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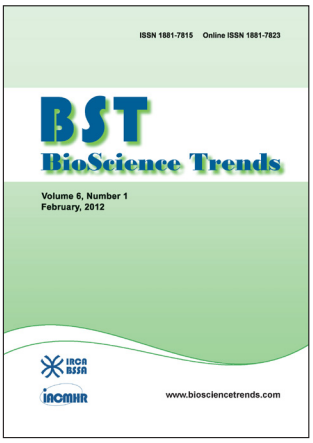

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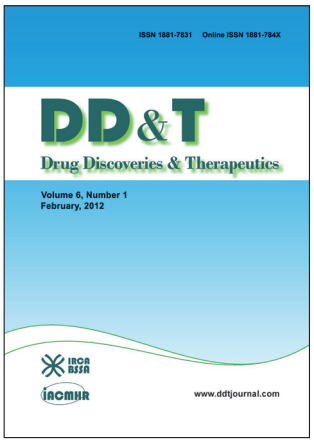

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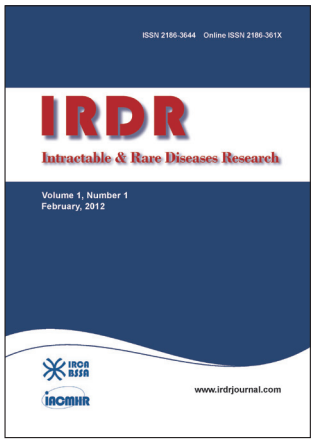

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Editorial and Head Office

Pearl City Koishikawa 603, 2-4-5 Kasuga, Bunkyo-ku,
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E-mail: office@irdrjournal.com
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Review

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Perspectives on urological care in multiple sclerosis patients

Mohamad Moussa[1], Mohamad Abou Chakra[2,*], Athanasios G. Papatsoris[3], Baraa Dabboucy[4], Michael Hsieh[5,6], Athanasios Dellis[7], Youssef Fares[8]

[1] Chairman of Urology Department, Lebanese University & Al Zahraa Hospital, University Medical Center, Beirut, Lebanon;
[2] Urology Department, Lebanese University, Beirut, Lebanon;
[3] 2nd Department of Urology, School of Medicine, Sismanoglio Hospital, National and Kapodistrian University of Athens, Athens, Greece;
[4] Department of Neurosurgery, Lebanese University, Beirut, Lebanon;
[5] Division of Urology, Children's National Hospital, Washington, USA;
[6] Department of Urology, The George Washington University, Washington, USA;
[7] Department of Urology/General Surgery, Areteion Hospital, Athens, Greece;
[8] Department of Neurosurgery, Neuroscience Research Center, Faculty of Medical Sciences, Lebanese University, Beirut, Lebanon.

SUMMARY Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system. Lower urinary tract dysfunction due to MS includes a dysfunction of the storage phase or dysfunction of the voiding phase or a detrusor-sphincter dyssynergia. Baseline evaluation includes a voiding chart, an ultrasound scan of the urinary tract, urine culture, and an urodynamic study. For storage symptoms, antimuscarinics are the first-line treatment, and clean intermittent catheterization (CIC) is indicated if there is concomitant incomplete bladder emptying. Intradetrusor injections with botulinum toxin A (BTX-A), are recommended for refractory cases. Urinary diversion is rarely indicated. For patients with voiding symptoms, CIC and alpha-blockers are usually offered. Sexual dysfunction in patients with MS is multifactorial. Phosphodiesterase type 5 inhibitors are first-line therapies for MS-associated erectile dysfunction in both male and female patients. This review summarizes the epidemiology, pathogenesis, risk factors, genetic, clinical manifestations, diagnostic tests, and management of MS. Lastly, the urologic outcomes and therapies are reviewed.

Keywords multiple sclerosis, urology, lower urinary tract dysfunction, neurogenic bladder

1. Introduction

Multiple sclerosis (MS), the most prevalent neurological disability, is an autoimmune-mediated disorder that affects the central nervous system (CNS) and often leads to severe physical or cognitive incapacitation as well as neurological problems in young adults (*1*). It is traditionally viewed as a two-stage disease, with early inflammation responsible for relapsing-remitting disease and delayed neurodegeneration causing non-relapsing progression. MS is more common in females. There is a genetic influence on MS susceptibility; about one in eight patients have a family history of MS. The main genetic risk associated with MS resides in HLA -DRB1*15 (*2*). Approximately, 2.5 million individuals are affected worldwide, and young individuals aged between 20 and 40 years are mainly affected. MS is regarded as a T cell-mediated autoimmune disorder with a predominance of CD8-positive T cells ($CD8^{+}$) cells compared with other T-cell subsets, B cells or plasma cells (*3*). The McDonald's diagnostic criteria for MS routinely undergo revisions aligning the criteria with advancements in imaging technologies. Diagnosis is based on parameters such as medical history and neurological exam, as well as paraclinical parameters such as magnetic resonance imaging (MRI), cerebrospinal fluid showing oligoclonal banding, and evoked potentials. MRI remains the most sensitive tool available for determining events that meet diagnostic criteria for dissemination in time and space. The prevalence of MS is highest in North America, Western Europe and Australasia (> 100 cases per 100,000 population), and lowest in countries centered around the equator (< 30 cases per 100,000 population) (*4*).

Effective management requires a multifaceted approach to control acute attacks, manage progressive worsening, and remediate bothersome or disabling symptoms associated with this illness. The emergence of higher-efficacy drugs requiring less frequent administration has made these preferred options in terms of tolerability and adherence (*5*). The goals of therapy with disease-modifying agents in patients with MS include shortening the duration of acute exacerbations, decreasing their frequency, and providing

symptomatic relief. It is common practice to treat acute relapses of MS with a short course of a corticosteroid (*6*). For newly diagnosed treatment-naive patients, multiple therapies are indicated and could include the injected medications (interferons (IFN), glatiramer acetate (GA)), oral therapies (fingolimod, dimethyl fumarate (DMF) and teriflunomide), and even monoclonal antibodies (ocrelizumab, natalizumab, and, alemtuzumab). In patients who have ongoing disease activity despite treatment with so-called platform therapies (interferons, GA, or teriflunomide) switching to therapies that may be more effective (fingolimod, DMF, ocrelizumab, natalizumab and alemtuzumab) might result in better control of relapsing disease activity (*7*). Based on the mechanisms of action and efficacy and safety profiles of these drugs, for most patients, one of the two treatment approaches is normally used. The first approach, which focuses on safety, is escalation and starts with lower risk disease-modifying therapies (DMTs) and shifting to higher risk treatment if disease activity occurs. The second approach, which prioritizes efficacy, is induction, in which a strong immune intervention is used from diagnosis of disease. Several factors have a role in treatment decisions for individual patients with MS, including disease activity, patient characteristics, treatment burden, and cost (*8*).

Although the genitourinary consequences of MS are rarely life-threatening, they can cause significant morbidity and patient frustration. The majority of patients with MS have symptoms ranging from urgency, urge incontinence, and frequency to urinary retention (*9*). Most patients with progressive MS will develop voiding dysfunction. Although there are many treatment options for patients with neurogenic bladders, therapy must remain conservative and initially reversible because symptoms from MS tend to wax and wane over time (*10*). The location of MS plaques will profile unique features of lower urinary tract dysfunction (LUTD). Lesions of the relevant suprapontine or spinal pathways regulating the lower urinary tract functions affect the storage phase, resulting in reduced bladder capacity and detrusor overactivity. The patient might report varying degrees of urinary urgency, frequency, nocturia, and incontinence (overactive bladder (OAB) symptoms). Injury to the suprasacral spinal pathways also results in loss of coordinated activation of the detrusor and inhibition of the urethral sphincter during voiding. Instead, there is a simultaneous contraction of the detrusor and urethral sphincter, known as detrusor-sphincter dyssynergia (DSD). Lesions of the sacral cord or infrasacral pathways result in voiding dysfunction associated with poorly sustained or absent detrusor contractions and/or non-relaxing sphincter (*11*). Nearly 90% of patients with MS experience some degree of voiding dysfunction and/or incontinence. LUTD rarely presents as the primary MS manifestation and usually appears 6-8 years after the initial diagnosis. There is a paucity of trials that guide treatment of urologic dysfunction in MS patients. Usually, a multidisciplinary team composed of general practitioners, rehabilitation specialists and neurologists, and urologists can properly manage those patients. The management of bladder dysfunction is individually tailored according to the pattern of LUTD (*12*). Management of LUTD focuses, primarily, on improvement of patients' symptoms and quality of life and, secondarily, on the preservation of the upper urinary tract and avoidance of urological complications. First-line treatments include fluid management, pelvic floor muscle training (PFMT), and medical therapies (antimuscarinic agents), and second-line treatments include Botulinum toxin A (Botox) intradetrusor injections, intravesical therapies, invasive and non-invasive neuromodulation, and catheterization (*13*).

We performed a narrative review to discuss briefly the epidemiology, pathogenesis, risk factors, genetic contribution, clinical course, diagnosis, and treatment of MS. The urologist had a crucial role in the management of urologic manifestations of patients with MS. We reviewed the current literature regarding the urological outcomes and management of patients with MS.

2. Overview of MS

2.1. Epidemiology of MS

MS is the most frequently demyelinating disease, with a prevalence that varies considerably, from high levels in North America and Europe (> 100/100,000 inhabitants) to low rates in Eastern Asia and sub-Saharan Africa (2/100,000 population) (*14*). The highest prevalence rates have been estimated for the age group 35-64 years for both sexes and all countries (*15*). In 2016, there were 2,221,188 prevalent cases of MS globally, which corresponded to a 10.4% (9.1 to 11.8%) increase in age-standardized prevalence since 1990. It was demonstrated that a strong latitude gradient for the prevalence of MS has occurred, with an increase in prevalence of 1.03 times per degree of latitude. A north to south decrease in prevalence by latitude gradient has been recognized in North America and Western Europe (*16*).

2.2. Pathogenesis of MS

Over the years, MS has been considered to be an autoimmune disorder where myelin-specific T cells initiate an inflammatory process that results in CNS demyelination. These autoreactive T cells are activated in the periphery and upregulate adhesion molecules that allow these T cells to interact with and cross the blood-brain-barrier and finally establish an inflammatory response directed against myelin (*17*). Within the CNS, these T cells re-encounter specific antigens and set up an inflammatory process that resembles delayed-

type hypersensitivity, dominated by lymphocytes and microglia. Various immunological effector mechanisms are initiated, which include cytotoxic T-cell proliferation, antibody production, and activation of microglia. The myelin-oligodendroctye unit is damaged, saltatory conduction breaks down and the symptoms of MS follow (*18*). In recent years, it has become more evident that axonal damage is the major morphological substrate of permanent clinical disability of MS patients. There was a significant correlation shown between the extent of axon damage and the numbers of CD8-positive cytotoxic T cells and macrophages/ microglia (*19*). The role of the B-lymphocytes (B cells) in MS pathogenesis is also important. It contributes twofold. First, as autoreactive B cells, they produce autoantibodies, secrete cytokines, clonally replicate memory B cells, and long-living plasma cells, which serve to advance the disease state by their constant production of autoantibodies. Second, as antigen-presenting cells, they activate autoreactive T cells (*20*).

Insulin-like growth factor I (IGF-I) promotes myelin production by oligodendrocytes. IGF-I and type 1 IGF receptors (IGF1R) are upregulated at the edges of the demyelinated plaque. It has been shown that oligodendrocytes within MS lesions have reduced IGF1R expression. The ablation of brain IGF1R prevents remyelination in this animal model (*21*). Immature oligodendroglia in areas of demyelination exhibited increased expression of the IGF-1 receptor during early recovery. Since IGF-1 is an essential factor involved in proper myelinogenesis and neurotrophic effects, this molecule holds potential for applications aimed at repairing tissue in MS (*22*).

In-depth analysis of spinal cord molecular neuropathology in MS patients was found that areas of incomplete demyelination extend a distance away from plaque borders and are characterized by a TGF-beta 1 (transforming growth factor-beta 1) progliotic signature. Based on the identification of astrocytes *vs.* oligodendrocyte gene co-expression networks, it was proposed that TGF-beta 1, while preventing acute inflammation, could promote gliosis and alter the translation of myelin genes (*23*). Also, TGF-β plays a vital role in the development and function of $CD4^+$ regulatory T cells (Tregs). It was found that TGF-β receptor II expression was reduced in MS patients' CD4 T cells. It is well-documented that there is a defect in the Treg population in MS patients, so modulation of TGF-β signaling may be used to correct this defect (*24*). It is believed that T helper 17 cells (Th17 cells), along with Th1 cells, play a role in MS pathogenesis, which, if not crucial, is at least significant. A majority of $CD4^+$ T cells in acute MS lesions produce IL-17A and hence can be classified as Th17 cells. Certain types of MS, such as opticospinal MS, have a dominant signature of Th17-driven pathology, including a large proportion of granulocytes among CNS-infiltrating cells (*25*).

Damage to axons is taken as a key factor for disability in MS, but its pathogenesis is largely unknown. Axonal injury is believed to occur as a consequence of demyelination and was recently shown to be a feature even of the early disease stage. Axonal injury is, therefore, at least in part, independent of demyelinating activity, and its pathogenesis may be different from demyelination (*26*). The factors that contribute to the pathogenesis of MS are summarized in Figure 1.

2.3. Risk factors of MS

Vitamin D deficiency, the season of birth, Epstein Barr virus (EBV) infection, and smoking behavior are strongly implicated and able to influence genetic predisposition to MS. Furthermore, these factors appear

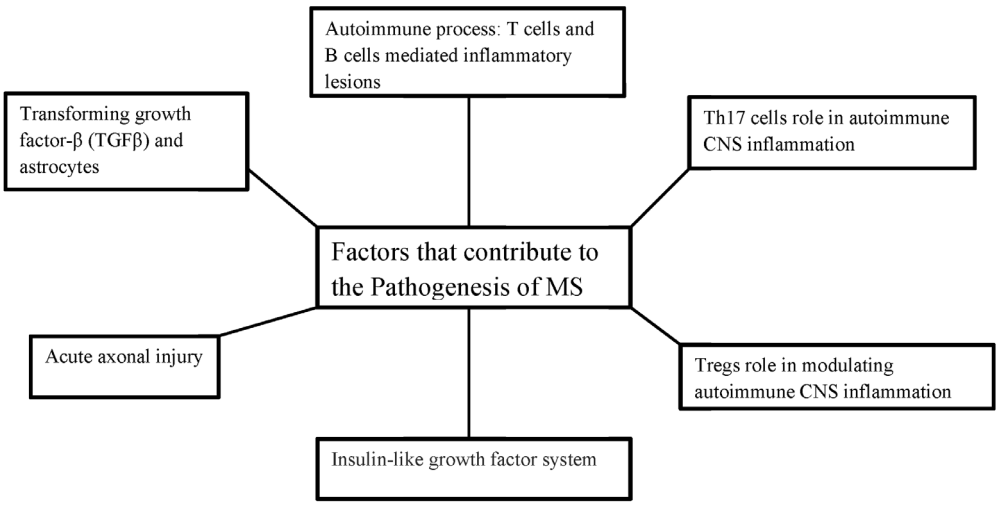

Figure1. The Pathogenesis of MS. MS, multiple sclerosis; Tregs, Regulatory $CD4^+$ T cells; CNS, central nervous system.

to act synergistically and the risk of MS in individuals exposed to more than one factor combines (*27*). The strongest known risk factor for MS is infection with EBV. Compared with uninfected individuals, the hazard of developing MS is approximately 15-fold higher among individuals infected with EBV in childhood and about 30-fold higher among those infected with EBV in adolescence or later in life. There is growing evidence that vitamin D insufficiency is a risk factor for conversion from clinically isolated syndrome to MS and MS progression (*28*). There is strong evidence that pregnancy does not worsen long-term prognosis despite an increased risk of relapses in the early postpartum period. Exclusive breastfeeding (no supplemental feedings for at least 2 months) reduces this risk of postpartum relapses, whereas nonexclusive breastfeeding appears to have no effect. Obesity or obesity-related comorbidities have been associated with worse outcomes among people with MS (*29*). It has been suggested that alcohol consumption was associated with mood disorders, disability, and even onset of MS, but a common theme is lacking (*30*). Physical and emotional stressors continue to be studied as potential MS risk factors. Important issues to be considered are the definitions of stress used, and the period of exposure thought to be relevant. Evidence for the role of emotional stress in MS etiology was weak, but left open the possibility that emotional stressors could be causal factors (*31*).

2.4. Genetic contribution to MS

The largest and first identified genetic risk factor for MS is an allele from the major histocompatibility complex (MHC) class II HLA-DRB1 gene, HLA-DRB1*15:01, which increases the risk about threefold. The HLA-DRB1 gene is expressed in antigen-presenting cells, and its protein functions in presenting particular types of antigen to CD4 T cells. The *HLA-DRB1*15:01* allele is associated with phenotypic features of the disease including female sex and the presence of cerebrospinal fluid-restricted oligoclonal bands (*32*). As anticipated, the MHC was definitively associated with MS susceptibility; however, beyond the MHC only two other loci were identified. These loci (the first non-MHC loci that were definitely associated with MS risk) encoded genes involved in immune regulation: the interleukin-2 receptor (IL2Ra) and the interleukin-7 receptor (IL7Ra) (*33*).

Genome-wide association studies (GWAS) have identified more than 150 single nucleotide polymorphisms (SNPs) associated with MS susceptibility. The odds ratio (OR) associated with the majority of these is small, around 1.1-1.2. Many of these SNPs lie close to genes associated with immune function, typically in regulatory rather than coding regions. Functional variants identified include those within IL7R, IL2RA, TNFR1, BAFF, and CYP2R1 (*34*). Overall, GWAS data support the long-held idea that susceptibility to MS is affected by the action of common (those with a risk allele frequency > 5%) sequence allelic variants in multiple genes. Meta-analysis has now brought the total number of associations to more than 200. The sum of each MS-associated allele (weighted by its effect size) is defined as MS genetic burden and can be calculated for each individual. A similar measure has also been computed to quantify risk at the HLA region; a high HLA-genetic burden is associated with a few demographic (young age at onset) and imaging characteristics. Besides, environmental factors have been shown to interact with genetic risk loci (smoking and HLA), therefore increasing the risk of developing MS (*35*).

There are significant differences between mild relapsing-remitting MS (mRRMS) and primary progressive MS (PPMS) gene expression. Surprisingly, the differentially expressed genes were mostly involved in immunological and inflammatory pathways, suggesting that the difference in MS phenotypes is caused primarily by a difference in immune responses (*36*).

