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X Chromosome Inactivation in Carriers of Barth Syndrome

Karen Helene Ørstavik,¹ Ragnhild E. Ørstavik,¹ Anna K. Naumova,^{2,*} Patrizia D'Adamo,³ Agi Gedeon,⁴ Pieter A. Bolhuis,⁵ Peter G. Barth,⁶ and Daniela Toniolo³

¹Department of Medical Genetics, Ullevål University Hospital, Oslo; ²The Fels Institute of Cancer Research, Temple University, Philadelphia; ³Istituto di Genetica Biochimica ed Evoluzionistica, Pavia, Italy; ⁴Department of Cytogenetics and Molecular Genetics, Women's and Children's Hospital, North Adelaide, Australia; and Departments of ⁵Neurology and ⁶Pediatrics, University of Amsterdam, Amsterdam

Summary

Barth syndrome (BTHS) is a rare X-linked recessive disorder characterized by cardiac and skeletal myopathy, neutropenia, and short stature. A gene for BTHS, *G4.5*, was recently cloned and encodes several novel proteins, named "tafazzins." Unique mutations have been found. No correlation between the location or type of mutation and the phenotype of BTHS has been found. Female carriers of BTHS seem to be healthy. This could be due to a selection against cells that have the mutant allele on the active X chromosome. We therefore analyzed X chromosome inactivation in 16 obligate carriers of BTHS, from six families, using PCR in the androgen-receptor locus. An extremely skewed X-inactivation pattern ($\geq 95:5$), not found in 148 female controls, was found in six carriers. The skewed pattern in two carriers from one family was confirmed in DNA from cultured fibroblasts. Five carriers from two families had a skewed pattern (80:20–<95:5), a pattern that was found in only 11 of 148 female controls. Of the 11 carriers with a skewed pattern, the parental origin of the inactive X chromosome was maternal in all seven cases for which this could be determined. In two families, carriers with an extremely skewed pattern and carriers with a random pattern were found. The skewed X inactivation in 11 of 16 carriers is probably the result of a selection against cells with the mutated gene on the active X chromosome. Since BTHS also shows great clinical variation within families, additional factors are likely to influence the expression of the phenotype. Such factors may also influence the selection mechanism in carriers.

Introduction

Barth syndrome (BTHS; MIM 302060) is a rare X-linked inherited disorder characterized by cardiac and skeletal myopathy, short stature, and neutropenia (Barth et al. 1983). The gene was recently mapped to Xq28 (Bolhuis et al. 1991; Adès et al. 1993), and a novel gene, *G4.5*, responsible for BTHS was cloned (Bione et al. 1996). BTHS is a severe disorder that is often fatal in childhood and may be due to an acyltransferase deficiency (Neuwald 1997). Unique mutations have been reported (D'Adamo et al. 1997; Johnston et al. 1997). BTHS has proved to be allelic to a lethal myocard disorder (Bleyl et al. 1997).

Female carriers of BTHS seem to have no symptoms of the disorder, although a systematic clinical examination of carriers has not been reported. This could be related to a nonrandom X chromosome inactivation. In female mammals, one of the two X chromosomes in each cell is inactivated in early embryonic life (Lyon 1961). The inactivation is believed to be random, and most females therefore have an ~50:50 distribution of the two cell populations. This is in agreement with the finding of a mildly affected phenotype in many carriers of X-linked disorders. However, in a number of severe X-linked disorders, such as Wiskott-Aldrich syndrome (WAS), α -thalassemia/mental retardation syndrome, and Bruton X-linked agammaglobulinemia, female carriers have a completely normal phenotype. Obligate carriers of these disorders have skewed X chromosome inactivation (Fearon et al. 1988; Gibbons et al. 1992; Allen et al. 1994). This is presumably the result of selection against cells with the X chromosome carrying the mutant allele as the active X chromosome. X chromosome-inactivation analysis therefore may be used in carrier detection for these disorders. The X-inactivation pattern in 20 carriers from six families with BTHS was analyzed in order to identify a possible selection mechanism in these families.

