Searching for Copy Number Changes in Nonsyndromic X-Linked Intellectual Disability

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Key Words

MECP2 • Multiplex ligation-dependent probe amplification • *TM4SF2/TSPAN7* • X-linked nonsyndromic intellectual disability

Abstract

Intellectual disability (ID) has a prevalence of 2–3% with 0.3% of the population being severely retarded. Etiology is heterogeneous, owing to numerous genetic and environmental factors. Underlying etiology remains undetermined in 75–80% of mildly disabled patients and 20–50% of those severely disabled. Twelve percent of all ID is thought to be X-linked (XLID). This study covers copy number analysis of some of the known XLID genes, using multiplex ligation-dependent probe amplification (MLPA) in 100 nonsyndromic patients. One of the patients was found to have duplication in all exons of *MECP2* gene, and another had duplication in the fifth exon of *TM4SF2/TSPAN7* gene. Affymetrix® 6.0 whole-genome SNP microarray confirmed the duplication in *MECP2* and showed duplication of exons 2–7 in *TM4SF2/TSPAN7*, respectively. *MECP2* duplication has recently been recognized as a syndromic cause of XLID in males, whereas duplications in *TM4SF2/TSPAN7* are yet to be determined as a cause of XLID. Being an efficient, rapid, easy-to-perform,

easy-to-interpret, and cost-effective method of copy number analysis of specific DNA sequences, MLPA presents wide clinical utility and may be included in diagnostic workup of ID, particularly when microarrays are unavailable as a first-line approach.

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Intellectual disability (ID) is defined as an intelligence quotient of 70 or lower together with performance lower than age- and culture-related standards in at least 2 areas of adaptive function, starting before 18 years of age [American Psychiatric Association, 2000]. ID is classified as mild, moderate, severe, and profound according to the intelligence quotient score [American Psychiatric Association, 2000]. ID prevalence is 2–3% with 0.3% of the population being severely retarded [Fishburn et al., 1983; Raymond, 2006; Shapiro and Batshaw, 2007].

Etiology of ID is highly heterogeneous. Genetic causes can be identified in only 5–10% of mild ID, whereas 22% of severe ID is due to chromosomal disorders and 21% is due to genetic syndromes [Strømme, 2000]. The underlying cause remains undetected in 75–80% of mild ID and 20–50% of severe ID [Shapiro and Batshaw, 2007].

Clinical workup of patients with ID should include karyotyping and, if clinically indicated, analysis for specific chromosome (Wolf-Hirschhorn, Angelman, Williams, etc.) or single gene (Noonan syndrome, Kabuki syndrome, etc.) disorders. Analysis for subtelomeric rearrangements and fragile X syndrome (FXS) should be routinely performed as these have a high etiological probability among patients with ID [Flint and Knight, 2003; Rooms et al., 2004; Raymond, 2006].

Single gene causes of ID constitute a large and heterogeneous group, as detected by means of a current OMIM search, which yields nearly 1,700 entries with ID. Many of the known genes lie on the X chromosome [Chelly et al., 2006]. X-linked ID (XLID) is responsible for 10–12% of ID in males [Mandel and Chelly, 2004; Ropers and Hamel, 2005]. FXS caused by full mutations of *FMR1* gene is alone responsible for 20–30% of XLID [Fishburn et al., 1983; Chiurazzi et al., 2008]. Although none of the remaining 90 X-linked genes known today are responsible for such a large proportion of ID [Raymond, 2006; Tarpey et al., 2009], current practice for ID in males involves analysis of known XLID genes. Patients highly suggestive of having XLID include those with pedigrees involving intellectually disabled males from at least 2 different generations related to each other through carrier females (42% yield); those with similarly affected brothers (17% yield), and those with idiopathic ID in whom previous analyses for karyotype, subtelomeric rearrangements and FXS have revealed normal results (10% yield) [de Brouwer et al., 2007; Rogers et al., 2008].

XLID has been traditionally classified as syndromic or nonsyndromic; however, as with FXS, clinical conditions initially classified as nonsyndromic XLID may be subject to reclassification as syndromic XLID, following revision of clinical experience and molecular data [Madrigal et al., 2007a; Nawara et al., 2008; Vandewalle et al., 2009; Fusco et al., 2010]. Examples include *ATRX*, *OPHN1*, *MECP2*, *ARX*, *PQBP1*, and *KDM5C*. Although nonsyndromic XLID was initially thought to be a result of point mutations, copy number changes of relevant genes have recently been described as causative. Examples include *OPHN1*, *GDI1* and *MECP2* [Madrigal et al., 2007a; Nawara et al., 2008; Vandewalle et al., 2009; Fusco et al., 2010]. Screening for copy number changes in XLID genes may be an important step in etiological testing, as suggested by previous studies [Madrigal et al., 2008]. In this study, the authors aimed to look for such changes in a group of nonsyndromic male patients with presumptive ID.

