Microphthalmia with linear skin defects syndrome (MLS): a male with a mosaic paracentric inversion of Xp

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Abstract. The microphthalmia with linear skin defects syndrome (MLS) is an X-linked dominant disorder with male lethality. In the majority of the patients reported, the MLS syndrome is caused by segmental monosomy of the Xp22.3 region. To date, five male patients with MLS and 46,XX karyotype ("XX males") have been described. Here we report on the first male case with MLS and an XY complement. The patient showed agenesis of the corpus callosum, histiocytoid cardiomyopathy, and lactic acidosis but no microphthalmia, and carried a mosaic subtle inversion of the short arm of the X chromosome in 15% of his peripheral blood lymphocytes, 46,Y,inv(X)(p22.13 ~ 22.2p22.32 ~ 22.33)[49]/46,XY[271]. By fluorescence *in situ* hybridization (FISH), we showed that

YAC 225H10 spans the breakpoint in Xp22.3. End-sequencing and database analysis revealed a YAC insert of at least 416 kb containing the genes HCCS and AMELX, and exons 2–16 of ARHGAP6. Molecular cytogenetic data suggest that the Xp22.3 inversion breakpoint is located in intron 1 of ARHGAP6, the gene encoding the Rho GTPase activating protein 6. Future molecular studies in karyotypically normal female MLS patients to detect submicroscopic rearrangements including the ARHGAP6 gene as well as mutation screening of ARHGAP6 in patients with no obvious chromosomal rearrangements will clarify the role of this gene in MLS syndrome.

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The microphthalmia with linear skin defects syndrome (MLS; MIM 309801) is a severe developmental disorder. Major clinical signs are localized dermal aplasia and microphthalmia. Occasional abnormalities include agenesis of the corpus callosum, sclerocornea, chorioretinal abnormalities, infantile seizures, congenital heart defect, and mental retardation. At least 30 patients have been described so far (Friedman et al., 1988; Al-Gazali et al., 1990; Donnenfeld et al., 1990; Temple et al., 1990; Allanson and Richter, 1991; Gericke et al., 1991; Thies et al., 1991; Lindor et al., 1992; Naritomi et al., 1992; Happle et al., 1993; Bird et al., 1994; Eng et al., 1994; Lindsay

et al., 1994; McLeod et al., 1994; Mücke et al., 1995; Camacho et al., 1997; Paulger et al., 1997; Cox et al., 1998; Ogata et al., 1998; Stratton et al., 1998; Zvulunov et al., 1998; Kono et al., 1999; Kayserili et al., 2001; Anguiano et al., 2003).

The MLS syndrome is considered to be an X-linked dominant male lethal disorder. Conventional and molecular cytogenetic studies of most MLS patients revealed an XX complement with a variety of X-chromosomal aberrations, including deletion of Xpter→p22 or unbalanced translocation between the X chromosome and an autosome or the Y chromosome, that all resulted in segmental monosomy of Xp22.3. Remarkably, four female patients with an apparently normal karyotype have also been described (Happle et al., 1993; Bird et al., 1994; Cox et al., 1998; Zvulunov et al., 1998) suggesting that submicroscopic rearrangements or "short length" mutations in a yet unidentified gene may also be responsible for the trait. To date, three affected males and twin brothers have been reported, all with an XX complement and a translocation between Xp and Yp (Lindsay et al., 1994; Paulger et al., 1997; Stratton et al., 1998; Kono et al., 1999; Anguiano et al., 2003). A sixth patient

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had ambiguous genitalia and a 46,XX karyotype (Camacho et al., 1997).