2.5. MS stages and clinical progression

MS expresses itself in four clinical forms: relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), primary progressive MS (PPMS), and progressive relapsing MS (PRMS). Approximately 87% of patients present with RRMS, characterized by acute attacks (relapses) followed by partial or full recovery (remission) (*37*). The earliest clinical presentation of relapsing-remitting MS (RRMS) is the clinically isolated syndrome (CIS). Predicting which CIS patients are at high risk for MS is complicated by the disparity between clinical attacks and the extent of axon pathology (*38*). With the increased knowledge base of MS pathology, the limitations of purely clinical phenotypes, lacking imaging and biological correlates, became evident. In 2012, the Committee (sponsored by the National Multiple Sclerosis Society (NMSS) and the European Committee for Treatment and Research in MS) reexamined the original clinical phenotypes to provide improved terminology while incorporating imaging, fluid biomarkers, and other assays. The Committee recommended retention of the basics of the original 1996 MS phenotypes but introduced new descriptors of activity and progression. Two new disease courses were added: radiologically isolated syndrome (RIS) and CIS (*39*).

RRMS can be further characterized as an active or not an active disease, worsening and stable disease. Active disease shows evidence of new relapses, new gadolinium-enhancing lesions, and/or new or enlarging T_2 lesions on MRI over a specified period. Not active disease is that showing no evidence of disease activity. The worsening state is defined as increased disability confirmed over a specified period following a relapse.

Stable disease is defined as no evidence of increasing disability over a specified period following a relapse. PPMS can be further characterized as an active or not an active disease, with progression or without progression. Disease with progression is defined as evidence of disease worsening on an objective measure of change, confirmed over a specified period, with or without relapses. Disease without progression is defined as disease with no evidence of worsening on an objective measure of change over a specified period. SPMS can be further characterized as an active or not an active disease, with progression or without progression. PRMS individuals who were previously diagnosed with progressive relapsing MS would now be considered primary progressive: active (at the time of relapses or new MRI lesions) or not active (*40*).

If the MRI is typical for MS but there are no clinical symptoms or signs suggestive of a demyelinating disorder, patients are diagnosed as having a radiologically isolated syndrome (RIS). Multiple studies have tried to identify common predictive factors for conversion of RIS to clinical MS, the strongest thus far being an asymptomatic cervical spinal cord lesion (*41*).

2.6. Diagnostic test for MS

The diagnosis of MS is based on neurological symptoms and signs, alongside evidence of dissemination of CNS lesions in space and time. MRI is often sufficient to confirm the diagnosis when characteristic lesions accompany a typical clinical syndrome, but in some patients, further supportive information is obtained from cerebrospinal fluid examination and neurophysiological testing (*42*). It is crucial to define the attacks correctly. In a patient presenting with an attack, the most important paraclinical test to confirm the diagnosis is MRI with intravenous contrast agent containing gadolinium. This can both present the nature of the lesions (inflammatory and demyelinating characteristics) for differential diagnosis, and the distribution of the lesions within the CNS (evidence of dissemination in time and space for the latest diagnostic criteria (*43*).

Since their introduction in 2001 up to the recent 2017 revision, the McDonald diagnostic criteria for MS are based on the number, size, and location of the brain and the spinal cord lesions believed to be typical of MS. Lesion assessment on conventional T_2-weighted and post-contrast T_1-weighted MRI sequences has allowed the definition of criteria that support the early diagnosis of MS (*44*). The 2017 revisions of the McDonald diagnostic criteria for MS were agreed on by an international expert panel. One major change is inclusion of cerebrospinal fluid (CSF) oligoclonal bands as evidence of dissemination in time (DIT) in a patient with dissemination in space (DIS) gathered by clinical or magnetic resonance examination. The distinction between asymptomatic and symptomatic lesions in accounting for evidence of dissemination in space or time in supra, infratentorial, and spinal cord syndrome has been abandoned. Finally, cortical lesions can be used to demonstrate dissemination in space (*45*).

CSF examination remains a valuable diagnostic test, particularly when clinical and MRI evidence is insufficient to confirm the diagnosis of MS. There has been a major change in the most recent MS diagnostic criteria in that oligoclonal bands in the CSF can be used as a surrogate marker of DIT to confirm the diagnosis of RRMS in people with CIS and MRI evidence of DIS. CSF findings are also important when there is a progressive course from onset (PPMS) and when there are any atypical clinical or imaging findings. Evidence of intrathecal antibody synthesis (oligoclonal bands in the CSF but not in a paired serum sample) supports the diagnosis of MS. An elevated CSF protein > 1.0 g/L or significant pleocytosis > 50 cells/mm³ or the presence of neutrophils would suggest an alternative diagnosis (*46*).

2.7. Management of MS

Intravenous methylprednisolone is an anti-inflammatory glucocorticosteroid that is indicated by the Food and Drug Administration (FDA) for the treatment of MS relapse. Repository corticotropin injection (RCI), is a subcutaneous (SC) or intramuscular (IM) injection of adrenocorticotropic hormone (ACTH) approved by the FDA for use in patients experiencing MS relapse. Plasmapheresis is a medical procedure that has been recommended for steroid-resistant acute MS relapses by the American Academy of Neurology and the American Society for Apheresis. Intravenous immunoglobulin (IVIG) is an administered treatment comprised of pooled immunoglobulin G (IgG) prepared from the fractionation of human plasma and is also used in the treatment of MS relapse despite not having an FDA indication for such use (47).

Seven DMTs are currently approved as first-line therapy in RRMS without any restrictions: (IFN)-beta 1a IM, IFN-beta 1a SC, IFN-beta 1b SC, Peginterferon-beta 1a, GA, teriflunomide, and DMF. In patients with highly active disease (HAD), fingolimod, siponimod, natalizumab, ocrelizumab, or cladribine may be initiated following careful risk stratification (serum anti-JCV antibody, prior immunosuppressant use, cardiac disease, diabetes mellitus, retinal disorders, and malignancies). In patients with rapidly evolving aggressive disease, natalizumab, ocrelizumab, or alemtuzumab are recommended after careful risk stratification. In patients with moderately active disease and suboptimal response to first-line therapies as defined above, treatment escalation to fingolimod, siponimod, natalizumab, ocrelizumab, or cladribine should be considered. In patients with HAD and suboptimal response to DMTs, treatment escalation to natalizumab, ocrelizumab, cladribine or alemtuzumab should be considered (*48*).

The treatment of both PPMS and SPMS collectively referred to as progressive MS, has proven quite challenging due to the multifactorial and poorly understood pathophysiology of MS in general, specifically that of progressive disease. Ocrelizumab is indicated for patients with early PPMS in terms of disease duration and level of disability, as well as inflammatory activity on MRI. The benefit of ocrelizumab in PPMS patients over age 55 years and more than 10-15 years disease duration is unclear (*49*). Siponimod is a newer-generation sphingosine 1 phosphate (S1P) receptor modulator that internalizes S1P1 receptors. The results of a phase-III study suggest that siponimod may be beneficial in secondary progressive MS, at least in patients with disease activity (*50*). Ibudilast slowed brain atrophy in PPMS and SPMS patients in a multicenter phase 2b study. Smaller early phase studies of alpha-lipoic acid and simvastatin each found slowing of the rate of whole-brain atrophy in SPMS patients. Reasons now exist for optimism in the search for DMTs for progressive MS (*51*). The summary of therapy for MS subtypes is summarized in Table 1.

3. Urologic outcomes of MS

Urological symptoms often may be present as one of the initial presenting symptoms of MS. It may be the initial presenting complaint in MS in 3-10% of patients. It appears that LUTD occurs secondary to spinal cord involvement and in 50-80% of the patients. This can result in significant morbidity and significant impairment of quality of life. Most patients have storage-phase symptoms (urgency, frequency, nocturia, and urgency urinary incontinence) at some point but many are also affected by voiding-phase symptoms (weak and interrupted stream, straining to urinate, double voiding, and sensation of incomplete bladder emptying after voiding). Storage-phase, OAB affects between 32% and 86% of patients with urgency urinary incontinence reported in 36-72% (*52*). The lateral and posterior cervical spinal tracts are the most common sites for demyelination from MS and are the exact site of the necessary spinal tract pathways used to coordinate normal voluntary voiding. A patient with progressive and advancing MS loses volitional and synergistic control of the micturition reflex. These patients void only when they experience an involuntary detrusor contraction. Detrusor hyperreflexia due to unmasking of the sacral micturition reflex center or removal of cerebral inhibitory pathways by suprapontine neural plaques (or both) is reported to occur in 50 to 90% of patients with MS. Of the patients with detrusor hyperreflexia, approximately half will also have coexisting external urinary sphincter dyssynergia. Because of the presence of a sacral plaque, approximately 20% of the patients will have detrusor areflexia (*53*).

The variation in LUTD experienced by patients with MS may be attributable to the variable locations of lesions in the CNS. Understanding this relationship is important to further elucidate the pathophysiological relationship between MS and LUTD (*54*). Table 2 summarizes the LUTD according to the site of lesion in MS patients. Two factors likely to influence the clinical presentation of LUTD in MS are the MS duration and the severity of the neurological deficiencies and disabilities. There appears to be a significant correlation between the MS duration and the presence and the severity of clinical LUTD but not with their clinical presentation. The prevalence of clinical LUTD appears to be correlated with the severity of the overall deficiencies. The correlation between urinary retention and neurological

Table 1. Treatment options according to each subtype of MS

Clinical subtype	Therapy
Acute MS relapse	Corticosteroids, repository corticotropin injection, plasmapheresis
RRMS	(IFN)-beta 1a IM, IFN-beta 1a SC, IFN-beta 1b SC, Peginterferon-beta 1a, GA, Teri-flunomide, DMF
RRMS; Highly active disease	Fingolimod, Siponimod, Natalizumab, Ocrelizumab, Cladribine
Active SPMS	Ocrelizumab or Siponimod
PPMS	Ocrelizumab
Refractory rapidly progressive SPMS	Cyclophosphamide, Methotrexate

MS, multiple sclerosis; RRMS, relapsing-remitting MS; IFN, Interferon; SC, Subcutaneous; IM, Intramuscular injection; GA, glatiramer acetate; DMF, dimethyl fumarate; SPMS, secondary progressive MS; PPMS, primary progressive MS.

Table 2. Lower urinary tract dysfunction according to the site of lesion in MS patients

Site of the lesion	Urologic dysfunction on UDS	LUTD type
Suprapontine	Detrusor overactivity	Mostly storage symp-toms
Spinal: Infrapontine, supra-sacral	Detrusor overactivity, detru-sor- sphincter dyssynergia	Storage and voiding symptoms
Spinal: Sacral and infrasacral	Detrusor underactivity	Mostly voiding symp-toms

MS, multiple sclerosis; LUTD, lower urinary tract dysfunction; UDS, urodynamic study.

status is still controversial (*55*).

The presence of LUTD emerges during the visit, either because it is reported by the patient, or because it is found by the neurologist. Initial evaluation consists of several protocols. A consensus of the Italian Multiple Sclerosis Study Group suggest (a) quantification of the neurological deficit using the Functional Systems (FS) Scale and the Expanded Disability Status Scale (EDSS) according to Kurtzke; (b) the use of dedicated psychometric instruments, to best evaluate the possible presence of urinary symptoms; (c) the execution of urine test with urine culture and the possible treatment of urinary tract infections (UTIs) based on the antibiogram; (d) the use of a bladder diary (volume/frequency card) filled out by the patient to obtain information about the frequency of urination and volume voided at each urination; (e) the determination of post-void residual volume (PVR) by ultrasound or bladder catheterization; (f) the performance of an ultrasound of the urinary apparatus (*56*). A United Kingdom (UK) consensus on the management of the bladder in MS recommends doing an initial assessment with urine testing and measurement of the PVR. Urodynamic study (UDS) with or without additional synchronous fluoroscopic screening (video urodynamics), is used in neuro-urological practice to plan management of refractory symptoms or to identify patients at risk of future complications, particularly upper urinary tract problems. However, it should be noted that upper tract deterioration is much less common in patients with MS than in spinal cord injury (*57*). The risk of a pathological result in urodynamics is especially high for patients with EDSS > 6.5, use of more than one incontinence pad per day, and an MS that is not of the relapsing-remitting type (*58*). The ideal moment and context to indicate UDS in MS patients remain controversial. UDS is not indicated in the initial assessment of all MS patients reporting LUTD. Indications to perform UDS in MS patients are one or more of these conditions: evidence for spinal cord disease, increasing degree of disability according to EDSS, hydronephrosis/deterioration of renal function, increased PVR, recurrent UTIs, failure of conservative treatment (*59*).

UTIs are a major concern for patients with MS and pose a challenge to the treating physician. UTIs are ranked among the top three reasons for hospitalization, being responsible for 30-50% of all in-patient admissions of people with MS. The occurrence of recurrent UTIs in those patients may indicate suboptimal management of underlying LUTD. Known facts are the usefulness of urinalysis using to exclude UTIs and the non-treatment of asymptomatic UTIs except in the context of an acute MS relapse (*60*). Anatomic upper urinary tract (UUT) abnormalities in MS are reported in 0-25% of patients, most of which are noted at presentation for the evaluation of LUTD. Adverse findings include: pyelonephritis (0-25%), hydronephrosis (0-25%), vesicoureteral reflux (0-15%) and nephrolithiasis (2-11%). The development of structural UUT changes is low in MS patients. One explanation is that the disease course of MS is marked by relapses and remissions, which may lead to fluctuations in LUTD, leading to less effect on the UUT (*61*). High EDSS is significantly associated with urodynamic risk factors for UUT damage and allows a risk-dependent stratification in daily neurological clinical practice. An EDSS of ≥ 5.0 identified almost 90% of patients at risk for UUT damage (*62*). Patients with MS have a high incidence of calcium phosphate stones and struvite stones. The method of bladder management appears to be a risk factor in the development of stone disease (*63*). Stratifying for sex and age, female patients with MS at the ages of 30 to 39 years and female patients with MS for more than 10 years exhibited an increased risk of bladder cancer, whereas in men the risk of bladder cancer was increased 1 to 9 years after MS diagnosis (*64*). In a matched cohort study, the incidence rates and mortality rates for bladder cancer were higher in the MS cohort than in the matched cohort, consistent with some, but not all prior studies. The increased risk of bladder cancer in the MS cohort may reflect the more frequent presence of risk factors such as recurrent UTIs or the use of chronic indwelling catheters secondary to the neurogenic bladder (*65*).

Sexual dysfunctions (SD) are highly prevalent in MS patients and include diminished desire, arousal/erectile dysfunction (ED), and orgasmic/ejaculatory dysfunction. SDs can be caused by damage to the brain, to the spinal cord areas, and the peripheral neurons engaged in sexual response (*66*). SD may not only be due to lesions affecting the neural pathways involved in physiological function (primary dysfunction) but also result from general physical disabilities (secondary dysfunction) or psychological and emotional issues (tertiary dysfunction) (*67*). The most common sexual complaint in an MS male is ED, which can be found in 23% to 91% of patients. It is generally known that MS substantially determines a generalized demyelination process that interrupts the continuity of the neural pathways and alters the neural function that is essential for normal erection function (*68*). As depression occurs very commonly in patients with MS, with a lifetime prevalence of around 50%, this contributes to the very high prevalence of SD in those patients (*69*). The Multiple Sclerosis Intimacy and Sexuality Questionnaire-19 (MSISQ-19) is a 19-item self-report tool that measures SD stemming from the primary, secondary, or tertiary domains and has been validated in the MS population. An advantage of the MSISQ-19 is that it is quick, taking approximately 2 minutes to complete, and can be done before any patient visit (*70*).

4. Urologic Management of MS

4.1. Management of the storage symptoms (OAB

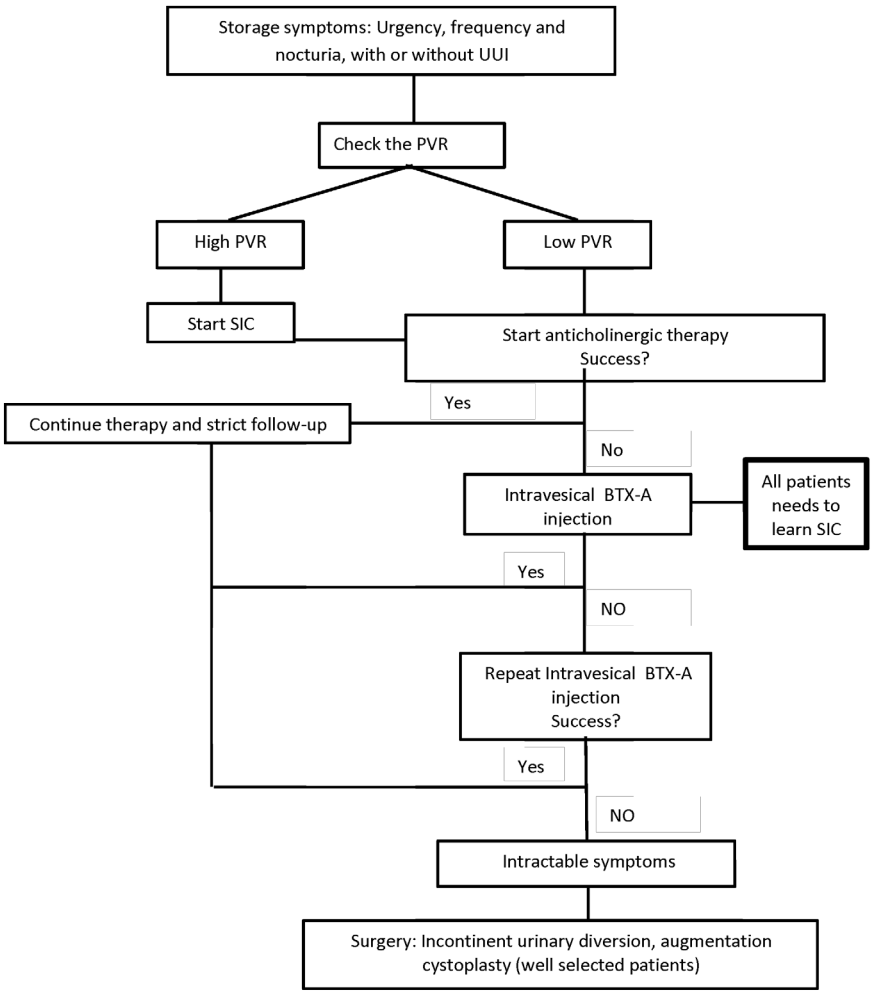

Figure 2. Algorithm for the management of storage symptoms in MS patients. MS, multiple sclerosis; UUI, urge urinary incontinence; SIC, self-intermittent catheterization; PVR, post void residual volume; BTX-A, Botulinum toxin A.

symptoms) in MS patients

A detailed algorithm for the management of the storage symptoms in MS patients is summarized in Figure 2.

