Short Report

Favorably skewed X-inactivation accounts for neurological sparing in female carriers of Menkes disease


Classical Menkes disease is an X-linked recessive neurodegenerative disorder caused by mutations in ATP7A, which is located at Xq13.1-q21. ATP7A encodes a copper-transporting P-type ATPase and plays a critical role in development of the central nervous system. With rare exceptions involving sex chromosome aneuploidy or X-autosome translocations, female carriers of ATP7A mutations are asymptomatic except for subtle hair and skin abnormalities, although the mechanism for this neurological sparing has not been reported. We studied a three-generation family in which a severe ATP7A mutation, a 5.5-kb genomic deletion spanning exons 13 and 14, segregated. The deletion junction fragment was amplified from the proband by long-range polymerase chain reaction and sequenced to characterize the breakpoints. We screened at-risk females in the family for this junction fragment and analyzed their X-inactivation patterns using the human androgen-receptor (HUMARA) gene methylation assay. We detected the junction fragment in the proband, two obligate heterozygotes, and four of six at-risk females. Skewed inactivation of the X chromosome harboring the deletion was noted in all female carriers of the deletion (n = 6), whereas random X-inactivation was observed in all non-carriers (n = 2). Our results formally document one mechanism for neurological sparing in female carriers of ATP7A mutations. Based on review of X-inactivation patterns in female carriers of other X-linked recessive diseases, our findings imply that substantial expression of a mutant ATP7A at the expense of the normal allele could be associated with neurologic symptoms in female carriers of Menkes disease and its allelic variants, occipital horn syndrome, and ATP7A-related distal motor neuropathy.

In order to maintain equivalent expression levels of X-linked genes between genders, mammals achieve dosage compensation by silencing one of the two X chromosomes in females, a mechanism termed X-chromosome inactivation (1). During the blastocyst stage in female embryos, expression of Xist RNA and transient pairing of the X-inactivation centers (Xic) of the two X chromosomes leads to heterochromatization of one X chromosome and resultant silencing of most of its genes (2). The pattern of X chromosome inactivation is generally random; however, when one X chromosome harbors a deleterious allele, X-inactivation can be non-random: cells that have the mutation-bearing chromosome active may be selected against over time, resulting in a predominance of cells with an active X chromosome containing the normal allele (3, 4). This favorably skewed X-inactivation may protect female carriers of X-linked recessive mutations from the phenotypic consequences of the mutation. In contrast, when an X chromosome carrying
a disease allele is not preferentially inactivated, female heterozygotes may manifest conditions usually restricted to males (4, 5). The complex relationship between X-inactivation status and clinical phenotype in carriers of certain X-linked disorders, including α-thalassemia/mental retardation syndrome, Bard syndrome, Duchenne muscular dystrophy, hypohidrotic ectodermal dysplasia, incontinentia pigmenti, nephrogenic diabetes insipidus, opotulatodigital syndrome, Ret syndrome, severe combined immunodeficiency disease, and Wiskott–Aldrich syndrome, is summarized in an excellent recent review (6).

Menkes disease and its allelic variants, occipital horn syndrome and ATP7A-related distal motor neuropathy, are X-linked recessive disorders caused by mutations in the copper-transporting P-type ATPase gene, ATP7A (7–11). Absence or severe reduction in ATP7A activity inhibits intestinal uptake of copper and transport of copper into the developing brain (12, 13). In addition, transport of copper into the trans-Golgi compartment for incorporation by certain copper-requiring enzymes is dependent on ATP7A (14). Thus, the activities of enzymes requiring copper as a cofactor, including dopamine-β-hydroxylase (15, 16) and peptidylglycine α-amidating monoxygenase (17), may be reduced. Males affected with classical Menkes disease have mutations associated with an estimated 0–15% of normal ATP7A activity and exhibit delayed neurodevelopment, dysmyelination, seizures, connective tissue abnormalities (18, 19), and premature death. However, early diagnosis and treatment with copper injections may markedly improve patients’ clinical outcomes, especially if some residual ATP7A activity is present (20). Patients with occipital horn syndrome typically have between 20% and 35% residual copper transport function and far less severe neurodevelopmental abnormalities, even in the absence of early diagnosis and treatment (10, 21). Males with ATP7A-related distal motor neuropathy have ATP7A mutations with an even greater quantity of residual copper transport activity and manifest a late-onset syndrome restricted to progressive distal motor neuropathy, without overt signs of systemic copper deficiency (11).