By mapping the breakpoints of MLS patients, an ~ 570-kb minimal region of monosomy has been identified in Xp22.3 (Wapenaar et al., 1993; 1994), distal to AMELX (amelogenin) (Lagerstrom et al., 1991), and cloned into overlapping cosmids (Cox et al., 1998). Three genes have been mapped and characterized in the critical deletion interval for MLS: MID1 (midline-1) mutated in the X-linked Opitz G/BBB syndrome (Quaderi et al., 1997), HCCS which encodes the human holo-cytochrome c-type synthetase (Schaefer et al., 1996), and ARH-GAP6 encoding a Rho GTPase activating protein (Schaefer et al., 1997). Mice deficient for the Rho GAP domain of *Arhgap6* did not show any detectable phenotypic or behavioural abnormalities (Prakash et al., 2000). In marked contrast, deletion of the *Hccs* gene in the mouse leads to lethality of the embryos early in development suggesting that loss of HCCS causes the male lethality of MLS syndrome (Prakash et al., 2002).

Another important factor in determining the MLS phenotype seems to be the pattern of X inactivation. It has been proposed that functional absence of the MLS gene caused by inactivation of the normal X chromosome in patients with Xp22 monosomy may play a critical role in the development of MLS (reviewed in Van den Veyver, 2001). Moreover, variable tissue-specific X-inactivation is likely to account both for the intra-familial variability and clinical divergence in sporadic cases (Allanson and Richter, 1991; Ballabio and Andria, 1992; Mücke et al., 1995).

In this report, we describe the first case of MLS syndrome in a male with an XY complement and a mosaic paracentric inversion of the short arm of the X chromosome in blood lymphocytes.

Materials and methods

Case report

The male infant was born to non-consanguineous parents by Cesarean section the 36th week of gestation. Ultrasound examination revealed intra-uterine growth retardation. There was no history of prenatal drug exposure or intrauterine infection. At birth, the patient was noted to have bilateral paranasal linear skin defects but no microphthalmia. Birth weight was low (1,620 g, -3 SD) and a severe postnatal metabolic acidosis developed. Ultrasound investigations revealed lissencephaly and agenesis of the corpus callosum as well as pericardial effusion and mild hypertrophic cardiomyopathy. Blood pyruvate concentration was normal with markedly elevated blood (6–18 mmol/l, normal <2) and CSF lactate values (10.7 mmol/l, normal <1.5), and a slightly elevated alanine level (8.5 mg/dl, normal <5.1). Lactic aciduria and ketonuria were present. Respiratory chain enzyme activities were in the normal range both in a muscle biopsy and in skin fibroblasts. The patient was treated symptomatically with bicarbonate buffer, a specific vitamin cocktail (containing carnitine, coenzyme Q, riboflavin, vitamin K1, and thiamine), and ketogenic diet without any obvious benefit. During follow-up studies, he showed failure to thrive, cardiomyopathy with supraventricular tachycardia and pericardial effusion, hepatic failure with cholestatic hepatopathy, and a deteriorating lactic acidosis. He died from multiple organ failures at the age of four months. Postmortem autopsy confirmed the agenesis of the corpus callosum and the severe liver disease with fatty degeneration and fibrosis. Histologic investigation of the heart showed histiocytoid/oncocytic cardiomyopathy and subendocardial myocardial necrosis.

Conventional cytogenetic and FISH studies

Chromosome preparations were made from PHA-stimulated peripheral blood lymphocytes. Cytogenetic analysis was carried out by an improved

high-resolution RBG-H banding method (700–1,000 band level) (Werner and Spiegler, 1988). Fluorescence in situ hybridization was performed as previously described (Bartsch et al., 1999). DOP-PCR products of YAC 225H10 were labeled using tetramethylrhodamin-6-dUTP. Chromosomes were counterstained using DAPI (Roche) and mounted in antifading solution (Vector Labs). Slides were analyzed by AxiophotTM epifluorescence microscopes (Zeiss) and the ISISTM digital imaging system (Meta Systems).

Derivation of YAC end-sequences

End-sequences of YAC clone 225H10 were obtained as described by Nehls et al. (1995). Briefly, total yeast DNA was isolated by standard protocols and digested with *Sau3AI*, *HpaII*, *TaqI*, *HinPI*, *AluI*, *BstUI*, *HaeIII*, *RsaI*, and *NlaIII*. DNA fragments were ligated to one of two vectorette “isoforms” and subsequently, end sequences were amplified selectively in primary and secondary PCR reactions. Purified amplicons (JETQUICK PCR purification kit, Genomed) were directly sequenced using the ABI Prism Dye Terminator Kit (PE Applied Biosystems) and the ABI Prism 377 automatic sequencer (PE Applied Biosystems).