4.1.1. General measures and physical treatment

Patients should be encouraged to keep a micturition diary, to drink adequately and regularly throughout the day (1.5-2l/day) but not late at night, and, if a pathological residual volume is present, not to delay micturition in case of urgency (*71*). Caffeinated fluid intake has a minimal effect on lower urinary tract symptoms in patients with MS. On average, patients with MS do not hydrate excessively and a considerable proportion restricts fluid intake to control urinary symptoms (*72*). Dietary modifications, with limitation of nighttime fluid intake, voiding before going to bed, and avoidance of caffeine, alcohol, and tea, should also be implemented before any pharmacological treatment for nocturia in patients with MS (*73*).

Physical therapy interventions can help reduce the negative effects of urge urinary incontinence by decreasing some of the symptoms. Treatment protocols vary, lacking a definitive technique (*74*). In patients with slight disability and OAB, PFMT may be beneficial. For PFMT to be effective, the neural pathways leading to the pelvic floor must be intact, and patients must be able to contract the pelvic floor muscles. PFMT is thought to strengthen the inhibition exerted on the detrusor by pelvic floor contraction. Several authors have demonstrated the beneficial impacts of PFMT on the course of the disease (*75*).

4.1.2. Antimuscarinic drugs

The treatment of urine storage problems in patients with MS has been attempted with antimuscarinic drugs such as oxybutynin, trospium chloride, propantheline, imipramine (particularly for nocturia), and solifenacin succinate. Efficacy results were varied but treatment was often associated with significant adverse effects

commonly associated with anticholinergic therapies such as dry mouth and constipation and, less frequently, impairment of accommodation with blurred vision (*76*). One particularly vulnerable population is the neurogenic bladder patients who maintain the ability to void. These patients can void but may have elevated residual urine, difficulty initiating voiding, or have detrusor hypocontractility. These patients can have urgency incontinence that would benefit from antimuscarinics but are at high risk of urinary retention, which is a known side effect (*77*). The chronic use of classical anticholinergic drugs for bladder symptoms may have a negative impact on cognitive functioning in MS patients. These potential cognitive side effects need to be considered both in clinical practice and research settings (*78*).

Anatomically and functionally anticholinergics have a potential benefit in MS but an important issue missed in a narrow randomized controlled trials-based assessment is the place of anticholinergics in the management of MS-related urinary symptoms, which has only been addressed thus far in consensus guidelines (*79*).

4.1.3. Botulinum toxin therapy

When anticholinergic therapy is insufficient or comes with too many side effects, intradetrusor injections with Botulinum toxin A (BTX-A) are recommended. BTX injection is recommended grade A in the European association of urology guidelines to be the most effective minimally invasive treatment to reduce neurogenic detrusor overactivity (NDO). These injections might increase PVR and might introduce the need for intermittent catheterization (*80*). A study was conducted by Deffontaines-Rufin *et al.* to assess the safety and efficacy of intravesical BTX-A injection in patients with MS and refractory NDO. Seventy-one patients with MS were included. 77% of the patients had clinical improvement or full success from the treatment with a reduction of their urgency and incontinence. Significant urodynamic improvement after treatment was shown (*81*). Mehnert *et al.* concluded in their study that OAB treatment in patients with MS using 100 Units (U) Botox intradetrusor injections seems to be effective and safe. Despite slightly impaired detrusor contractility most patients still voided voluntarily without symptoms (*82*). The dose of BTX-A commonly used to treat NDO in patients with MS is 200 U, even if in selected patients lower doses can be preferred. To be considered eligible for treatment, all patients should accept and be instructed to perform clean intermittent catheterization (CIC), since the risk of increased PVR volume and/or urinary retention after injection is high, especially with 200 UI of BTX-A (*83*). Repeated detrusor BTX-A for refractory NDO in patients with MS has a consistent effect on bladder control, resulting in sustained improvement in quality of life (*84*).

4.1.4. Sacral neuromodulation

Sacral neuromodulation (SNM) has shown promising results in MS patients who presented with DO. Usually, MS patients who are candidates for SNM should have stable disease without an expected requirement for frequent or routine MRI; patients with RPMS typically should not have SNM systems implanted (*85*).

4.1.5. Surgery

Surgical treatments of LUTD in patients with MS are appropriate in certain situations: when conservative therapies have failed; when intermittent self-catheterization (ISC) through the urethra is not possible; or in patients with serious complications such as sepsis, urethral or perineal fistula, renal failure or severe urinary incontinence. Augmentation cystoplasty can be done in highly-motivated patients. The goal of an augmentation cystoplasty procedure, which involves surgically enlarging the bladder, is to restore a low-pressure and compliant reservoir and, thus, achieve urinary continence. This procedure is often performed using a segment of detubularized ileon to augment the urinary bladder (*86*). Indications for augmentation cystoplasty include intractable (to conservative management, pharmacological treatment, and neuromodulation) DO or urgency urinary incontinence, and the procedure is performed either to protect renal function or to provide continence. Augmentation ileocystoplasty proved to be effective in neurological patients, including those with MS. Overall, a 77% success rate has been reported using ileum, and the use of ISC varies greatly among different series. Continence rate can be as high as 100%, although anticholinergics, bladder neck reconstruction, or continent diversion may be required to achieve it. Contraindications to augmentation cystoplasty include intrinsic bowel disease and inability to perform ISC. Long term results of augmentation cystoplasty suffer a variable rate of surgical revision (from 5% to 42%), although a 92% success rate has been reported for NDO (*87*).

Urethral catheters can be uncomfortable and can cause urethral and bladder neck trauma and should therefore only be considered as a temporary measure while waiting for a suprapubic catheter insertion. The patient should be referred to a urology service for suprapubic catheterization, which should be performed under both cystoscopic and ultrasound guidance to minimize the risk of accidental bowel injury. Once inserted, to allow adequate tract epithelialization, the suprapubic catheter should not be changed for up to 8 weeks, after which it can then be changed at intervals of up to 3 months. To minimize the risk of complications associated with long-term urinary catheterization, such as recurrent infections, encrustation, blockages,

and bleeding, catheters can be washed out at regular intervals (between changes) and attention can be given to cleanliness (*88*).

If all previous therapies are insufficient to control LUTD in MS patients and properly prevent complications, or inapplicable because of the disability associated with the disease that does not allow ISC, a surgical approach with non-continent urinary diversion may be an option. Large size studies are lacking to describe outcomes of this strategy and therefore define more accurately the targeted population as well as better inform patients before surgery (*89*).

4.2. Management of the voiding symptoms in MS patients

4.2.1. Clean intermittent catheterization and long term indwelling catheterization

CIC has been a staple treatment for neurogenic bladder patients with urinary retention and incomplete emptying. In the North American Research Committee on Multiple Sclerosis (NARCOMS) survey of over 9,000 MS patients, 11-15% of people reported currently using or past use of a catheter. Of these patients, over 80% performed intermittent catheterization (*90*). The frequency of catheterization will depend on factors such as bladder volume, fluid intake, PVR, and some urodynamic parameters. CIC is to be done in cases with persistent urinary retention but indwelling catheters should be avoided. UTI is the most frequent complication in patients performing CIC. Trauma from catheterization can occur but specific data in MS patients are lacking (*91*).

Indwelling urethral or suprapubic catheters are used by individuals with chronic urinary retention who are unable to perform CIC because of poor hand dexterity, no caregiver assistance, difficulty in using the bathroom, or in select cases of incontinence (*92*).

4.2.2. Alpha-blockers

Alpha 1-Adrenergic receptors are present at the bladder neck, where increased tone may be responsible for urinary retention and diminished flow rates. O'Riordan *et al.* conducted a randomized placebo-controlled study to test the hypothesis that blockade of these receptors using the selective alpha 1-adrenergic receptor antagonist would improve bladder emptying in patients with MS. 18 men with MS were included. There was a mean 41% improvement in peak flow rate in the actively treated group compared with a 7.4% deterioration in the placebo group ($p < 0.05$). Residual volume improved in both groups (*93*). Alpha-blockers may be effective and safe for treating neurogenic LUTD in female and male patients with MS but the studies were small and the overall quality of evidence was low. To make definitive conclusions, well designed randomized controlled trials are highly warranted (*94*).

4.3. Management of the detrusor sphincter dyssynergia in MS patients

Nearly half of the patients with untreated DSD will develop deleterious urologic complications, due to high intravesical pressures, resulting in urolithiasis, UTIs, vesicoureteral reflux, hydronephrosis, obstructive uropathy, and renal failure. The mainstay of treatment is the use of antimuscarinics and catheterization, but in those for whom this is not possible external sphincterotomy has been the last resort option (*95*). BTX-A injections for DSD have been primarily used in patients who were unable to perform CIC as an alternative to surgical sphincterotomy (*96*).

4.4. Management of sexual dysfunction in MS patients

The first-line therapies for male SD are phosphodiesterase type 5 inhibitors. Sildenafil and tadalafil are proven effective and well-tolerated therapy for ED in MS patients. Alprostadil (prostaglandin E1) and adrenoceptor antagonists (phentolamine) have been proven useful (*97*). Women with MS and SD are faced with limited treatment options including sildafenil and possibly estrogen replacement therapy, although more research is needed (*98*). Sildenafil is unlikely to help all patients with neurogenic female sexual dysfunction. Statistically significant improvement following sildenafil was only reported in the lubrication domain of the sexual function (*99*).

5. Conclusion

LUTD is common in MS and can be extremely disabling and embarrassing for patients. Depending on disease status and symptoms, MS urinary symptoms may respond to directed behavioral therapy, antimuscarinics, CIC, intravesical BTX injections, sacral neuromodulation, or surgical therapies. Due to the complexity and multifactorial nature of sexual dysfunction in MS patients, a multidisciplinary approach is necessary to provide them the best care. A close urology follow-up can reduce morbidity and improve the quality of life of those patients.

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Address correspondence to:
Mohamad Abou Chakra, Faculty of Medicine, Department of Urology, Lebanese University. Beirut, Lebanon.
E-mail: mohamedabouchakra@hotmail.com

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Original Article

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Surgical treatment of scoliosis in Ullrich Congenital Muscular Dystrophy: a case series of 3 patients

Cesare Faldini[1], Giovanni Viroli[1], Michele Fiore[1,*], Francesca Barile[1], Marco Manzetti[1], Luciano Merlini[2], Alberto Ruffilli[1]

[1] 1st Orthopaedic and Traumatologic Clinic, IRCCS Istituto Ortopedico Rizzoli (IOR), Bologna, Italy;
[2] Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy.

SUMMARY Scoliosis in Ullrich Congenital Muscular Dystrophy (UCMD) is very common, with a reported incidence of more than 50%, and it is rapidly progressive. There are no previous studies which specifically focus on scoliosis surgery in UCMD patients. This article reports three cases of scoliosis surgery in UCMD, focusing on operative course, clinical and radiological results achieved, fusion area and complications, with a 2-year follow-up. The surgical technique adopted for vertebral arthrodesis included: high-density pedicle screw systems, asymmetric rods contouring and direct vertebral rotation. The summary results shown a significative correction of the coronal deformity, with a reduction of the mean Cobb angle from 49° to 25° post-operatively. Mean pelvic tilt remained stable, while L5-tilt showed a decrease from 10° to 6°. Mean screw density was 1.92. None of the patients required extended fixation to S2. No major complications were reported, and patients maintained their pre-operative walking ability. All the patients reported a subjective improvement in quality of life, with a better sitting comfort. In conclusion, posterior spinal fusion with high-density pedicle screw systems and direct vertebral rotation may be safe and effective in surgical correction of scoliosis in UCMD. If pelvic obliquity and L5-tilt are less than 15°, could be possible to achieve an optimal spinal and pelvic balance even without sacral or pelvic fixation.

Keywords neuromuscular scoliosis, ullrich congenital muscular dystrophy, spine surgery

1. Introduction

Ullrich Congenital Muscular Dystrophy (UCMD) is a rare muscular dystrophy firstly described by Otto Ullrich in 1930 (*1*). It is caused by a mutation in one of the COL6A1-A2-A3 genes, which leads to a deficiency of collagen VI in the extracellular muscular matrix (*2-4*). The prevalence of UCMD is reported to be 1.3 per million (*5*) and scoliosis is present in more than 50% of them (*6*).

Surgical treatment of scoliosis in patients with UCMD is more challenging than idiopathic scoliosis, because of both the patients' respiratory comorbidities and the characteristics of the curve. In fact, scoliosis in UCMD has an early onset (reported average age of 6.5 years at diagnosis (*7*)) and is rapidly progressive (reported maximum progression rate of 16.2 ± 10° per year (*6*)). This eventually results in highly rigid, severe, and extended scoliotic curves, with high peri-operative risks and surgical complexity. Moreover, scoliosis in UCMD may be associated with pelvic obliquity, which makes sitting position difficult and worsens the disability of the patients. Therefore, long fusions to the sacrum and/or pelvis are often required to correct pelvic obliquity and to restore an optimal sitting balance. This poses an additional problem, since the poor bone quality and the large loads found in the lumbosacral region may lead to instrumentation failure and pseudoarthrosis.

Because of the rarity of UCMD, there are currently no studies that focused on the results of the surgical treatment of scoliosis in this specific disease. The aim of this article was to review the clinical and radiological results of scoliosis surgical treatment in 3 UCMD patients treated at our Institute over the last 5 years.

2. Patients and Methods

Three consecutive patients with UCMD diagnosis who underwent scoliosis surgery at our institution between 2015 and 2018 were included.

To make the initial diagnosis, genomic DNA from UCMD patients and unaffected parents was extracted from peripheral lymphocytes after informed consent following standard diagnostic methods (*8*): PCR primers

were designed to amplify all the 107 exons of COL6 genes and their flanking intronic regions. Skeletal muscle biopsies were collected during surgery and fresh frozen samples were used to confirm genetical disorder.

Patients were clinically and radiologically reviewed pre-operatively, post-operatively and after 2 years of follow-up.

Radiologic measurements were performed by two independent operators on the radiographs to evaluate the scoliotic deformity, obtaining the following parameters: Cobb angle, flexibility index, pelvic obliquity (using the Maloney method), L5-tilt (calculated as the angle between a line across the superior endplate of L5 and the intercristal line), thoracic kyphosis, lumbar lordosis, and vertebral rotation (according to Nash and Moe) (*9*). All measurements were done with the help of software (Carestream Health Italy, Inc., Genova, Italy).

All the patients received pre-operative echocardiography and pulmonary function tests: none of them had cardiac impairment, but all the patients had restrictive lung disease. All the patients received spinal CT-scan and MRI before surgery, to help the surgical planning and to rule-out any underlying neural axis abnormality.

All the patients underwent posterior instrumented spinal arthrodesis, performed by the same surgeon (C.F). The surgical technique used for all patients consisted of high-density pedicle screws constructs, various combinations of translation maneuver over differently shaped cobalt chrome rods (according to deformity) and direct vertebral rotation. Titanium rods were used in 2 cases, cobalt-chrome rods in 1 case (*10-12*).

During all surgeries, Somatosensory and Motor Evoked Potentials (SEPs and MEPs) were monitored.

After surgery, all the patients were monitored in intensive care unit. A full-time TLSO brace was prescribed to all the patients for two months, followed by progressive weaning.

3. Results

All the patients had a single large thoracolumbar curve: 2 patients presented the curve convexity on the right side, 1 patient on the left side. Average age at surgery was 13.3 years (range 10.8-15.6); pre-operative radiographic values are reported in Table 1. None of the patients showed intellectual impairment. Two patients had a limited deambulatory autonomy of a few meters; one had been never able to walk.

3.1. Case 1 (female): *De novo COL6A3 heterozygous c.6465G>A* (*novel mutation*)

The patient referred to our division presenting a thoracolumbar curve of 55°, convex to the right (Figure 1, A and B), with left coronal imbalance requiring two sub-

Table 1. Pre-operative radiographic measurements

PATIENT	FUSION AREA	POST-OPERATIVE					
		Cobb Angle	Thoracic Kyphosis	Lumbar Lordosis	Nash Moe	Pelvic Obliquity	L5-tilt
Case 1	T4-S1	21°	48°	61°	II	6°	9°
Case 2	T4-L4	11°	26°	68°	II	6°	2°
Case 3	T4-L4	43°	36°	54°	II	10°	8°
Mean Value	-	25°	36°	61°	II	7°	6°

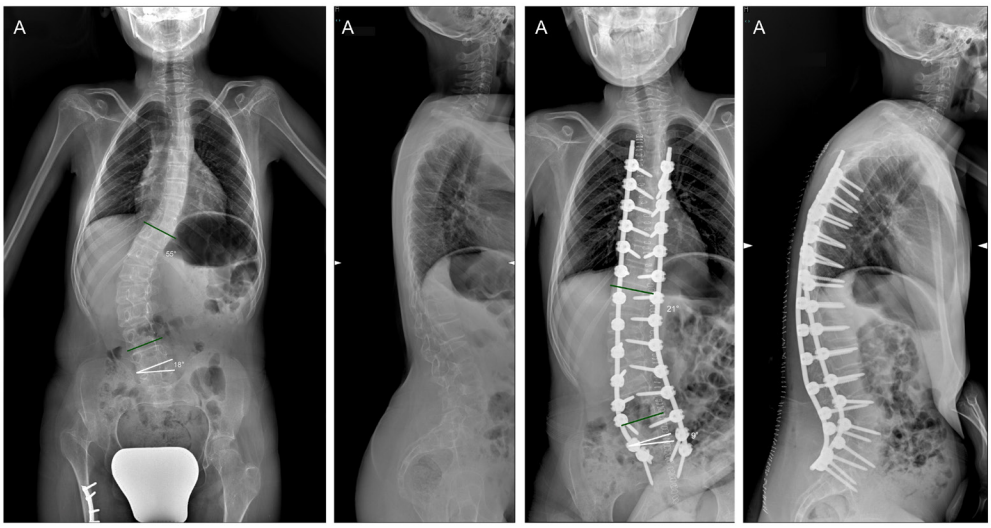

Figure 1. Case 1: Pre-operative radiographs, anteroposterior (A) and lateral (B) view; post-operative radiographs, anteroposterior (C) and lateral (D) view. Both pre- and post-operative Cobb angle (grey lines) and L5-tilt (white lines) measurement are reported.

axillary side pads when seated.

She had the first diagnosis of scoliosis at the age of 9, unsuccessfully treated with full-time bracing. In anamnesis she had developmental right hip dysplasia, torticollis and talus foot, both surgically corrected. She suffered from severe restrictive lung disease (Forced Vital Capacity FVC: 58% of the predicted value) and allergic asthma.

We performed a T4-S1 arthrodesis at the age of 13.6 years (Figure 1, C and D). S1 was chosen as the lowest instrumented vertebra because of the high L5-tilt (18°), despite the relatively low pelvic obliquity (8°). Screw density was 2.0; operative time was 210 minutes. No major complications occurred intra- and post-operatively. After 2 years, she reported a subjective improvement in quality of life, with a better sitting comfort and the same indoor walking ability.