Subjects and Methods

Subjects

Blood samples were obtained from 20 carriers and four noncarriers, from six BTHS families, after informed

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Address for correspondence and reprints: Dr Karen Helene Ørstavik, Department of Medical Genetics, Ullevål University Hospital, Box 1036, Blindern, 0315 Oslo, Norway. E-mail: k.h.orstavik@ioks.uio.no

* Present affiliation: Department of Genetics and Molecular Biology, School of Medicine, University of Pennsylvania, Philadelphia.

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consent. In all the families, a mutation in the BTHS gene had been identified and confirmed in the carriers (D'Adamo et al. 1997). All the families had unique mutations, except families 5 and 6, which had the same missense mutation in exon 10. The affected males from these two families had a clinical picture of endocardial fibroelastosis (EFE1). One family had a single carrier only; the remaining families had 2-6 carriers (fig. 1). The age at death or the age of the surviving male is indicated in figure 1. In family 2, skin fibroblasts and granulocytes were obtained from both carriers, in addition to blood. Further details of the families, including the age of the carriers at the time of blood sampling, are listed in table 1.

None of the carriers were known to have cardiac disease or short stature. The white-cell count was normal in carriers IV-20 and IV-21 (Barth et al. 1983) and in the two carriers from family 2. For the remaining carriers, a white-cell count was not available. Life expectancy of BTHS carriers is not known, but the mother of carriers III-1 and III-2 from family 1 was reported to be

alive at age 92 years. Blood was also obtained from 158 blood donors, aged 20-60 years.

DNA Isolation

Skin fibroblasts were subcultured in minimal essential medium. Granulocytes were separated from whole blood, and DNA was isolated from blood cells and fibroblasts, by standard procedures.

X Chromosome-Inactivation Analysis of the Androgen-Receptor (AR) Gene

The X-inactivation pattern was determined by PCR analysis of a polymorphic CAG repeat in the first exon of the AR gene (Allen et al. 1992). Methylation of sites close to this short tandem repeat has been found to correlate with X chromosome inactivation. The site is methylated on the inactive X chromosome and therefore resists cleavage by *HpaII*, and PCR products are obtained from the inactive X chromosome only. The PCR products were separated on an ABI 373A automated se-

