus be expected to have a relatively minor effect on the structure of the 3,685 residue protein and was not detected in the affected brother. In another affected boy from a multiplex family, a 381.6-kb duplication affected the 5′ end of the gene, including the start codon of the full-length isoform. It is difficult to predict what effect this mutation might have on *DMD* dosage or protein structure and no DNA was available from the affected brother to test segregation. Here, we focus on the third family (3019), which harbours an ~225-kb duplication involving the central portion of the gene and for which DNA was available from the affected brother for further testing.

Fine-mapping and segregation of *DMD* duplication in family 3019

In this third family, a maternally inherited duplication of Xp21.1 was detected in subject 3019.3 during a large

genome-wide scan for CNVs (Pinto et al. 2010). After manual inspection of the log R ratios and B allele frequencies in BeadStudio, the maximal and minimal duplicated region was determined to be from rs16990134 to rs5972556 and from rs1795588 to rs1555256, respectively (ESM Fig. 1a). At the proximal end, this resolution was not sufficient to determine which combination of exons had been duplicated. Therefore, qPCR was used to validate and resolve the mutation further. These data showed that the duplication involved exons 31–44 (Fig. 1a) and so is predicted to leave the reading frame intact. These qPCR results also indicated that the affected brother (3019.5) had inherited the same mutation from the mother, whilst the unaffected sister did not carry this variant (Fig. 1a).

A rare CNV disrupting *TRPM3* in the same family

Previous studies suggest that mutations at the 3′ end of *DMD*, disrupting the shorter brain-expressed isoforms (Dp140 and Dp71), are more likely to result in cognitive impairment (Taylor et al. 2010). As the mutation in family 3019 involved the middle section of the gene, we considered other potential contributory factors for the neurological phenotype. We therefore examined the genomic background, looking for other rare CNVs (present at <1% frequency and >500 kb in size) in this family, and identified a partial deletion of *TRPM3* on chromosome 9q21, from Illumina 1M data (Pinto et al. 2010). SNP array data showed that this CNV was present in the proband (3019.3) and inherited paternally. Manual inspection of the genotyping data determined the maximal and minimal duplicated region to be from rs1011308 to rs10781006 and from rs1317604 to rs17056622, respectively (ESM Fig. 1b).

The CNV was validated experimentally by long-range PCR using primers designed to flank the predicted breakpoints (ESM Table 1 and Fig. 1b). This confirmed the presence of the CNV in both the father and proband, as well as both siblings (Fig. 1b).

The segregation pattern seen for both rare CNVs in this family (Fig. 1c) was consistent with haplotype flow determined from data generated in a previous linkage study (Szatmari et al. 2007) and could suggest a multiple-hit mechanism whereby both rearrangements contribute to the autism phenotype.