3.2. Case 2 (female): *Inherited COL6A1 heterozygous c.896G > A*

She referred to our division presenting a curve of 42°, convex to the left side, complaining recurrent low back pain and discomfort in the sitting position in her wheelchair. Notably, the patient's stepsister was affected by UCMD carrying the same genotype, but never required spine surgery. Conversely, the father was asymptomatic, strongly suggesting the occurrence of paternal germline mosaicism (*13*).

Physical examination of the patient revealed generalised muscle weakness, predominantly in trunk and proximal limb musculature, joint contracture of elbows, knees and ankles, and a single large thoracolumbar scoliotic curve. In anamnesis, bilateral clubfoot (left foot corrected with a triple arthrodesis). She was able to walk for a few meters. She suffered from severe restrictive lung disease (FVC 44%) treated with mechanical insufflation-exsufflation machine ("cough machine") twice a day and non-invasive ventilation during night-time.

We performed T4-L4 arthrodesis at the age of 15.6 years. Screw density was 1.92; operative time was 300 minutes. Neither intra-operative nor post-operative complications occurred. After 2 years, the patient reported a more balanced sitting posture and maintained her indoor walking ability.

3.3. Case 3 (male): *De novo COL6A2 homozygous c.2572C>T.*

The patient referred to our division presenting a 52° thoracolumbar curve with convexity to the right, diagnosed at the age of 8 and unsuccessfully treated with bracing.

At the physical examination, the patient showed a trunk and proximal limb muscles weakness, lower and upper limbs areflexia, right knee joint flexion contracture and hyperlaxity of the wrists. In anamnesis, he had bilateral developmental right hip dysplasia treated with Pavlik harness and then corrective surgery. He had never walked, although at 36 months he was able to stand erect with aid: unfortunately, this ability was lost after a few months. He suffered from an early onset restrictive lung disease, with an FVC of 29% of the predicted value. Therefore, since the age of 4, he used non-invasive ventilation during night-time and mechanical insufflation–exsufflation machine twice a day.

We performed T4-L4 fusion at the age of 10.8 years. Screw density was 1.84 and operative time was 170 minutes. No intra-operative complication occurred. After extubation, the patient required prolonged respiratory support with non-invasive ventilation. On post-operative day 2, fever and productive cough occurred, therefore an empirical antibiotic therapy with Ceftriaxone was administered, suspecting a post-operative pneumonia. The patient was discharged after 17 days, after general conditions returned optimal. After 2 years, the patient reported better quality of life, with an improvement in sitting posture comfort.

Average operative time was 226 minutes (range 170-300), and average blood loss was 1190 mL (range 642-1580 mL). Average screw density was 1.92. Post-operative results are reported in Table 2. A satisfactory reduction of Cobb angle was obtained, from the average pre-operative value of 49° to the post-operative value of 25°, with an average reduction of 48% (24°) of the pre-operative value. Average lumbar lordosis improved from 56° to 61° and Nash-Moe decreased from III to II in all the patients. Pelvic obliquity remained stable, with a slight decrease to an average value of 7°. L5-tilt was reduced to a mean value of 6°. None of the patients required fixation to S2 nor to the pelvis.

Two patients were extubated after surgery in the

Table 2. Post-operative radiographic measurements

PATIENT	AGE AT SURGERY	RISSER STAGE	POST-OPERATIVE						
			Cobb Angle	Flexibility index	Thoracic Kyphosis	Lumbar Lordosis	Nash Moe	Pelvic Obliquity	L5-tilt
Case 1	13.6	3	55°	43%	38°	67°	III	8°	18°
Case 2	15.6	5	42°	38%	14°	59°	III	5°	3°
Case 3	10.8	0	52°	45%	33°	42°	III	11°	10°
Mean value	13.3	2.7	49°	42%	28°	56°	III	8°	10°

operating room, with a smooth transition to spontaneous breathing; Case 3 required prolonged non-invasive respiratory support. Also, none of the patients had mechanical or septic complications. No neurological injury occurred.

Patients were discharged after an average hospital stay of 15 days. At 2-year follow-up, no long-term complications, and no deformity progression or loss of correction occurred. All patients who had a pre-operative walking capacity, although limited, maintained it.

4. Discussion

Scoliosis in UCMD is characterized by an early onset, a rapid progression and resistance to brace treatment (*14,15*). This is confirmed by the low Risser stage (average value 2.66) that our patients had at the time of surgery. Regarding curve type, all the patients had a single large thoracolumbar curve. These characteristics are different from those seen in in Adolescent Idiopathic Scoliosis (AIS) and are more similar to those described by Karol *et al.* (*16*) in Duchenne Dystrophy. A large, gentle curve develops, with the apex at the thoracolumbar junction; with its rapid progression, the curve involves the whole thoracic and lumbar spine, leading to pelvic obliquity (*16*). This highly progressive scoliosis accelerates the progression of physical disability, making even simple activities such as standing, sitting, and walking difficult. On the other hand, by reducing chest wall compliance, scoliosis contributes to the restrictive respiratory dysfunction that usually afflicts these patients. For these reasons, scoliosis treatment in UCMD is crucial.

In our case series, we adopted a surgical technique derived from AIS surgery, achieving satisfactory clinical and radiological results. Infact, while sublaminar instrumentation and hybrid constructs are often used in neuromuscular scoliosis surgery, we preferred high density pedicle screws. Pedicle screws provide strong and stable 3-column fixation, allowing to perform strong corrective maneuvers such as direct vertebral rotation, achieving better curve correction, reduced blood loss and reduced surgical time compared to hybrid constructs (*17,18*). Moreover, as Hitchcon *et al.* (*19*) demonstrated, pedicle screws offer greater pull-out strength compared to sublaminar instrumentation. High-density screw constructs allow to distribute the corrective forces to every instrumented vertebra, avoiding pedicle breakage and screw pull-out during the corrective maneuver. This is crucial, considering that patients with neuromuscular scoliosis tend to have osteoporosis and osteopenia due to D-hypovitaminosis (*20*).

There are no studies in current literature that specifically focus on the surgical treatment of scoliosis in UCMD. Only one study conducted by Takaso *et al.* (*21*) described scoliosis surgery in a series of 10 patients affected by various congenital muscular dystrophies: 3 of their patients had UCMD. We believe that the rarity of UCMD is the reason why there are no previous studies focusing on scoliosis surgery in these patients: in fact, the vast majority of research on neuromuscular scoliosis involves patients with more common neuromuscular diseases such as Duchenne Dystrophy, Cerebral Palsy and Spinal Muscular Atrophy. On the other hand, research on neuromuscular scoliosis is less developed than that on AIS because surgery in neuromuscular scoliosis was developed later, due to cardiological and respiratory comorbidities. Moreover, many patients still have a certain, albeit limited, degree of motor autonomy and they refuse surgery for fear of losing it. This is particularly true in UCMD, in which scoliosis onset typically precedes the loss of ambulation, as Yonekawa *et al.* (*6*) and Nadeau *et al.* (*7*) studies revealed; on the contrary, scoliosis in Duchenne Dystrophy usually develops after patients become wheelchair dependent (*22*).

In our case series, 2 patients were able to walk for a few meters in an indoor setting and the surgery did not compromise motor autonomy in any of the cases. Indeed, post-operatively, all patients reported an increased comfort in the sitting position and higher confidence in walking, due to the restoration of a better global spinal balance, both in sagittal and coronal planes.

Regarding the deformity correction, we obtained a 48% coronal correction of the curve, while Takaso *et al.* (*21*) reported a higher 76% coronal correction. This can be explained by comparing the flexibility of the curves: in Takaso's case series, the curves had a flexibility index of 75%, whereas our patients had stiffer curves, with a flexibility index of 42%. It is also important to remember that deformity correction in neuromuscular scoliosis is less important than in patients with AIS, therefore higher radiological correction of the deformity may not be clinically relevant.

Instead, pelvic obliquity is a keypoint in neuromuscular scoliosis surgery: in order to correct or prevent it, routinely long fusion to the sacrum is a mainstay for many authors (*23-26*). Conversely, considering the challenges that sacropelvic fixation poses in these patients (including instrumentation failure due to osteoporosis and biomechanical stress, increased blood loss and longer operative time) others (*27-31*) support shorter fusions. In particular, Modi *et al.* (*31*) and Takaso *et al.* (*27*) reported excellent results with fusion up to L5 in patients with pelvic obliquity less than 15° and L5-tilt less than 15°. As we support this view, we performed fusion up to S1 only in one patient, whose L5-tilt was 18°. Infact, pelvic obliquity is often caused by supra-pelvic effects of scoliosis, such as asymmetric retraction of the muscles connecting trunk and pelvis (*32*): therefore, correction of the scoliotic curve may also reduce the pelvic obliquity, as Frischhut *et al.* (*33*) noted. In our experience, pelvic obliquity remained stable, but patients reported improved quality of life, with a

balanced and comfortable sitting position.

Respiratory complications have been reported as the most frequent complication following neuromuscular scoliosis surgery (*34-36*) and their frequency was found to be related to pre-operative FVC (*37,38*). In particular, Kang *et al.* (*37*) reported that patients with a pre-operative FVC of < 39.5% of the predicted value are more likely to develop a post-operative pulmonary complication. In our series, one patient (whose FVC was 29%, the lowest in our series) developed a suspected post-operative respiratory infection, which was successfully treated with Ceftriaxone. This is consistent with the Literature and shows how a multidisciplinary approach can be successful in controlling respiratory complications. None of our patients had infectious, cardiological, neurological, or implant-related complications.

The present study is affected by several limitations. First, the sample size is small, but UCMD is an extremely rare disease. Second, the follow-up period is too short to detect long-term complications. Further research is needed in order to assess the impact of scoliosis surgery on long-term pulmonary function of these patients as well.

5. Conclusion

Posterior spinal fusion with high density pedicle screws and direct vertebral rotation is safe and effective in the surgical correction of scoliosis in UCMD. If pelvic obliquity and L5-tilt are less than 15°, optimal balance could be achieved even without sacral or pelvic fixation, avoiding the risks of instrumentation failure, increased blood loss and operative time involved in sacropelvic fixation.

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Address correspondence to:
Michele Fiore, 1st Orthopaedic and Traumatologic Clinic, IRCCS Istituto Ortopedico Rizzoli (IOR), via G. C. Pupilli 1, 40136, Bologna, Italy.
E-mail: michele.fiore@ior.it

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Original Article

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The prevalence and patterns of chromosome abnormalities in newborns with major congenital anomalies: A retrospective study from Saudi Arabia

Lama Mohammed El-Attar[1,*], Ahmed Abdelrahman Bahashwan[2], Ameen Deen Bakhsh[2], Yasser Mohammed Moshrif[2]

[1] Department of Human Genetics, Medical Research Institute, Alexandria University, Alexandria, Egypt;
[2] Main Laboratory and Blood Bank, Madinah Maternity and Children Hospital (MMCH), Al-Madinah Al-Munawarah, Saudi Arabia.

SUMMARY Congenital anomalies are a worldwide health problem that places a burden on the family and society. Chromosome abnormalities are one of the leading causes for congenital anomalies in newborns. Despite the remarkable development in cytogenetic services in the past years, still there are limited data from Middle East countries. The current study aimed to evaluate the prevalence and patterns of chromosomal aberrations in newborns admitted to the neonatal intensive care unit (NICU) with major congenital anomalies at Medina province in the western region of Saudi Arabia. Out of 2,541 live births, 150 newborns were selected based on the presence of major birth defects. Demographic and clinical data were collected from hospital medical records and statistically analyzed. The prevalence of major congenital anomalies was 10.7/1,000 live births (95% CI: 9.076-12.583). The most common congenital anomalies in descending order were congenital heart disease, musculoskeletal and chromosome abnormalities. The birth prevalence of chromosome abnormalities was 4.22/1,000 live births (95% CI: 3.211-5.441). The most common chromosome abnormality was Down syndrome-nondisjunction type (66%). Advanced parental age was strongly associated with chromosome aberrations ($p < 0.001$) while consanguinity was evident in cases with normal karyotype ($p < 0.001$). High birth prevalence of chromosome abnormalities in newborns with congenital anomalies in Al Madinah was evident and advanced parental age is a potential risk factor. A local registry system for congenital anomalies is highly recommended to provide proper health services to high risk families.

Keywords congenital anomalies, chromosome abnormalities, birth prevalence, newborns, Down syndrome

1. Introduction

Congenital anomalies or Birth defects are defined as abnormalities of structure or function, including metabolic that are present at birth. Congenital anomalies in children vary from minor to major anomalies, and despite remarkable development in various treatment services, they remain an important cause of infant mortality and childhood disability (*1,2*).

From WHO reports, major congenital anomalies have been recorded in about three million newborns per year with a prevalence rate of 3% (*1*). On the other hand, there are some variations in the prevalence of congenital anomalies between different countries. Generally, in developed countries it ranged between 45-50/1,000 live births *(3,4*) while in the middle east and Africa it was between 20-30/1,000 live births (*5-9*). High numbers of deaths in low and middle income countries have been reported (*3,10*). Indeed underestimation in underdeveloped countries is mainly related to lack of local registry systems and records of actual cases.

There are several etiological factors of congenital anomalies like genetic, chromosomal, environmental and multifactorial. However; in many cases the cause is unknown (idiopathic). Chromosomal abnormalities have their impact on general health and wellbeing causing multiple problems including either mental retardation and/or physical disabilities. An underlying chromosome aberration is found to cause gross phenotypic anomalies in conjunction with mental retardation (*11-13*).

These facts encouraged governmental health authorities all over the world to establish proper cytogenetics diagnostic facilities in collaboration with well-trained genetic counseling services to provide

information and increase awareness in the community to guide high risk families.

Although there are several perinatal studies on the prevalence of congenital anomalies in different regions of Saudi Arabia; up until now there are no available data on the birth prevalence of chromosomal abnormalities in newborns with congenital anomalies at Medina province in the western region of Saudi Arabia.

The present study aimed to assess the prevalence and patterns of chromosomal abnormalities in newborns with major congenital anomalies delivered at a tertiary care maternity hospital in Medina province in the western region of Saudi Arabia.

2. Materials and Methods

2.1. Materials

The current retrospective descriptive study was conducted at Al Madinah Maternity and Children Hospital (MMCH) in Al-Madinah Al-Munawarah, the capital of Medina province. MMCH is considered the main tertiary care hospital that provides integrated medical care to pregnant mothers at Al Madinah in the western region of Saudi Arabia.

The study started January 2019 and was conducted for a period of twelve months based on data extracted from hospital medical records concerning newborns aged from one to 28 days with major congenital anomalies and admitted to the neonatal intensive care unit (NICU). Newborns with minor congenital anomalies, inborn errors of metabolism, home delivered or referred from outside the hospital were excluded.

Out of 2,541 live births admitted to the NICU, 150 newborns had major congenital anomalies. Clinical assessment by a specialist and full investigations regarding Echo, abdominal ultrasound, CT brain, X-ray and referral for chromosomal analysis were done routinely to all cases and data were fed to hospital medical records.

Congenital anomalies were categorized according to body parts affected based on the International Statistical Classification of Diseases and Related Health Problems (ICD-10) classification (*14*). Chromosome aberrations were categorized according to the International System for Human Cytogenetic Nomenclature (ISCN) 2016 (*15*).

In the current research demographic and clinical data regarding gender, consanguinity, family history of the presence of any inherited/genetic disorders and medical history of any congenital anomalies like central nervous system (CNS), congenital heart diseases (CHDs), gastrointestinal anomalies and limb anomalies were collected and statistically analyzed.

The study approval was obtained from the local ethical committee (IRB 533; H-03-M-048). The study protocol was in agreement with the Declaration of Helsinki guidelines 1975, as revised in 2013.

2.2. Statistical analysis

Prevalence per 1,000 live births was calculated and descriptive statistics were used to represent the qualitative data and the frequency of numerical and structural chromosome abnormalities. Comparison between variables was done by Chi square test or Fisher Exact test. Odds ratio (OR) at 95% confidence interval (95% CI) was used for assessment of association between chromosome anomalies and some demographic variables. *P* value was considered statistically significant at $p \leq 0.05$.

3. Results

Out of 13,988 live births delivered at the gynecology and obstetrics department in maternity and children hospital; total admissions in NICU were 2,541 newborns, major congenital anomalies were detected in 150 newborn infants (3.7%) in which 74 (49.3%) were males and 76 (50.7%) were females. The overall birth prevalence rate was 1.07% (10.7/1,000 live births). The most common congenital anomalies were related to the circulatory system (congenital heart diseases), followed by musculoskeletal system and then suspected chromosome anomaly (Figure 1).

Chromosome abnormalities were found in 59 newborn infants with congenital anomalies (39.3%) accounting for 2.3% of total NICU admissions. The prevalence rate of chromosomal abnormalities was 0.42% (4.22/1,000 live births).

Normal karyotype was found in 91 (60.7%), 43 newborns had normal male karyotype (46,XY) and 48 newborns had normal female karyotype (47.35% and 52.75%; respectively). On the other hand, abnormal

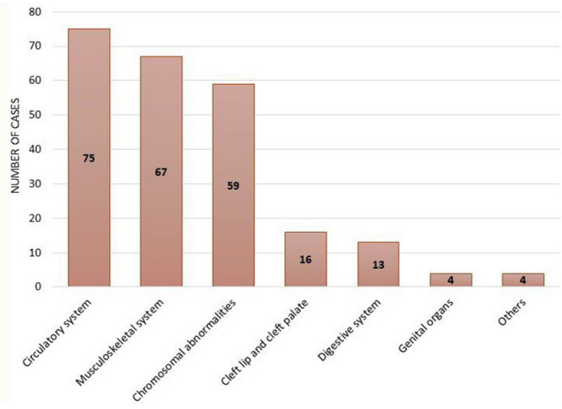

Figure 1. The frequency of newborns with birth defects according to body parts affected. The most frequent congenital anomalies in the studied cases were congenital heart diseases (75 cases) followed by musculoskeletal anomalies (67 cases), chromosome abnormalities (59 cases), cleft lip and palate (16 cases), gastrointestinal anomalies (13 cases), genital anomalies (4 cases), and others (4 cases). The frequency of congenital anomalies in the above figure exceeds the total number of studied newborns as one single infant may contain more than one anomaly.

Table 1. Birth prevalence of chromosome abnormalities in newborns with congenital anomalies

Characteristic	Total	Male	Female	M:F ratio	*p*	per 1,000 live births	95% CI
Newborns with congenital anomalies	150 (100%)	74 (94.3%)	76 (50.7%)	0.97:1	-	10.723	9.076-12.583
Normal karyotype	91 (60.7%)	43 (47.35%)	48 (52.75%)	0.9:1	0.527	6.51	5.238-7.987
Chromosomal abnormalities	59 (39.3%)	31 (52.5%)	28 (47.45%)	1.1:1		4.22	3.211-5.441
Numerical abnormalities	47 (79.7%)	27 (57.45%)	20 (42.55%)	1.35:1	0.135	3.36	2.469-4.468
Structural abnormalities	12 (20.3%)	4 (33.3%)	8 (66.7%)	0.5:1		0.858	0.443-1.498

M:F ratio male to female ratio.