A total of 10 females affected with Menkes disease have been reported (22, 23), among whom one was mosaic for Turner (XO) syndrome and five had X-autosome translocations interrupting the ATP7A gene. As predicted for the latter circumstance (24), the normal X chromosomes in these five patients were preferentially inactivated, avoiding haploinsufficiency of the autosomal genes involved but resulting in the expression of Menkes disease.

Female carriers of Menkes disease may exhibit subtle clinical manifestations, including patchy skin hypopigmentation (25) and pili torti of scalp hair (26). Biochemically, uncloned fibroblast cultures from Menkes disease obligate heterozygotes showed reduced copper transport compared with normal female controls (27). These findings indicate that mosaicism for the ATP7A mutations, mediated by X-chromosomal inactivation patterns, may be clinically relevant in this condition.

To our knowledge, X-inactivation patterns in cytogenetically normal females heterozygous for ATP7A mutations have not been reported (6). To begin to evaluate this issue, we studied a large family that included numerous at-risk females of child-bearing age.

Methods

Subjects

Ten members of a family with a history of Menkes disease were evaluated. The study was approved by the institutional review boards of the University of Utah, the Eunice Kennedy Shriver National Institute of Child Health and Human Development, and the National Institute of Neurological Disorders and Stroke.

Case history

The proband was born at 36-week gestation by cesarean section for breech presentation to a 22-year-old gravida 3 para 2 aborta 1 mother, after an uneventful pregnancy. The Apgar scores were 5, 8, and 9 at 1, 5, and 10 min, respectively. Jaundice and temperature instability were noted during the first week of life. Due to a family history of Menkes disease in a maternal uncle, plasma neurochemical levels (15, 16, 20) were obtained shortly at birth and were diagnostic for this condition. The infant began copper injection treatment under an NIH protocol (ClinicalTrials.gov identifier NCT00001262) at 8 days of age. At 6 weeks of age, failure to thrive and suspicion of clinical seizure activity were noted. Pulmonary insufficiency consistent with emphysema developed subsequently, requiring chronic oxygen administration. The infant died at 5 3/4 months of age from respiratory distress while receiving hospice care at home. The parents declined a postmortem pathological examination.

The mother’s medical history was notable for brief episodes of abnormal movement considered to be minor motor seizures at 11 months of age.
Desai et al.

She also had scattered regions of hypopigmentation on her trunk, as did one younger sister.

DNA extraction and analysis

Genomic DNA obtained from each family member was isolated from peripheral blood samples using the Gentra Autopure system and Puregene DNA Purification kit. ATP7A mutation analysis in the proband was performed by multiplex polymerase chain reaction (PCR) (28) of the infant’s genomic DNA and revealed deletion of ATP7A exons 13 and 14. The effects of this deletion on translation of the gene product were analyzed using macvector software. Long PCR was performed on proband and wild-type genomic DNA with primers spanning the 3’ end of exon 12 and the 5’ end of exon 15 of ATP7A: 12–15 long F: 5’-GGACATTCTATGGTAGATGAGTCCC-3’ and 12–15 long R: 5’-CTGTGATAGGCTTGGA-AAAGCAATCG-3’. PCR was carried out for 30 cycles at 98°C for 30 s, 94°C for 15 s, 60°C for 20 s, and 68°C for 20 min. The PCR product obtained from the proband was analyzed by automated DNA sequencing (ABI 377 Prism; Applied Biosystems, Foster City, CA, USA) from both ends until the breakpoint was identified.

Junction fragment assay

To provide a method for screening prospective carriers for the family mutation, we designed PCR primers to flank the putative breakpoints, which reproducibly generated a 664-bp PCR product in the proband and in two obligate heterozygotes. The primers and sequences were: 50071F: 5’-GGACATTCTATGGTAGATGAGTCCC-3’ (forward) and 56291R: 5’-CTGTGATAGGCTTGGA-AAAGCAATCG-3’ (reverse). PCR was carried out for 30 cycles at 94°C for 1 min, 60°C for 30 s, and 72°C for 90 s. As a control for PCR fidelity, exon 13 of ATP7A with its associated splice donor and splice acceptor junctions (291-bp fragment) was amplified as previously described (28).

