

## Stepwise partitioning of Xp21: a profiling method for XK deletions causative of the McLeod syndrome

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**BACKGROUND:** McLeod syndrome (MLS) is hematologically defined by the absence of the red blood cell (RBC) antigen Kx on the transmembrane RBC protein, XK, representing a highly specific diagnostic marker. Direct molecular assessment of XK therefore represents a desirable diagnostic tool. Whereas pathogenic point mutations may be simply identified, partial and complete deletions of XK on Xp21.1, eventually covering adjacent genes and causing multifaceted “continuous gene syndromes,” are difficult to localize.

**STUDY DESIGN AND METHODS:** Three different McLeod patient samples were tested using 16 initial positional polymerase chain reaction (PCR) procedures distributed over an approximately 2.8-Mbp Xp-chromosomal region, ranging telomeric from *MAGEB16* to *OTC*, centromeric of XK. The molecular breakpoint of one sample with an apparent large Xp deletion was iteratively narrowed down by stepwise positioning further PCR procedures and sequenced. Two mutant XK genes, one previously published and serving as a positive control, were also sequenced.

**RESULTS:** We confirmed the positive control as previously published and listed as XK\*N.20 by the International Society of Blood Transfusion (ISBT). The other XK showed a novel four-nucleotide deletion in Exon 1, 195-198delCCGC (newly listed as XK\*N.39 by the ISBT). The third sample had an approximately 151-kbp X-chromosomal deletion, reaching from Exon 2 of *LANCL3*, across XK to Exon 3 of *CYBB* (newly listed as XK\*N.01.016 by the ISBT). Carrier status of the patients' sister was diagnosed using a diagnostic “gap-PCR.”

**CONCLUSIONS:** The stepwise partitioning of Xp21.1 is pragmatic and cost-efficient in comparison to other diagnostic techniques such as “massive parallel sequencing” given the rarity of MLS. All males with suspected MLS should be considered for molecular XK profiling.

The McLeod syndrome (MLS) is a multisystem disorder with involvement of the central and peripheral nervous system, as well as neuromuscular, cardiologic, and (immuno-) hematologic manifestations predominantly in males.<sup>1,2</sup> Neurologic symptoms comprise a Huntington disease-like phenotype with choreatic movement disorder, cognitive decline, and psychiatric manifestation with an onset ranging between 25 and 60 years of age (mean onset, 30-40 years) and a presumably high penetrance. In the context of a contiguous X-chromosomal deletion, associated symptoms may include Duchenne muscular dystrophy (DMD), chronic granulomatous disease (CGD), retinitis pigmentosa, and ornithine transcarbamylase deficiency.<sup>3,4</sup> Accurate diagnosis

**ABBREVIATIONS:** ABSCT = autologous blood stem cell transplantation; CGD = chronic granulomatous disease; DMD = Duchenne muscular dystrophy; ENA = European Nucleotide Archive; MLS = McLeod syndrome.

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This work was exclusively financed by the Blood Transfusion Service Zürich, Swiss Red Cross (SRC), Zürich-Schlieren, Switzerland.

Received for publication September 8, 2016; revision received April 13, 2017; and accepted April 23, 2017.

doi:10.1111/trf.14172

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TRANSFUSION 2017;00:00–00

TABLE 1. Currently reported large genomic deletions involving *XK* on Xp21.1

First author and reference number	Publication year	Index patient	<i>DMD</i>	<i>PRRG1</i>	<i>XK</i>	<i>CYBB</i>	<i>RPGR</i>	<i>OTC</i>	Extent of deletion
Francke <sup>19</sup>	1985	BB	(del)*	del*	del*	del*	del*	post†	~7.15 Mbp
Bertelson <sup>20</sup>	1988	Sand H	post†	(??)	(del)*	post†	post†	post†	~380,000 bp
Bertelson <sup>20</sup>	1988	SS	(del)*	del*	del*	post†	post†	post†	~6.6 Mbp
Frey <sup>21</sup>	1988	OM	post†	post†	del*	del*	del*	post†	~580,000 bp
de Saint-Basile <sup>22</sup>	1988	SB	post†	post†	del*	del*	del*	post†	~580,000 bp
Ho <sup>18</sup>	1994	MT	post†	post†	del*	post†	post†	post†	~50,000 bp
El Nemer <sup>23</sup>	2000	Be	(del)*	del*	del*	del*	post†	post†	~5.0 Mbp
El Nemer <sup>23</sup>	2000	Bi	post†	post†	del*	del*	del*	post†	~580,000 bp
El Nemer <sup>23</sup>	2000	Lh	post†	post†	del*	del*	post†	post†	~120,000 bp
Singleton <sup>24</sup>	2003	NA	post†	post†	del Exon 2, parts of introns*	post†	post†	post†	7,454 bp
Peng <sup>3</sup>	2007	Case 1	pos	del*	del Exons 1 and 2*	post†	post†	post†	~1.12 Mbp
Peng <sup>3</sup>	2007	Case 2	(del)*	del*	del*	del*	post†	post†	~5.65 Mbp
Al-Zadjali <sup>25</sup>	2015	Patient 1	pos	del*	del*	del*	post†	post†	~870,000 bp

\* Deleted genomic regions.  
† Undeleted genomic regions.

