Tandem Duplication of Exons 1–7 Neither Impairs *ATP7A* Expression Nor Causes a Menkes Disease Phenotype

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Abstract *ATP7A* duplications are estimated to represent the molecular cause of Menkes disease in 4–10% of affected patients. We identified a novel duplication of *ATP7A* exons 1–7 discovered in the context of a challenging prenatal diagnostic situation. All other reported *ATP7A* duplications ($n = 24$) involved intragenic tandem duplications, predicted to disrupt the normal translational reading frame and produce nonfunctional *ATP7A* proteins. In contrast, the exon 1–7 duplication occurred at the 5' end of the *ATP7A* gene rather than within the gene and did not correspond to any known copy number variants. We hypothesized that, if the exon 1–7 duplication was in tandem, functional *ATP7A* molecules could be generated depending on promoter selection, mRNA splicing, and the proximal and distal duplication breakpoints and that Menkes disease would be averted. Here, we present detailed molecular characterization of this novel duplication, as well as 2-year postnatal clinical and biochemical

correlations. The case highlights the ongoing need for cautious interpretation of prenatal genetic test results.

Introduction

Menkes disease (MIM# 309400) is a lethal infantile X-linked recessive disorder of copper metabolism caused by mutations in *ATP7A* (NCBI accession number: NM_000052.5), which is located at Xq21.1 and encodes a copper-transporting ATPase (Kaler and Packman 2013). This condition is characterized by male gender, early-onset cerebral and cerebellar neurodegeneration, failure to thrive, seizures, hypotonia, coarse hair, and connective tissue abnormalities. Death typically occurs by 3 years of age. Biochemical features include decreased activities of copper-dependent enzymes such as dopamine-beta-hydroxylase, cytochrome c oxidase, and lysyl oxidase (Kaler 2011). Affected individuals manifest low copper and ceruloplasmin levels in plasma or serum, as well as in cerebrospinal fluid (Donsante et al. 2010). Even in healthy newborns, serum copper and ceruloplasmin levels remain low for several weeks and thus are not reliable for diagnosis of the illness until at least 6–8 weeks of age (Kaler et al. 1993a, b, c). Prenatally, chorionic villus and amniocyte copper accumulation offer useful biochemical markers of the disease (Kaler and Tumer 1998).

On a molecular basis, the spectrum of *ATP7A* mutations causing the Menkes disease clinical and biochemical phenotype includes gene deletions and duplications, as well as missense and splice junction alterations (Moizard et al. 2011; Mogensen et al. 2011; Tümer 2013). While *ATP7A* genotype is generally predictive of response to early copper replacement therapy (Kaler 1996; Kaler et al. 2008), ambiguous situations involving novel molecular alterations in this gene may occur, as we recently reported (Schoonveld

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et al. 2013). Specifically, the latter case involved an unborn fetus with a novel duplication (exons 1–7) of the *ATP7A* gene detected prenatally. Based on the molecular context, we posited that the alteration did not preclude transcription and translation of functional ATP7A species and that the fetus would likely not be affected with Menkes disease (Schoonveld et al. 2013). Here, we present molecular, biochemical, and 2-year clinical follow-up data for this infant.

Materials and Methods

Human Subjects Protection: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from the parents of the patient included in this study.

Fibroblast Cell Culture: We cultured fibroblasts from the subject, from a known Menkes disease patient, and from a normal control CRL-2076 (ATCC, Manassas, VA, USA) in Dulbecco's Modified Eagles Media (DMEM) with 10% fetal calf serum and antibiotics in a 5% CO₂ incubator at 37°C.

