Novel pathogenic \textit{XK} mutations in McLeod syndrome and interaction between \textit{XK} protein and chorein

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Abstract

Objective
To identify \textit{XK} pathologic mutations in 6 patients with suspected McLeod syndrome (MLS) and a possible interaction between the chorea-acanthocytosis (ChAc)- and MLS-responsible proteins: chorein and \textit{XK} protein.

Methods
Erythrocyte membrane proteins from patients with suspected MLS and patients with ChAc, ChAc mutant carriers, and normal controls were analyzed by \textit{XK} and chorein immunoblotting. We performed mutation analysis and \textit{XK} immunoblotting to molecularly diagnose the patients with suspected MLS. Lysates of cultured cells were co-immunoprecipitated with anti-\textit{XK} and anti-chorein antibodies.

Results
All suspected MLS cases were molecularly diagnosed with MLS, and novel mutations were identified. The average onset age was 46.8 ± 8 years, which was older than that of the patients with ChAc. The immunoblot analysis revealed remarkably reduced chorein immunoreactivity in all patients with MLS. The immunoprecipitation analysis indicated a direct or indirect chorein-\textit{XK} interaction.

Conclusions
In this study, \textit{XK} pathogenic mutations were identified in all 6 MLS cases, including novel mutations. Chorein immunoreactions were significantly reduced in MLS erythrocyte membranes. In addition, we demonstrated a possible interaction between the chorein and \textit{XK} protein via molecular analysis. The reduction in chorein expression is similar to that between Kell antigens and \textit{XK} protein, although the chorein-\textit{XK} interaction is a possibly noncovalent binding unlike the covalent Kell-\textit{XK} complex. Our results suggest that reduced chorein levels following lack of \textit{XK} protein are possibly associated with molecular pathogenesis in MLS.
Glossary

MLS = McLeod syndrome; ChAc = chorea-acanthocytosis; NA = neuroacanthocytosis.

Neuroacanthocytosis (NA) syndromes are rare neurodegenerative disorders exhibiting neurologic abnormalities and erythrocyte acanthocytosis. The core NA syndromes are characterized by degeneration of the striatum and huntingtonism. They comprise 2 main diseases: chorea-acanthocytosis (ChAc) and McLeod syndrome (MLS). ChAc is caused by loss-of-function mutations in VPS13A, leading to an absent or markedly reduced level of the encoding protein, chorein. MLS is caused by loss-of-function mutations in the XK, leading to absent XK protein. Although later onset and cardiomyopathy may occur predominantly in MLS, the 2 diseases share almost their entire symptomology in the CNS and erythrocyte membrane. Although molecular interactions are assumed to exist between these diseases, no studies have as yet established a direct association.

Methods

Human samples and mutation analysis
All 6 patients with suspected MLS were Japanese males with clinically suspected NA (table). Six healthy male controls and 6 male patients with ChAc were matched to suspected MLS cases by age. A further 6 heterozygous ChAc mutant carriers were used for the analyses. Lymphoblastoid cell lines from MLS cases and a healthy control were established by SRL (Tokyo, Japan).

Coding and flanking regions of XK (NC_000009.11) and VPS13A (NC_000009.11) were analyzed by Sanger sequencing on an ABI PRISM 3100 Avant Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA). In the case of MLS_6, we performed a whole-genome sequence, long-range PCR covering the deletion region, and Sanger sequencing.

Immunoprecipitation and immunoblot analysis
Co-immunoprecipitation (co-IP) and reverse co-IP assays were performed using Dynabeads Protein G (Thermo Fisher Scientific). K562 and HEK293 cells that stably overexpressed chorein were lysed with Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA). K562 cells that were subcultured at 1 × 10⁶ cells/mL and incubated for 24 hours were used. The cell lysates (input) were used for the Dynabeads-antibody complex and Dynabeads-IgG complex. The cell lysate was diluted 5 times with 1× Tris-buffered saline because delicate surfactant conditions were required to maintain the IP interaction. The cell lysate and each bead were incubated for 2 hours at room temperature.