Results

Conventional and molecular cytogenetic studies

Cytogenetic investigation of peripheral blood lymphocytes by high resolution RBG-H banding revealed two cell lines, one with an apparently normal (46,XY) karyotype and a second one with an aberrant karyotype. The normal karyotype was observed in 271 (~ 85 %) of the 320 metaphases analyzed (Fig. 1a, b). In 49 metaphases, an aberrant R-banding pattern was found at the telomeric part of the short arm of the X chromosome (Fig. 1a, c). A terminal or an interstitial deletion was unlikely for both the normal and rearranged X chromosomes had the same length. Fluorescence in situ hybridization (FISH) analysis with cosmid 34F5, containing exons 1–5 of the SHOX gene located in the pseudoautosomal region (PAR), and with cosmid 2.1, that maps about 1,700 kb proximal to SHOX, yielded a single signal on the terminal end of the X chromosome in all metaphases analyzed (data not shown). Taking the cytogenetic and FISH data together, the rearrangement observed was interpreted as a paracentric inversion with the distal breakpoint in Xp22.32 ~ p22.33 and the proximal breakpoint in Xp22.13 ~ p22.2, karyotype mos 46,Y,inv(X)(p22.13 ~ 22.2p22.32 ~ 22.33)/46,XY.

To map the putative inversion breakpoint in Xp22.3 more precisely, we performed FISH analysis with YAC clone 225H10, which contains part of the previously defined MLS critical region (Wapenaar et al., 1993) and which has been found to be deleted in three patients with MLS syndrome (Lindsay et al., 1994). In the majority of the 38 metaphases analyzed with YAC 225H10, we observed one distinct signal on the short arm of the X chromosome (Fig. 2a). However, in three metaphases, this YAC produced two signals of similar intensity (Fig. 2b), one in Xp22.3 and another one located more proximally, suggesting that this clone is bridging the breakpoint.

YAC 225H10 contains exons 2–16 of the ARHGAP6 gene in Xp22.3

By using vectorette PCR, we determined the end-sequences of YAC 225H10 and initiated BLASTN database searches at the NCBI. YAC endmarker A was located at position 11,514 bp in BAC clone GS-602M16 (GenBank acc. no. AC003657) and

Fig. 1. Mosaic paracentric inversion of the short arm of the X chromosome [inv(X)(p22.13 ~ 22.2p22.32 ~ 22.33)] in the patient with MLS syndrome. **(a)** Ideogram of a high resolution RBG-banded X chromosome. The upper arrow indicates the breakpoint in Xp22.33 ~ p22.32, whereas the lower one points to that in Xp22.2 ~ p22.13. **(b)** Three RBG-H-banded normal X chromosomes and **(c)** three rearranged X chromosomes of the patient. The two short lines point to the centromere and the arrow in **(c)** marks the inverted region.

Fig. 2. Characterization of the inversion breakpoint in Xp22.3. **(a, b)** Fluorescence in situ hybridization with YAC clone 225H10 on metaphase spreads of the patient. **(a)** In the majority of the metaphases analyzed, YAC 225H10 gives one signal on the X chromosome. **(b)** In contrast, YAC 225H10 produces two signals in a small number of metaphases with the rearranged X chromosome of the patient. The probe is labeled by tetramethylrhodamin (depicted in green). Chromosomes are counterstained with DAPI (depicted in red). **(c)** Schematic representation of the critical breakpoint area in Xp22.3. The uppermost arrow indicates the proximal part of the critical region of the MLS syndrome. The second line represents a region in Xp22.3

in telomere (Xpter) to centromere (cen) orientation, that is not drawn to scale. Microsatellite marker loci and the deletion breakpoint of MLS patient BA325 (zigzag line), that defines the proximal boundary of the MLS critical region, are shown. Middle lines indicate genomic contig sequences and PAC, BAC, or cosmid clones that are completely sequenced. The bottom line represents the insert of YAC 225H10. Below the YAC insert, exons of the genes ARHGAP6, HCCS, and AMELX are shown by thin vertical bars which are partially numbered. White boxes indicate untranslated regions of the ARHGAP6 gene. The bracket at the very bottom shows the putative breakpoint region in the patient.