Total RNA Preparation: The PureLink RNA mini-kit (Invitrogen) and DNase I (Qiagen) were used to isolate fibroblast total RNA from the subject, from a known Menkes disease patient, and from a normal control.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR): RT-PCR was performed on fibroblast total RNA using the Enhanced Avian RT First Strand Synthesis Kit (Sigma #STR-1) with random nonamer primers, Platinum Taq DNA Polymerase High Fidelity (Invitrogen #11304-011), and ATP7A-specific primers 7eF:GAATGACGTGT

GCCTCCTGCGTACATA; 1eR, GAGCTACGCA-GACCGTGGCAGCGAT; 3bF, AAAATTTACCTCA-GAAAGAAGTGT; and 4aR, CAATGCATGCCATCAAT.

GATG, as described in the text.

Western Blotting: Western blots were prepared by electrophoresis of fibroblast proteins, transferring to PVDF membranes and probing with a reliable carboxyl-terminus anti-ATP7A antibody or an anti-beta-actin antibody, as previously described (Haddad et al. 2012).

Immunofluorescence Confocal Microscopy: The patient's fibroblasts were examined by confocal microscopy (Zeiss 510) and images captured using META software, as previously described (Yi et al. 2012).

Fluorescent in situ Hybridization: Metaphase spreads from fibroblast cell lines were prepared by standard air-drying technique, and FISH (fluorescent in situ hybridization) performed with labeled DNA BAC clones, essentially as described (Dutra et al. 1996). We chose two

BAC clones predicted to encompass both duplication breakpoints using the UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly. To cover the proximal breakpoint, we used labeled BAC clone RP11-637B20 (chrX: 77,067,633-77,221,610), which covers the genomic region upstream of *ATP7A*, *ATP7A* exon 1, and most of *ATP7A* intron 1 (size of BAC clone: 153,978 bp). For the distal breakpoint, we concurrently used labeled BAC clone RP11-776014 (chrX: 77,242,535-77,414,058), which extends from *ATP7A* intron 2 until the end of the *ATP7A* locus (size of BAC clone: 171,524 bp).

On each slide, 50 ng of labeled probe was applied. Repeat sequences were blocked with Cot-1 (10X excess). A 10 µL hybridization mixture containing the labeled DNA in 50% formamide, 2x SSC, and 10% dextran sulfate were denatured at 75°C for 10 min and then incubated at 37°C for 30 min for pre-annealing. Slides were then denatured and hybridized for at least 18 h and counterstained with DAPI-Antifade.

Results

Clinical and Biochemical Findings

When examined at 7 months of age, the infant was well nourished and well developed. He weighed 8.85 kg (50–75th percentile) and his head circumference was 45.3 cm (75th percentile). His hair was normal in color and texture and his skin showed no excess laxity. Neurologically, he smiled, had excellent head control, rolled from front to back and back to front, sat independently, and transferred objects. His overall muscle tone was normal and there were no focal neurological deficits. Serum copper and ceruloplasmin levels were normal (Table 1). His plasma catechol levels (Kaler et al. 1993a, b) also were normal at 7 months, as at birth (Table 1). Microscopic examination of 25 hair shafts showed no *pili torti*. He walked independently at 13 months of age, and at 2 years of age, his neurodevelopment was entirely age appropriate.

Molecular Analysis

The patient had been diagnosed prenatally as having a duplication of exons 1–7 of the *ATP7A* gene. We hypothesized that, if the *ATP7A* promoter region had not been interrupted by meiotic crossover, at least three potential ATP7A molecules could be generated depending on promoter choice, mRNA splicing, and position of the 5' breakpoint and inferred that at least one would be functional (Schoonveld et al. 2013). Potential transcripts were calculated to encode a 623 amino acid ATP7A

Table 1 Blood biochemical data

Age	DOPA pg/mL	DOPAC pg/mL	DA pg/mL	NE pg/ml	DHPG pg/mL	DOPA:DHPG	DOPAC:DHPG	DA:NE	Cu µg/dL	Cp mg/L
1 day	4474	4120	29	428	1903	2.35	2.17	0.068	NA	NA
7 months	2499	941	10	215	901	2.77	1.04	0.047	148	344
Normal values ^{a, b}	2488 ± 526	2144 ± 319	4 ± 4	748 ± 106	1021 ± 100	2.36 ± 0.25	2.17 ± 0.38	0.04 ± 0.03	40–170	190–420
Menkes disease ^{a, b}	5346 ± 468	4832 ± 1528	199 ± 45	341 ± 92	458 ± 71	14.28 ± 2.59	11.73 ± 1.74	0.83 ± 0.71	10–53	NA