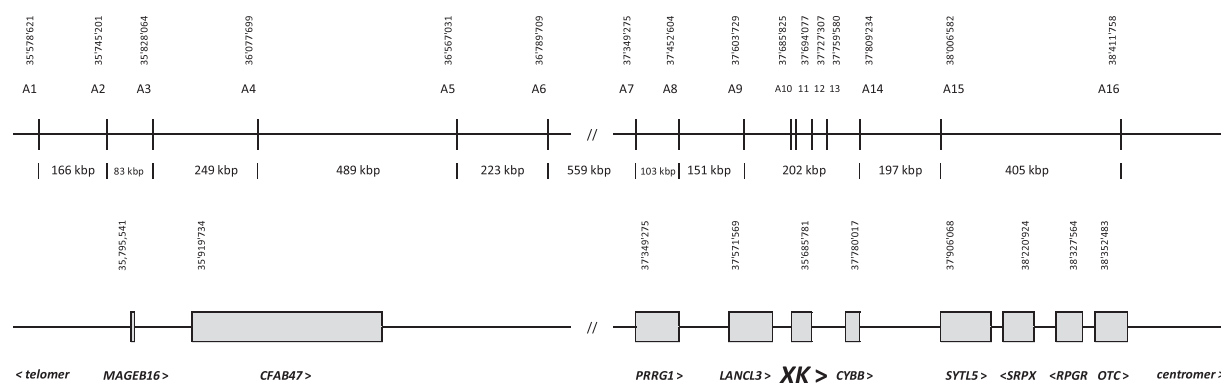
of MLS remains difficult and critical when based on symptoms and phenotypic features alone.<sup>5,6</sup> Red blood cell (RBC) acanthocytosis is an unreliable finding unless specific techniques are used.<sup>7</sup> Although elevated creatine kinase levels are present in almost all MLS patients, this finding is not specific.<sup>4</sup> Furthermore, associated symptoms may be confounding with other neuropsychiatric disorders such as Huntington disease or choreoacanthocytosis, suggesting an unknown number of unreported cases.<sup>8,9</sup>

Kx is expressed on the 444-amino-acid multipass transmembrane RBC XK protein constituting the International Society of Blood Transfusion (ISBT) 019 blood group system. In all ethnicities, the frequency of Kx is more than 99.9% defining the only known public antigen of *XK* and marking Kx- individuals as sought-after, rare blood donors.<sup>10-12</sup> In the RBC membrane, *XK* exists as heterodimer together with the membrane Kell protein explaining the weakened expression of all Kell antigens in Kx-deficient individuals.<sup>13-15</sup> Today, the pathophysiologic link between Kx negativity and the onset of MLS is still unclear<sup>1</sup> although Rivera and colleagues<sup>16</sup> showed that deficiency of *XK* and *XK*/Kell proteins led to altered transmembrane transport of divalent cations.<sup>17</sup> Absent Kx RBC antigen is pathognomonic for patients with MLS and therefore represents the diagnostic marker of highest specificity.<sup>18</sup> However, beside one murine anti-Kx with questionable specificity when employed in FACS analysis, typing sera are commercially not available and polyclonal anti-Kx from sensitized McLeod individuals are very rare and therefore less suitable for donor and patient typing.

Consequently, molecular assessment of Xp-chromosomal recessively inherited *XK* remains an attractive diagnostic alternative. Single pathogenic point mutations may be approached simply by Sanger sequencing. Pathogenic *XK* variants may also involve large

deletions on Xp21.1, including centromeric and telomeric genes and extending up to 5.65 million base pairs (bp, million = Mbp) in length, covering up to 20 different genes and thereby constituting the continuous gene syndrome.<sup>3,18-25</sup> Exact molecular diagnosis of large deletions would also add valuable information to explain symptoms associated with MLS such as X-linked CGD, retinitis pigmentosa, ornithine transcarbamylase deficiency, and *DMD* encoded by the respective genes *CYBB*, *RPGR*, and *OTC*, all located centromeric, or *DMD* located telomeric from Xp21.1, respectively.<sup>26</sup> Such diverse and large deletions are often inaccessible by utilizing “flanking” primers, with deleted (inexistent) sites to prime from.

So far, 29 different mutants of *XK* have been described and were termed *XK*\*N.01 to *XK*\*N.29 by the Red Cell Immunogenetics and Blood Group Terminology Committee of the ISBT, all publically available (*XK*, ISBT019, Version v3.0 160622).<sup>27</sup> The majority of unexpressed “*XK* null” alleles recognized by the ISBT are located within the gene itself and are caused by single nucleotide, multiple nucleotide, small deletions, or insertions, as well as punctual nonsense, alternative splicing and missense mutations (*XK*\*N.06 to *XK*\*N.29).<sup>27</sup> A minority of *XK* null mutant haplotypes display a remarkably different molecular background, including large Xp-chromosomal deletions, which may lack all three exons of *XK*, or may be deficient for one of the three exons (*XK*\*N.01 to *XK*\*N.05). There are more reports of Xp-chromosomal haplotypes with large deletions including *XK* than currently recognized by the ISBT (Table 1).<sup>27</sup> This may be explained by the challenge to assess large deletions in general. We therefore devised a pragmatic approach not only for diagnosis of mutant *XK* genes, but also for exact breakpoint detection in deletional Xp-chromosomal haplotypes. Three male index cases with clinically suggestive phenotypes for X-chromosomal



**Fig. 1.** Schematic representation of the 2.8-Mbp genomic region of Xp21.1 investigated using the presented method. Vertical lines mark positions of 16 PCR amplicons near *XK* on Xp21.1 in the top part of the figure. PCR amplicons are numbered A1 (telomeric) to A16 (centromeric of *XK*). Amplification primers are given in Table 2A. Genetic distances in between these amplicons are given in kilobase pairs (kbp) below and as indicated. Genes with known expression of proteins are displayed as gray boxes below. Their name is given and their orientation of transcription is indicated by arrows. Locations according to build GRCh38.p7 primary assembly for chromosome X are given for the telomeric end of the 16 PCR amplicons and the initiation of transcription of the respective genes.

defects associated with MLS were assessed using the presented stepwise partitioning of Xp21.1.