at position 4,255,725 bp in human genome contig NT_011812.10 (Fig. 2c). We mapped YAC endmarker B to position 29,485 bp in clone GSHB-590J6 (GenBank acc. no. AC004554) and to position 4,821,848 bp in contig NT_011812.10 (Fig. 2c). Thus, an insert size of 566,123 bp was determined for YAC 225H10 that agreed well with the size of 560 kb estimated previously by PFGE analysis (Ferrero et al., 1995). By compiling recent data, we found three genes, HCCS, AMELX, and ARHGAP6, present on YAC 225H10, which contains the putative inversion breakpoint of the patient's rearrangement in Xp22.3 (Fig. 2c).

ARHGAP6 consists of 16 exons and spans a region of 528 kb. With the exception of exon 1, that is located 373 kb centromeric to the previously designated first exon (currently exon 2), all exons of the ARHGAP6 gene are present on the insert of YAC 225H10. The fact that exon 1 of ARHGAP6 is not covered by the YAC bridging the putative breakpoint suggests that the ARHGAP6 gene is disrupted by the inversion in Xp22.3 in ~15% of the patient's lymphocytes. Moreover, the equal intensities of the split signals obtained by FISH (Fig. 2b) suggest that the breakpoint is located approximately in the middle of the insert, i.e. in intron 1 of ARHGAP6 (Fig. 2c).

In an attempt to further characterize the breakpoint, we performed 3' RACE experiments on cDNA prepared from fibroblast RNA of the patient using a "forward" primer located in exon 1 of ARHGAP6 in combination with a polyT primer. Although we did several rounds of RACE-PCR, cloned the PCR products, and sequenced the inserts of about 70 clones, we failed to identify any aberrant ARHGAP6 transcript consisting of the first exon of ARHGAP6 fused to a sequence originating from the proximal inversion breakpoint in Xp22.13 ~ p22.2 (data not shown). Possibly, the presence of the chromosomal inversion in only a distinct portion of the patient's cells has significantly hampered the identification of such aberrant fusion transcripts.

Discussion

In the present paper, we describe the first 46,XY male with MLS syndrome, who carries a mosaic of a paracentric inversion of Xp with one breakpoint in Xp22.32 ~ p22.33 and the other one in Xp22.13 ~ p22.2 in peripheral blood lymphocytes. In addition to MLS, he showed histiocytoid cardiomyopathy and a deteriorating lactic acidosis. To our knowledge, the patient described here is the first MLS case with an X-chromosomal rearrangement other than a terminal deletion or translocation resulting in monosomy of Xp22.3.

We mapped the telomeric inversion breakpoint in Xp22.32 ~ p22.33 to a region of ~500 kb, most likely in intron 1 of ARHGAP6, i.e. our data suggested that the ARHGAP6 gene was disrupted in a portion of the patient's lymphocytes. Several lines of evidence suggest that ARHGAP6 is an attractive candidate gene for MLS syndrome. First, ARHGAP6 is expressed in fetal brain and retina (Schaefer et al., 1997). Various protein isoforms are generated by extensive alternative splicing (Prakash et al., 2000; Kayserili et al., 2001) including three that contain amino acids encoded by exon 1 (GenBank