± values represent standard deviation

^a Kaler et al. (1993b)^b Kaler et al. 2008

DOPA plasma dihydroxyphenylalanine, DOPAC dihydroxyphenylacetic acid, DA dopamine, NE norepinephrine, DHPG dihydroxyphenylglycol, Cu serum copper, Cp ceruloplasmin, NA not available

Fig. 1 FISH analysis indicates X chromosomal localization of *ATP7A* exon 1–7 duplication. Metaphase spread of chromosomes from a normal male control fibroblast cell line (**a**) and from the patient's fibroblasts (**b**) hybridized with DNA BAC clones containing segments of the *ATP7A* exon 1–7 region. The patient's metaphase (**b**) shows a more intense signal on the long arm of the X chromosome compared to normal, consistent with Xq21.1 cytogenetic localization, and no autosome signal is evident. Arrows indicate X chromosome centro-

meres. (**c**) Diagram of BAC clones RP11-637B20 (*lavender*) and RP11-776014 (*red*) employed, depicting coverage in relation to the *ATP7A* locus (*green*) and in the context of an exon 1–7 tandem duplication (*light blue*). Vertical black lines denote approximate locations of the 23 exons within the 140 kB *ATP7A* gene. Intron 1 is large (≈ 60 kB) and not drawn to scale. Please see “Materials and Methods” for detailed probe descriptions

fragment (nonfunctional), the normal 1,500 amino acid ATP7A, and a 2,176 amino acid version of ATP7A, requiring activation of a cryptic splice acceptor site at the downstream (3') exon 1 (see Figure 1, Schoonveld et al.

2013). A fourth product, 2,130 residues in length, was also theoretically possible, if exon skipping were to join exon 7 of the duplicated segment to the downstream exon 2, given the weak exon 1 splice acceptor. These larger versions

Fig. 2 (continued)

would retain the proper reading frames for ATP7A but would include 53 and 7 extraneous amino acids, respectively, between the duplicated and parent segments.

To investigate the chromosomal localization of the duplicated ATP7A fragment, we performed FISH analysis on the patient's fibroblasts. Compared to a normal male control (Fig. 1a), the patient's metaphase showed increased signal on the long arm of the X chromosome using DNA BAC probes encompassing the duplicated segment (Fig. 1b, c). No signal was detected on any other chromosome(s). These data indicate that the exon 1–7 duplication occurred adjacent to the *ATP7A* locus on the X chromosome.

Also utilizing the patient's cultured fibroblasts, we evaluated *ATP7A* transcripts in this infant. We sought to determine the presence of cDNA species predicted by mRNA transcripts containing the tandem duplication (Fig. 2a). We purified total RNA from cultured fibroblasts and generated cDNA using reverse transcription-polymerase chain reaction (RT-PCR). These RT-PCR assays failed to detect any RNA transcripts that supported inclusion of the duplicated segment (Fig. 2b). Western blots of the patient's fibroblast protein showed ATP7A protein of the normal size and amount, with no larger version(s) evident (Fig. 2c). Immunofluorescence confocal microscopy of the

patient's fibroblasts revealed normal ATP7A quantity and *trans*-Golgi localization, as well as normal intracellular trafficking in response to increased copper concentration (Fig. 2d).