## MATERIALS AND METHODS

### Phenotypic investigations by serology and flow cytometry

Expression of Kx and Kell antigens as well as the presence of Kell membrane protein were examined by serologic and flow cytometric approaches as described earlier.<sup>28</sup>

### Polymerase chain reaction procedures and Sanger sequencing

Conventional polymerase chain reaction (PCR) procedures were designed to test the Xp-chromosomal genomic region in between locations 35,578,621 and 38,411,758 (build GRCh38.p7), including *XK* and covering a total length of approximately 2.8 Mbp (Fig. 1, Table 2). Basic and BLAST algorithms from the National Center for Biotechnology Information were used for retrieval and analysis of genomic sequences and identity comparisons.<sup>29</sup>

Four sets of PCRs (Steps 1 to 4) with the respective primers, their names, sequences, and amplification product lengths are given in Table 2 (A, B, C, and D, respectively). The final reaction volume of all PCR procedures was 10  $\mu$ L, containing PCR buffer (Ready, Inno-train Diagnostik GmbH) and 0.4 units of DNA polymerase (Ampli-Taq, Applied Biosystems, Thermo Fisher Scientific, Life Science Group). Concentration of specific primers was 300 nmol/L in all reactions, except in PCRs A15, A10, A11, and A12 the primer concentrations were 600, 400, 200, and 200 nmol/L, respectively. Amplification control primers were as described previously, had a concentration of

80 nmol/L in all reactions and amplified a 434-bp product of *GHI*.<sup>30</sup> Cycling conditions for all PCR procedures were described previously.<sup>30</sup> Step 4 PCR corresponding to a “gap PCR” (Table 2, D, “Step 4”) was performed with adapted cycling conditions.<sup>31</sup> Amplifications of PCR products for sequencing (Reactions A10, A11, and A12 for *XK*) were identical as described in Table 2 and done in 2.5 times scaled-up reaction volumes and sequenced by standard Sanger sequencing procedures using primers as given (Table 2, E) by Microsynth AG. One additional set of primers was used to diagnose carrier status of a sister to Ankara-H-00058 (Table 2, D, “diagnostic”). All PCR procedures were performed on an automated thermocycler (either GeneAmp PCR system 9700 or the Verity Dx, Applied Biosystems). PCR amplicons were visualized by agarose gel electrophoresis and documented by digital imaging. Allele and contiguous gene syndrome-specific sequences of the three patients described here were submitted to the European Nucleotide Archive (ENA) and the ISBT terminology committee for the assignment of allele names to the newly discovered *XK* mutants comprising deletion breakpoint.<sup>27,32</sup>

## RESULTS

### Three male patients with suspected MLS

#### Patient Solothurn-H-00192

Propositus Solothurn-H-00192 was identified as asymptomatic carrier of a 895C>T mutation causing MLS associated neuropsychiatric phenotype in male mutation carriers of his large family.<sup>33</sup> The 895C>T mutation in Exon 3 of *XK* established a stop codon leading to a truncated Kx protein at Gln299 and prototypic RBC McLeod phenotype.<sup>2</sup> The allele is listed as *XK*\*N.20 by the ISBT.<sup>27</sup>

**TABLE 2. PCR amplification and Sanger sequencing primers used in this study**

Reaction	Primer name	Primer forward (F) Primer reverse (R)	Genomic location of 3' primer end (GRCh38.p7)	Amplicon size (bp)	Genomic distance to next amplicon (bp)
<b>A</b>					
<i>First-step PCRs A1 to A16</i>					
1	nX-1212KB-F nX-1212KB-R	TGTTCTGCCACCAGGTGATCCC CAAGTGAGAAGTTGAACCAGTACAAATG	35,578,621 35,578,758	138	166,442
2	XChrom-16 + 165kB-F XChrom-16 + 165kB-R	GCCTCCTTTGTAGAAACTCTTTTCTATAGTATGATG ATTGGGAAAAGCAAGGAAACATACAACTGTCACTG	35,745,201 35,745,434	234	82,629
3	X-0962KB-F X-0962KB-R	TACTGTTCCACATCTTAATGAGCCAGG AGAGTGCAGTCAAGTCAATAGATGGATGGT	35,828,064 35,828,367	304	249,331
4	nX-0711KB-F nX-0711KB-R	GAAGTCATTTGTCAGTTACAGCAATCTCTGG CACCCACCTCAGCCTCCCAAG	36,077,699 36,077,864	166	489,166
5	nX-0210KB-F nX-0210KB-R	CTTCCCTCAGGCTTGCCAGAAACTC TGAGTGGAGCCAGAGCAGAAAGG	36,567,031 36,567,174	144	222,534
6	USA1MB-511-F USA1MB-418-R	TCACAAAAGATGCCATGTAAGTTGCTG GACCTGTAGAGCATGAGGTATCCCAAGAAC	36,789,709 36,789,858	150	559,416
7	X-PRRG1-139-F X-PRRG1-i1 + 117R	TCITTCACCTCCCTCCAGTCCG GTGCTACCACGCTACCGCATATC	37,349,275 37,349,536	262	103,067
8	X-PRRG1-i3-509-F X-PRRG1-i3-396-R	TGGAGAGGGAGCTGAGGAGGAATG CCAACTACAAAGATTCATCACATGATCCC	37,452,604 37,452,769	166	150,959
9	X-Lan+31KB-F X-Lan+31KB-R	TTTTCAGACAAATGATTAGTGGGGAT GCAGGGAAGCTGGCATTGTTTCA	37,603,729 37,603,981	253	81,843
10	X-XK-120-F X-XK-i1 + 576-R	TGGGGGGCTGCCAGAG TTGCCAGGGCCGGAAGACATTTCTA	37,685,825 37,686,806	982	7,270
11	X-XK-i1-185-F X-XK-i2 + 171-R	CTCATCAATCCCATGGCCGTCT TTGAGCCCTGCCTAGAATGCAGATC	37,694,077 37,694,744	668	32,562
12	X-XK-i2-300-F X-XK+1426-R	ATGCCTCAGAGATTTCTCTGTAGGATG GCTCACAAAGGTTAACAGCTAATCTCCTGC	37,727,307 37,728,584	1278	30,995
13	X-CYBB-50KB-F X-CYBB-50KB-R	GGTTCATTGCCAAATAGCACATGAGC GGCCTGGTCCAGTCCATTTAATTGG	37,759,580 37,759,885	306	49,348
14	X-CYBB-i11-305-F X-CYBB-i11-124-R	CATCCATCAATGCCTGACTCAATACTGC CTGCTGGATGCTGAAAGGTAAACAAA	37,809,234 37,809,468	235	197,113
15	X-SYTL5-412-F X-SYTL5-i1 + 5-R	CACAAGGTCAAGTAGTCCAGCATTTACAGT TCACACTGGAGATCCCTAAACGCATTAGTC	38,006,582 38,006,702	121	405,055
16	X-OTC-i8-78-F X-OTC-i9 + 1-R	ATTGATGTTCTGACTACTGGCCATGTGT AGAACTTATCCTCCATTCCTTTTCTGCTTAC	38,411,758 38,412,033	276	
<b>B</b>					
<i>Second-step PCRs B9.1 to B9.5 and B13.1 to B13.4</i>					
9.1	X-Lan+48KB-F X-Lan+48KB-R	AAAGGCAGATATTCAGGCCCTGTGATAGA TTGGAATTTCTGGTTCTGGAAGTGGGG	NA NA	252	
9.2	X-Lan+63KB-F X-Lan+63KB-R	TCCAGACAGCTAAAGTAACTTGCCCAA TAAGGACAAAATTTGGGGGAGAACAT	NA NA	267	
9.3	X-Lan+78KB-F X-Lan+78KB-R	GTACATGTGCACAATGTGCAGGTTCTGT GGATGAAGCTGGAAACCCATCATTCTG	NA NA	264	
9.4	X-Lan+92KB-F X-Lan+92KB-R	AGCACCATCCATGTTCTGGACATA TTAGTGTATCAGGAGAGACCCTAAAGGAGGG	NA NA	248	
9.5	X-Lan+106KB-F X-Lan+106KB-R	TAAATGTCATCTTCCAGTGGCAGC TACTACAGTGGCCCTAAAAGTGGCA	NA NA	254	
13.1	X-CYBB-40KB-F X-CYBB-40KB-R	CCAAAGTGGCCGAATAGGAACAGC GTCCGCTGTGCACCCCTTCTTTAAC	NA NA	252	
13.2	X-CYBB-30KB-F X-CYBB-30KB-R	GGGGTGCATGACACAATCTCGG CGTGGCTCACGCCCTGTAATCC	NA NA	241	
13.3	nX-CYBB-20KB-F	GTCCAGCACCCAGTTGCTGGGTT	NA	237	