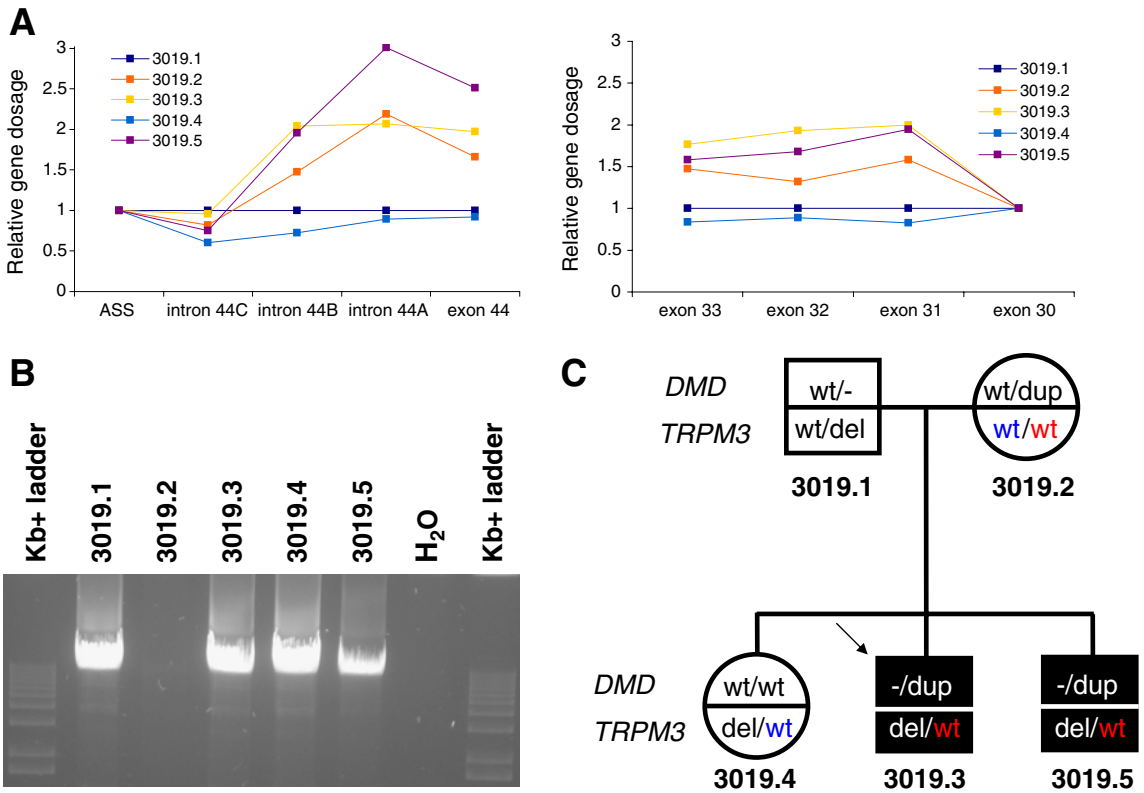

Fig. 1 Segregation of two rare CNVs in family 3019. **a** qPCR results for fine mapping distal and proximal ends of the *DMD* duplication. Results are normalised to a qPCR assay from outside the duplicated region and then compared to the father for whom the 1M SNP array indicated did not have the duplication. *ASS* alternative start site. **b** Agarose gel showing long-range PCR products. Presence of the *TRPM3* deletion is indicated by an ~8-kb fragment. **c** Pedigree summarising CNV status. *Shaded symbols* correspond to autism. *Blue* and *red fonts* indicate most likely maternal *TRPM3* haplotype flow, as determined using Merlin analysis of 10K SNP data. Birth order has been switched to ensure family anonymity

Sequence analysis of *TRPM3* and microRNA MIR204

Haplotype flow also indicated that the non-deleted (maternal) version of *TRPM3* and the microRNA MIR204 was shared identical-by-descent in the two affected sons (Fig. 1c), and so we considered whether the deletion might be unmasking a rare point mutation in *trans*. However, Sanger sequencing of all 25 *TRPM3* exons and intron/exon boundaries uncovered only one non-synonymous variant (rs6560142) on this shared chromosome, situated near the 3′-untranslated region and reported to have an average heterozygosity of 0.449 ± 0.152. There were also no rare variants found in MIR204.

Further characterisation of both CNVs

Although the SNP array and qPCR confirmed that exons 31–44 of *DMD* were duplicated, they could not distinguish whether the extra segment of DNA was situated in tandem with the normal copy or at a different genomic locus. Fibre FISH using BAC and fosmid probes confirmed that the duplication was in a direct tandem repeat orientation (Fig. 2a) and therefore that the functional copy of *DMD* was disrupted.

In order to validate the *TRPM3* deletion by an independent method, additional interphase and metaphase FISH was performed for the proband, with chromosome spreads from the mother used as an undeleted control. These results confirmed the presence of the 9q21 deletion in the proband (Fig. 2b).