acc. nos. AF012272, AF022212, and AF117067). Expression of a variant in the tissues affected in MLS syndrome would suggest that this isoform plays a role in the normal development and/or function of these organs. However no data exist so far on the tissue-specific expression of these ARHGAP6 transcripts/isoforms. Second, at least exons 6–16 of ARHGAP6 are deleted in all MLS patients. The proximal boundary of the MLS critical region has been located in intron 1 of ARHGAP6 by defining the deletion breakpoints of the female patient BA325 (Wapenaar et al., 1994) and of the patient described by Kayserili et al. (2001), both presenting with characteristic clinical features of MLS syndrome. In the patient reported here, the mRNAs transcribed from the aberrant X chromosome would not produce any functional ARHGAP6 variant containing the first 196 amino acids encoded by exon 1. However, other transcripts with exon 2 as the first exon might be normally expressed from the rearranged X chromosome. Thus, one can hypothesize that the absence of an ARHGAP6 isoform containing the amino acids encoded by exon 1 is the cause of the MLS syndrome in the patient. Clearly, we can not exclude that another not yet identified gene present on YAC insert 225H10, possibly located in the >373-kb intron 1 of ARHGAP6, is implicated in the pathogenesis of MLS syndrome.

The function of ARHGAP6 has been investigated by generating *Arhgap6*-deficient mice and in vitro expression studies (Prakash et al., 2000). The targeted *Arhgap6* allele encodes a putative protein of 470 amino acids that lacks the Rho GAP domain and the C-terminus. Remarkably, loss of Rho GAP function did not cause any detectable phenotypic or behavioural abnormalities in the mutant mice. The N-terminus of ARHGAP6 has been shown to be required for the regulation of cell morphology and actin polymerization (Prakash et al., 2000) and thus, it seems critical for proper ARHGAP6 function. This again indicates that mutated ARHGAP6 isoforms containing an N-terminus encoded by exon 1 may be implicated in MLS syndrome.

Mutation analysis of ARHGAP6 in MLS patients has not been performed because this gene was always included in the terminal Xp22 deletions. Several MLS syndrome patients have been reported with obviously normal karyotypes (Happle et al., 1993; Bird et al., 1994; Cox et al., 1998; Zvulunov et al., 1998; Peter Meinecke, personal communication). However, these patients may carry chromosomal rearrangements that escaped detection by routine cytogenetic analysis. Further studies, including FISH and Southern blot, will help to elucidate the presence of small rearrangements in the above mentioned patients. Finally, mutation screening of ARHGAP6 would be of great interest in cases with no chromosomal rearrangements.

In FISH analysis, YAC 225H10 produces split signals on the short arm of the rearranged X chromosome in three metaphases of the patient analyzed suggesting that this YAC spans the breakpoint in the patient. Nonetheless, alternative interpretations of the results obtained are also possible although not very likely. A complex rearrangement might have occurred with a duplication in addition to the inversion. Thus, split signals may result from a duplication comprising (part of) the YAC insert at the proximal inversion breakpoint in Xp22.2 ~ p22.13. Indeed, characterization of an X/10 translo-

cation by FISH revealed that, in addition to the translocation, a duplication of at least 500 kb occurred at the breakpoint region in 10q24 (I. Martinez-Garay and K. Kutsche, unpublished data). Low-copy repeats (LCRs) represent a new class of repetitive DNA element that has been recently identified in the human genome. Intrachromosomal duplications are typically found on a single chromosome or in a single chromosomal band (Mazzarella and Schlessinger, 1998; Ji et al., 2000). Two families of LCRs, CRI-S232 and G1.3 (DXF22S), also known as FAM9 gene family, were found to be interspersed on Xp22.3 (Bardoni et al., 1988; Ballabio et al., 1990; Martinez-Garay et al., 2002). Thus, the presence of a segmental duplication on the short arm of the X chromosome may also account for the split signals seen in the patient.