Table 2: Continued

Reaction	Primer name	Primer forward (F) Primer reverse (R)	Genomic location of 3' primer end (GRCh38.p7)	Amplicon size (bp)	Genomic distance to next amplicon (bp)
13.4	nX-CYBB-20KB-R X-CYBB-10KB-F X-CYBB-10KB-R	GGTGGCTGGTTGGAAAGGTGGAGG GGACACTTGGGATTCATCTTTGAGC ACTTCTGCCACCACATGATGCC	NA NA NA	249	
C		<i>Third-step PCRs C9.2.1 to C9.2.4 and C13.3.1 to C13.3.4</i>			
9.2.1	X-Lan+64KB-F X-Lan+64KB-R	GGAGTGACTATGATGCAGTCCATTAGAA GTAGAAAAGCATCTTAGAGGATGCTTGG	NA NA	276	
9.2.2	X-Lan+66KB-F X-Lan+66KB-R	CTCACCTCTTTTTCATTTTCATGACATC ATATCTATTACAGTGCATCAACATCCAAAGG	NA NA	248	
9.2.3	X-Lan+70KB-F X-Lan+70KB-R	CCCAAAGTGACTTTAAAGGATTTGAGGAT TTTAAAGTGGGGCTGACTGCCTGAG	NA NA	263	
9.2.4	X-Lan+74KB-F X-Lan+74KB-R	TTTTCTGCTCATGATGTTCTCTGGAGG TGGTTAAATTTTACTTCTATATCTTTGGGG	NA NA	220	
13.3.1	X-CYBB-21KB-F X-CYBB-21KB-R	ATTTCTTCCCAGTCTCATTTTACCCCTC CTCATTTAATGCAACAAGGTAATCTTAATGTCC	NA NA	257	
13.3.2	X-CYBB-2356B-F X-CYBB-2356B-R	AAAAAAGTCTTGGTTGCATGTGTAAT AAATACAGCCCTTCTTGCACTGACACTAC	NA NA	247	
13.3.3	X-CYBB-26KB-F X-CYBB-26KB-R	AAGGTTGGTGGGGGTGGCT CCAAAGAAAAGTGGTAAGAGAGTAGGGAATAGG	NA NA	254	
13.3.4	X-CYBB-30KB-F X-CYBB-30KB-R	GATGCTGCATTTATGTTCTTGAAGTCTACTC CTGAAGCTTACAAGAAGTGCCTTATTTGTG	NA NA	274	
D		<i>Fourth-step gap and diagnostic PCRs</i>			
Step 4	X-Lan+64KB-F X-CYBB-21KB-R	GGAGTGACTATGATGCAGTCCATTAGAA CTCATTTAATGCAACAAGGTAATCTTAATGTCC	NA NA	~2300	
Diagnostic	X-LANCL3-11-17964-F X-CYBB-i3-2627-R	CCTGTTGTTTGGATTAAGCCACTAGAAGTGAG ATGACTTATAATCTCCTCCCTTGAGTTTGG	NA NA	224	
E		<i>Sequencing primers</i>			
Sequencing	ORH-Break-F2 ORH-Break-R2 XK-Exon 1-R XK-Exon 2-F XK-Exon 3-F nXK-Exon 3-F	GTCAGGTAATAATGCTAGGG TTCTTATATAGCCCTCCTTGTG GGTCAATTTCAAGTTGAATGTTGGC GCCAAGTCAAGGCTTTAAGAATC GCTAACAACTGGAAAGTCAGGCT CCTCTTCTGGTGCAGTGGTTCC	NA NA NA NA NA NA		



Therefore, and to illustrate the process of *XK* gene sequencing described, the respective patients' sample material was included as a positive control into this study.