X-inactivation studies

X-inactivation patterns in this kinship were assessed for skewing with the human androgen-receptor locus (HUMARA) methylation assay (29). The assay was performed under standards and conditions certified according to the Clinical Laboratory Improvement Amendments which include quality assurance and use of normal control specimens. Digestion of genomic DNA with the methylation-sensitive restriction endonuclease, HpaII, and subsequent PCR amplification of the HUMARA polymorphic CAG repeat was used to determine X-inactivation status in female family members of child-bearing age. Inactivation ratios less than 80:20 were considered random. Ratios greater than or equal to 80:20 and less than or equal to 90:10 were considered moderately skewed. Ratios greater than 90:10 were considered highly skewed.

Results and discussion

Deletion breakpoint characterization and junction fragment screening

Using a multiplex PCR assay that amplifies each of the 23 exons of ATP7A (28), we detected deletion of exons 13 and 14 in the proband (data not shown). Deletion of these coding regions, which include 155 and 135 bp, respectively, predicts a translational reading frame shift: translation analysis (macvector) of the mutant sequence revealed substitution of two amino acids (LQ) after residue 876 (G), followed by a premature stop codon (UAG) at position 879 of the 1500-amino acid gene product. The actuator domain of ATP7A includes residues 806–924 and is required for the phosphatase step of the molecule’s catalytic cycle. The recently solved solution structure of this region (30) indicates protrusion of a catalytically important TGE loop (residues 875–877) from this region for interaction with the phosphorylated site in the ATP-binding domain. Deletion of exons 13 and 14 disrupts the TGE loop, converting this highly conserved motif to TGL and introducing the premature stop codon at position 879. Even if alternative splicing occurred through microRNAs or small nucleolar RNAs, the absence of a large portion of the actuator domain, disruption of the TGE phosphatase loop, and removal of an entire transmembrane segment (31), components which are all encoded by exons 13 and/or 14, would prohibit bioactivity of the mutant copper ATPase. Characterization of a similar mutation, deletion of ATP7A exons 20–23, which eliminates an ATP-binding domain and two transmembrane segments, showed no residual copper transport capacity in a yeast complementation assay (20). Based on these combined analyses and comparisons, we therefore propose that the mutant ATP7A with deletion of exons 13 and 14 is completely non-functional. This is consistent with the suboptimal clinical outcome in the proband despite early identification and treatment, as noted in other patients with Menkes disease having severe ATP7A mutations (13, 20, 32).

Given that exons 12 and 15 were each detected in the proband via exon-specific PCR, the gene
Favorably skewed X-inactivation accounts for neurological sparing
deletion appeared to involve the approximately 15-kb segment of ATP7A between these two coding regions (31). We utilized long PCR of genomic DNA from a normal control and the proband, using a primer pair that spanned the 3' end of exon 12 to the 5' end of exon 15. The PCR product obtained in normal control DNA was larger than a 12.2-kb DNA marker and in the proband was approximately 11 kb (data not shown). The proband's PCR product was sequenced from both ends in stepwise fashion until the breakpoint was encountered. The deletion comprised 5557 bp in total (Fig. 1a), beginning at base 50,387 and ending at base 55,943 as referenced in a X-chromosome clone (RP3-465G10, GenBank accession number AL645821.1).

PCR using primers flanking the breakpoint (Fig. 1b) produced a 664-bp junction fragment in the proband (III-1, Fig. 2a) and two obligate heterozygotes (I-1 and II-5, Fig. 2a). Testing for
the junction fragment (Fig. 2b) indicated that four of six at-risk females (II-4, II-6, II-7, and II-8) carried the deletion and two (II-1 and II-3) did not. As expected, exon 13 was amplified in all individuals tested except the proband (III-1). We subsequently used this junction fragment assay to exclude the diagnosis of Menkes disease in an at-risk male newborn (offspring of II-6, data not shown).

X-inactivation results

The proband’s maternal grandmother (I-1) showed HUMARA allele sizes of 269 and 287 bp (Fig. 3a, top left panel). Analyses from the deletion-carrying maternal aunts (II-4, II-6, II-7, and II-8) showed that they all inherited a 281-bp HUMARA allele from their father and the 269-bp allele from their mother (I-1). In contrast, the proband’s maternal aunts who do not carry the ATP7A deletion (II-1 and II-3) each inherited the 281-bp paternal allele and the other (287 bp) maternal allele (data not shown), documenting that the 269-bp HUMARA allele originates from the same X chromosome as the ATP7A exon 13/14 deletion.