Patients and Methods

A total of 100 male patients considered likely to have nonsyndromic XLID were included in the study. Gross chromosomal disorders, subtelomeric rearrangements (using FISH) and FXS were previously excluded in all, using methods previously described [Fu et al., 1991; Oberle et al., 1991; Rousseau et al., 1991; Utine et al., 2009]. Where possible, patients with pedigrees suggestive of XLID (affected males from more than one generation related through females or affected brothers) were included. The study was approved by the Ethics Committee of Hacettepe University Faculty of Medicine.

MLPA was performed according to manufacturer's instructions (Salsa P106 and P015, MRC, Holland). Data analysis was undertaken using Coffalyser MLPA software (www.mlpa.com). This software allows the drawing of visual fragment analysis diagrams, known as electropherograms, following normalization of data using reference DNA. Copy number alterations were calculated as ratios between normalized peak areas and the corresponding mean of normalized peak areas from a male reference data set. Genomic copy number analysis is normal in samples with normalized peak areas of 0.7–1.3 times that of reference DNA samples, whereas outlying results show either a loss of normal genomic copy number (deletion) or a gain in copy number (duplication).

P106 MRX kit (2005 version) includes 43 probes for coding regions in 14 XLID genes: *FMR2*, *ARX*, *ARHGEF6*, *TM4SF2/TSPAN7*, *RPS6KA3*, *OPHN1*, *GDI1*, *PQBP1*, *SLC6A8*, *FACL4 (ACSL4)*, *DCX*, *IL1RAPL1*, *PAK3*, and *AGTR2*. P015 kit (2005 version) includes 8 probes for *MECP2* gene and 18 control probes for autosomal and X-linked regions.

SNP microarray analysis was performed in order to validate the findings. The Affymetrix Genome-Wide Human SNP Array 6.0 arrays (Affymetrix, Santa Clara, Calif., USA) were performed according to manufacturer's instructions. The average probe spacing of the 6.0 array is 0.7 kb. SNP copy number was assessed using Affymetrix Genotyping Console 3.0 software.

X chromosome inactivation (XCI) pattern was studied by genotyping the highly polymorphic CAG repeat in the *AR* gene, as previously described [Allen et al., 1992; Özbalkan et al., 2005]. DNA samples from a male control, a female control with random XCI pattern and a female control with an extremely skewed XCI pattern were used.

Results

Two patients out of 100 (2%) had copy number variations of XLID genes: one brother had a duplication of exon 5 of *TM4SF2/TSPAN7* gene and another patient (see family 2) had duplication of the entire *MECP2* gene.

Family 1

A moderately retarded 9-year-old patient and his similarly affected 11-year-old brother had infantile hypotonia and delayed motor development (fig. 1). They did not acquire language

abilities, but were able to communicate well with gestures; both were silent and pleasant in character. There was no history of seizures, stereotypical or aggressive behavior. No dysmorphic characteristics or malformations were present. No other family member was affected. Probe for the exon 5 of *TM4SF2/TSPAN7* gene was duplicated in the patient and his similarly affected brother (fig. 2). SNP microarray analysis revealed duplication of a 143 kb long segment at Xp11.4 at 38.37–38.5 Mb. Consistent with the MLPA results, the duplication excluded exon 1, but included all the remaining 6 exons (fig. 3, 4). The mother, who was healthy, had 3 copies of the same region (fig. 3, 4). XCI analysis of the mother was done for this family and was found noninformative (fig. 5).

Family 2

An 8-year-old boy had severe intellectual disability and infantile hypotonia leading to a delay in motor function development. He experienced recurrent respiratory infections and displayed autistic behavior with stereotypical hand movements, accompanied by very limited speech. He also displayed brachycephaly, hypotonic facies, very short and infrequent eye contact, and drooling (fig. 6). He did not experience seizures, but an epileptic pattern was detected in the EEG. The daughter of a maternal aunt suffered from ID, and the son of another maternal aunt showed speech delay without ID. MLPA identified duplications of all probes for *MECP2* gene as well as probes for the

flanking *IRAK1* and *LICAM* genes (fig. 7). SNP microarray analysis confirmed the duplication of a 509 kb long segment localized at Xq28 between 152.8–153.3 Mb (fig. 8). The mother, who was healthy, had the same duplication, leading to 3 copies of the region (fig. 8).

Fig. 1. Facial characteristics of the 9- (on the left) and 11-year-old brothers in family 1.

Fig. 2. MLPA showing duplication of the probe for exon 5 of *TM4SF2/TSPAN7*.

Fig. 3, 4. SNP microarray analysis showed duplication of Xp11.4 (38.37–38.5 Mb), including exons 2–7 and excluding exon 1 of *TM4SF2/TSPAN7*. The mother had 3 copies of the same region.

Fig. 5. XCI analysis of the mother in family 1 (marked as 'sample DNA') showing noninformative results.