Discussion

All prior reported ATP7A duplications ($n = 24$) involved intragenic tandem duplications predicted to disrupt the normal translational reading frame and produce nonfunctional ATP7A proteins (Moizard et al. 2011; Mogensen et al. 2011; Tümer 2013). In contrast, the exon 1–7 duplication occurred at the 5' end of ATP7A rather than within the gene. While the parents considered pregnancy termination following the prenatal genetic diagnosis, they elected to continue after careful consideration of the risks and the unknown genotype-phenotype correlation (Schoonveld et al. 2013). An apparently healthy male infant was delivered at 36 weeks gestation and showed neither biochemical nor clinical evidence of disturbed copper metabolism (Kaler et al. 1993a, b, c) (Table 1). He has achieved normal neurodevelopment throughout infancy up to his current age (24 months),

Fig. 2 Normal *ATP7A* transcript and protein in subject with duplication of *ATP7A* exons 1–7. **(a)** If the patient's cells produced a messenger RNA containing the tandem duplication (exons 1–7 + exons 1–23), we predicted amplification of a 262 bp product (red) by RT-PCR using the primer pair Exon7eF/Exon1eR and a 2.549 kb product (blue) with the primer pair Exon3bF/Exon4aR. The latter primers would also produce a 515 bp product, both from the putative mutant transcript and the normal transcript (blue). **(b)** RT-PCR resulted in amplification of only the 515 bp fragment (lane 1) and neither of the specific products predicted from a mutant transcript with the duplication (262 bp, 2.549 kb) were detected (lanes 2 and 1, respectively). The absence of a PCR product in lane 2 also excluded an inverted duplication. **(c)** Western blot of protein extracted from patient's fibroblasts shows only the normal-sized ATP7A. (Extra

bands of approximate size 95 kDa represent nonspecific interaction with this antibody that we have observed previously.) A well-characterized fibroblast cell line from a Menkes disease patient with deletion of *ATP7A* exons 20–23 showed no ATP7A, as expected. **(d)** Confocal imaging of fibroblasts from the patient (dup exon 1–7) and a normal control (wild type) illustrates normal quantity, *trans*-Golgi localization, and intracellular trafficking of ATP7A. Arrows indicate intense perinuclear signal in the patient's cells after staining with anti-ATP7A (green) under basal copper concentration (0.5 μ M). Middle panels show staining with the *trans*-Golgi marker, TGN46 (red). Merged images illustrate co-localization (yellow signal). Under exposure to elevated copper (200 μ M), the ATP7A signal is no longer evident in the *trans*-Golgi, consistent with intracellular trafficking to the periphery, as expected. Scale bars = 10 μ m

without copper replacement treatment (Sheela et al. 2005), and his biochemical phenotype has remained normal (Table 1).

Postnatally, we evaluated whether this patient's fibroblasts produced *ATP7A* mRNA and protein that included the exon 1–7 duplication and did not find evidence to support these theoretical possibilities. Instead, we found only normal results in our molecular and cellular functional analyses, implying that this duplication is a benign copy

number variant, presumably due to its position at the 5' region of *ATP7A* rather than at an intragenic location. We have submitted this apparently benign copy number variant to the *ClinVar* database (http://www.ncbi.nlm.nih.gov/clinvar/docs/submit/#min_content).

This case highlights the ongoing need for cautious interpretation of prenatal molecular genetic test results that involve previously uncharacterized alterations, including copy number variants.

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Compliance with Ethics Guidelines

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from the parents of the patients included in the study.

This article does not contain any studies with animal subjects performed by the any of the authors.

Eun-Young Choi, Keyur Patel, Marie Reine Haddad, and Ling Yi performed the molecular and cell biological experiments described in this article. Courtney Holmes and David S. Goldstein performed the neurochemical analyses. Amalia Dutra and Evgenia Pak performed fluorescence in situ hybridization (FISH) experiments. Eun-Young Choi and Stephen Kaler planned the studies and wrote the manuscript.

All authors (Eun-Young Choi, Keyur Patel, Marie Reine Haddad, Ling Yi, Courtney Holmes, David S. Goldstein, Amalia Dutra, Evgenia Pak, and Stephen Kaler) declare that they have no conflict of interest.

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