Table 2. The birth prevalence according to type of chromosome abnormality

Type of chromosome abnormality	Number	Percentage	per 1,000 live births	95% CI
Autosomal chromosome abnormalities	53	89.8%	3.789	2.837-4.956
Down syndrome	39	66%	2.77	1.983-3.811
Trisomy 21	37	62.7%	2.61	1.862-3.646
Translocation Down syndrome	2	3.4%	0.143	0.017-0.517
Trisomy 18	5	8.5%	0.357	0.116-0.834
Trisomy 13	3	5.1%	0.215	0.044-0.627
Others	6	10.2%	0.429	0.157-0.934
Sex chromosome abnormalities	6	10.2%	0.429	0.157-0.934
Classic Turner syndrome	2	3.4%	0.143	0.017-0.517
Others	4	6.8%	0.286	0.078-0.732
Total cases of chromosome anomalies	59	100%	4.22	3.211-5.441

Table 3. The karyotype patterns detected in newborns with congenital anomalies

Chromosome abnormality	Number	Percentage
47,XY,+21	24	40.7
47,XX,+21	13	22.03
47,XX,t(14;21)+21	1	1.7
47,XY,t(21;21)+21	1	1.7
47,XY,+18	3	5.1
47,XX,+18	2	3.4
47,XX,+13	3	5.1
45,X	2	3.4
46,X,i(Xq)	1	1.7
45,X/46,XX	1	1.7
46,X,del(Xp)(p11)	1	1.7
46,XX,t(X;3)	1	1.7
46,XY,t(13;14)	1	1.7
46,XY,inv(9)	1	1.7
46,XX,del(13)	1	1.7
46,XX,del(18q)	1	1.7
46,XY,del(18q)	1	1.7
46,XX,del(p5)	1	1.7
Total	59	100

karyotypes were found in 59 (39.3%) newborn infants. According to gender, males were more prone to chromosome abnormalities especially numerical abnormalities than females, however the difference was statistically non-significant ($p = 0.527$) (Table 1).

Autosomal chromosome abnormalities were more often detected in the enrolled infants than sex chromosome abnormalities (89.8%% and 10.2%; respectively). Moreover, numerical chromosome abnormalities were recorded in 47 (79.7%) cases while structural abnormalities were seen only in 12 (20.3%) newborns. The most common autosomal abnormality was non-disjunction Down syndrome; trisomy 21 (62.7%) while classic Turner syndrome (45, X) was the noticeable sex chromosome abnormality (3.4%) (Table 2 and Table 3).

Chromosome abnormalities were more often noticed in full term infants ($p = 0.050$) and significantly associated with maternal age above 35 years and paternal age more than 40 years ($p < 0.001$). Despite seventy-nine newborn infants that were born to consanguineous parents (52.7%), chromosome abnormalities were more common in infants delivered from non-consanguineous parents than consanguineous parents ($p < 0.001$) (Table 4).

4. Discussion

Congenital anomalies are one of the leading causes of neonatal deaths and childhood disabilities creating a high burden on the family and community. In Saudi Arabia, prematurity and its complications, together with congenital anomalies, account for 85.5% of all causes of neonatal mortality (*16,17*).

One of the main causes of congenital anomalies is chromosome aberrations whether inherited or de novo. There is a paucity of chromosomal studies in the western region of Saudi Arabia especially the Medina province. The current study was directed to estimate the frequency of chromosome anomalies among newborns with major congenital anomalies delivered at the main tertiary care hospital in the country to which many pregnant mothers

Table 4. The demographic characteristics of newborn infants included in the study

Demographic data	Normal karyotype (no.91)		Abnormal karyotype (no.59)		OR (95% CI)	*p*
	No.	%	No.	%		
Sex:						
Males	43	47.3	31	52.5	1.235 (0.641-2.382)	0.527
Females	48	52.7	28	47.5	0.809 (0.420-1.560)	
Gestational age (weeks)						
Preterm (< 37)	3	3.3	7	11.9	3.949 (0.978-15.937)	0.050*
Full term (37-42)	88	96.7	52	88.1	0.253 (0.063-1.022)	
Post term (> 42)	0	0	0	0	-	
Maternal age (years)						
≤ 35	68	74.7	19	32.2	0.161 (0.078-0.331)	< 0.001*
> 35	23	25.3	40	67.8	6.224 (3.023-12.820)	
Paternal age (years)						
≤ 40	70	76.9	12	20.3	0.077 (0.034-0.170)	< 0.001*
> 40	21	23.1	47	79.7	13.056 (5.867-29.05)	
Consanguinity						
Present	71	78.02	8	13.56	0.044 (0.018-0.108)	< 0.001*
Absent	20	21.97	51	86.44	22.63 (9.244-55.407)	
Family history of genetic disease						
Positive	5	5.5	2	3.4	0.603 (0.113-3.218)	0.705
Negative	86	94.5	57	96.6	1.657 (0.311-8.835)	

*Statistically significant at $p \leq 0.05$.

are admitted.

In the present study birth prevalence rate of congenital anomalies was 1.07% (10.7/1,000 live births), which was consistent with other national and regional studies from Al Ahsa at Saudi Arabia (1.14%) (*18*), Kuwait (1.25%) (*19*), Oman (1.2%) (*20*), Iran (1.12%) (*21*), Morocco (1.02%) (*22*), Pakistan (1.14%) (*23*), and Northeast India (1.2%) (*8*). However, birth prevalence rates of congenital anomalies were higher in other reports than that in the present study (*4,9,24*). In comparison to other Saudi studies, the rate was higher in Riyadh (3.9%) (*25*), Hofof (2.27%) (*26*), and Jeddah (2.8%) (*27*). Sallout *et al.* found a relatively high prevalence of congenital anomalies in the KFMC (King Fahd Medical City) population in Riyadh, more specifically 46.5 cases per 1,000 live births that may be related to a high consanguinity rate (*7*).

In the current study, the consanguinity rate was 52.7% in newborns with congenital anomalies and was strongly associated with congenital anomalies in newborns with normal karyotype. On the other hand, chromosomal abnormalities were more common in infants delivered from non-consanguineous parents than consanguineous parents ($p < 0.001$). The overall consanguinity rate is high in middle east countries. In the Kingdome of Saudi Arabia it was estimated to be 57.7 % as it is part of the customs and traditions of society (*28*). Close association between consanguineous mating as a risk factor and birth defects especially congenital heart diseases have been previously reported (*29,30*).

In addition to consanguinity, other factors may cause discrepancy between different studies like study design, inclusion and exclusion criteria and environmental exposure to teratogens.

The relatively low birth prevalence rate of congenital anomalies in our study could be related to the fact that it included only live births from Medina delivered in the assigned hospital with exclusion of referred or home delivered cases. Also enrolled newborns were admitted to NICU with major congenital anomalies with exclusion of cases with minor congenital anomalies or inborn errors of metabolism.

In the current study birth prevalence of chromosome abnormalities was 4.22/1,000 live births. In addition, numerical chromosome abnormalities were more prominent than structural chromosome abnormalities and Down syndrome (trisomy 21) was the most common recorded anomaly. In comparison with other studies, birth prevalence of chromosome abnormalities among newborns in Medina was within international rates, however it was relevantly higher compared to national studies (*25,31*). Table 5 summarizes the comparison between our study and some national and international studies.

Using a literature review, there was no sufficient data regarding the prevalence of chromosome abnormalities in newborns in the western region of Saudi Arabia especially Medina province. Variations between different studies could be related to criteria of selection of cases and methods used for chromosome analysis. One of the influencing factors is the availability of advanced prenatal diagnostic techniques and the extent to which the society is aware of their importance, especially in light of religious regulations.

Another factor we have to take into account is the frequency of certain types of suspected chromosome

Table 5. Comparison between the birth prevalence of chromosome abnormalities in the current study and other national and international studies

Study region	Total Cases	Congenital anomalies	Chromosome abnormalities	per 1,000 live births	*Ref.*
Current study	13,988	150	59	4.22	-
Riyadh, Saudi Arabia	28,376	296	79	2.78	(*25*)
Jedda, Saudi Arabia	98	-	78	2.8	(*31*)
Aljahraa, Kuwait	7,739	97	17	2.18	(*19*)
Assiut, Upper Egypt	5,000	103	28	5.6	(*9*)
Tokyo, Japan	14,835	-	93	6.27	(*32*)
Goa	8,551	166	40	4.7	(*33*)
Denmark	77,520	2,089	291	3.75	(*34*)
Wales, England	466,475	20,374	1,269	2.73	(*35*)

anomaly in the studied population like, for example, most of the cases involved here were Down syndrome (66%), which directs towards numerical aberrations and aneuploidy as the most common abnormality in the studied cases.

Parents' age is also an important factor that must be taken into account. Maternal age above 35 years and paternal age above 40 years were highly associated with chromosome abnormalities in newborns with congenital anomalies enrolled in the present study while their association was non-significant in newborns with normal karyotypes. These findings match previous studies (*36,37*). It is well established that advanced maternal age is a risk factor for non-disjunction during chromosome segregation and aneuploidy. However, the exact mechanism is not yet well understood. In addition, some studies found an association between advanced paternal age (above 40 years) and impaired male reproduction in which chromosome aneuploidy and impaired chromatin integrity have been detected in male sperm. These changes could explain the association between advanced paternal age and some genetic disorders and chromosomal abnormalities like Trisomy 21 especially when it is taken along with advanced maternal age (*38,39*). Among most cases advanced paternal age is accompanied with advanced age of the mother making the association between paternal age and aneuploidy difficult to be assessed. Hence more precise studies are needed to find the exact role of paternal age.

Like other studies this study is not without limitations. Indeed, although our study was the first one that established the prevalence of chromosome abnormalities in newborns with birth defects, a retrospective design was preferred to suit the nature of this research and the information available. In addition; chromosomal abnormalities were diagnosed by standard karyotype methods, but some submicroscopic chromosome aberrations need more advanced techniques like chromosomal microarrays, which were not accessible.

In conclusion, birth prevalence of chromosome abnormalities in Medina province in the western region of Saudi Arabia was 4.22/1,000 live births. Congenital heart diseases were the most frequent congenital anomalies seen in newborn infants while Down syndrome was the most frequent chromosome anomaly. Consanguinity is a respectable risk factor for congenital anomalies in affected newborns with normal karyotypes whereas advanced maternal and paternal ages were evident risk factors for chromosomal abnormalities. We were trying to shed light on chromosome abnormalities as a cause of congenital anomalies in newborns. This study also makes it clear that there is a need to establish an updated national registry system for congenital anomalies and trends over time. In addition, cytogenetic examination should be afforded to newborns with congenital anomalies for proper diagnosis and genetic counseling for high risk families in Al Madinah in the west of Saudi Arabia.

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**Address correspondence to:*
Lama Mohammed El-Attar, Department of Human Genetics, Medical Research Institute, Alexandria University, 165 ElHoreya Rd, Al Ibrahimeyah Qebli; Alexandria, Egypt.
E-mail: lami.elatar@alexu.edu.eg

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Brief Report

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Novel mutations of epidermolysis bullosa identified using whole-exome sequencing in Indonesian Javanese patients

Suci Widhiati[1,2], Retno Danarti[3], Niken Trisnowati[3], Dewajani Purnomosari[2,4], Tri Wibawa[2,5], Hardyanto Soebono[2,3,*]

[1] Department of Dermatology and Venereology, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia;
[2] Doctoral Study Program, Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada, Yogyakarta, Indonesia;
[3] Department of Dermatology and Venereology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia;
[4] Department of Histology and Cell Biology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia;
[5] Department of Microbiology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia.

SUMMARY Epidermolysis bullosa (EB) is a group of inherited blistering skin diseases known to have heterogenicity of phenotypes and genotypes. There are four main types of EB: simplex, junctional, dystrophic, and Kindler syndrome, which are further classified into 34 distinct subtypes. Twenty different gene mutations are responsible for the loss of function and integrity of the basal membrane zone. In limited-resource settings such as Indonesia, diagnoses of hereditary skin disease often rely on clinical features. This limitation was managed by using the Clinical Diagnostic Matrix EB for clinical diagnosis support and whole-exome sequencing for genetic analysis. This study is the first whole-exome sequencing analysis of Javanese Indonesian patients with EB. The genetic analysis from four patients with EB identified all novel mutations unreported in the dbSNP database. There are Kindler syndrome with *FERMT1* frameshift mutation in exon 4, at c.388A (p.I130fs), which causes truncated protein; junctional EB generalized intermediate (JEB-GI) subtype with missense mutation at *LAMB3* gene position c.A962C (p.H321P); and recessive dystrophic EB (RDEB) a missense mutation at *COL7A1* gene position c.G5000T (p.G1667V). The whole-exome sequencing was further verified by Sanger sequencing. The new mutations' finding is possibly due to the limited genetic database in the Malayo-Polynesian ethnic group. Indonesia has hundreds of ethnic groups, and the Javanese is the largest ethnic group that populates Indonesia. Genetic data of these ethnic groups is important to be established in the international genetic database. This combination of clinical diagnostic and genetic analysis tools with whole-exome sequencing confirmed the challenging diagnosis of epidermolysis bullosa.

Keywords clinical diagnostic matrix, Kindler syndrome, junctional epidermolysis bullosa, dystrophic epidermolysis bullosa, Malayo-Polynesian ethnic group

1. Introduction

Epidermolysis bullosa (EB) is an inherited skin disorder characterized by flaky skin and blisters after light trauma or friction. The wounds tend to occur repeatedly and may become chronic, which causes high morbidity. This disease's clinical manifestations vary, ranging from local wounds on the hands and feet to generalized blisters or erosion and failure to thrive (*1*).

Epidermolysis bullosa comprises four major subtypes: EB simplex, junctional EB (JEB), dystrophic EB (DEB), and Kindler syndrome (KS). Each subtype is classified based on the molecular structure mutation in the dermis-epidermis (*2*). Before high-throughput next-generation sequencing (NGS) was developed, diagnosis of EB required an invasive procedure such as skin biopsy followed by microscopic examination and/or direct immunofluorescence to determine the mutation locations and finally, the results were confirmed by Sanger sequencing (SS) as the gold standard of diagnosis (*3*).

Caused by mutations in 20 different genes, EB involves the loss of function and integrity of the basal membrane zone structure. These genes code structured proteins in the intermediate filaments (keratin 5 and 14), focal adhesions, and desmosome complexes (desmoplakin, plakophilin, and plakoglobin). They are also vital to form intraepidermal adhesions and the

dermo-epidermal anchoring complex in the basement membrane zone of the mucous membrane and skin (integrin 64, collagen type XVII, laminin 332, collagen type VII, integrin three subunits, and kindlin-1) (*4*). Unfortunately, SS can only test single or a few genes at a time; thus, it is time-consuming and costly to do SS in cases when multiple genes need to be tested. Recently, direct procedures with NGS or targeted NGS have been performed to overcome the previous procedure's limitations. NGS screens all the suspected genes involved in EB using a single sample (*3*).

The NGS technology can simultaneously sequence the peptides/proteins coded by a known gene in the genome called whole-exome sequencing (WES). Even though WES can only sequence 1.5% of the entire human genome, 85% of the pathogenic mutations occur in regions encoded by protein, where WES is preferable. In recent years, the decreased cost of WES has developed a potential preference to use WES as a clinical practice (*5*).

In a limited-resource setting such as Indonesia, EB and EB subtypes' diagnosis depends on clinical findings and hematoxylin-eosin staining biopsy results. Electron microscopic and immunofluorescence examinations are not routinely done because they are rarely available in many centers. Thus, this condition implies a holistic approach to care for patients with EB. WES sequencing for analyzing all genes simultaneously to find the pathogenic variants will have a tremendous impact on both the novel search for disease gene discoveries and the efficient diagnosis of known genetic diseases in Indonesia.

Novel mutations or novel variants can be identified when they are not present in unrelated healthy control samples that were obtained from the exome sequence databases Exome Aggregation Consortium (ExAC) browser (*https://gnomad.broadinstitute.org*) and other databases such as dbSNP, 1000 Genomes Project (*http://www.1000genomes.org*), and Genome-Wide Association Studies (GWAS).

Javanese comprises the most prominent traits in Indonesian people who populate Java Island, and genetic testing with WES for the inherited disorder to the researchers' knowledge has never been done before in Javanese Indonesians. The prevalence of EB in Indonesia remains unknown. Based on case reports between 2013-2020, there were nine cases in Javanese traits, which is less than expected. Diana *et al.* reported around 31 EB cases in Indonesia in 2018 based on the Epidermolysis Bullosa Community Indonesia database (*6*). Even though genetic variants have been reported extensively in other populations, a study in the Javanese Indonesian population is lacking.

This study explores the use of WES to diagnose EB cases in Javanese Indonesians. This study highlights the first-tier use of WES to analyze genetic variation in Javanese EB cases. It will hopefully become a standard diagnostic tool for EB and other genetic disorders in Indonesia.

2. Materials and Methods

The clinical subtype diagnosis of EB was performed using a clinical matrix diagnostic tool (*7*), consisting of nine of the most often identified subtypes of EB. From these, the clinicians chose the most appropriate clinical findings that matched the available matrix.

DNA extraction from patients, parents, and healthy controls were obtained after informed consent for genetic analyses and medical information disclosure. Ethical clearance was completed following the Declaration of Helsinki with approval from the Universitas Gadjah Mada Medical and Health Research Ethics Committee. Four patients with EB, five unaffected parents, and ten healthy controls were included in this study.

Genomic DNA was purified using the AllPrep DNA/RNA Mini Kit (QIAGEN, Hilden, Germany). The concentration and quality of genomic DNA (gDNA) extracted from peripheral blood were examined with NanoDrop ND-100 (Thermo Scientific) and agarose gel electrophoresis.

Whole-exome sequencing was performed at Novogene (Hongkong, China). DNA was captured using the Agilent SureSelect Human All Exon kit and sequenced on an Illumina HiSeq platform. The sequence was aligned to the human genome reference sequence (hg38) using the Burrows-Wheeler Aligner (v0.7.8-r455). Duplicate reads were removed using Picard (v1.111), single nucleotide polymorphisms (SNPs) and Indels (insertions and deletions) were identified (SAMtools v1.0). The detected variants were then annotated using ANNOVAR (*8*). We defined database annotation variants with reported allele frequencies of 0.5% or greater in one of the following databases: dbSNP, 1000 Genomes Project (*http://www.1000genomes.org*), Genome-Wide Association Studies (GWAS), and Exome Aggregation Consortium (ExAC). We focused on variants of known causal genes in EB diseases, including *KRT5*, *KRT14*, *DST*, *EXPH5*, *DSP*, *PLEC*, *LAMA3*, *LAMB3*, *ITGA3*, *LAMC3*, *ITGB4*, *ITGA6*, *JUP*, *COL7A1*, *COL17A1*, *EXPH5*, *FERMT1*, *PKP1*, and *TGM5*. From those variants, we filtered deleterious predicted variants according to the following criteria: frameshift, in-frame insertion and deletions, stop codon changes, missense unless predicted innocuous by SIFT, polyphen-2, Mutation Taster, likelihood ratio test (LRT), Mutation Assessor, and Functional Analysis Through Hidden Markov Models (FATHMM).