#### *Patient Brisbane-H-00183*

The sample material came from an Australian index patient of Caucasian ethnicity. He suffered from chorea, seizures, and cognitive decline. His two brothers died in their 40s, one from a car accident and one from presumed sudden cardiac death. Both brothers had a son, including one with sudden cardiac death at the age of 47. The index patients' two sisters did not manifest features of MLS. One sister had an apparently healthy son, a healthy daughter, and one son who unexpectedly died in his 40s with "blood clot." The other sister of the index patient had three unaffected daughters. The mother of the patient reportedly showed some involuntary movements and diabetes.

The Australian Red Cross Laboratory completed the serologic analysis on the index patients' blood revealing RBCs that were Kx<sup>-</sup>, K<sup>-</sup>, and weakly positive for k and Kp<sup>b</sup>. Weak positivity was found by adsorption-elution techniques. Molecular analysis for *KEL* confirmed the patient's *KEL\*01/02* heterozygous genotype. The direct antiglobulin test reacted negative and search for antibodies was negative. Creatinine kinase levels were mildly elevated at 337 U/L and his blood smear demonstrated the occasional presence of acanthocytes. Molecular analysis of Xp21.1 was started without any information on the expected *XK* mutation.

#### *Patient Ankara-H-00058*

Patient Ankara-H-00058 originated from Turkey and was diagnosed with X-linked CGD (X-CGD) by the age of 10 years. The case has been reported in abstract format.<sup>34</sup> The patient suffered from therapy-refractory pulmonary aspergillosis and was referred to the Children's University Hospital in Zurich for stem cell transplantation. He was treated by *CYBB* (gp91phox) gene corrected autologous blood stem cell transplantation (ABSCT).<sup>34</sup> Before ABSCT, he received repeatedly allogeneic granulocyte transfusions. After ABSCT, he became transfusion dependent and received 19 units of RBCs. On pretransfusion compatibility testing he presented with high-titer (>1:8000) anti-Kx and anti-Km preventing allocation of random compatible allogeneic RBCs from blood storage repository. Search of rare donor data files provided one Swiss and several international Kx<sup>-</sup> donors who donated the RBCs necessary for transfusion support. Although after engraftment of ABSCT the patient achieved immune competency to clear aspergillosis infection, the Kx<sup>-</sup> RBC phenotype remained, as expected. Eight months after ABSCT, the patient presented with progressive cytopenia with loss of Chromosome 8 indicating treatment-induced myelodysplasia requiring allogeneic SC transplantation. Six months after allotransplantation, he achieved complete chimerism with

extinction of McLeod RBC phenotype and disappearance of anti-Kx/Km alloantibodies.

Using array comparative genomic hybridization, the expected *XK* mutation had been characterized as a large deletion involving *XK* and *CYBB*.<sup>34-36</sup> However, the exact break point was not identified, nor were DNA sequences submitted, nor has an allele-(haplotype)-name been assigned by the ISBT for this apparently large deletion at Xp21.1 defining a contiguous gene syndrome.

#### **First-step analysis of three patient samples for large deletions on Xp21.1**

The Xp-chromosomal region in between genomic location 35,578,621 near *MAGEB16* and 38,411,758 near *OTC* (build GRCh38.p7) with a total length of approximately 2.8 Mbp of the three patient samples was investigated with first-step PCRs A1 to A16 (Fig. 1). Distances between the PCRs ranged from 7270 to 559,416 bp with a mean of 189 kbp and a median of 151 kbp. The start codon of the *Homo sapiens* Dystrophin gene, *DMD*, is located approximately 2.4 Mbp telomeric from the position of PCR 1, hence not covered by the presented analysis. The *XK* gene is located in between genomic location 37,685,825 and 37,728,584 (build GRCh38.p7) and its three exons are detected individually by PCRs 10, 11, and 12, respectively (Fig. 1).

Patient samples Solothurn-H-00192 and Brisbane-H-00183 showed a reaction pattern of first-step PCRs A1 to A16 comparable to those of normal female and male control DNA samples (Fig. 2). Large deletions of Xp21.1 could therefore be excluded in these cases a priori. Patient sample Ankara-H-00058, however, showed negative results for first-step PCRs A10, A11, A12, and A13, indicating a large deletion of the Xp-chromosome (Fig. 2) with its maximal extent in between genomic locations 37,603,729 and 37,809,234. The extent of the deletion ranged in between a maximum length of 205.7 kbp and a minimum of 74.1 kbp, with both extremes predicted to include *XK* and parts of *CYBB*.

#### ***XK* sequencing of Solothurn-H-00192 and Brisbane-H-00183**

Amplicons of first-step PCRs A10, A11, and A12 covering *XK* Exons 1, 2, and 3, and the respective adjacent noncoding sequences, were sequenced and delivered one mutation each for both patients. The allelic variant of the *XK* gene of Solothurn-H-00192 had previously been published and was termed *XK\*N.20* by the ISBT.<sup>27,33</sup> By our approach, we confirmed the 895C>T mutation in Exon 3, leading to the Gln299X stop codon of *XK*. DNA sequence of *XK\*N.20* has been submitted to ENA Accession Number LT838808.