Discussion

Over the last 10 years, copy number changes in XLID genes as causes of syndromic and nonsyndromic ID, including *MECP2*, *GDI1*, *IL1RAPL1*, and *OPHN1*, have been discussed [Madrigal et al., 2007a; Nawara et al., 2008; Vandewalle et al., 2009; Fusco et al., 2010]. Syndromic nature of these genomic diseases have already been established for some genes (*MECP2*, *GDI1*, *OPHN1*) [Meins et al., 2005; van Esch et al., 2005; Friez et al., 2006; Madrigal et al., 2007a; Lugtenberg et al., 2009]. For others, it is still under investigation whether copy number changes, particularly duplications, are causative for ID [Bauters et al., 2008; Madrigal et al., 2008; Gijsbers et al., 2011].

Whether copy number changes in other X-linked genes cause XLID may become evident with future research. *MECP2* duplication in affected males was first reported in 2005 [Meins et al., 2005; van Esch et al., 2005], *GDI1* duplication in 2007 [Madrigal et al., 2007a] and *IL1RAPL1* deletion in 2008 [Nawara et al., 2008]. Severe intellectual disability, infantile hypotonia, delay in motor function development, recurrent respiratory infections, autistic behavior, stereotypical hand movements, limited speech with hypotonic facies, short and infrequent eye contact, and drooling in patient 2 are consistent and well-known features of *MECP2* duplication. However, copy number changes in *TM4SF2/TSPAN7* are yet unclear as causes of ID.

ID, autism and schizophrenia are 3 pathological conditions which probably share common biological path-

Fig. 6. Facial characteristics of the 8-year-old patient in family 2.

ways for neurotransmission, synapse formation and maintenance, involving common gene products [Gulimatre et al., 2009; Piton et al., 2010]. Some recent studies showed evidence against the causative role of *TM4SF2/TSPAN7* duplications in autism spectrum disorders and schizophrenia [Cai et al., 2008; Noor et al., 2009; Piton et al., 2010]. In ID, a causative deletion in *TM4SF2/TSPAN7* gene was previously described in 1 male patient [Madrigal et al., 2007b] and duplications in *TM4SF2/TSPAN7* were reported in 2 families [Froyen et al., 2007]. In the

Table 1. Duplication in *TM4SF2/TSPAN7* in the present family and in the literature

Feature	Present family		Literature (Froyen et al., 2007)	
	dup	dup	dup	dup/del
Copy number state	dup	dup	dup	dup/del
Size	143 kb	143 kb	15.5 Mb	0.3 Mb/0.4 Mb
Involved genes	<i>TM4SF2</i>	<i>TM4SF2</i>	<i>TM4SF2</i> + 41 genes	<i>TM4SF2/OPHN1</i>
MR	moderate	moderate	moderate	moderate/severe
Hypotonia	+	+	?	?
Absent speech	+	+	?	+
Autistic behavior	–	–	?	+
Hyperactivity	–	–	?	?
Ataxia	–	–	+	?
Malformations	–	–	+	+
Dysmorphism	–	–	+	+
Epilepsy	–	–	+	+
Stereotypies	–	–	?	?

latter report, the affected individuals included 2 brothers in one family and a single male patient in a second family. The 2 brothers, who had moderate-severe ID, autism, epilepsy, hypogenitalism, and cerebellar hypoplasia, had a complex cytogenetic rearrangement with a coexistent deletion in *OPHN1* gene, which was apparently causative for the clinical findings. In the second family, a cytogenetically visible large deletion of 15.5 Mb at Xp11.4p21.3 spanning 41 genes, including *IL1RAPL1*, was present and was likely to be causative for ID. His mother, who had borderline ID, also had the same duplication of *TM4SF2/TSPAN7*. It was difficult to conclude that in these 2 families the *TM4SF2/TSPAN7* duplication was causative. Similarly, large duplications of Xp11p21 involving many XLID genes were shown in 2 patients in 2008; the roles of individual genes in ID, not unexpectedly, being unclear [Tzschach et al., 2008]. The isolated duplication of *TM4SF2/TSPAN7* in both brothers reported in this study demonstrated by SNP array analysis might suggest the presence of a clinical phenotype with ID in cases where such duplications occur, although this observation is certainly inadequate for making a definitive conclusion. Unfortunately, XCI study of maternal DNA revealed nonin-

formative results because of homozygosity in CAG repeat number in the *AR* gene. Features of both brothers in our study and the patients from Froyen et al. [2007] are presented in table 1.

MLPA procedure identifies copy number changes for multiplex short DNA sequences efficiently, rapidly and reliably. The procedure is easy to perform and easy to interpret and has a wide variety of uses in clinical laboratories. Although nonsyndromic ID may be highly heterogeneous in etiology and the diagnostic yield of MLPA in MRX may be very low, as seen in the current study, for families in which affected males from different generations who are related to each other through females, the technique may provide diagnostic results as high as 5–10% [Madrigal et al., 2007a, b, 2008].

The current consensus across many laboratories is to perform microarrays initially in patients displaying ID with or without malformations/dysmorphisms. Array CGH is desirable as a first-line approach, but in cases where it is not easily available, MLPA screening may be considered a cost-effective prescreening, as there are recently shown examples of causative copy number changes in XLID genes.

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