The SS was performed to confirm the pathogenic variants in the patient, parents, and the control group using an ABI PRISM ®BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences of each of the suspected pathogenic variant genes

FERMT1 and *LAMB3* were chosen based on previous studies (*9,10*), the *COL7A1* exon 53-54 were modified from Cristianto *et al.* in 1997 with forward 5'CCTTGAGAACTGCTTGCTTC3' and reverse 5'GAAGATTGGGAGGGTTTAGC3' (*11*). The sequence reads using 4peaks application (*https://nucleobytes.com/4peaks*).

To identify the novelty of EB variants in Indonesia, the literature search was conducted using PubMed and Google Scholar with the term "epidermolysis bullosa" "southeast Asia" "Indonesia" "Javanese" and the full texts of retrieved literature were read carefully. Personal communication with clinicians was also established to find EB cases in Central Java and Yogyakarta. Novel variants are considered when the variant annotation is not found in the databases: dbSNP, 1000 Genomes Project (*http://www.1000genomes.org*), Genome-Wide Association Studies (GWAS), and the Exome Aggregation Consortium (ExAC).

3. Results and Discussion

Four patients with clinically confirmed EB were included in this study. The clinical diagnosis was made with support from clinical matrix diagnostic tools, and all patients underwent genetic analysis. Two patients are not related, but Cases 2 and 3 were siblings. Two patients are male, and two are female with an age range of 10 to 22 years. Three from 4 subjects were missense mutations; one subject was a frameshift mutation. The subtypes of EB were varied from Kindler syndrome, JEB-GI, and RDEB-GS. All patients are genetically of Javanese ethnicity.

Each of the pathogenic EB variants was found to be deleterious by its rare or low frequency. We documented novel mutations in all subjects with WES (Table 1). The targeted EB variants in our study were not reported in the dbSNP, GWAS study, 1000 Genomes Project (released in August 2015) nor in the ExAC. Evaluation of 10 unrelated control individuals by SS failed to demonstrate all the mutations found in this study. Thus, we identified pathogenic novel mutations in the *FERMT1*, *LAMB3*, and *COL7A1* genes, which confirmed Kindler syndrome, JEB-GI, and RDEB, respectively.

Clinical WES is a valuable diagnostic tool to pinpoint rare genetic alterations in an unbiased and efficient way to confirm a diagnosis despite extensive testing and workup. The use of WES as a first-tier test would substantially reduce the time to diagnosis, and it is only a half to one-quarter the cost of traditional testing (*12*). WES has a consistently higher diagnostic yield than routine clinical SS. However, the Sanger method is still considered the "gold standard" for clinical DNA sequencing. Nevertheless, SS has limitations because it can only test single or a few genes at a time and becomes costly when multiple genes are tested before reaching a diagnosis (*13*). Hamilton *et al.* in 2016 demonstrated that WES has high concordance with SS with 97.3% of variants concordant (*13*).

However, WES has some limitations in poor targeting by the exome capture kit, high guanine-cytosine (GC) content, and the presence of repetitive sequences, leading to inadequate coverage or sequencing inaccuracy. The exome capture kit used to define allele frequencies in our study was using the 1000 Genomes Project, GWAS, ExAC, and dbSNP. Unfortunately, none of the commercial arrays for SNPs databases were created from polymorphism data generated in Southeast Asian Malays. In the future, it is essential to develop population-specific databases as a country-based molecular variation database since different genetic backgrounds may have different susceptibility to the haploid in the sufficiency of variants or mutations. SNPs may contribute to the genetic disorder, or they may be in linkage disequilibrium with other causal variants and mutations (*14*).

Indonesia, located in Southeast Asia, comprises more than 17,000 islands, including the five biggest islands, which are Sumatra, Java, Borneo (Kalimantan),

Table 1. EB associated variants identified with whole-exome sequencing in-silico analysis

Characteristic variant	Case 1	Case 2	Case 3	Case 4
Annotation	Indels	SNP	SNP	SNP
Gene name	*FERMT1*	*LAMB3*	*LAMB3*	*COL7A1*
	NM_017671	NM_001017402	NM_001017402	NM_000094
	exon 4	exon 9	exon 9	exon 54
Amino acid change	p.I130fs	p.H321P	p.H321P	p.G1667V
cDNA	c.388dupA	c.A962C	c.A962C	c.G5000T
Known variant	Novel*	Novel*	Novel*	Novel*
SIFT	-	Deleterious (0.002)	Deleterious (0.002)	Deleterious (0)
Polyphen2	-	Probably damaging (0.999)	Probably damaging (0.999)	Probably damaging (1.0)
Mutation taster	-	Disease-causing	Disease-causing	Disease-causing
LRT	-	Deleterious	Deleterious	Deleterious
FATHMM	-	Tolerated (-0.2)	Tolerated (-0.2)	Tolerated (-4.62)

*Variant not found in dbSNP, 1000 Genomes Project, GWAS, ExAC, nor ESP database.

Sulawesi, and Papua. Indonesia is the world's 4th most-populous country with over 267 million people, and Java is the most populous island. Indonesia consists of hundreds of distinct native ethnic and linguistic groups, with the largest being Javanese (*https://en.wikipedia.org/wiki/Indonesia*). Our study is located in Central Java and Yogyakarta, which is mostly populated by people with Javanese ancestry.

Case 1, a 12-year-old boy with clinical manifestation of skin brittle, poikiloderma, meatal stenosis, aphthous ulcer, and history of hernia inguinal (Figure 1). On genetic analysis, we identified a homozygous mutation in the location of 389 FERMT1, an insertion of A-base, that converts an isoleucine codon (ATT) at codon 130 to asparagine codon (AAT) and leads to a premature termination codon (PTC) at codon 132 (p.I130fs). The parents were heterozygous for the mutation and were considered obligate carriers. The location of the mutation is at the F1 domain, which is considered highly conserved. We confirmed the diagnosis of Kindler syndrome. The mutation of c.388dupA p.I130fs is the first reported in the literature. The duplication of *FERMT1* in exon 4 has been reported previously (*15*), but the correlation between duplication and severity of the clinical findings is still unclear. The mutation in our study is in the F1 segment of *FERM*, which is predicted to cause loss of the FERM domains of the protein, thus resulting in the detached adhesin bond and membrane matrix (*16*). Furthermore, the isoleucine protein in the F1 domain is a highly conserved domain (*17*). This mutation appears to contribute to the pathogenesis of several organs, not only manifesting in skin atrophy and poikiloderma but also including dysfunction of the oral, intestinal, and urinary systems.

Cases 2 and 3 are siblings with various clinical manifestations (Figure 2). The chronic insole ulcers

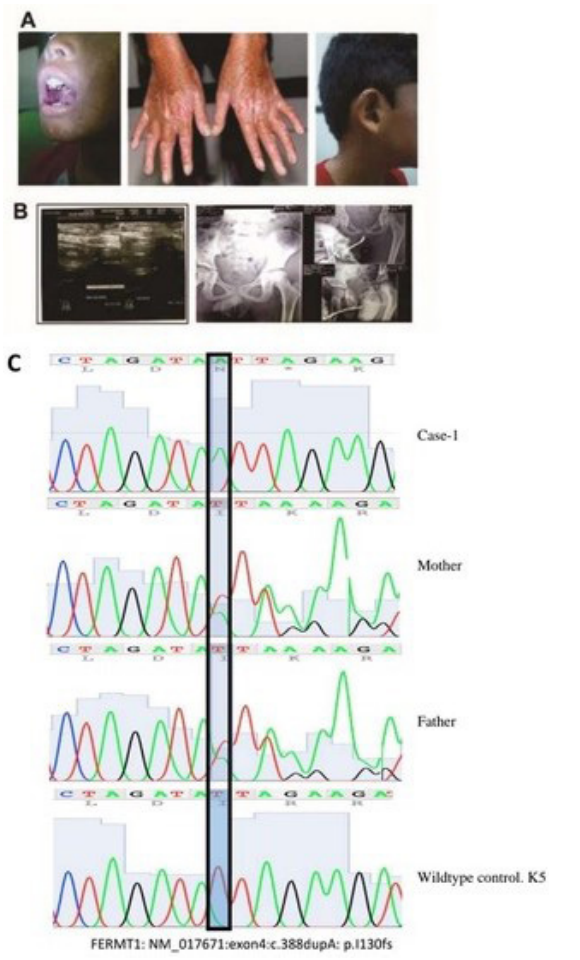

Figure 1. Clinical manifestations of Kindler syndrome and electropherogram of *FERMT1* mutation. (A) Poikilodermas on the face and neck and hands; aphthous ulcer of buccal gingiva; atrophic skin on hands; **(B)** Ultrasonography imaging: bowel defect in the left inguinal region during Valsalva test; contrasted urethrography image: stenosis of the urethra. **(C)** A frameshift mutation of *FERMT1* gene duplicated T base resulting in changes of amino acid and premature stop codon; heterozygous mutation on father and mother; and normal wildtype control.

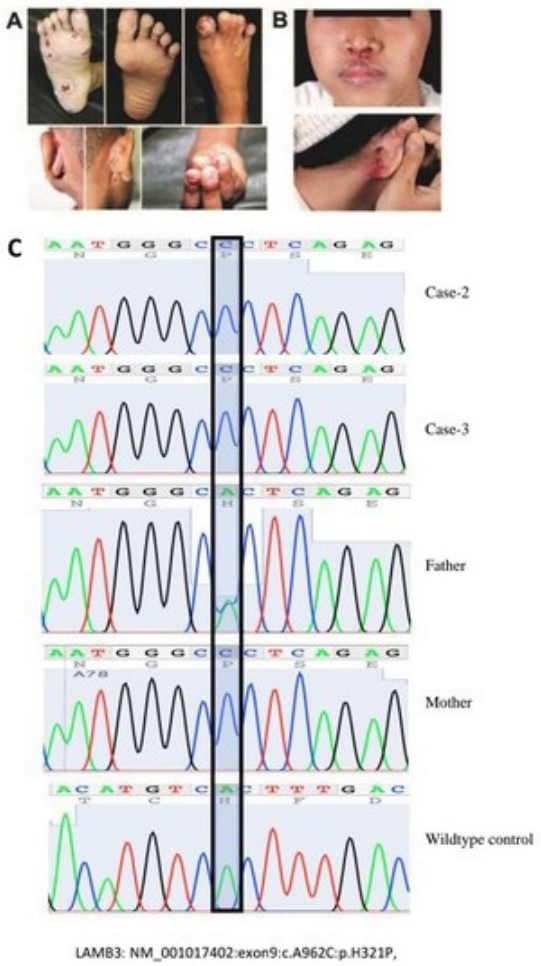

Figure 2. Clinical manifestations of JEB and electropherogram of *LAMB3* mutation. (A) Chronic ulcer on the insole, back of the ear, and hand fingers of Case 2; **(B)** Chronic ulcer on the nose philtrum and back of the ear of Case 3; **(C)** sequencing of *LAMB3* of Case 2 and Case 3. A single-base substitution of c.962A>C in brother (Case 2) and sister (Case 3) was observed, and it was also apparent in mother while father is heterozygous carrier, and the wildtype control shows no mutation.

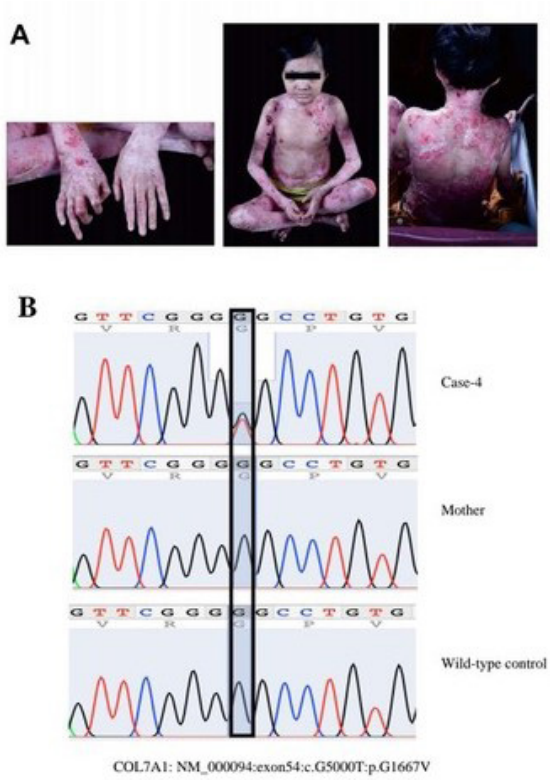

Figure 3. Clinical manifestations of RDEB and electropherogram of *COL7A1* mutation. (A) skin erosion and right point finger contracture in Case 4; and **(B)** heterozygous missense mutation at c.5000G>T, which was not shown in wildtype control nor the mother.

and finger ulcers were seen in the 10-year-old younger brother, but not in his sister, instead, a chronic ulcer at the philtrum was seen. Both of them have ulcers behind their ear, and unsurprisingly they have the same homozygous mutation in the location of 962 LAMB3 A>C. The histidine codon (CAC) converts to a proline residue (CCC). The mother also has the same homozygous variant, but interestingly she did not have any clinical manifestations. The father has a heterozygous variant in the same allele. The diagnosis confirmed junctional EB intermediate. Mutation of the *LAMB3* gene is the hallmark of JEB, the critical encoding component of the hemidesmosome-anchoring filament complex that links the keratin cytoskeleton to the lamina densa of the dermo-epidermal junction (*18*). In the majority of the JEB cases, 80% of mutations predominantly affect the *LAMB3* gene. However, the clinical spectrum is highly variable depending on the mutation's type and position (*19*). We identified siblings with the same mutation of *LAMB3* at the same loci c.A962C (p.H321P), but they demonstrated different phenotypes. Other polymorphisms in each person may correlate with various clinical manifestations. However, this possible explanation needs to be addressed in future studies.

In Case 4, the result of WES and SS had high concordance. The WES presented a mutation in the G>T exon 54 COL7A1, and SS reported a heterozygous mutation at codon 5000 in the affected child (Figure 3). However, we did not find the variant in the mother nor wildtype control. Unfortunately, paternal DNA cannot be defined. Thus, we diagnosed the patient with recessive dystrophic epidermolysis bullosa. *COL7A1* is responsible for dystrophic EB with dominant and recessive genetic patterns, though genetic analyses of the *COL7A1* gene in affected individuals revealed that most mutations detected are recessive DEB (RDEB) (*20*). The majority of severe RDEB cases are caused by PTC mutations resulting from nonsense, frameshift, or splice-site mutations on both *COL7A1* alleles, which result in either nonsense-mediated decay of the mRNA or truncated polypeptides, and the result is unable to assemble the functional protein and the mRNA degraded within the cell. Milder cases of RDEB are often caused by compound heterozygous mutations: one PTC mutation and a missense mutation (*21*). Our patient had a heterozygous missense mutation of *COL7A1* at exon 54 substitution 5000G>T that substitutes glycine for valine. Mallipeddi *et al.* in 2003 pointed out the need to search for further mutations when they found glycine substitution in sporadic cases (*22*). However, our case did not reveal other mutations on *COL7A1*. The heterogeneous mutations in our case caused disease phenotypes and led to moderate-to-severe DEB forms, unlike in other articles, which need to be further examined (*23*).

Murata *et al.* in 2003 demonstrated that the presence of recurrent mutations could be classified into specific ethnic groups and worldwide mutations (*20*). Our four cases revealed novel mutations that were not recorded in any public SNP database (dbSNP). Our novel finding is probably because of the limited number of EB Malay traits in reported articles, resulting in a lack of identified recurrent specific ethnic group mutations in Malay ancestry. Abu Sa'd *et al.* in 2006 also stated that diagnostic strategies based upon molecular epidemiological features that are determined in Western origin populations might not apply to Middle Eastern populations (*24*). However, two studies reported two Sundanese Indonesian families with dystrophic EB. They reported the mutations in *COL7A1* at c.6218G>A; pGly2073Asp, c.5945G.T; pGly1982Val and c.6253G>T; p.Gly2085Trp (*25,26*). We did not find other articles associated with EB genetic examination in Indonesian traits.

As a limitation, the number of subjects was too small to analyze a high diagnostic yield in WES compared to standard genetic testing. The low incidence of cases may hamper subject recruitment.

In conclusion, we demonstrated a novel mutation in Kindler syndrome on c.388A (p.I130fs), junctional epidermolysis bullosa intermediate on c.A962C (p.H321P), and recessive dystrophic epidermolysis bullosa on 5000G>T (p.G1667V) as Javanese traits. In

these cases, practical genetic analysis was best performed using whole-exome sequencing, a less invasive genetic diagnostic tool, with a reduced time to diagnosis.

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**Address correspondence to:*
Hardyanto Soebono, Department of Dermatology and Venereology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Gedung Radiopoetro lt3, Jl. Farmako Sekip Utara, Sleman, Yogyakarta 55281, Indonesia
E-mail: hardyanto@ugm.ac.id

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Brief Report

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A case series of adult patients affected by EAST/SeSAME syndrome suggests more severe disease in subjects bearing *KCNJ10* truncating mutations

Yoko Suzumoto[1,§], Valeria Columbano[2,§], Luciano Gervasi[3,§], Rosa Giunta[3], Teresa Mattina[4], Gabriele Trimarchi[4], Giovanna Capolongo[2], Mariadelina Simeoni[2], Alessandra F. Perna[2], Miriam Zacchia[2], Gianpiero Toriello[5], Rosa M. Pollastro[2], Francesco Rapisarda[3], Giovambattista Capasso[1,2], Francesco Trepiccione[1,2,*]

[1] Biogem Research Institute, Ariano Irpino, Italy;
[2] Department of Translational Medical Sciences, University of Campania "L. Vanvitelli", Naples, Italy;
[3] School of Nephrology, Department of Clinical and Experimental Medicine, University of Catania, Italy;
[4] Department of Biomedical and Biotechnological Sciences, University of Catania, Italy;
[5] Nefrology Unit, "L. Curto Hospital", Polla, Italy.