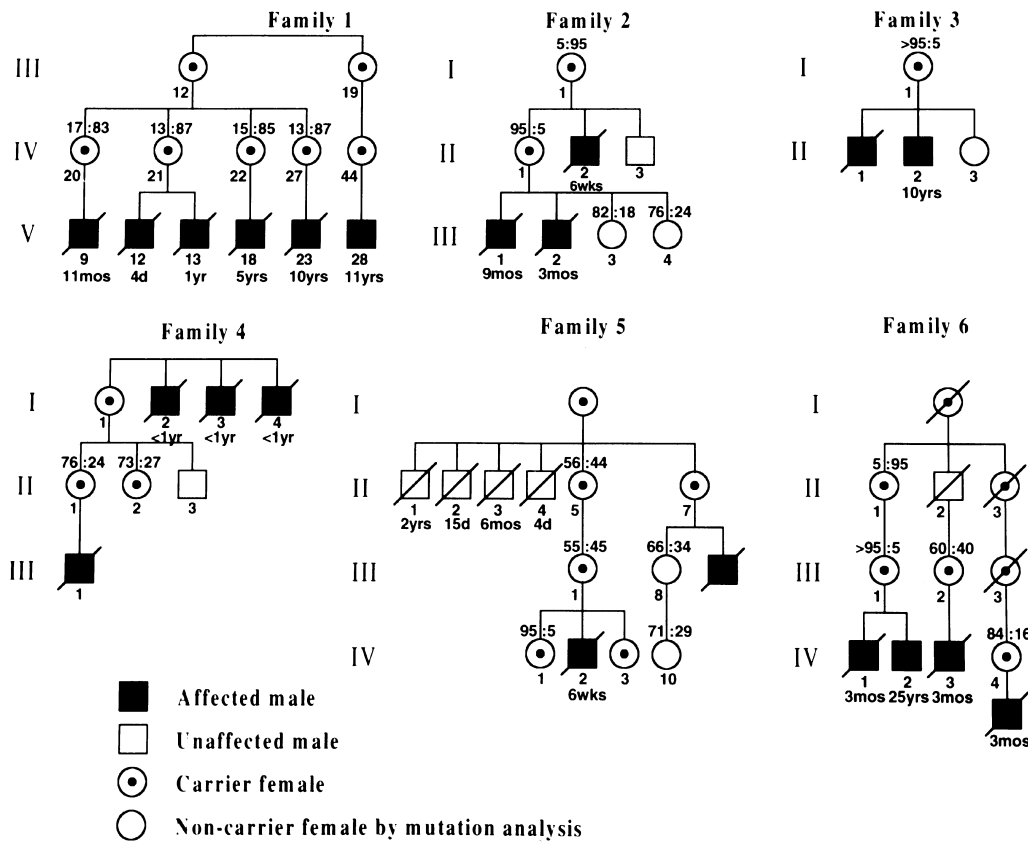


Figure 1 Pedigrees of families, as reported previously (family 1 by Barth et al. [1983]; family 2 by Ørstavik et al. [1993]; families 3, 4, and 6 by D'Adamo et al. [1997]; and family 5 by Lindenbaum et al. [1973]). The X-inactivation pattern is indicated as a ratio, for informative carriers. Age at death or present age is indicated for affected males.

Table 1**X Chromosome Inactivation in Females from BTHS Families**

Family and Carrier	Age at Blood Sampling (years)	Reference	Mutation	X Inactivation	Origin of Inactive X
1:					
III-12	66	Barth et al. (1983)	Y51X exon 2	Homozygous	
IV-20	42		Y51X exon 2	17:83	Maternal
IV-21	39		Y51X exon 2	13:87	Maternal
IV-22	38		Y51X exon 2	15:85	Maternal
IV-27	33		Y51X exon 2	13:87	Maternal
IV-44	29		Y51X exon 2	Homozygous	
2:					
I-1	60	Ørstavik et al. (1993)	F178I exon 6	5:95	Maternal
II-1	35		F178I exon 6	95:5	
III-3	7		No mutation	82:18	
III-4	4		No mutation	76:24	
3:					
I-1	35	D'Adamo et al. (1997)	428 del13 exon 2	>95:5	
4:					
II-1	36	D'Adamo et al. (1997)	G197R exon 8	76:24	
II-2	34		G197R exon 8	73:27	
5:					
II-5		Lindenbaum et al. (1973)	Missense exon 10	56:44	
II-7			Missense exon 10	Homozygous	
III-1		D'Adamo et al. (1997)	Missense exon 10	55:45	Maternal
IV-1	Child		Missense exon 10	95:5	
IV-4			Missense exon 10	Homozygous	
III-8			No mutation	66:34	
IV-10	Child		No mutation	71:29	
6:					
II-1	75	D'Adamo et al. (1997)	Missense exon 10	5:95	
III-1	50		Missense exon 10	>95:5	
III-2	47		Missense exon 10	60:40	
IV-4	18		Missense exon 10	84:16	Maternal

quencer and were analyzed by GeneScan software (Applied Biosystems) (Pegoraro et al. 1994). X-inactivation patterns were classified as random (ratio 50:50–<65:35), moderately skewed (ratio 65:35–<80:20), skewed (ratio 80:20–<95:5), or extremely skewed (ratio \geq 95:5).

X Chromosome–Inactivation Analysis of the FMR1 Gene

X inactivation was also analyzed in one carrier, by PCR of the *FMR1* gene (Fu et al. 1991). The methylation of the *HpaII* sites in the 5' region of the *FMR1* gene, located upstream of the polymorphic GGC repeat, has been found to correlate with inactivation of the X chromosome. Quantitation of the results of the PCR-methylation assay was performed by use of phosphorimaging analysis, as described elsewhere (Naumova et al. 1996).