Mutant *XK* allele of Brisbane-H-00183, however, showed a small deletion of four nucleotides, 195-198delCCGC, within Exon 1 of the *XK* gene and is not

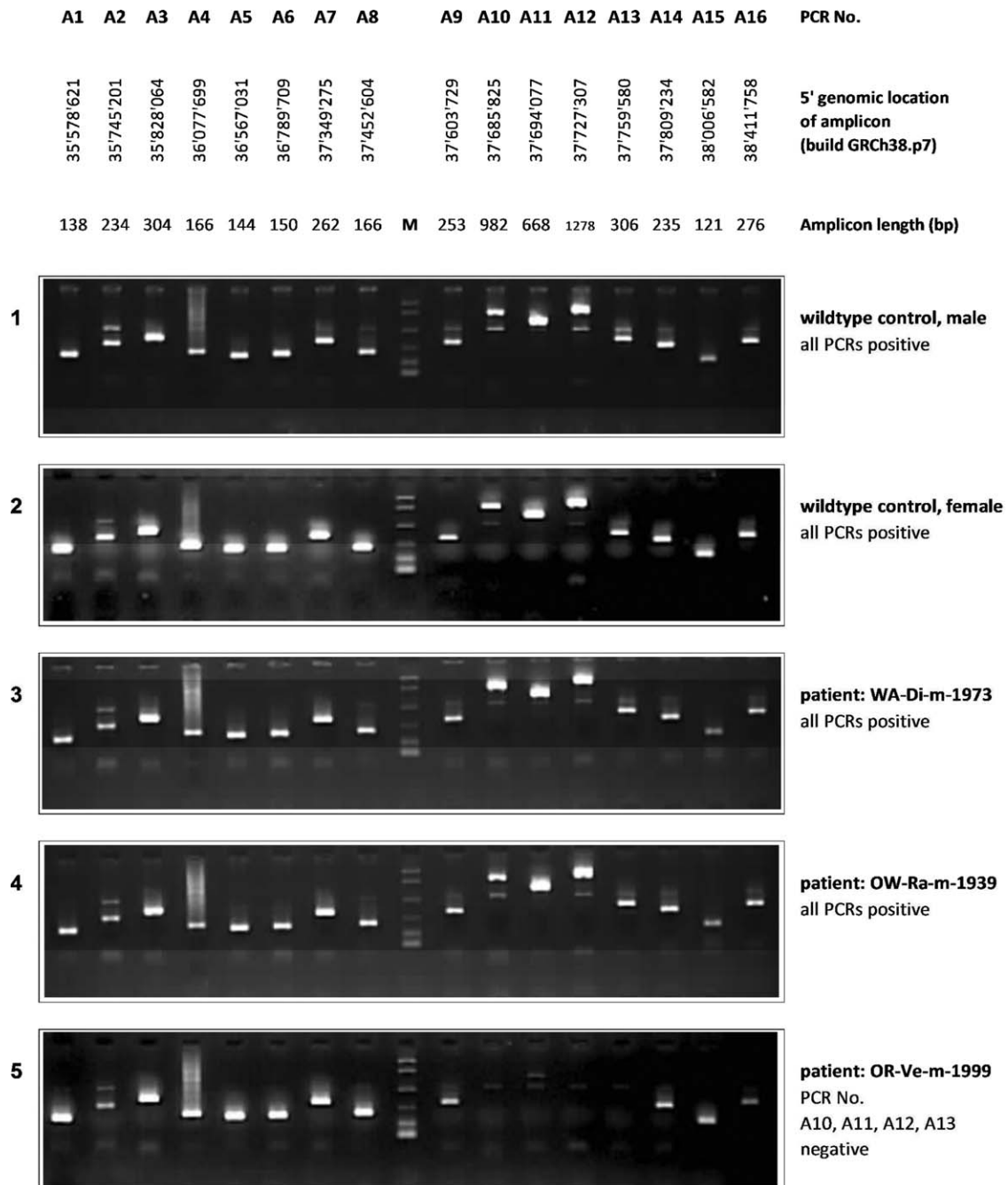
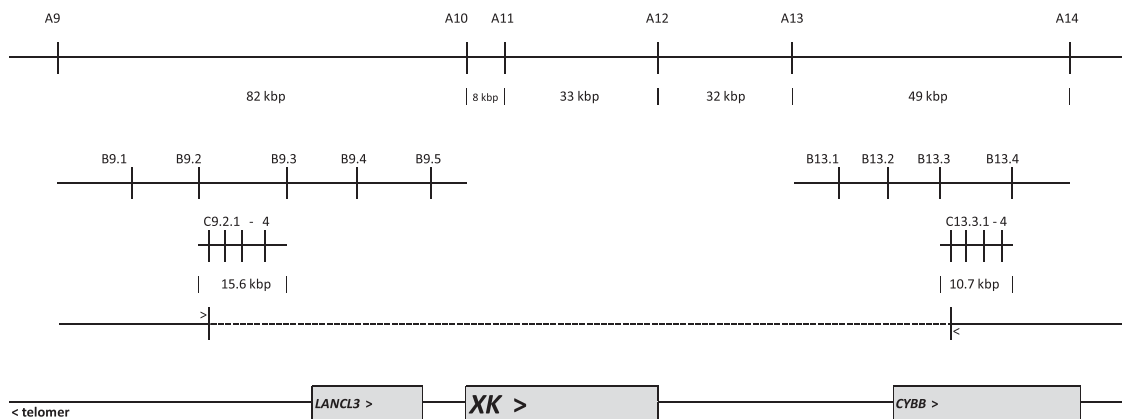


Fig. 2. PCRs A1 to A16: first-step analysis in the stepwise partitioning of Xp21.1. Pictures of agarose gel electrophoresis for five selected DNA samples with first-step PCRs A1 to A16 are shown (see Table 2A). Reactions A1 to A16 are all positive in the presence of a normal Xp-chromosomal haplotype. Specific negativities may occur in cases of large Xp-chromosomal deletions in males, for example, for first-step PCRs A10 to A13 in sample Ankara-H-00058 at the bottom of the figure. Mutant *XK* genes with single-nucleotide substitutions or small insertions and deletions will not become visible using this approach, for example, Solothurn-H-00192 and Brisbane-H-00183. *XK* sequencing as described will be needed for molecular diagnosis in such cases. In each reaction, a 434-bp *GHI* control PCR product is included as a positive amplification control. Due to competition, the 434-bp control amplicon may disappear in n cases of a specific amplification. M = molecular size marker with fragments of 50, 100, 200, 400, 850, and 1500 bp, respectively.

published up to now. The observed frameshift predicted a mutant *XK* protein after amino acid Arg66 with a premature stop codon at prior Leu129. The respective DNA

sequence has been submitted to ENA under Accession Number LT838808 and was assigned ISBT allele name *XK\*N.39*.



