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Novel pathogenic *XK* mutations in McLeod syndrome and interaction between XK protein and chorein

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Abstract

Objective

To identify *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 XK protein.

Methods

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

Conclusions

In this study, *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 XK protein via molecular analysis. The reduction in chorein expression is similar to that between Kell antigens and XK protein, although the chorein-XK interaction is a possibly noncovalent binding unlike the covalent Kell-XK complex. Our results suggest that reduced chorein levels following lack of XK protein are possibly associated with molecular pathogenesis in MLS.

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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*,[1,2] leading to an absent or markedly reduced level of the encoding protein, chorein.[3,4] MLS is caused by loss-of-function mutations in the *XK*, leading to absent XK protein.[5] Although later onset and cardiomyopathy may occur predominantly in MLS,[6] 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_1[7] and a healthy control were established by SRL (Tokyo, Japan).

Coding and flanking regions of *XK* (NC_000023.10) and *VPS13A* (NC_000009.11) were analyzed by Sanger sequencing on an ABI PRISM 3100 Avant Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA).[3] 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[8] were lysed with Mammalian Protein Extraction Reagent (Thermo Fisher Scientific). K562 cells that were subcultured at 1×10^6 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 (HPA021662; 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

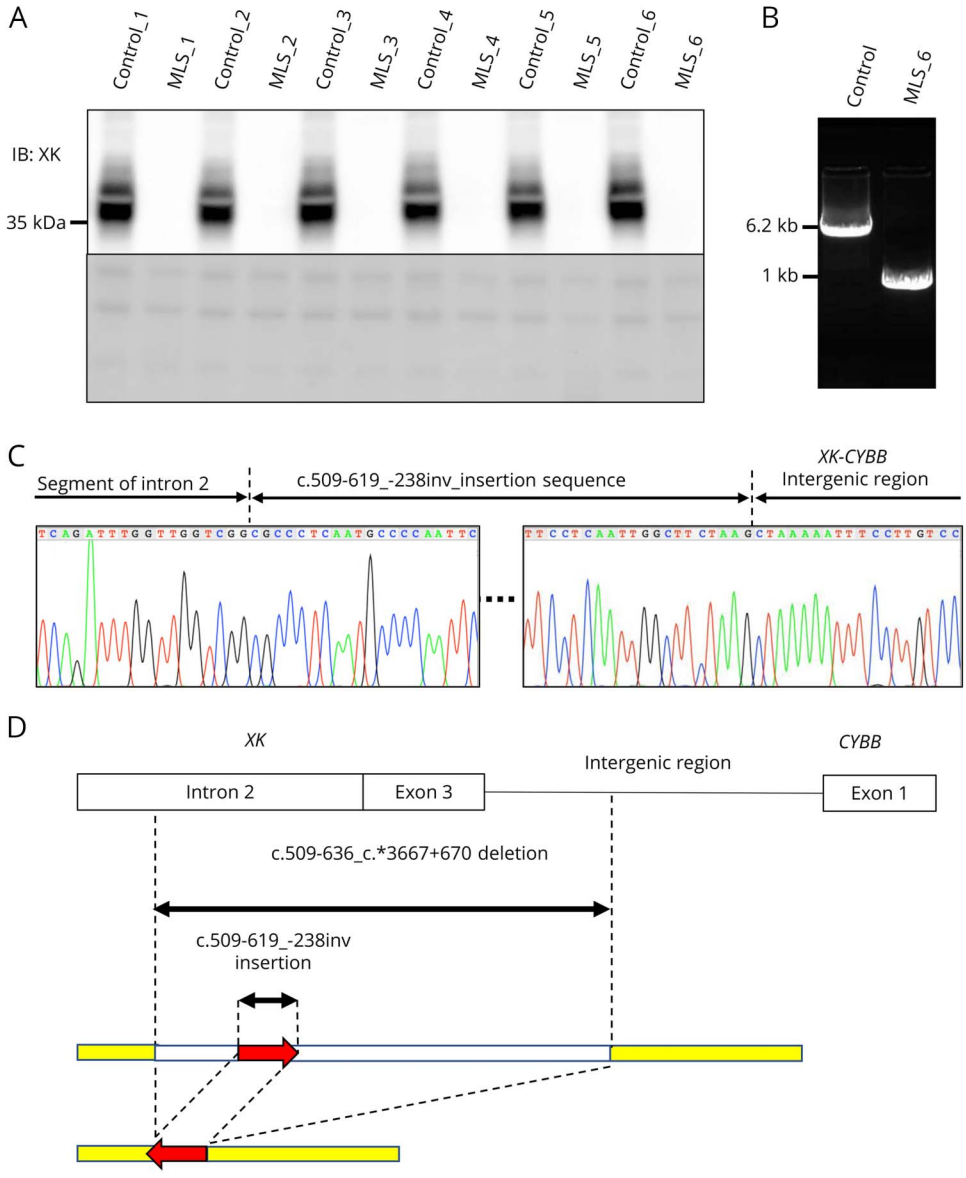
We found a marked reduction in chorein immunoreactivity in all patients with MLS (figure 2A). The mean density level of patients with MLS was significantly lower ($p = 0.00127$, d = 2.6) at 0.55, relative to controls (figure 2D). The average reductions in the levels of chorein immunoreactivity in the 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