Statistical Methods

The distributions of the X-inactivation pattern in the BTHS carriers and the female controls were compared by use of the Mann-Whitney test. The frequencies of the

extremely skewed X-inactivation pattern were compared by use of Fisher's exact test.

Results

The results of X-inactivation analysis of the carriers are shown in table 1. Four of the 20 carriers were homozygous for the CAG repeat at the AR locus and therefore were not informative. In family 1, the four informative carriers had an almost identically skewed pattern (15:85) in the AR assay. However, the pattern in this family was difficult to interpret, since the two alleles in all the heterozygotes differed by one CAG repeat only. Therefore, one carrier from this family also was tested in the *FMR1* assay, and an extremely skewed pattern (95:5) was found. All four carriers had the maternally inherited X chromosome, which carried the mutation, as the inactive X chromosome.

In family 2, the two obligate carriers, I-1 and II-1, had an extremely skewed pattern in the AR assay (fig. 2A). The skewed pattern in peripheral blood cells was confirmed in DNA from granulocytes and cultured fi-

broblasts. II-1 also had the maternally inherited X chromosome as the inactive X chromosome. The two daughters of II-1, III-3 and III-4, lacked the mutation and had a skewed and moderately skewed X-inactivation pattern, respectively. In this family, therefore, the extremely skewed X-inactivation pattern segregated with the mutation. The only carrier from family 3 also had an extremely skewed X-inactivation pattern, whereas a moderately skewed pattern was found in the two carriers, II-1 and II-2, from family 4.

Families 5 and 6 included five and four informative carriers, respectively. In these families, carriers with both an extremely skewed X-inactivation pattern and a random pattern were found. In skewed carriers IV-1 from family 5 and IV-4 from family 6, the origin of the inactive X chromosome was maternal.

The X-inactivation pattern was analyzed in the 158 female controls by use of the AR assay. Ten females were not informative. Of the remaining 148 females, 55% had a random pattern, 38% had a moderately skewed pattern, and 7% had a skewed pattern (table 2). A similar distribution previously had been found in 30 normal females (Ørstavik et al. 1996b). No female in either sample had an extremely skewed X-inactivation pattern. The difference between the distributions of the X-inactivation pattern in the BTHS carriers and the female controls was highly significant ($P < .0001$; Mann-Whitney test). The finding of an extremely skewed X-inactivation pattern in six carriers and in none of the female controls also was highly significant ($P < .00001$; Fisher's exact test).

Table 2

Distribution of X-Inactivation Pattern in BTHS Carriers and Female Controls

X-Inactivation Pattern	No. (%) of BTHS Carriers	No. (%) of Female Controls
Random (50:50-<65:35)	3 (19)	81 (55)
Moderately skewed (65:35-<80:20)	2 (12)	56 (38)
Skewed (80:20-<95:5)	5 (31)	11 (7)
Extremely skewed ($\geq 95:5$)	6 (38)	0 (0)
Total	16	148

NOTE.— $P < .0001$ (Mann-Whitney test).

Discussion

The X-inactivation pattern in the 16 carriers varied both between and within the six families (table 1). An X-inactivation pattern of 95:5 or more—as was found in the six carriers from families 2, 3, 5, and 6—was not found in any of 148 female controls. The X-inactivation pattern in BTHS carriers therefore clearly differs from that in female controls. Furthermore, a skewed pattern was found in significantly more carriers than controls (table 2).