The patient had bilateral paranasal linear skin defects but no microphthalmia and thus showed an "incomplete" phenotype of MLS syndrome. Certainly, the mosaicism present in our patient, as in patient 2 described by Lindsay et al. (1994), may have contributed to the milder phenotype. Four female patients with MLS but without microphthalmia have also been reported. Cytogenetic analysis revealed a deletion of Xpter→p22.2 in one patient (Allanson and Richter, 1991), a 45,X/46,X,del(X)(pter→p22.2) mosaicism in the second one (patient 2 in Lindsay et al., 1994), and a normal karyotype in the two remaining cases (patient BA529 in Cox et al., 1998; Zvulunov et al., 1998). Thus, although two patients lacked larger portions of Xp, they did not show the complex phenotype suggesting that the MLS phenotype was not necessarily caused by a larger deletion. Instead, different patterns of X inactivation may account for the variability of the phenotype. The finding of two karyotypically normal patients (Cox et al., 1998; Zvulunov et al., 1998) suggests that submicroscopic gene defect(s) together with a preferentially inactivated normal X chromosome may also cause the MLS phenotype in females.

The patient presented with severe lactic acidosis, in addition to MLS syndrome. Defects in the pyruvate dehydrogenase (PDH) complex are an important cause of primary lactic acidosis (Lissens et al., 2000). The great majority of PDH complex deficiencies result from mutations in the PDHA1 gene (Dahl et al., 1992; Lissens et al., 2000) which is located in Xp22.2→p22.1 (Brown et al., 1989). PDHA1 encodes the α -subunit of the pyruvate dehydrogenase (E1), one of three components of the PDH complex. Remarkably, the proximal inversion breakpoint of the patient was localized to Xp22.2 ~ p22.13 suggesting that the PDHA1 gene could be functionally impaired by the rearrangement.

Somatic mosaicism is becoming increasingly recognized for explaining the phenotype of surviving male patients carrying

X-linked dominant mutations, e.g. in incontinentia pigmenti (IKBKG), Rett syndrome (MECP2), or chondrodysplasia punctata (EBP) (Metzenberg et al., 1999; Clayton-Smith et al., 2000; Armstrong et al., 2001; Kenwrick, 2001; Topçu et al., 2002). We assume that the paracentric inversion of the X chromosome occurred postzygotically in a particular subset of the patient's cells. Furthermore, it is likely that the inversion was also present in a significant portion of cells of skin, brain, and heart for the patient showed typical features of MLS syndrome, in addition to a histiocytoid/oncocytic cardiomyopathy. Two female patients with MLS syndrome and oncocytic cardiomyopathy (Bird et al., 1994) or hypertrophic cardiomyopathy (Happle et al., 1993) have been reported showing similarities in physical findings and clinical course. Both had normal karyotype suggesting that the genetic defect that caused the MLS syndrome might also be implicated in histiocytoid cardiomyopathy. It is of interest to note that the gene for holocytochrome *c*-type synthetase, HCCS, resides in the critical region for MLS syndrome (Schaefer et al., 1996). It has been speculated that a defect of the HCCS enzyme activity could give rise to a nuclear-encoded respiratory chain defect (Schwarz and Cox, 2002) which, in turn, may lead to (histiocytoid) cardiomyopathy in MLS cases. Remarkably, a missense mutation in the mitochondrial cytochrome b (MTCYB) gene was found in a patient with histiocytoid cardiomyopathy (Andreu et al., 2000) suggesting that a mitochondrial respiratory chain enzyme is involved in the pathogenesis of (one form of) this heart phenotype.

To understand the contribution of the genes located in the critical region for MLS syndrome, and in particular that of HCCS, mouse models were created with overlapping deletions similar to the human MLS deletion (Prakash et al., 2002). The complete loss of *Hccs* leads to embryonic lethality that can be rescued by overexpressing the human HCCS gene from a BAC. These data suggest that male lethality in MLS is caused by deficiency of HCCS (Prakash et al., 2002). The distal inversion breakpoint of the patient maps at least 170 kb proximal to the 3' end of HCCS suggesting that this gene is not directly impaired by the chromosomal rearrangement. Although it seems not likely, we cannot exclude with certainty an involvement of the HCCS gene in the phenotypic features of MLS syndrome in the patient. Further studies are required to unravel the role of HCCS and ARHGAP6 in this syndrome.

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