Finally, to determine whether these two rare CNVs may have been mediated by repetitive elements, we mapped the breakpoints at base pair resolution by sequencing the long-range PCR amplicons. For the *DMD* duplication, the sequencing electropherogram further confirmed that the tandem duplication was in a direct orientation and resolved the genomic location as chrX:32,097,213-32,321,945, inclusive (NCBI build 36; Fig. 2a, c), with a size of 224,733 bp. Sequencing the *TRPM3* junction fragment amplicon using an internal sequencing primer determined

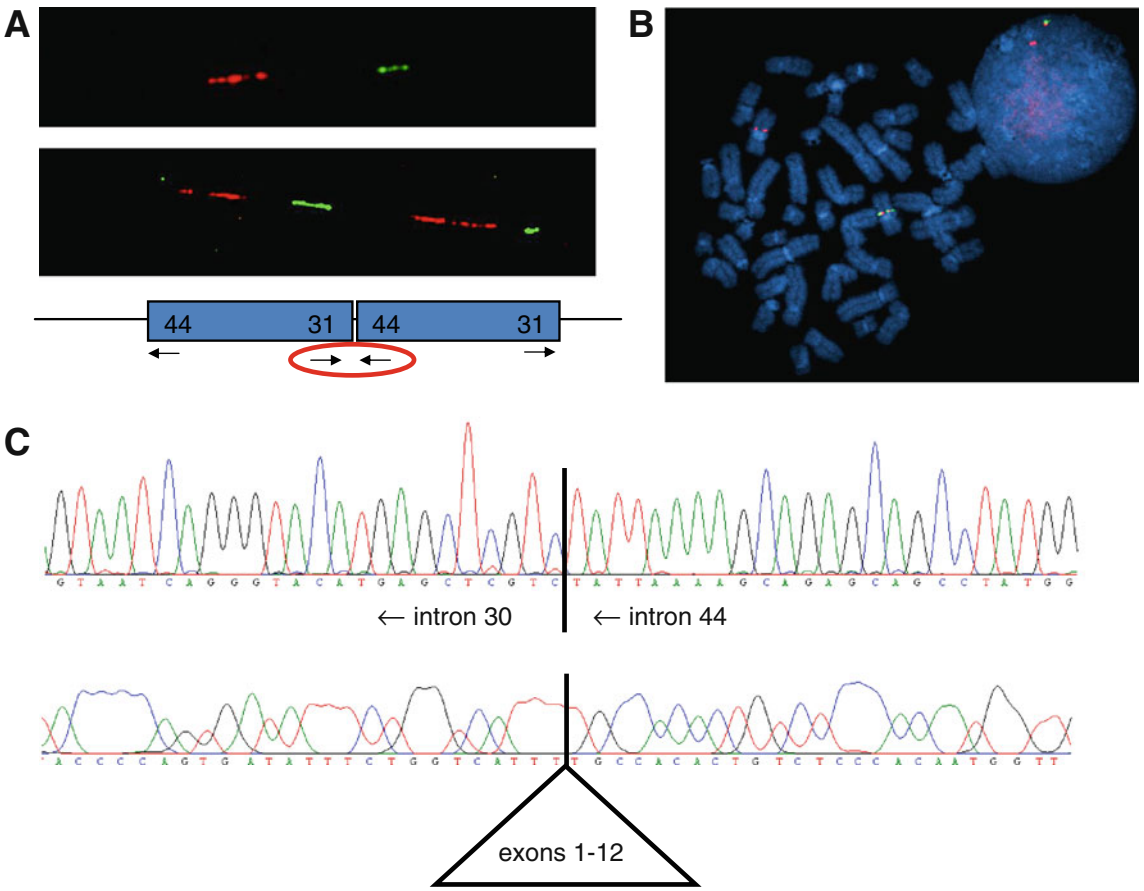

Fig. 2 Further molecular characterisation of both rare CNVs. **a** Fibre FISH images from normal X chromosome from mother (*upper*) and duplicated X chromosome from proband (*lower*). Schematic shows *DMD* duplicons alongside position of primers used for long-range PCR. **b** Interphase and metaphase FISH images from proband for the *TRPM3* locus. The deleted chromosome 9 is missing the signal from the RP11-89K20 probe (*green*). **c** Electropherograms with breakpoint-spanning sequences across the *DMD* duplication (*upper*) and *TRPM3* deletion (*lower*)

the deleted bases to be chr9:72,480,413-73,064,196 (Fig. 2c) and therefore 583,784 bp in size. The deletion thus removes the 5′ end of *TRPM3* spanning the first nine exons of the gene and the microRNA MIR204. There was no obvious homology at each end of either rearrangement, ruling out non-allelic homologous recombination and other homology-based CNV mechanisms.