SUMMARY EAST/SeSAME syndrome is a rare disease affecting the Central Nervous System (CNS), inner ear, and kidney. The syndrome is due to loss-of-function mutations in the *KCNJ10* gene encoding the inward-rectifying potassium channel Kir4.1. EAST/SeSAME syndrome is mainly diagnosed during childhood with a tonic-clonic seizure being the usual first symptom. Due to a limited number of patients and recent identification of the disease, few data are available on the clinical progress of this disease in adulthood. In particular, neurologic and nephrological outcomes have not been reported. We present a case series of 4 adult patients harbouring homozygous missense mutation p.Ala167Val and homozygous frameshift mutations p.Asn232Glnfs*14 and p.Gly275Valfs*7. Effects of these mutations were predicted by *in silico* modelling and bioinformatic tools. Patients with truncating mutations were associated with more severe outcomes, both in tubulopathy severity and neurological symptomatology. Conversely, either missense or truncating mutations were correlated with similar severity of epilepsy, with a long free-of-event period up to 20 years old. No eGFR decline was documented. Modelling predicted that truncating mutations lead to complete Kir4.1 dysfunction. Finally, all patients had a mild increase in urinary protein excretion. Our study indicates that the prognosis of patients suffering from EAST/SeSAME syndrome is related to the severity of the mutation causing the disease. As predicted by *in silico* modelling, truncating mutations of *KCNJ10* are associated with more severe disease, with recurrence of symptomatic hypokalemia and more severe neurological phenotype. The type of mutation should be considered for the therapy tailored to patients' phenotype.

Keywords Kir4.1, potassium channel, tubulopathy

1. Introduction

EAST/SeSAME syndrome is a rare inherited disorder affecting the Central Nervous System (CNS), inner ear and kidney. The first description of the syndrome was in 2009, in two independent studies (*1,2*), where the syndrome was called EAST syndrome (Epilepsy, Ataxia, Sensorineural deafness and Tubulopathy) or SeSAME syndrome (Seizures, Sensorineural deafness, Ataxia, Mental retardation and Electrolyte imbalance). In both reports, loss-of-function mutations in *KCNJ10* were described.

KCNJ10 gene encodes Kir4.1, a potassium channel mainly expressed in oligodendrocytes and basolateral membrane of the distal nephron including the cortical thick ascending limb of Henle's loop (TAL), distal convoluted tubule (DCT) 1/2, connecting tubule (CNT) and cortical collecting duct (cCD). Kir4.1 forms a hetero tetramer with another potassium channel, Kir5.1, to conduct inwardly-rectifying potassium currents (*3*). *KCNJ16* gene encoding Kir5.1 is mainly expressed in the kidney and loss-of-function mutations cause a Gitelman like tubulopathy (*3*).

EAST/SeSAME syndrome is an ultra-rare disease

affecting 1:1,000,000 (*4*). Tonic-clonic seizures responsive to common anticonvulsants is a usual presenting symptom and the syndrome is difficult to distinguish from primary epilepsy. Later on, ataxia and deafness manifest in the majority of patients. Other cerebellar symptoms and mental retardation may variably be present. Children can have neurodevelopmental delay, especially in the acquisition of motor and language skills (*4*). Whether this is related to the frequency and severity of seizures, or due to the disease itself is unclear. Impaired function of astrocytes and oligodendrocytes is likely responsible for the development of neurological symptoms (*5*). Since the Kir4.1 channel plays a crucial role in the production of endolymph and endochoclear potential (*4*), Sensorineural Hearing Loss (SHL) can be variably present and often requires the implantation of an acoustic device. Renal involvement is characterized by salt-losing nephropathy resembling Gitelman syndrome, with hypokalemic metabolic alkalosis, normo-hypomagnesemia and hypocalciuria.

EAST/SeSAME syndrome is usually diagnosed during infancy and consequently the data on this disease are mainly from childhood. Indeed, among the 54 cases reported on PubMed database identified as "EAST syndrome" or "EAST/SeSAME syndrome", none was centered on adult patients prognosis and clinical progress (*2,6-8*). Thus, several questions on the prognosis and evolution of renal and neurologic phenotype are still open. Here we present a case series of 4 adult patients with EAST/SeSAME syndrome and the correlation between their symptomatology and genetic background.

2. Patients and Methods

2.1. Patient data

We report retrospective data from a cohort of patients affected by genetically confirmed diagnosis of EAST/SeSAME syndrome. Studies on patients were conducted according to the ethical standards of the Institutional Committee of the University of Campania and University of Catania, under the 1964 Helsinki Declaration and later amendments or comparable ethical standards. Informed consent was obtained from all participants and/or their legal guardians for the anonymous publication of the data.

2.2. *In silico* analysis

Homology model of human Kir4.1 (sequence 25 - 349) was generated by the Swiss-Model protein structure homology-modelling server (*https://swissmodel.expasy.org*), using the crystal structure of Kir3.1-prokaryotic Kir channel chimera (*9*) (PDB code : 2QKS) as a template. Sequence alignment between Kir4.1 and the template using PROMALS3D server (*http://prodata.swmed.edu/promals3d/promals3d.php*) showed 35.6% sequence identity. The model was refined by the 3Drefine server (*http://sysbio.rnet.missouri.edu/3Drefine*). Modelling of the Kir4.1-Ala167Val variant was performed similarly using refined model as a template. Molecular visualization was performed with PyMol software. Effects of the Ala167Val mutation were predicted by online bioinformatic tools PolyPhen-2, Mutation taster, PROVEAN and SIFT using default settings.

3. Results and Discussion

3.1. Patient presentation

We report four clinical cases of adult patients affected by EAST/SeSAME. Patient 1 and 2 are siblings and their parents share a common ancestor (Figure 1). Patient 1 is a male younger sibling of patient 2. He was born at full-term. Like his sister, epilepsy was the first sign of the disease, which occurred when he was 9 months old (m/o). His head magnetic resonance imaging (MRI) was normal and he was misdiagnosed as idiopathic epilepsy responsive to valproate. Tonic-clonic seizures occurred again when he was 5 and 10 years old (y/o), although he was under anti-epileptic drug treatment. When he started primary school, slight cognitive disabilities with oral and written comprehension were diagnosed. Since no clear symptoms of psychiatric disorder or tubulopathy developed during adolescence, he never received potassium and/or magnesium salt replacement therapy until the age of 16, when he was screened for tubulopathy as part of a clinical work-up of his sister. At 14 y/o he was diagnosed with SHL, requiring an acoustic device. His last blood and urine electrolyte profile is shown in Table 1.

Patient 2 is a female, born by full-term delivery. Her psychomotor development was considered normal, being able to walk at around 15 m/o. At 9 m/o, she experienced her first seizure. Epilepsy was effectively controlled by valproate. This treatment was withdrawn at age 4 due to a paucity of events. Brain MRI ruled out morphological alterations causing epilepsy. She was free from seizures

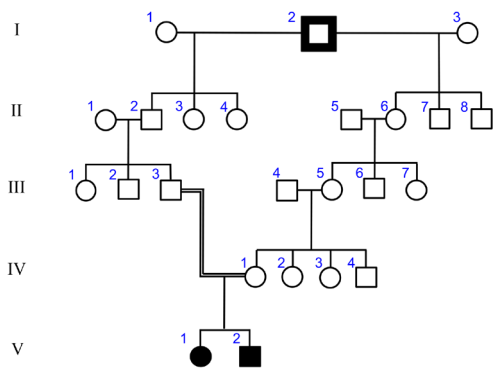

Figure 1. Pedigree of patient 1 and 2. Two siblings affected by Ala167Val mutation are indicated by filled symbols.

Table 1. Blood and 24h Urine parameters

Patients		1	2	3	4
Age		16	20	32	33
Blood					
Creatinine	μM	44	44	57.2	70.4
eGFR	mL/min/1.73m^2	161	140	129	118
Na^+	mM	141	137	132	140
K^+	mM	3.1	2.7	3.1	3.5
Cl^-	mM	99	101	94	99
Ca^{2+}	mg/dL	8.8	10.6	9.9	9.4
Mg^{2+}	mM	0.66	0.66	0.66	0.58
pH		7.41	7.41	7.42	7.38
HCO_3^-	mM	27.5	26.2	31.9	32.5
pCO_2	mmHg	46.5	45.9	50.3	53.9
Urine					
Creatinine	mM	4.5	3.53	3.71	8.3
Na^+	mM	120	82	78	94
K^+	mM	63	60	74	100
Cl^-	mM	168	120	121	129
Ca^{2+}	mg/dL	2	5.8	3.3	11
Phosphate	mg/dL	3.9	2.7	3.9	2.6
Mg^{2+}	mM	3.55	3.05	4.2	11
FE Na^+	%	0.83	0.75	0.91	0.57
FE K^+	%	19.92	27.78	30.95	24.32
FE Cl^-	%	1.66	1.48	2.00	1.11
FE Mg^{2+}	%	5.27	5.78	9.85	16.14
FE Ca^{2+}	%	0.22	0.68	0.52	1.04
Ca^{2+}/Creatinine	mg/dL/mg/dL	0.04	0.15	0.08	0.12
PCR	mg/mmol	17.87	24.48	24.26	20.48

and off treatment until age 9 when she experienced a new tonic-clonic seizure. At 10 y/o, laboratory examinations revealed hypomagnesaemic and hypokalaemic metabolic alkalosis associated with salt-losing tubulopathy (low blood pressure and secondary aldosteronism). These electrolyte abnormalities suggested a syndromic disease and genetic testing was thus performed when she was 11 y/o. At this time, as part of a clinical work up for EAST/SeSAME syndrome, she was found to be affected by SHL and moderate intellectual disability. However, she also had psychiatric symptomatology that is not usually part of the disease. Indeed, from 11 y/o and throughout adolescence she progressively manifested psychomotor agitation, panic attacks and outbursts of anger. This clinical scenario did not prevent her from attending school. Currently at 20 y/o, she has never experienced symptomatic hypokalaemia requiring hospital admission and her epilepsy is under control with no events for about 10 years. No clear signs of ataxia have been diagnosed so far.

Patient 3 is a male, born at term from consanguineous parents (first cousins). At 3 m/o he was admitted for tonic-clonic seizures and treated with carbamazepine and phenobarbital. At this age he was also affected by normokalaemic hypomagnesaemia and mild hypocalcaemia. At 6 m/o, when he was hospitalized for another seizure and obstinate constipation, hypokalemia was detected. He was started on a potassium and magnesium salt supplement alongside carbamazepine and phenobarbital. At 20 m/o, during an episode of dehydration and hypokalemia, spironolactone was introduced. However, this therapy did not prevent a hospital admission at 2 y/o for symptomatic hypokalaemia, after an episode of fever. Developmental delay was diagnosed at 1 y/o when he was unable to maintain upright position and progressively worsened until 6 y/o when ataxia was diagnosed. Diffuse muscle hypotrophy became evident with adolescence. At 3 y/o he was diagnosed with SHL. Genetic testing was negative for most common hypokalemic tubulopathies during infancy. EAST/SeSAME syndrome was confirmed only at 28 y/o when genetic analysis was extended to the *KCNJ10* gene. During his childhood and adolescence he presented with salt craving symptoms and received *ad libitum* salt intake. Epilepsy was increasingly better controlled with no major events registered. The last severe episode of seizure was at 8 y/o with development of coma after discontinuation of carbamazepine. Currently, he is 32 y/o and his last hospitalization for hypokalaemia-related symptoms occurred at 27 y/o. Therapy with potassium and magnesium supplements coupled with spironolactone was reinforced accordingly. His last clinical blood and urine electrolyte profile is shown in Table 1.

Patient 4 is a male, born prematurely from consanguineous parents (first grade cousins). Hypomagnesemia (Mg^{2+} 1.4 mg/dL) and hypokalaemia (K^+ 2.8 mM) were diagnosed at birth and replacement therapy was started. Pregnancy was not affected by polyhydramnios. A detailed report of his neurologic findings was previously described (*8*). Briefly, he presented with tonic-clonic seizures at 3 m/o that recurred almost weekly, despite therapy with valproate and phenobarbital. An audiogram revealed normal hearing during childhood. His psychomotor development was delayed. At 4 y/o he was admitted to an intensive care unit for 14 days because of severe status epilepticus unresponsive to normal therapy. It is difficult to assess the impact of this event on the progression of his neurologic condition. However, he experienced progressive loss of motor ability and cerebellar symptoms together with myoclonus and dystonia of cervical muscles and the trunk up to 23 y/o, when he became wheel-chair bound. Currently he is 33 y/o and his epilepsy is well-controlled with valproate and phenobarbital with no major events reported in the last 10 years. His potassium level was well-controlled during childhood, but he experienced symptomatic hypokalemia requiring hospital admission 3 times in the last 9 years. As evident from the last clinical check (Table 1), no alteration in the estimated glomerular filtration rate (eGFR) was reported thus far.

3.2. Gene mutation modelling

Patients 1 and 2 carried a homozygous *KCNJ10* c.500C>T variant, resulting in p.Ala167Val amino acid change. Patients 3 and 4 have rare homozygous variants c.693dup and c.822delG, producing truncated

Table 2. Clinical hallmarks over time

Patients	1	2	3	4
Age (years)	16	20	32	33
Mutation	Missense	Missense	Frame-shift	Frame-shift
Protein variant	p.Ala167Val	p.Ala167Val	p.Asn232Glnfs*14	p.Gly275Valfs*7
Genetic variant	NM_002241.5: c.500C>T	NM_002241.5: c.500C>T	NM_002241.5: c.693dup.	NM_002241.5: c.822delG
Human (GRCh37/h19)	Chr1:160.011.823	Chr1:160.011.823	Chr1:160.011.630	Chr1:160.011.500
Disease Onset (months)	9	9	3	at birth
Symptomatic Hypokalemia	NO	NO	YES	YES
Ataxia	NO	NO	YES	YES
Seizures (frequency)	Low	Low	High	High
Speaking ability	YES	YES	NO	NO
Motor ability	YES	YES	Limited	NO
SHL	YES	YES	YES	NO
Psycosis	NO	YES	NO	NO
KCl dose (mmol/kg/day)	0.17	1.1	2.72	1.44
MgCl dose (mmol/kg/day)	0.17	1.1	--	1.43

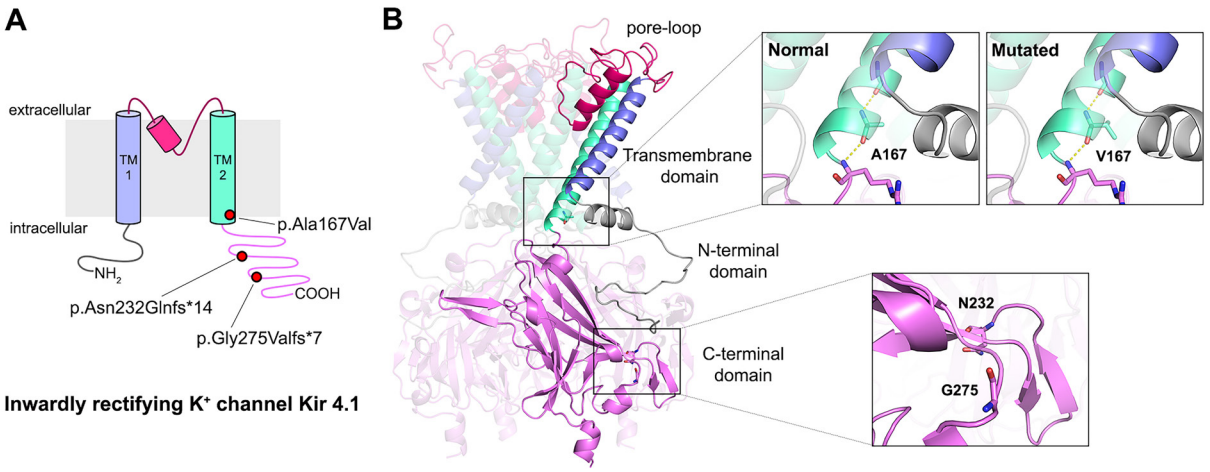

Figure 2. Mutations of the inwardly rectifying potassium channel Kir4.1 reported in this study. (A) Topology diagram of Kir 4.1 including the location of the sites of the three mutations. **(B)** Three-dimensional homology model of human Kir 4.1 homotetramer generated through Swiss-Model protein structure homology-modelling server (*http://swissmodel. expasy.org*). Molecular visualization was performed by PyMol software.

Kir4.1 p.Asn232Glnfs*14 and p.Gly275Valfs*7, respectively (Table 2). Homology modelling of human Kir4.1 revealed that Ala167Val at the junction between transmembrane domain 2 (TM2) and the C-terminus is predicted not to interrupt the sequence of the hydrogen bonds, thus not altering TM2 alpha-helix structure (Figure 2). Furthermore, effects of Ala167Val mutation were evaluated as "probably damaging" by PolyPhen-2 (score : 0.999) and "disease causing" by the Mutation taster tool with 0.999 probability, while PROVEAN and SIFT predicted Ala167Val as neutral (score : -2.45, cut-off = -2.5) and tolerated (score : 0.240, cut-off = 0.05), respectively. The frameshift mutations carried by patients 3 and 4, instead, confer a considerable loss of the C-terminal region of Kir4.1 resulting in a truncated protein which is highly likely nonfunctional.

3.3. Symptoms severity

A complete overview of the last serum and urine electrolytes profile (Table 1) revealed hypokalemic metabolic alkalosis associated to hypomagnesemia and hypocalciuria are still present in adulthood. Only patient 3 currently takes potassium-sparing diuretics on top of electrolyte replacement therapy, due to frequent episodes of symptomatic hypokalemia. None of the patients have eGFR below 100 mL/min/1.73 m^2, suggesting that even at the latest observed time point (33 y/o) no advanced signs of renal insufficiency can be detected. However, patients 3 and 4 present with severe muscle wasting and the use of Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula could be overestimating GFR. Singularly, all patients presented with a mild increase in urinary protein to creatinine ratio (PCR) independent of age, since similar values were observed during childhood. Whether this is an unfavourable prognostic factor for renal function, as well as a direct measure of GFR remains to be determined.

All patients reported here had seizures as a symptom of disease onset. Although Patient 4 had hypokalemia

and hypomagnesemia at birth, no further diagnostic or therapeutic evaluation was completed before 3 m/o when the first seizure was diagnosed and a therapeutic program started. However, patients 1 and 2 had their first seizure later than patients 3 and 4 (Table 2, 9 *vs.* 3 m/o). Furthermore, during childhood and adolescence, the neurologic involvement was also milder for patient 1 and 2 based on both frequency of epileptic events and appearance of other symptoms including ataxia, mental retardation and maintenance of language and motor abilities (Table 2). Indeed, patients 3 and 4 progressively lost their speaking and motor abilities. Preserved speaking and ambulatory ability of patients 1 and 2, at similar ages, suggests a potential influence of the type of mutation on the severity of phenotype. For all the patients reported here, it is of note that tonic-clonic seizures were very well-controlled by anti-epileptics during adulthood, with patients free of events for up to 10 years (Table 2). Finally, the severity of electrolyte imbalance secondary to salt-losing tubulopathy mainly manifest with the symptoms of hypokalemia. The patients carrying truncating mutations experienced more severe disease, as evaluated by the number of hypokalemic episodes requiring hospitalization and the dose of the supplement received (Table 2).