**Fig. 3. PCR analysis Steps 2 to 4, narrowing down Xp21.1 deletion of patient Ankara-H-00058 to the actual position and extent. Vertical lines of the topmost line marks positions of first-step PCRs A9 to A14 adjacent to XK on Xp21.1. Second-step PCRs B9.1 to B9.5 and B13.1 to B13.4 and third-step PCRs C9.2.1 to C9.2.4 and C13.3.1 to C13.3.4 are given below (see Table 2A-2C). Positions of LANCL3, XK, and CYBB are displayed as gray boxes at the bottom of the figure and their respective orientation of transcription is indicated by arrows. Dashed line represents final finding of the deletion observed on the X-chromosome of patient OR-Ve-m-1999.**

### Second-, third-, and fourth-step analysis of the large Xp-chromosomal deletion predicted for Ankara-H-00058

Second-step PCRs B9.1 to B9.5 and B13.1 to B13.4 were located in between first-step PCRs A9 and A10 (81.8 kbp) and A13 and A14 (49.3 kbp), respectively (Fig. 3). The resulting reaction pattern unambiguously located the “telomeric start” of the deletion in between second-step PCRs B9.2 and B9.3 (15.6 kbp). Centromeric of XK, however, second-step PCRs B13.1, B13.2, and B13.4 all typed positive, thereby delivering contradictory results. All second-step B13.1 to B13.4 PCR product sequences were searched against nucleotide databases (BLAST) and delivered at least six non-X-chromosomal sequence identities higher than 91% for B13.1 and B13.2 (Table 3). Consequently, second-step PCRs B13.1 and B13.2 were considered as false positives, whereas the correct “centromeric end” of the deletion was expected to lie in between B13.3 and B13.4 (10.7 kbp).

Third-step PCRs were C9.2.1 to C9.2.4 and C13.3.1 to C13.3.4 for the expected telomeric start and centromeric end of the investigated deletion, respectively. For both sides, results were unambiguous, further narrowing down the candidate area of the deletion in between C9.2.1 and C9.2.2 (1944 bp) and in between C13.3 and C13.3.1 (1081 bp).

Fourth-step PCR combined the forward primer of C9.2.1 and the reverse primer of C13.3.1, for example, sequences expected to be located adjacent to the telomeric and centromeric ends of the large deletion, respectively. Testing succeeded in a gap-PCR product with an approximate length of 2300 bp. The product was only found to be positive in the sample material of Ankara-H-

00058, but in none of the six control DNA samples tested simultaneously (data not shown). The respective product of the gap-PCR was sequenced and delivered the exact sequence of the deletion, with the Xp-chromosomal telomeric start reading TTTCTCTGTGAAGTTTCGTC and the centromeric end reading TAAAGTCAACTTGGGACTTT, whereas the first and last nucleotides of the deletion are underlined, respectively. The Xp-chromosomal deletion in Ankara-H-00058 has a predicted length of 151,464 bp and reaches from genomic location 37,637,812 to 37,789,276 (GRCh38.p7; Fig. 3). The deletion covers Exons 2 to 6 of LANCL3, all three exons of XK, and Exons 1 to 3 of the total 13 exons of CYBB. Carrier status of the sister of Ankara-H-00058 was proven using a “diagnostic PCR” (Table 2D, Fig. 4) covering the X-chromosomal breakpoint sequence. The respective DNA sequence has been submitted to ENA under Accession Number LT838809 and the assigned ISBT allele name is *XK\*N.01.16*.

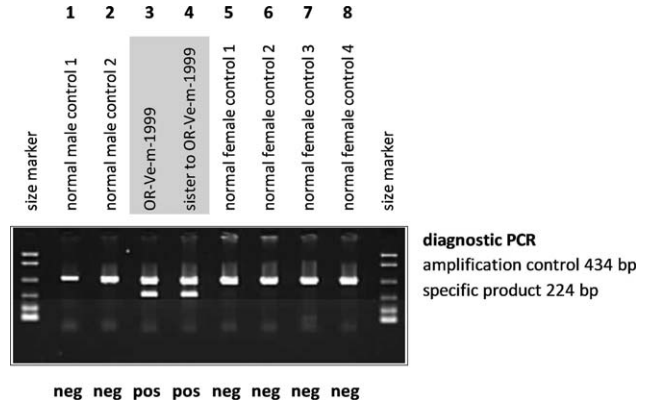
## DISCUSSION

Most known XK mutant alleles are caused by single-nucleotide substitutions and short insertion or deletion polymorphisms. However, a minority of XK null mutant haplotypes represent large Xp-chromosomal deletions. The presented stepwise partitioning of Xp21.1 represents a pragmatic and cost-efficient approach for an unambiguous characterization of these large Xp-chromosomal deletions in patients with proven or suspected MLS. The described profiling method for XK deletions could easily be extended to include other clinically relevant genes next to the investigated chromosomal region, for example, DMD. Beside their duties, however, it must be kept in