Discussion

In a recent genome-wide scan for CNVs, three male probands from 996 ASD families harboured exonic CNVs involving *DMD*, all being detected by multiple CNV calling algorithms and inherited maternally. In contrast, a single exonic CNV involving *DMD* was detected in one female subject out of the 1,287 controls assessed (Pinto et al. 2010). These data are consistent with a degree of comorbidity between DMD/BMD and ASD, as described previously (Hendriksen and Vles 2008; Young et al. 2008). In this study, a combination of molecular methods has been used to characterise two rare CNVs within one of these families. The first CNV was a 225-kb duplication in the *DMD* gene, found in the two individuals with autism and their mother. It has previously been reported that *DMD* duplications sometimes lead to unexpected splicing patterns (White et al. 2006), and so we cannot be sure that the exon 44 donor splice site is compatible with the exon 31 splice acceptor. The reading frame rule should thus be applied with caution. As this patient was diagnosed with autism and had not manifested any obvious signs of muscular dystrophy at the time of inclusion in the study, no muscle biopsy material is available to test what effect the mutation has on the dystrophin protein. Nevertheless, the duplication observed leaves the reading frame intact and is predicted to result in an additional 735 amino acids in the middle of the dystrophin protein. The production of an elongated but partially functional form of dystrophin is predicted to result in a Becker phenotype. If the variant had a more severe impact on *DMD*, the patients would likely have presented with muscular dystrophy when evaluated as young adults. Interestingly, a similar duplication involving exons 31–44 has been previously identified in an individual with BMD, referred for molecular testing aged 14 years (Stockley et al. 2006); Stockley, January 2010, personal communication), consistent with our interpretation.

The second CNV identified in the family deleted the 5′ portion of *TRPM3* (transient receptor potential melastatin 3). This variant was paternally inherited by all three children and is particularly rare, being found in only this one family in the AGP study of 996 ASD families and in none of the 1287 control samples (Pinto et al. 2010). In addition, there are no similar multi-exonic CNVs listed in the Database of Genomic Variants (Iafrate et al. 2004), March 2010 freeze. Although to date no clear link has been established between this gene and ASD susceptibility, it is an intriguing candidate, representing a potential second hit contributing to the autism phenotype in this family. *TRPM3* encodes a calcium permeable ion channel (Naylor et al. 2010) and is expressed in the brain. Of the seven other paralogous members of the TRPM subfamily, *TRPM3* is most closely related to *TRPM1*, a gene localised within the ASD-associated 15q13.3 deletion, providing further evidence for a potential role in susceptibility in this family. *TRPM3* is localised to neurons prior to myelinisation and to oligodendrocytes during differentiation (Hoffmann et al. 2010). The gene has multiple splice forms with different functional properties, although how this affects their physiological role is unclear (Nilius & Voets 2008). The neurosteroid pregnenolone sulphate is known to improve synaptic function, neuroprotection, myelinisation and hippocampal neurogenesis. *TRPM3* is directly activated by this steroid and, as the gene is expressed in the brain, provides a possible mechanism by which pregnenolone mediates its effects. However, it has yet to be shown that pregnenolone has such an effect on *TRPM3* under physiological conditions (Nilius and Voets 2008). A deletion involving *TRPM3* has previously been found in a patient with Kabuki syndrome, a rare, clinically recognisable congenital mental retardation syndrome (Kuniba et al. 2009). However, the relevance of this finding is not clear, given that a recent study identified mutations in the *MLL2* gene in over 60% of patients with Kabuki syndrome (Ng et al. 2010).

The segregation pattern and rarity, together with background literature on the genes disrupted, have led us to hypothesise that these genomic variants might act as two separate “hits” influencing autism risk in this multiplex ASD pedigree. However, it must be noted that the majority of pedigrees are also likely to harbour multiple rare or even private CNVs. As the numbers of subjects undergoing high-resolution genome-wide CNV analysis increases, the roles of these two genes in ASD susceptibility will become clearer. Future studies should formally assess whether individuals with ASD and *DMD* mutations are more likely to have a second hit, in *TRPM3* or other brain-expressed genes, compared to individuals with *DMD* mutations but no autism.

