Case report

Elevated urinary β2 microglobulin in the first identified Japanese family afflicted by X-linked myopathy with excessive autophagy ☆

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Abstract

Here we report what is to our knowledge the first identified Japanese family afflicted by X-linked myopathy with excessive autophagy. The index case is a 52-year-old man with almost 40 years of progressive proximal muscle weakness. High urinary β2 microglobulin, normal serum β2 microglobulin, autophagic vacuoles with sarcolemmal features, and a hemizygous c.164–7T>G mutation in the *VMA21* gene were found. His two maternal uncles had similar clinicopathological findings. High urinary β2 microglobulin without obvious renal dysfunction might result from decreased urine acidification in the distal convoluted tubules caused by the *VMA21* gene mutation. These findings might prove to be useful as a preliminary marker suggestive of X-linked myopathy with excessive autophagy.
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1. Introduction

X-linked myopathy with excessive autophagy (XMEA) is a slowly progressive, inherited, autophagic, vacuolar myopathy (AVM) defined by proximal muscle weakness of the extremities beginning in early childhood. Clinically, there is no cardiac muscle involvement, cognitive impairment, or other organ manifestation [1,2].

Electromyography (EMG) findings are characterized by abundant myotonic and high-frequency discharges with no clinical myotonia, even in clinically unaffected muscles [2]. Muscle MRI has revealed fatty degeneration [2] and muscle biopsy specimens have revealed so-called autophagic vacuoles with sarcolemmal features (AVSFs) by light microscopy and a multi-layered basal membrane by electron microscopy [1–3]. AVSFs contain cell debris (degenerating organelles, granular material, and membrane whorls), which is also present between the layers of the multiplied basal lamina, and both compartments stain positively for acetylcholine esterase, acid phosphatase and other lysosomal enzymes such as lysosome-associated membrane protein 2 (LAMP2) [2,3]. Recent findings have revealed that vacuolar membrane ATPase activity 21 (*VMA21*) is a causative gene of XMEA [4].

VMA21 is one of the factors required for assembly of the vacuolar (V-) ATPase V_0 domain in the endoplasmic

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reticulum (ER) [5]. VMA21 interacts with subunit c′ of the V_0 domain and directs assembly of the proteolipid ring and subunit d of the V_0 domain. A complex of other factors stabilizes subunit a of the V_0 domain and, along with the remainder of the V_0 complex, facilitates the assembly of V-ATPase. VMA21 accompanies the V_0 and V_1 complex to the Golgi apparatus, where it is released and then recycled to the ER [5]. Lysosomal degradation by V-ATPase requires a low pH; a pH that is too high might decrease protein degradation in the autolysosomes of XMEA patients, which might lead to up-regulation of multivesicular body formation and exocytosis. V-ATPases are also located at the plasma membrane of several types of cells, which include renal intercalated cells in distal tubules and collecting ducts, and osteoclasts in bone. Dysfunction of V-ATPase may also cause renal dysfunction and osteopetrosis [5].

XMEA families have been identified in Europe and North America [2,4], but to our knowledge none has been identified previously in Asia, in spite of previous reports of severe cases [6,7]. In this report, we describe a Japanese XMEA family identified by examining and interviewing an index patient, and suggest that the elevation of urinary β2 microglobulin (β2 MG) may be a useful preliminary marker suggestive of XMEA.

2. Case reports

The pedigree chart of the afflicted family is presented in Fig. 1A.