Clinical symptoms												*XK* pathogenic mutations		
Case	Age at sampling	Age at onset	Initial symptom	Main psychiatric symptom	Acanthocytes	Chorea	EEG abnormality	CK (IU/L)	DTRs	Cardiomyopathy	Atrophy of the corpus striatum on MRI	Exon	DNA change	Protein change
MLS_1	64	47	Muscle weakness	Insomnia	+	+	Intermittent theta wave	5465	Absent	−	+	Exon 3	c.del669_673TGTAGinsGGTCCTCTTTACC	p.V225Lfs*12
MLS_2	61	53	Involuntary movement	?	?	+	?	?	[a]Hyperreflexia	?	+	Exon 3	c.1013delT	p.F338Sfs*70
MLS_3	69	43	Muscle weakness	Persecutory delusion	+	?	?	920	Absent	+	?	Exon 2	c.451dupC	p.Q151Pfs*47
MLS_4	56	33	Involuntary movement	Depression	+	+	3–4 Hz slow wave	821	Normal	−	+	Exon 2	c.370C>T	p.Q124*
MLS_5	65	50	Involuntary movement	Cognitive decline	+	+	?	2,422	?	?	?	Exon 2	c.397C>T	p.R133*
MLS_6	70	55	Involuntary movement	Obsessiveness	+	+	?	1,052	Absent	−	−	Exon 3	Gross deletion	Unknown

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).
[a] When DTR tests were performed, MLS_2 was affected with bacterial meningitis.

Figure 1 Molecular diagnosis of 6 MLS cases



(A) The results of XK immunoblotting revealed a lack of XK immunoreactivity in all patients with MLS. Equal loading was shown by staining with MemCode reversible protein stain (Pierce), shown in the lower panel. (B) In the case of MLS_6, the *XK* gene mutation was predicted to be a gross deletion including exon 3 based on the results of gDNA amplification. To identify the breakpoints of this mutation, we performed a whole-genome analysis. Based on the results, we performed a long-range PCR covering the deletion region. The results of long-range PCR for MLS_6 showed a gross deletion mutation that was approximately 5500 bp in size. (C) Sanger sequencing results revealed a combination of a gross deletion, from intron 2 (c.509–636) to an *XK-CYBB* intergenic region (c.*3667 + 670), and an insertion of a complementary sequence of 380 bp in intron 2. (D) The schematic shows the structure of the gross deletion region in MLS_6.

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).