Protein samples were analyzed by immunoblotting using rabbit anti-chorein (HPA019036; Atlas Antibodies, Bromma, Sweden) and rabbit anti-XK protein (HPA019036; Atlas Antibodies) primary antibodies, which show no cross-reactivity with spectrin. Donkey anti-rabbit IgG, HRP-linked whole Ab (GE Health care, Little Chalfont, England) and VeriBlot for IP Detection Reagent (HRP) (ab131366; Abcam, Cambridge, UK) were used as secondary antibodies. Proteins were visualized using ECL Prime Western Blotting Detection Reagent (GE Health care), and images were recorded with a digital analyzer (FUSION-SOLO.7S.WL; Vilber Lourmat, Marne-la-Vallée, France).

Standard protocol approvals, registrations, and patient consents
Genomic DNAs and/or proteins from peripheral blood samples were taken from all participants who provided written informed consent. The research protocol and consent form were approved by the Institutional Review Boards of Kagoshima University.

Data availability statement
The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Results

Molecular diagnosis and clinical features of MLS cases
For all suspected MLS cases, XK protein immunoreactivity was lacking in the immunoblot analysis of the erythrocyte membrane (figure 1A). Clinical symptoms and pathologic XK mutations are presented in the table. In MLS_6, comprehensive mutation analysis revealed a mutation, which was a combination of a gross deletion and an insertion (figure 1, B–D).

Chorein immunoreactivity reductions in all MLS cases
We found a marked reduction in chorein immunoreactivity in all patients with MLS (figure 2A). The mean density level of patients with MLS was significantly lower ($p = 0.00127, d = 2.6$) at 0.55, relative to controls (figure 2D). The average reductions in the levels of chorein immunoreactivity in the erythrocyte membranes of MLS patients were equivalent to those found in heterozygous ChAc mutation carriers (figure 2, C and D), although no pathogenic mutations were identified in VPS13A in any patients with MLS. On the other hand, the average density levels of the XK immunoreactions did not significantly differ between ChAc and ChAc mutant carriers and healthy controls in either the immunoblot or densitometric analyses (figure 2, B–E). Chorein immunoreactions of
## Table
Profile of patients with MLS in this study

<table>
<thead>
<tr>
<th>Case</th>
<th>Age at sampling</th>
<th>Age at onset</th>
<th>Initial symptom</th>
<th>Clinical symptoms</th>
<th>Main psychiatric symptom</th>
<th>EEG abnormality</th>
<th>CK (IU/L)</th>
<th>DTRs</th>
<th>Cardiomyopathy</th>
<th>Atrophy of the corpus striatum on MRI</th>
<th>XK pathogenic mutations</th>
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<tr>
<td>MLS_1</td>
<td>64</td>
<td>47</td>
<td>Muscle weakness</td>
<td>Acanthocytes</td>
<td>+</td>
<td>+</td>
<td>5465</td>
<td>Absent</td>
<td>−</td>
<td>+</td>
<td>Exon 3 c.del669_673TGTAGinsGGTCCCTTTACC p.V225Lfs*12</td>
</tr>
<tr>
<td>MLS_3</td>
<td>69</td>
<td>43</td>
<td>Muscle weakness</td>
<td>Persecutory delusion</td>
<td>+</td>
<td>?</td>
<td>920</td>
<td>Absent</td>
<td>+</td>
<td>?</td>
<td>Exon 2 c.451dupC p.Q151Pfs*47</td>
</tr>
<tr>
<td>MLS_4</td>
<td>56</td>
<td>33</td>
<td>Involuntary movement</td>
<td>Depression</td>
<td>+</td>
<td>+</td>
<td>821</td>
<td>Normal</td>
<td>−</td>
<td>+</td>
<td>Exon 2 c.370C&gt;T p.Q124*</td>
</tr>
<tr>
<td>MLS_5</td>
<td>65</td>
<td>50</td>
<td>Involuntary movement</td>
<td>Cognitive decline</td>
<td>+</td>
<td>+</td>
<td>2,422</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>Exon 2 c.397C&gt;T p.R133*</td>
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<tr>
<td>MLS_6</td>
<td>70</td>
<td>55</td>
<td>Involuntary movement</td>
<td>Obsessiveness</td>
<td>+</td>
<td>+</td>
<td>1,052</td>
<td>Absent</td>
<td>−</td>
<td>−</td>
<td>Exon 3 Gross deletion Unknown</td>
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Abbreviations: CK = creatine kinase; DTR = deep tendon reflex.
Novel mutations were identified in MLS_4 and MLS_6.
Age at onset: age when first signs or symptoms appeared (yr).
* When DTR tests were performed, MLS_2 was affected with bacterial meningitis.
the lymphoblastoid cell lysates from MLS_1 and the control were equivalent. In addition, there was no immunoreaction corresponding to the XK protein band in both control and MLS_1 lymphoblastoid cells (figure 2F).