2.1. Patient 1

The index case (III-1) had learned to walk normally, but at the age of 6 years was unable to keep pace with his companions while running. The muscle weakness had progressed slowly, so that at age of 52 years he was still ambulatory without assistance and able to work as a medical laboratory technician. On presentation at age of 52 years, cardiac and respiratory functions were normal.
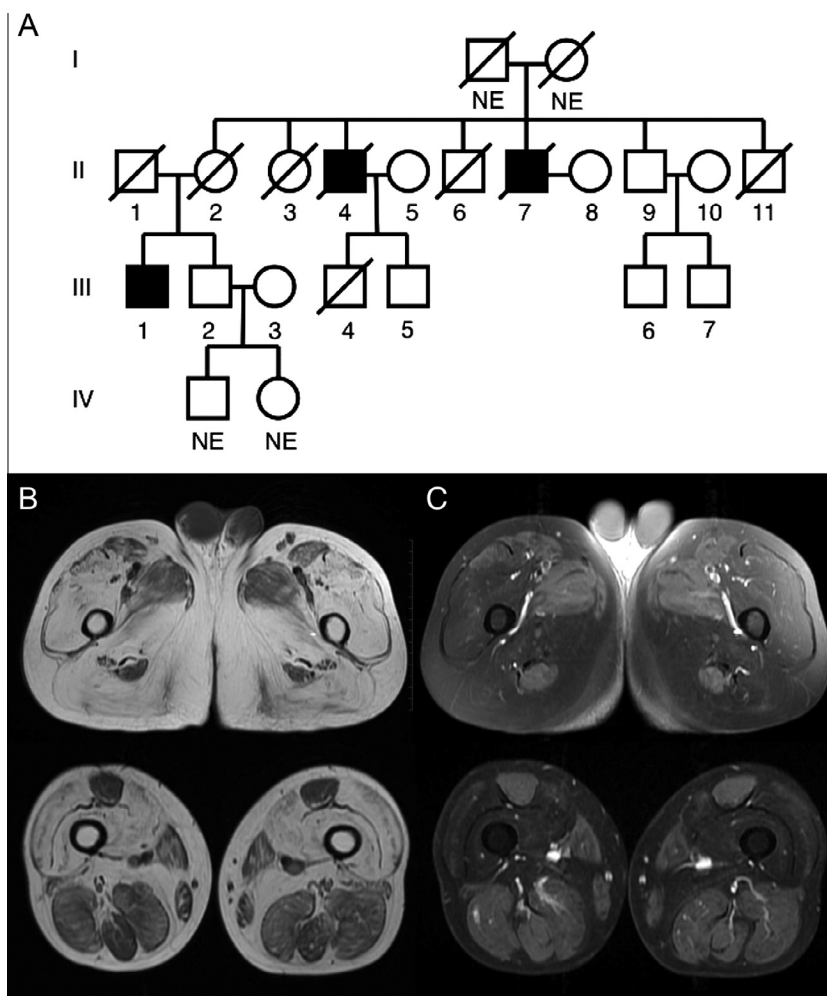