The frequency of skewed X inactivation increases in older females (Busque et al. 1996; Gale et al. 1997), possibly owing to an X-linked gene that regulates hematopoietic stem-cell kinetics (Abkowitz 1998). However, of the six females with an extremely skewed X-inactivation pattern, one was a child and two were young females. Furthermore, although skewed carriers

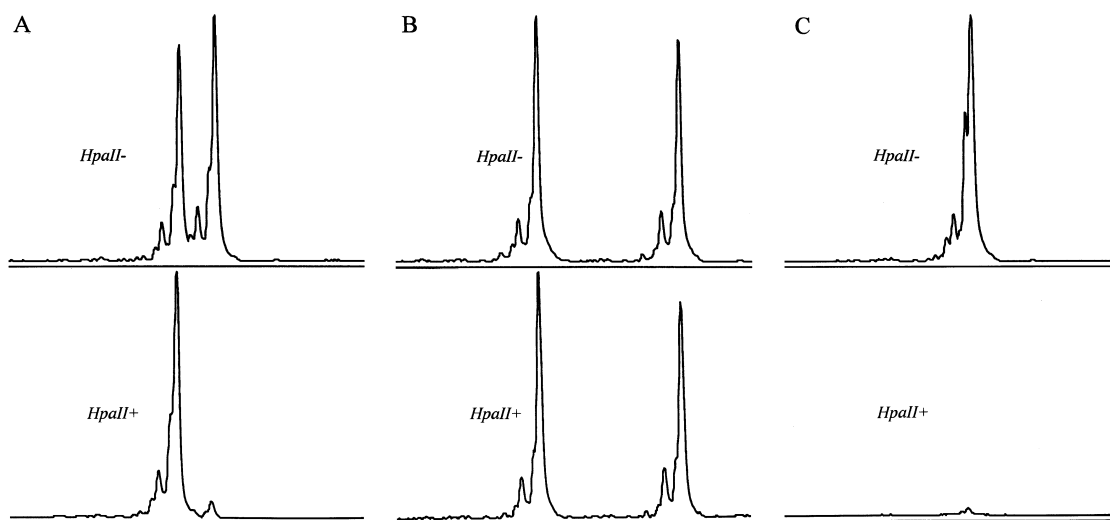


Figure 2 X-inactivation analysis. A minus sign (–) indicates without *HpaII* predigestion, and a plus sign (+) indicates with *HpaII* predigestion. A, Results for II-1 of family 2 (X-inactivation pattern, 95:5). B, Results for II-5 of family 5 (X-inactivation pattern, 56:44). C, Results for a male control. Note the lack of PCR product after *HpaII* digestion.

I-1 from family 2 and II-1 from family 6 were 60 and 75 years of age, respectively, at the time of blood sampling, they both had daughters who also were extremely skewed. The skewed pattern found in I-1 of family 2 also was found in cultured fibroblasts, in which age-related increased skewing of X inactivation is not expected. The extreme skewing found in the six BTHS carriers therefore is unlikely to be related to age.

A skewed X-inactivation pattern may be mainly due to three different mechanisms (Puck and Willard 1998). The most frequent cause of a skewed X-inactivation pattern is a chance occurrence arising from the small number of cells at the time of X inactivation. This is a highly unlikely explanation for the skewing in the BTHS carriers, both because of the large number of females with an extremely skewed pattern, in this carrier population, and because of the occurrence of more than one extremely skewed carrier in the same family.

A second possibility is a genetic influence on the process of X inactivation in these families that acts independently of the BTHS mutation. Many families—both normal families and families with X-linked disorders—have been reported to have more than one female with a skewed X-inactivation pattern (Hoffman and Pegoraro 1995; Belmont 1996; Naumova et al. 1996; Ørstavik et al. 1996a). Genetic factors are most likely to influence the choice of which X chromosome will be inactivated initially. Some females therefore will be predisposed to skewing (Plenge et al. 1997).

A mutation in the *XIST* gene recently was reported for a carrier of X-linked ichthyosis who had skewed X inactivation (Plenge et al. 1997). This *XIST* minimal-promoter mutation was found in only 1 of 1,166 independent X chromosomes studied. Therefore, this rare mutation is unlikely to have occurred by chance in five of six BTHS families. Furthermore, in all the families, the extremely skewed X inactivation was found to be associated with carrier status, since none of the four females who were excluded as carriers had the extremely skewed X-inactivation pattern. Therefore, a genetic mechanism acting independently of the BTHS mutations is not a likely explanation for the skewed X-inactivation pattern in the carriers.