**Chorein-XK protein interaction in cultured cells**

Cell lysates extracted from K562 cells were immunoprecipitated with anti-XK antibody. In the subsequent immunoblot analysis, positive chorein bands were detected in the XK immunoprecipitants (figure 2G). Because the endogenous chorein level was low, XK protein immunoreactivity was not visually observed in chorein immunoprecipitants. Therefore, co-IP and reverse co-IP assays were conducted in a similar manner using the lysate extracted from chorein stably overexpressing HEK293 cells. Signals positive for chorein and XK protein were detected in the XK and chorein immunoprecipitants, respectively (figure 2H).

**Discussion**

In the present study, we analyzed 6 cases with MLS and confirmed the molecular diagnosis, as well as identifying 2 additional novel pathogenic mutations (table). The profile of 6 cases of MLS in this study was similar to those reported previously. In our MLS cases, the average onset age was 46.8 ± 8 years, which is approximately 13 years older than that of patients with ChAc. The disease duration for MLS may be longer than 30 years, which is typically longer than for ChAc.
In the present study, semiquantitative chorein immunoblotting using erythrocyte membranes from all patients with MLS revealed significantly reduced chorein immunoreactivity compared with age- and sex-matched healthy controls (figure 2A). Chorein immunoreactivities in heterozygous ChAc mutation carriers are also reduced to the same level as in patients with MLS (figure 2, C and D). These findings were demonstrated in at least triplicate independent experiments. Some ChAc mutation carriers exhibit partial symptoms of NA such as acanthocytosis.9 Taken together, the later onset and slower progression found for MLS compared with ChAc suggest that the chorein level reductions found in MLS may be directly associated with MLS molecular pathology. The erythrocyte membrane from 1 patient with MLS and lymphoblastoid cells from another patient with MLS showed normal chorein levels in previous study.4 In that study, chorein immunoblotting of heterozygous ChAc mutant carriers showed normal chorein levels, suggesting that the results of immunoblotting analysis might be unavailable for semiquantification.

In the present study, XK immunoblotting of lymphoblastoid cell lysate from healthy controls showed no XK protein band, suggesting no expression of XK protein in lymphoblastoid cells. This may account for the normal chorein immunoreactivity found by chorein immunoblotting of lymphoblastoid cell lysate from MLS_1, although further investigation is required.

XK protein covalently interacts with Kell antigens, which are remarkably reduced in erythrocyte membranes of MLS patients.6 In this study, based on the finding of reduced chorein in the erythrocyte membranes of patients with MLS, we hypothesized that the XK protein directly or indirectly interacts with chorein. In the present study, we performed IP assays, which revealed the possible interaction. In erythrocyte...
membranes, the absence of XK led to reduced chorein levels, although the absence of chorein was unrelated to XK levels. Computational analysis revealed a number of the corresponding impaired phosphorylation pathways in MLS and ChAc, suggesting a common molecular background bridging the generation of acanthocytes. In the present study, protein staining on blotting membranes revealed upshift in band 3 from both MLS and ChAc (data not shown), suggesting results of phosphorylation. Taken together, these results suggest that reduced chorein is associated with MLS phosphorylation-related molecular pathology in the erythrocyte membranes. However, the direct mechanisms of reduced chorein in erythrocytes of MLS are unknown. In addition, our study did not include molecular investigations of the CNS. Further studies are needed to elucidate the molecular mechanism of NA.

**Acknowledgment**
The authors thank the patients with MLS, patients with ChAc, ChAc mutant carriers, and healthy controls for their participation, and Ms. Kyoko Meguro for her technical assistance.

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**Disclosure**
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### Appendix Authors

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References
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