Fig. 1. (A) Pedigree of XMEA family. Shaded boxes represent affected members. Symbols having diagonal lines represent decreased individuals. Unaffected family members exhibited no abnormalities during the course of their medical examinations. (B) T2-weighted MRI and (C) fat suppression MRI of bilateral thighs revealed atrophy and fatty degeneration of femoral muscles, apart from the rectus femoris that was spared in both patients. Muscle biopsies were performed for individuals II-7 and III-1. *NE:* not examined.
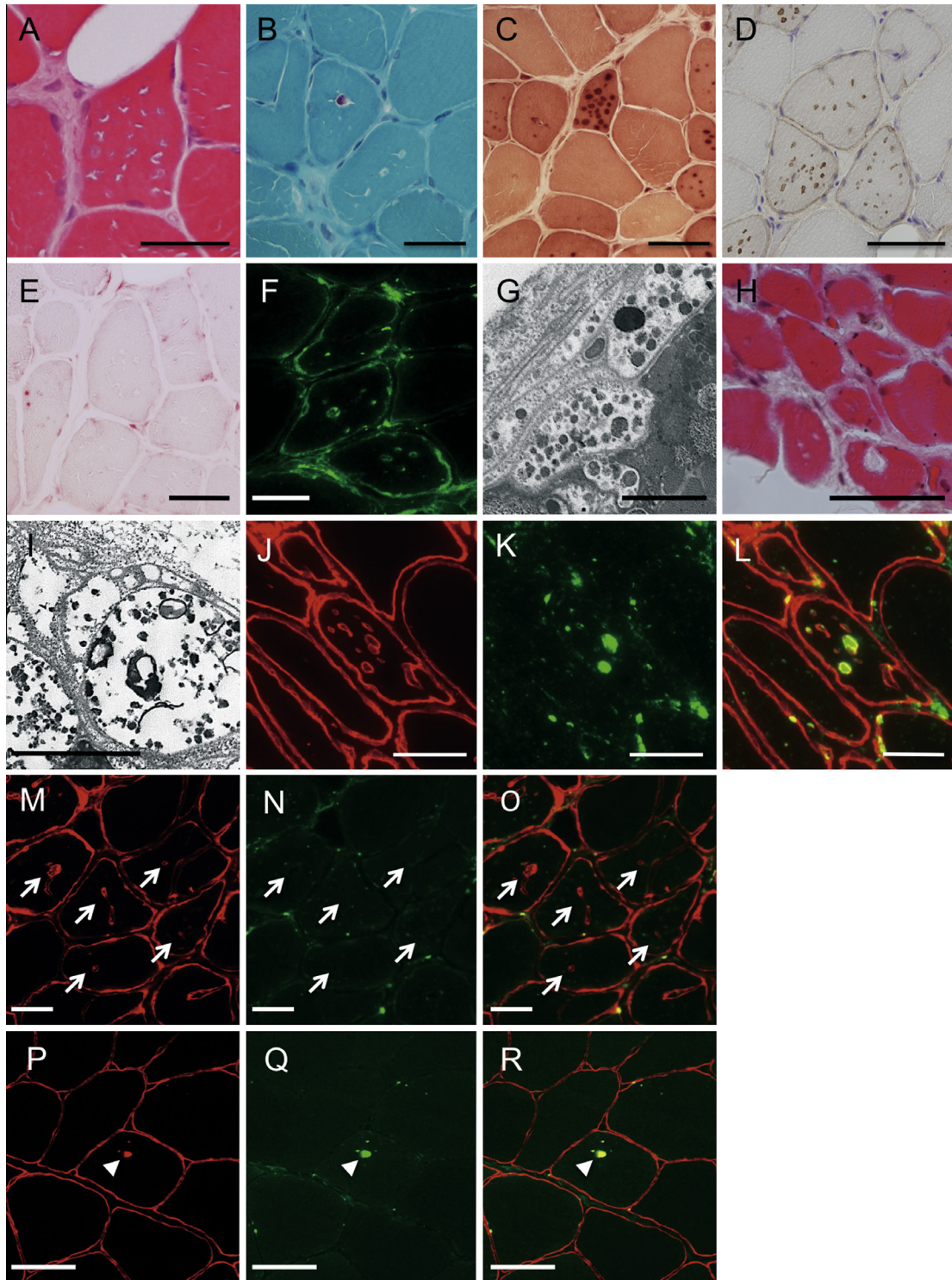
Fig. 2. Histological analysis of muscle biopsy specimens. Sections from individuals (A–F) III-1 and (H) II-7. (A) By hematoxylin and eosin staining, mild variation in fiber size was observed. Many fibers had vacuoles containing basophilic granules. (B) By modified Gomori trichrome staining, the vacuoles contained red or purple material. The vacuoles displayed high non-specific enolase (C) and acetylcholine esterase (D) activity. (E) Acid phosphatase activity in the vacuoles was variable and (F) the immunoreactivity against lysozyme was positive. (G) By electron micrography, the section from individual III-1 contained many vacuoles surrounded by a single-layer membrane, in which debris was seen. A multilayered basal lamina along the sarcolemma was observed. (H) The hematoxylin and eosin stained section revealed mild variation in fiber size and vacuoles in many fibers. (I) By electron micrography, the section from individual II-7 also contained many vacuoles surrounded by a single-layer membrane, in which debris was seen. A multilayered basal lamina along the sarcolemma was observed. Immunohistochemical analysis revealed that the vacuoles observed in frozen sections from individual III-1 were immunopositive for all sarcolemmal proteins assayed, including dystrophin (J) and merosin (M, P). (J–L) Dystrophin-positive vacuoles were also immunopositive against LAMP2 (J). (M–O) Merosin-positive and VMA21-negative vacuoles (arrow) were scattered. (P–R) Sections from individuals with various neuromuscular diseases (see text) reveal AVSFs having strong immunoreactivity for VMA21. Scale bars: (A–F, H, J–R) 50 μm; (G, I) 1 μm.