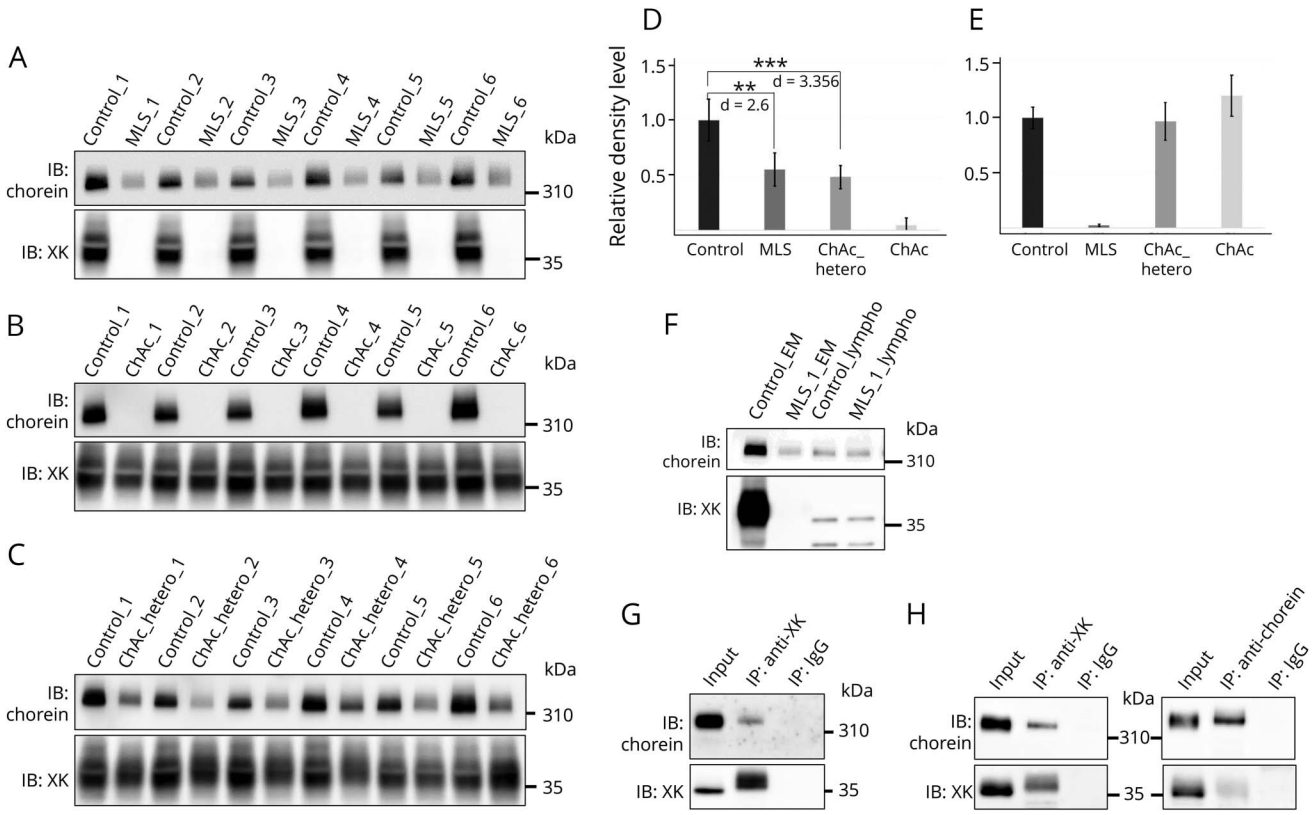
Chorein-XK protein interaction in cultured cells

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.[6] 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.[3] The disease duration for MLS may be longer than 30 years, which is typically longer than for ChAc.[6]

Figure 2 Immunoblotting of erythrocyte membranes, lymphoblastoid cells, and co-immunoprecipitants



(A and C) Chorein immunoblotting results revealed reduced chorein immunoreactivity in the erythrocyte membranes of patients with MLS, which are equivalent to the heterozygous mutant carriers of ChAc (ChAc_hetero). (B and C) XK immunoblotting results revealed normal XK immunoreactivity in the erythrocyte membranes of ChAc and ChAc_hetero. (A–C) Each imaging was performed by underexposed condition for semiquantitative analysis. (D) The histograms show the chorein relative density ratio in patients with MLS and ChAc and ChAc_hetero. Each band density was normalized by protein density staining with MemCode reversible protein stain. Error bars represent the SD (each n = 6); 2-sample Student *t* tests were performed accordingly on different sets of data. $^{**}p < 0.01$; $^{***}p < 0.001$. d shows the effect size (Cohen's *d*). (E) The histograms show the XK protein relative density ratio in patients with MLS and ChAc and ChAc_hetero. Each band density was normalized by protein density staining with memcode reversible protein stain. (F) Chorein immunoblotting results revealed equivalent chorein immunoreactivity in the lymphoblastoid cells of MLS_1 and the control (MLS_1_lympho and Control_lympho, respectively). The XK immunoblotting of lymphoblastoid cells shown in the lower panel of figure 2F reveals a lack of XK immunoreactivity. The Control_EM and MLS_EM lanes show the erythrocyte membrane for control and MLS, respectively. (G) Co-immunoprecipitation (IP) assay using K562 cells was performed with anti-XK antibody. Immunoblot analyses used anti-chorein (upper panel) and anti-XK antibodies (lower panel). (H) Co-IP and reverse co-IP assays using human embryonic kidney 293 (HEK293) cells stably overexpressing Myc-DDK–tagged chorein confirmed an interaction between XK protein and chorein. Immunoblot analyses used anti-XK protein and anti-chorein antibodies.

In the present study, semiquantitative chorein immunoblotting using erythrocyte membranes from all patients with MLS revealed significantly reduced chorein immunoreactions 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 immunoreaction 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.[10] 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 suggests 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.

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Disclosure

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Appendix *(continued)*

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