By using the high-resolution 1M SNP data to design nine qPCR arrays and then reusing two combinations of these same primers for long-range PCR amplification of junction fragment, base pair resolution of the duplicated sequence was obtained in under 4 weeks. For the *TRPM3* deletion, SNP array data were sufficient to render the intermediate qPCR step unnecessary. These data demonstrate the ease with which CNV breakpoints can now be mapped. The latest developments in next-generation se-

quencing are now starting to mean that CNV discovery and identification of breakpoints happen simultaneously.

The increasing resolution and availability of genome-wide microarrays means that the likelihood of unexpected genetic diagnoses is escalating. For example, a recent array CGH scan studying the genetics of learning disability identified a de novo deletion of 17p13.1 involving the gene encoding the tumour suppressor P53 (Schwarzbraun et al. 2009). This unanticipated genetic diagnosis of Li–Fraumeni syndrome led to increased cancer surveillance in this subject. The two individuals described in our case report also raise similar ethical issues about unexpected findings arising from research studies. Information provided during the IMGSAC consent procedure states that "if any significant abnormality is found on either physical examination or laboratory testing, we would inform you and your GP of the results, their potential significance and advise on any further investigations that may be required". We considered this duplication of *DMD* to be an "abnormal" finding of potential clinical relevance, worth reporting to the family. Although BMD is currently incurable, confirmation by a diagnostic laboratory and further clinical assessment could lead to better management of the condition in these two individuals, the potential for genetic counselling and increased surveillance for heart conditions which are associated with BMD. A recent study of 30 individuals with BMD and dilated cardiomyopathy suggests that measurement of the variations in beat-to-beat intervals can act as a further predictor of cases at risk of sudden death and so may help suggest which BMD patients may benefit most from treatment with ACE inhibitors or, in the future, implantable defibrillators (Ammendola et al. 2006). For family 3019, it is worth noting that a distant maternal cousin (male) was described as having died suddenly of an unknown cause.

Before recommending that these two individuals be tested for creatine kinase levels, we decided to first characterise this duplication in more detail. Whilst qPCR was used to determine the exact combination of exons that were involved, other molecular methods were used in order to confirm that the duplication was in tandem and thus disrupting the functional copy of *DMD*. These additional experiments thus allowed a more complete picture of the genomic disruption to be communicated to the relevant clinicians.

There has been much recent discussion about whether research findings such as these should be communicated to participants on an individual basis (Miller et al. 2010). Even in the context of fully accredited clinical genetics testing, a recent study has shown that relatives of patients often prefer to postpone testing, depending on their personal circumstances (Dancyger et al. 2010). For families which are part of large research cohorts and for whom consent was taken a long time ago (~14 years in the case of family 3019), this issue is particularly difficult. Subjects will likely have forgotten the information provided at the time of consent and so any genetic information communicated to them may come as a surprise, especially if it confers risk for a condition other than that for which the individual came to clinical attention. In cases like this, we believe that the family's doctor is best placed to make a decision on how best to inform the parents. However, our experience with family 3019 also highlights another issue associated with the passage of time—our records were out of date and so it was initially difficult to trace the family's doctor.

In conclusion, we describe the phenotypic and molecular characterisation of a multiplex autism family found to have two rare CNVs disrupting the *DMD* and *TRPM3* genes. Although various studies propose a link between the location of the mutation within the *DMD* gene and cognitive ability, there are insufficient data to carry out a similar analysis specifically for DMD and ASD. As well as raising important issues about the communication of research findings, the family described in more detail here, taken in the context of other studies proposing two-hit CNV mechanisms (Girirajan et al. 2009; Pagnamenta et al. 2010; also AGP family 5126 in which a *IL1RAPL1* duplication is found in *cis* with the *DMD* variant; Pinto et al. 2010), suggests that genomic background may also play an important role in modulating neurological outcomes in individuals with *DMD* mutations.

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Conflict of interest statement The authors report no conflicts of interest.

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