Laboratory studies revealed elevation of serum creatine kinase (CK) (450 IU/l; normal range ⩽210 IU/l) and urinary $\beta2$ MG (1.51 μg/ml; normal range ⩽0.20 μg/ml). Serum $\beta2$ MG (0.90 μg/ml) was within the normal range (⩽1.20 μg/ml). The urinary pH was between 5.5 and 6.0 (normal range ⩽6.5). Chest X-ray (CXR) revealed no bone abnormalities and bone mineral density was normal. Needle EMG showed abundant myotonic discharges in the muscles. Muscle MRI revealed adipose degeneration (Fig. 1B and C). Muscle biopsy from the left biceps brachii was performed at age 52; light microscopic examination revealed muscle fibers with vacuoles containing delicate basophilic debris (Fig. 2A and B). The vacuoles had the characteristic high non-specific enolase and acetylcholine esterase activity of AVSFs, consistent with previous reports (Fig. 2C and D) [2,3]. The AVSFs had high acid phosphatase (Fig. 2E) and lysozyme (Fig. 2F) activity, consistent with a previous report [1]. Electron microscopy showed that the AVSFs were surrounded by a single membrane, and that there were vacuolated fibers containing granular debris between redundant folds of the basal lamina as well as in the AVSFs (Fig. 2G). Immunohistochemical analysis confirmed that the AVSFs were immunopositive for lysozyme, all sarcolemmal proteins assayed, including dystrophin (Fig. 2J) and merosin (Fig. 2M), and LAMP2 (Fig. 2K), consistent with a previous report [3]. Importantly, double immunofluorescence revealed that the AVSFs were less immunopositive for VMA21 than they were for merosin, and areas of reduced VMA21-immunoreactivity were scattered throughout the AVSFs (Fig. 2M–O). On the other hand, the AVSFs observed in cases having other diseases had VMA21 immunoreactivity (Fig. 2 P–R).

To assess the specimens, samples were subjected to 20% SDS–polyacrylamide gel electrophoreses and then electrotransferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), as previously described [8]. In sarkosyl-soluble fractions of the muscle biopsy specimens, we observed a single ~15-kDa band, which is in accordance with the previously reported molecular weight of VMA21 [4]. The muscle biopsy specimen of individual III-1 contained a weaker VMA21 band than did that of an age-matched healthy control (Fig. 3A). Genetic analysis of *VMA21* was performed for III-1 after informed consent was provided as a part of the diagnostic procedure; a hemizygous mutation was revealed (c. 164–7T>G) (Fig. 3B).