Digestion of genomic DNA from the deletion carriers with the methylation-sensitive restriction endonuclease, HpaII, and subsequent PCR amplification of the HUMARA CAG repeat showed marked predominance of the 269 bp allele, representing the methylated, inactive X, which was protected from HpaII digestion. Four of the female mutation carriers (I-1, II-4, II-6, and II-7) showed highly (≥95:5) skewed X-inactivation (Figs. 2a and 3), and the other two carriers (II-5 and II-8) showed moderate (≥87:13) skewing (Fig. 2a). In contrast, the X-inactivation patterns in the

Fig. 3. X-inactivation analyses. (a) The proband’s grandmother (I-1) has HUMARA allele sizes of 269 and 287 bp (top left panel). Individual II-4, and all other of the proband’s maternal aunts who are heterozygous for the exon 13/14 deletion, inherited allele 281 from their father and allele 269 from their mother (top right panel). In contrast, the non-carrier females in this family (II-1 and II-3) inherited the 287-bp HUMARA allele from their mother (data not shown). These results indicated that the 269-bp HUMARA allele originates from the X chromosome harboring the ATP7A exon 13/14 deletion. (b) Digestion of genomic DNA from each subject with the methylation-sensitive restriction endonuclease, HpaII, prior to polymerase chain reaction amplification yields only allele 269, representing the methylated, inactive X chromosome (bottom panels) and indicating completely skewed (100:0) inactivation of the X chromosome bearing the ATP7A deletion.
Favorably skewed X-inactivation accounts for neurological sparing

non-carrier females (II-1 and II-3) were random (inactivation ratios = 71:29 and 62:38, respectively; Fig. 2a).

This represents the first report of X-inactivation patterns in cytogenetically normal females heterozygous for mutation at the ATP7A locus. Our analysis revealed that all six female heterozygotes showed skewed X-inactivation, with preferential silencing of the mutant X chromosome in each instance. The phenomenon of favorable skewing in female carriers of deleterious traits has been observed for other X-linked diseases, including α-thalassemia/mental retardation syndrome, Wiskott–Aldrich syndrome, dyskeratosis congenita, X-linked agammaglobulinemia, severe combined immunodeficiency disease, and the MECP2 duplication syndrome, although exceptions have also been noted (6). Because there may be tissue-specific differences in X-chromosome inactivation (33, 34), we acknowledge that our results, obtained from peripheral blood, may not reflect the X-inactivation status of other organs and tissues in these subjects.

Several mechanisms can lead to skewed X-chromosome inactivation. The first is selection, whereby cells with a specific active X chromosome develop a selective growth advantage (4, 6, 27). This phenomenon has been documented in cultured cells from obligate carriers of Menkes disease (27, 35). Alternatively, one of the two X chromosomes per female cell may be silenced coordinately during early embryogenesis, with cis-acting alleles mediating gene repression (36, 37). Because the murine homolog of ATP7A, atrp7a, appears to partially escape transcriptional silencing, with loss of epigenetic gene repression increasing over time (37, 38), it is possible that female carriers of Menkes disease could express mutant ATP7A from an inactivated X chromosome in some cells. This phenomenon could potentially modify clinical phenotypes in female carriers. Because ATP7A plays multiple roles in human physiology, including central nervous system development, neuronal activation, synaptogenesis, axon targeting, myelination, and motor neuron function, as well as contributing to hair, bone, and connective tissue integrity (11–13, 26, 39, 40), the scope of phenotypic manifestations could be quite broad.

Additional studies of X-inactivation patterns in obligate female carriers of ATP7A mutations, with careful clinical correlations, will be useful to determine whether the extreme skewing documented in this family is standard for this gene, and, in cases where it is not, whether deviation from this pattern has phenotypic consequences in females with a family history of Menkes disease, or its allelic variants, occipital horn syndrome (10), and ATP7A-related distal motor neuropathy (11).

Acknowledgements

We are grateful to the family for their participation and to Sarah Godwin for mutation identification in the proband. This work was supported by the NIH Intramural Research Program.

Conflicts of interest

No conflicts of interest related to this work are noted by any of the six authors.

References