**TABLE 3. Genomic reference sequences with high homology to PCR Amplicons 13.1 and 13.2**

	Identity to second step PCR B13.1 amplicon sequence (%)	Identity to second step PCR B13.2 amplicon sequence (%)
<i>Second step PCR B13.1 amplicon sequence identity to</i>		
NG_027957.1	95	NA
NG_031962.1	94	NA
NG_017155.1	94	NA
NG_011403.1	94	NA
NG_047064.1	94	NA
NG_034120.1	94	NA
NG_017044.1	94	NA
NG_017196.2	93	NA
<i>Second-step PCR . B13.2 amplicon sequence identity to</i>		
NG_009065.1	NA	100
NG_013249.1	91	93
NG_008835.1	NA	92
NG_029857.1	NA	92
NG_028144.1	NA	92
NG_028144.1	NA	92
NG_021196.1	NA	92
NG_029153.1	NA	92
<i>H. sapiens phosphodiesterase 4D (PDE4D), RefSeqGene on Chromosome 5</i>		
<i>H. sapiens synaptotagmin 14 (SYT14), RefSeqGene on Chromosome 1</i>		
<i>H. sapiens Dmx-like 2 (DMXL2), RefSeqGene on Chromosome 15</i>		
<i>H. sapiens coagulation factor VIII (F8), RefSeqGene on Chromosome X</i>		
<i>H. sapiens neural EGFL like 1 (NELL1), RefSeqGene on Chromosome 11</i>		
<i>H. sapiens HFM1, ATP-dependent DNA helicase homolog (HFM1), RefSeqGene on Chromosome 1</i>		
<i>H. sapiens solute carrier family 1 member 1 (SLC1A1), RefSeqGene on Chromosome 9</i>		
<i>H. sapiens armadillo repeat containing, X-linked 4 (ARMCX4), RefSeqGene on Chromosome X</i>		
<i>Second-step PCR . B13.2 amplicon sequence identity to</i>		
<i>H. sapiens cytochrome b-245 beta chain (CYBB), RefSeqGene (LRG_53) on Chromosome X</i>		
<i>H. sapiens tenascin transmembrane protein 1 (TENM1), RefSeqGene on Chromosome X</i>		
<i>H. sapiens cadherin-related 23 (CDH23), RefSeqGene on Chromosome 10</i>		
<i>H. sapiens carbohydrate sulfotransferase 8 (CHST8), RefSeqGene on Chromosome 19</i>		
<i>H. sapiens staufer double-stranded RNA binding protein 1 (STAU1), RefSeqGene on Chromosome 20</i>		
<i>H. sapiens WD repeat containing planar cell polarity effector (WPCP), RefSeqGene on Chromosome 2</i>		
<i>H. sapiens disco interacting protein 2 homolog B (DIP2B), RefSeqGene on Chromosome 12</i>		
<i>H. sapiens Sp3 transcription factor (SP3), RefSeqGene on Chromosome 2</i>		



**Fig. 4. Diagnostic PCR for 151,464-bp deletion of Xp21.1 also covering XK and observed in patient Ankara-H-00058 and his sister. In each reaction, a 434-bp GHI control PCR product is included as a positive amplification control. M = molecular size marker (see legend to Fig. 2).**

mind that most transfusion laboratories are not in the position to provide genetic counseling on the effect of coinherited diseases, such as, for example, DMD or CGD. As a matter of course, national legislations with respect to genetic testing need to be followed. In the context of McLeod diagnosis, well-established interdepartmental cooperation between immunohematologists and neurologists is highly desirable and offers benefits to patients entering the health system from either side. Among three investigated patient samples in total, one of them, Ankara-H-00058, showed such a large deletion of approximately 151 kbp involving not only all three exons of XK but also Exons 2 to 6 of LANCL3 and Exons 1 to 3 of CYBB. The large deletion could be defined unambiguously down to the scale of single nucleotides. Patient Solothurn-H-00192 had a single-nucleotide 895C>T nonsense mutation causing a predicted premature stop codon at prior Gln299. Patient Brisbane-H-00183 showed a 4-bp deletion, 195-198delCCGC, within Exon 1 of the XK gene, leading to a predicted premature stop codon at prior Leu129. None of the three specific sequences had been deposited with any searchable nucleotide database before and could therefore be considered as “new.” XK mutant 895C>T(Gln299X), however, had been reported in an abstract and had been assigned an ISBT allele name, XK\*N.20, previously.<sup>27,34</sup>

The terminology committee of the ISBT currently does not include all Xp-chromosomal deletions involving XK, particularly larger deletions, representing a deficiency in nomenclature.<sup>27</sup> The inclusion of all known deletions and precise molecular characterization of deletions, however, is highly helpful for the specific genetic detection of carrier status in female family members, for expanded pedigree analysis, and for the interpretation of associated symptoms, caused by additionally deleted genes.

The presented stepwise partitioning of Xp21.1 is somewhat tedious but pragmatic and cost-efficient. The proposed iterative incremental partitioning by positional PCRs, narrowing down the genomic region of the suspected deletions to a specific gap-PCR including the actual breakpoint sequence, requires several repetitive steps. Other techniques, for example, next-generation sequencing, also known as “massive parallel sequencing,” may not represent an advantageous alternative, since almost equally time-consuming and requiring a large number of samples analyzed in parallel, for optimized cost-efficiency. However, if any, next-generation sequencing approaches addressing the whole genome of a patient could probably deliver the most significant results. Since MLS is rare, number of demands for molecular diagnosis of its causative *XK* mutations will stay low in the foreseeable future. Cases with irregular findings in RBC phenotyping such as weakened or absent Kell antigens, Kx negativity, and acanthocytosis should be considered for molecular McLeod profiling.

#### ACKNOWLEDGMENTS

The authors thank A. Danek for bringing the concise word “eponymous” to the first author’s attention. Usage example: “Fifteen different *XK* mutations were found, nine of which were novel, including the one of the eponymous case McLeod.” CG designed the technical approach, analyzed data, and wrote the paper; CB, YM, SS, and EM conducted research and analyzed results; MM performed genomic analysis and submitted sequences to the European Nucleotide Archive; JO contributed sample material and data of patient Brisbane-H-00183; HHJ contributed sample material and data of patient Solothurn-H-00192; BMF initiated the study and provided helpful discussions; and all authors reviewed the data, provided comments, and approved the final manuscript.

#### CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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