2.2. Patient 2

This individual (II-7) is a maternal uncle of the index patient, and a younger brother of *Patient 3* (II-4). He learned to walk normally and exhibited normal intelligence. However, muscle weakness became evident during his childhood. He could not walk without support but was able to work as a taxi driver. He was examined
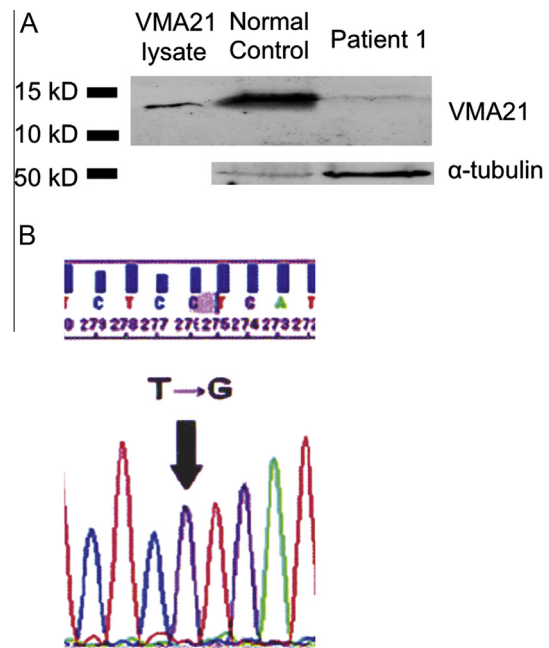

Fig. 3. Western blot and gene analysis. (A) The sarkosyl-soluble fractions and HEK293T lysate (sc-112091, Santa Cruz, CA, USA) were subjected to SDS–polyacrylamide gel electrophoresis followed by immunoblotting with anti-VMA21 or anti-alpha tubulin (loading control) antibodies. (B) *VMA21* gene analysis showed a hemizygous c.164–7T>G *VMA21* mutation.

at 43 years of age. At that time, cardiac function was normal but restrictive respiratory impairment was observed because of pulmonary emphysema due to heavy smoking (Brinkman Index [9] = 810). His laboratory data included elevations of serum CK (300 IU/l) and urinary $\beta2$ MG (0.90 μg/ml). Serum $\beta2$ MG was normal (0.51 μg/ml). The urinary pH was between 5.5 and 6.0. CXR revealed a flattened diaphragm due to pulmonary emphysema but no bone abnormalities. Needle EMG disclosed abundant myotonic discharges. These findings prompted us to perform a muscle biopsy. Examination of formalin-fixed sections by light microscopy revealed AVSFs (Fig. 2G). Electron microscopy revealed that the AVSFs were surrounded by a single membrane, and that there were vacuolated fibers containing granular debris between redundant folds of the basal lamina as well as in the AVSFs (Fig. 2H). This patient died of pulmonary emphysema during his fifth decade.

2.3. Patient 3

This individual (II-4) is a maternal uncle of III-1 and an older brother of II-7. He learned to walk normally and exhibited normal intelligence. Muscle weakness became evident during his childhood. He was examined at age 47, when II-7 was examined. He was still ambulatory without assistance and was able to work as a farmer. Cardiac involvement was not observed. His laboratory data included elevation of urinary $\beta2$ MG (2.00 μg/ml). Serum

Table 1
Demographic and clinical features of Japanese XMEA patients.

Patient	III-1	II-7	II-4
Age at onset	Childhood	Childhood	Childhood
Gender	Male	Male	Male
Symptom at onset	Lower muscle weakness	Lower muscle weakness	Lower muscle weakness
Progression	Very slow	Slow	Very slow
Cognitive symptom	None	None	None
Other clinical features	None	Pulmonary emphysema	Pulmonary emphysema
Serum CK (IU/l; normal $\leqslant$ 210 IU/l)	450	300	N.E.
Serum β2 microglobulin (μg/ml; normal $\leqslant$ 1.20 μg/ml)	0.90	0.51	1.10
Urinary β2 microglobulin (μg/ml; normal $\leqslant$ 0.20 μg/ml)	1.51	0.90	2.00
Age at biopsy	52	43	N.E.
Muscle biopsy	Left biceps brachii muscle	Left biceps brachii muscle	N.E.
Cause of death	Alive	Pulmonary emphysema	Choking to death
Genetic analysis	c.164–7T>G in *VMA21*	Unavailable	Unavailable

CK: creatine kinase, N.E.: not examined.