The most likely explanation for the skewed X inactivation in the BTHS carriers is a postinactivation selection mechanism (Migeon 1993). This implies a growth disadvantage for the cells having the mutated BTHS gene on the active X chromosome. Postinactivation cell selection is expected to be restricted to a single target tissue, as is found in WAS carriers, in whom the skewed X inactivation is found in hematopoietic cells but not in fibroblasts and buccal-smear cells (Fearon et al. 1988). In family 2, both carriers also were shown to have a skewed pattern in cultured skin fibroblasts. However, since respiratory-chain abnormalities have been

found in cultured fibroblasts from BTHS patients (Barth et al. 1996), the skewed pattern in fibroblasts is not in conflict with a selection mechanism.

In family 1, all four carriers had a skewed pattern in the AR assay, and one carrier had an extremely skewed pattern in the *FMR1* assay. In the four carriers, the X-inactivation pattern was identical, and the maternal X carried the mutation as the inactive X chromosome, in most cells. Therefore, the pattern is likely to be related to carrier status, and a selection is likely to have taken place in family 1. Unfortunately, there were no noncarrier females available from this family, to confirm that the skewed pattern segregated with the disorder. The selection hypothesis is supported by the finding of a maternal origin of the inactive X chromosome in seven carriers with skewed and extremely skewed X inactivation. Family 4, however, in which both carriers had a moderately skewed X-inactivation pattern, demonstrates that an extremely skewed X inactivation is not a general phenomenon in BTHS carriers.

The most striking finding of our study is the X-inactivation pattern in families 5 and 6, in which both a skewed and a random pattern were found. Affected males in these two families had EFE1 and a G→A change in exon 10, causing a G→R change in the sequence of the protein (G240R) (D'Adamo et al. 1997). Of particular interest is the intrafamilial variation in affected males in family 6. Although three patients from family 6 died in infancy, one patient (IV-2) survived and is now normal at age 25 years. Another male (II-2) from this family must have transmitted the gene to his daughter. No further information on this male was available.

The phenotype of BTHS varies both between and within families and cannot be explained by the nature of the mutation alone (Christodoulou et al. 1994; D'Adamo et al. 1997; Johnston et al. 1997). It therefore has been suggested that additional factors modify the expression of the BTHS phenotype. Such additional factors also may modify the selection mechanism in the BTHS carriers. The function of the *G4.5* gene is unknown. The sequence of the tafazzins is highly conserved, and, therefore, the tafazzins likely have important functions. Since a few patients who survived the disorder in childhood became healthy adults, the function of tafazzins appears to be substituted in later life.

One possible explanation for the presence of both a skewed and a random X-inactivation pattern in BTHS carriers may be a different degree of growth disadvantage during early development. In some carriers, the growth disadvantage of the cells with the BTHS mutation on the active X chromosome may result in extreme skewing. In other carriers, additional factors that modify the expression of the BTHS phenotype also could modify the growth disadvantage to such a degree that the selective pressure is reduced or does not take place at all.

The X-inactivation patterns in families 5 and 6 were analyzed in DNA from peripheral blood cells only. The possibility exists that the X-inactivation pattern in carriers with a random pattern may be skewed in other tissues, such as fibroblasts, in which respiratory-chain abnormalities have been found. Unfortunately, skin fibroblasts were not available from any of the carriers from families 5 and 6.

To our knowledge, this is the first report of both a very skewed and a random pattern of X inactivation in unaffected carriers of an X-linked disorder. It would be of interest to see if carriers with a random pattern differ from carriers with an extremely skewed pattern—for instance, by having minor signs of BTHS, such as shorter stature and a lower white-cell count.

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Electronic-Database Information

Accession number and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim> (for BTHS [MIM 302060])

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