β2 MG was normal (1.10 μg/ml). The urinary pH was between 5.5 and 6.0. EMG showed abundant myotonic discharges. CXR revealed no bone abnormalities. This patient choked to death during his fifth decade.

Other family members have never experienced symptoms of muscle disease. II-9 has been receiving treatment for chronic glomerulonephritis and renal cell carcinoma for 10 years. Table 1 presents the clinical features, including urinary and serum β2 MG, of patients III-1, II-4, II-7, and II-9.

In addition, 45 patients with Becker muscular dystrophy, polymyositis, sporadic inclusion body myositis, distal myopathy with rimmed vacuoles or late-onset Pompe disease were examined. Their laboratory data included normal levels of urinary and serum β2 MG.

3. Discussion

XMEA is a slowly progressive inherited AVM characterized pathologically by membrane-bound sarcoplasmic vacuoles [1,2]. XMEA is caused by hypomorphic alleles of the *VMA21* gene, which is an essential assembly chaperone of the vacuolar ATPase (V-ATPase), the principal mammalian proton pump complex [4]. The patients presented here had proximal muscle weakness with fatty degeneration, and abundant myotonic and high frequency discharges on EMG, which are common among XMEA patients. We diagnosed XMEA using pathological findings, western blot analysis, and *VMA21* gene analysis, which revealed the same mutation found in a previously-reported French family [4].

VMA21 is one of the assembly factors of V-ATPase's V$_0$ domain in the endoplasmic reticulum (ER) [5]. VMA21 interacts with subunit c' of V$_0$ domain and directs assembly of the proteolipid ring and subunit d of V$_0$ domain. VMA21 accompanies the V$_0$ and V$_1$ complex to the Golgi, where it is released and then recycled to the ER [5]. Hemizygous mutation of *VMA21* causes to decrease V-ATPase and raise lysosomal pH. This might decrease protein degradation in the autolysosomes of

XMEA patients, which might lead to up-regulation of multivesicular body formation and exocytosis. V-ATPases is also located at the plasma membrane of several types of cells, which include renal intercalated cells in distal tubule and collecting duct, and osteoclasts in bone, so dysfunction of V-ATPases may also cause to renal dysfunction, osteopetrosis [5].

The current XMEA patients had increased urinary, and normal serum, β2 MG, unlike non-affected family members and patients with other neuromuscular disorders. In addition, other laboratory data indicated that these XMEA patients had normal renal function. β2 MG is secreted into the urine in the renal glomerulus, and 99% of secreted β2 MG is reabsorbed in the proximal convoluted tubules [10]. Increased urinary β2 MG is indicative either of increased serum β2 MG or dysfunction of the proximal convoluted tubules. Serum β2 MG is increased in patients with malignant tumors, autoimmune disease, infection (including with hepatitis B virus, infectious mononucleosis, and human immunodeficiency virus), or glomerular dysfunction [10,11]. All current affected family members were free from malignant tumors, autoimmune disease, and infection, so in these cases, the increased urinary β2 MG may have been attributed to dysfunction of the proximal convoluted tubules; however, other markers of renal function suggested that this was unlikely. β2 MG has been widely studied in clinical settings, but its instability in urine poses a major problem [12]. β2 MG is unstable in urine at room temperature when the pH is less than 5.5, and appreciable loss of β2 MG occurs at body temperature if the pH is less than 6.0 [10]. Urine is acidified in the distal convoluted tubules in association with acid–base homeostasis by Na$^+$ uptake depending on the Na$^+$/H$^+$ exchange in intercalated cells, which regulate acid–base homeostasis by V-ATPases [13]. Elevated urinary β2 MG without obvious renal dysfunction in XMEA patients could be due to less urinary acidification in the distal convoluted tubules caused by decreased V-ATPase, which might be concomitant with dysfunction

of VMA21. Therefore, elevated urinary β2 MG in the absence of obvious renal dysfunction may suggest XMEA, and these findings may potentially be useful as a preliminary marker suggestive of this condition.

Acknowledgements

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