Neurologic phenotype has a major impact on the quality of life in patients affected by EAST/SeSAME syndrome. However, electrolyte disorders are potentially life-threatening throughout the patients' lives. Recently several studies have reported on the physiological role of Kir4.1 in the distal nephron that helps to explain mechanisms underlying the tubulopathy and suggest alternative treatments.

The distal nephron is heterogeneous in cellular composition (*10*), allowing fine control of sodium reabsorption and potassium secretion (*11,12*) by different cell types sharing common precursors (*13*) and a high degree of plasticity (*14,15*). Kir4.1 is expressed all along the distal nephron (from TAL to CD) (*16,17*) and is thus potentially crucial for a large number of cells. However, the renal clinical phenotype of EAST/SeSAME patients is consistent with defective DCT and activated CD function resembling Gitelman syndrome. Indeed, heteromer Kir4.1/Kir5.1 is essential for the activity of DCT and principal cells since it is the predominant potassium channel in the basolateral membrane and the main determinant of membrane potential. In DCT, Kir4.1 works as a sensor of circulating potassium levels. In response to hyperkalemia, inhibition of Kir4.1 leads to membrane depolarization and increased intracellular chloride (*18*). This in turn mediates a With No lysine (K) serine-threonine kinase (WNK)-dependent inhibition of sodium chloride co-transporter (NCC) on the apical side of the DCT (*19*), allowing increased delivery of sodium and chloride to the CNT and CD, and promotes potassium secretion into the urine secondary to epithelial sodium channel (ENaC) dependent sodium reabsorption. This ultimately restores the serum potassium concentration back to a normal level. Genetically encoded Kir4.1 dysfunction, as in EAST/SeSAME syndrome, results in a condition in which DCT wrongly senses hyperkalemia causing a sodium and potassium losing phenotype, despite hypokalemia and metabolic alkalosis. Defective activity of Kir4.1 in principal cells increases activity of the ENaC and renal outer medullary potassium channel (ROMK) further stimulating urinary potassium loss (*20*).

Homozygous Ala167Val and compound heterozygous with Arg297Cys were previously described as mild to moderate EAST/SeSAME syndrome (*2,21*). Patients 1 and 2 presented here belong to the same kindred showing a milder phenotype compared to patients 3 and 4 harboring truncating mutations. Ala167Val variation was evaluated as likely pathogenic by only two out of four bioinformatic prediction tools. This is mainly due to the type of substitution, which does not alter the TM2 alpha-helix structure (Figure 2), since alanine and valine share similar physical and chemical properties. Although Ala167 in Kir4.1 is highly conserved across various species (*2*), some members of Kir channels possess other hydrophobic, non-polar amino acids including valine in Kir3.4 at this position (*22*). Ala167Val retained approximately 60% of channel activity compared to wild-type (*23*), thus reduced channel function is not the sole cause of pathologies associated with Ala167Val. Indeed, mis-trafficking of Kir4.1-Ala167Val/Kir5.1 prevents the interaction between Kir4.1 and anchor protein MAGI-1 on the basolateral membrane, resulting in reduced basolateral potassium channels in the DCT leading to salt-wasting (*24*). Furthermore, Ala167 is located at the vicinity of the Kir4.1 channel gate, according to the crystal structure of Kir3.1 (*9*). Thus, reduced permeability to K^+ flux cannot be excluded in Ala167Val mutants.

Other missense mutations including Ala201Thr and/or Ile209Thr and Ile60Thr, despite an earlier time of presentation than patient 1 and 2 (before 7m/o), were associated with milder neurologic phenotypes compared to our truncating mutations (*25,26*). Finally, no signs of tubulopathy and hearing loss were diagnosed up to 3 y/o in patients with the Ala201Thr and/or Ile209Thr mutations and up to 20 y/o in patients carrying the Ile60Thr mutation. This is in line with patients bearing Thr57Ile showing signs of tubulopathy between 5 and 8 y/o (*27*). In our cohort of patients, tubulopathy was identified together with the first seizure (3 m/o) or even earlier for truncating mutations, but much later in two siblings bearing Ala167Val. Since patient 1 had hypokalemic and hypomagnesemic metabolic alkalosis diagnosed accidentally at 16 y/o, one can infer that not only the onset is delayed but also the severity of tubular defect is less.

Two frameshift mutations Asn232Glnfs*14 and Gly275Valfs*7 lack large parts of cytoplasmic C-terminus

thus the channel is likely nonfunctional. Deletion of 47 amino acids from the C-terminus was sufficient to abolish channel activity of Kir4.1 (*28*), and Val259Ter and Arg199Ter with deletions of similar or larger parts of C-terminus compared to our truncating mutations result in nonfunctional channel as well (*29*). This could explain the severe neurological and renal phenotype found in our patients and more generally, an increase in patient phenotype severity when present as compound heterozygosity. Indeed, patients with compound heterozygosity for Arg199Ter and Arg65Pro, namely a nonsense mutation associated with a missense mutation that was biochemically characterized by residual channel function (*30*), present with a phenotype more severe than patients carrying homozygous Arg65Pro, at least as observed for serum potassium level, 2.9 *vs.* > 3 mM (*1,2*). Further follow-up studies on adult patients will be required for comprehensive prognostic evaluation of the disease.

Our study has certain limitations. First, this is a retrospective analysis on a small cohort of four Italian patients. Second, as often occurs with ultra-rare diseases, there are few reports of adult patients for a comprehensive comparison. A registry based multi-center prospective study will be necessary for compelling clinical evolution and prognostic evaluation of renal and neurologic phenotype.

4. Conclusion

We report here one of the largest cohorts of adult patients affected by EAST/SeSAME syndrome. The evolution of clinical picture and prognosis fit with the severity of mutations causing the disease. As predicted by *in silico* modelling, truncating mutations of *KCNJ10* are associated with more severe adult prognosis in terms of recurrence of symptomatic hypokalemia and neurological phenotype, suggesting that the type of mutations should be taken into consideration in tailoring the electrolytes replacement and anticonvulsants therapy.

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[§]These authors contributed equally to this work.
**Address correspondence to:*
Francesco Trepiccione, Department of Translational Medical Sciences University of Campania "L.Vanvitelli", Via Pansini n5, 80131 Naples, Italy.
E-mail: Francesco.trepiccione@unicampania.it

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Brief Report

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A novel *BICD2* mutation of a patient with Spinal Muscular Atrophy Lower Extremity Predominant 2

Munkhtuya Tumurkhuu[1,2,*], Uranchimeg Batbuyan[2], Satoru Yuzawa[3], Yanjinlkham Munkhsaikhan[2], Ganbayar Batmunkh[4], Wataru Nishimura[1]

[1] Department of Molecular Biology, International University of Health and Welfare, School of Medicine, Narita, Chiba, Japan;
[2] Department of Genetics and Molecular Biology, School of Bio-Medicine, Mongolian National University of Medical Sciences, Mongolia;
[3] Department of Biochemistry, International University of Health and Welfare, School of Medicine, Narita, Chiba, Japan;
[4] Laboratory of Medical Genetics, National Center of Maternal and Child Health, Mongolia.

SUMMARY The bicaudal D homolog 2 (*BICD2*) gene encodes a protein required for the stable complex of dynein and dynactin, which functions as a motor protein working along the microtubule cytoskeleton. Both inherited and *de novo* variants of *BICD2* are reported with autosomal dominant spinal muscular atrophy with lower extremity predominance (SMALED2). Here, we report a male patient with a novel mutation in the *BICD2* gene caused by a heterozygous substitution of arginine with cysteine at residue 162 (Arg162Cys); inherited from his asymptomatic mother. The patient showed typical clinical symptoms of SMALED2, which was genetically confirmed by sequencing. The Arg162Cys mutant clusters with four previously reported variants (c.361C>G, p.Leu121Val; c.581A>G, p.Gln194Arg; c.320C>T, p.Ser107Leu; c.565A>T, p.Ile189Phe) in a region that binds to the dynein-dynactin complex (DDC). The BICD2 domain structures were predicted and the Arg162Cys mutation was localized in the N-terminus coiled-coil segment 1 (CC1) domain. Protein modeling of BICD2's CC1 domain predicted that the Arg162Cys missense variant disrupted interactions with dynein cytoplasmic 1 heavy chain 1 within the DDC. The mutant did this by either changing the electrostatic surface potential or making a broader hydrophobic unit with the neighboring residues. This hereditary case supports the complex and broad genotype-phenotype correlation of *BICD2* mutations, which could be explained by incomplete penetrance or variable expressivity in the next generation.

Keywords novel mutation, Arg162Cys, dynein-dynactin complex, coiled coil structure

1. Introduction

Spinal muscular atrophy (SMA) is a diverse group of diseases characterized by muscle weakness and atrophy. This is caused by the degeneration of anterior horn cells in the spinal cord, which is also a leading genetic cause of infant death (*1*). The most common form of SMA is caused by the survival of motor neuron 1 (*SMN1*) genes homozygous mutations or deletions in 5q13.1. These are inherited in an autosomal recessive manner and termed SMA5q (*2*).

The SMA5q disease has been classified into four types by order of increasing age and decreasing clinical severity: childhood-onset of SMA type I (MIM #253300), type II (MIM #253550), type III (MIM #253400), and adult-onset of SMA type IV (MIM #271150). Type I SMA is the most severe form with patients having generalized muscle weakness or hypotonia, and a disease onset within the first 6 months of life. Disease severity of SMA5q is found to correlate with oligomerization of SMN and the number of SMN2 gene copies methylated at positions −290 and −296 (*3,4*). In contrast, non-5q SMAs are genetically heterogeneous and phenotypically diverse. They are usually classified by their mode of inheritance (autosomal dominant, recessive, or X-linked) and through their distribution of muscle weakness (proximal, distal, or bulbar) (*5*).

Spinal muscular atrophy with lower limb predominance (SMALED) is an early onset static or slowly progressive disorder. It is best characterized by proximal muscle weakness and atrophy that predominantly affects the lower extremities, with no detectable upper extremity weakness or atrophy (*6,7*). Autosomal dominant SMAs account for < 2% of cases, including SMALED type I and II caused by heterozygous mutations in dynein cytoplasmic 1 heavy chain 1 (*DYNC1H1*) and *BICD2*, respectively (*7,8*). Both genes encode proteins that are part of the dynein-

dynactin complex (DDS). Muscle weakness and atrophy predominantly affect the proximal lower limbs, although involvement of the upper limb and distal lower limb may also occur. In addition, some patients may also demonstrate mild upper motor signs and foot deformities such as high-arched foot, rotated toes, or areflexia of the lower limbs (*9-11*). To date, 30 families with SMALED2 have been reported, carrying 17 missense mutations, including four different mutations in the CC1-dynein binding region (*8-10,12-14*).

Here, we describe a boy who has been clinically diagnosed with a proximal SMA at the age of five, *via* genetic analysis. Molecular analysis revealed a novel missense variant (c.484C>T, Arg162Cys) in exon 3 of *BICD2* in both the boy's and mother's samples.

2. Materials and Methods

2.1. Clinical characteristics of the patient

The proband was a five-year-old boy with movement difficulties, whose family had approached the Department of Genetics and Molecular Biology, in the Mongolian National University of Medical Sciences (MNUMS), to seek medical consultation five years ago. He was born to healthy non-consanguineous parents, and his biological brothers were not clinically affected. The proband's age at symptom onset, walking pattern, and family history were investigated. He has had frequent falls and difficulty in running, jumping, and climbing stairs since he was three years of age. While walking, he showed a waddling gait and Gowers' sign (Figure 1A, 1C).

Intellectual development and physical growth were within normal range. Neurological examination revealed dystrophy of the lower distal limb muscles, reduced deep tendon reflex at the knee, and absent deep tendon reflex at the ankle. The big toe rotated and pointed towards the other toes, and lumbar hyperlordosis was observed (Figure 1A, 1B). Due to the clinical symptoms; electroencephalography, spinal magnetic resonance imaging (MRI) and electromyography were performed. His myelography and spinal MRI results showed normal findings. Motor nerve conduction studies, including speed and amplitude of motor nerve conduction velocity (NCV) and compound muscle action potential, showed results within the normal range. Electromyography showed chronic neurogenic changes in the tibialis anterior and abductor hallucis muscles.

Based on these findings, he was diagnosed clinically with a sporadic SMA and was followed up once a year for the last five years. A recent examination was done in October, 2020 at the age of 10, which revealed a worsening phenotype; the patient was unable to walk without support nor stand-alone for more than ten seconds (Figure 1C). Now, he uses walking aids in two hands.

2.2. Genome isolation and sequencing

After obtaining informed consent, peripheral blood samples were collected from the proband's family, including the parents and two brothers. Both parents of the affected child and unaffected siblings were voluntarily accepted to participate in this study and publish it. All procedures were reviewed and approved by the Ethical Review Committee of MNUMS. All the clinical investigations are in accordance with the Declaration of Helsinki.

Genomic DNA was isolated from 5 mL of the participants' peripheral blood, using a QIAamp DNA mini kit (QIAGEN, Hilden, Germany). Control DNA was obtained from 50 healthy Mongolian individuals. Primer pairs were designed to amplify exons, exon and intron boundaries, and short intron flanking stretches based on primer 3.0 application. The products were sequenced on an ABI Prism 310 automated DNA sequencer (Applied Biosystems, Foster City, CA).

2.3. In-silico analysis of protein structure

The clinical significance of the detected variation was screened using the single nucleotide polymorphism database and the Human Gene Mutation Database. To predict possible effects of the protein mutations, the PolyPhen-2, Sorting Intolerant from Tolerant (SIFT), and Mutation taster software were used in this study. Clinical symptoms of this variant were submitted to ClinVar in March 2020 (accession number VCV000916026.1). The

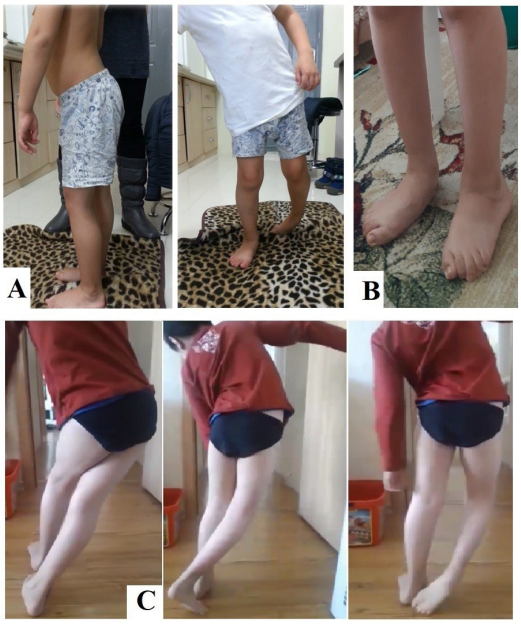

Figure 1. Clinical features of the proband. (A) The proband at the age of 5 years. Marked atrophy of the lower limb muscles, lumbar lordosis and a waddling gait. **(B)** The proband at age of 10 years. Big toe rotated and pointed towards other toes. **(C)** Step-wise movement of the proband at the age of 10. Waddling gait and Gowers' sign were deepened.

prediction of coiled coil domains in BICD2 proteins was performed using Coiled Coils ExPASy. Protein modeling of BICD2 was performed based on 1c1g (protein data bank -PDB) and interactions with the dynein-dynactin complex were revealed based on the cryo-electron microscopic structure of dyneins bound to dynactin and BICD1R (PDB code 6F1T) or BICD2 (PDB code 6F3A) using the SWISS-MODEL and *PyMOL* web servers (*15,16*). Solvent accessible surface area (SASA) of each residue was calculated using the GetArea program (Sealy Center for Structural Biology, USA) and the location within the heptad structure was modeled by MARCOIL (MPI BioInformatics toolkit).

3. Results and Discussion

We first analyzed all coding regions of the *SMN1* gene in the proband's sample and did not detect any mutations. Next, sequencing was performed by screening for a *BICD2* mutation in exons 2, 3, and 6; where mutations have previously been reported (*5,8-10,17*). A novel heterozygous missense variant, c.484C > T (Arg162Cys) in the *BICD2* gene was found in exon 3. This variant was predicted to be pathogenic by susceptibility prediction sites such as SIFT (0-deleterious), PolyPhen-2 (0.9175-possibly damaging), Mutation Taster (disease-causing mutation with probability- 0.9999), and Grantham Matrix (chemical dissimilarity as radical-180 scores). The Arg162Cys variant was inherited from his mother, with his siblings and father do not have the heterozygous allele. This variant is a novel mutation of *BICD2* and it was not found in 50 Mongolian control subjects.

The Bicaudal D gene is evolutionarily conserved from flies to humans and is localized in chromosomal region 9q22.3, and encodes BICD proteins (*8*). To date, 17 pathogenic missense *BICD2* variants have been described in the medical literature (*10,11,18*) and 95 heterozygous *BICD2* variants with an SMA phenotype have been reported in ClinVar mutants (Figure 2A). These SMA-related cases are equally distributed among domains of *BICD2*, and clinical characteristics of the disease could vary widely, ranging from asymptomatic to lethal congenital manifestations within the same family (*10,18,19*). Our proband showed a typical SMALED2 phenotype; slow progressive weakness affecting only the lower extremities were observed in his early childhood. However, the mother did not show any symptoms.

In mammals, there are two *BICD* homologs (*BICD1* and *BICD2*), whereas there is only one in invertebrates (*10,20*). BICD proteins are comprised of three putative coiled-coil domains, with multiple heptad repeats (*21*). BICD2 coiled-coil domains were predicted using Coiled Coil ExPASy software, showing five potential coiled-coil domains. These were CC1a residues 97-131, CC1b residues 161-310, CC2a residues 381-450, CC2b residues 482-569, and CC3 residues 712-845. Moreover, the coiled-coil probabilities of the domains were 0.99 (CC1a), 0.99 (CC1b), 0.97 (CC2a), 0.82 (CC2b), and 0.87 (CC3) (Figure 2A). The Arg162 residue was localized in the CC1b domain, which in turn was included in the N-terminal domain (residues 25-400) of BICD2. As reported previously, the CC1 domain involves direct binding to both dynein heavy chain-1 and dynactin subunit 2 and promotes the stable interaction between dynein and dynactin. This stability in the DDC complex forms a highly processed microtubule motor (*21,22*). The c.484 C>T (Arg162Cys) in the CC1b domain in BICD2 has previously not been described as a spinal muscular atrophy case, moreover, it is not clear how this mutation results in the interaction of BICD2 within the DDC. To further explore this issue, we constructed a model of the CC1 domain of BICD2 protein using the SWISS model, which we based on the PDB structure 1c1g (Figure 3C, 3D). This protein structure comprised of the CC1 region of the BICD2 protein, consisting of amino acids 15-263. Previously, the physical properties of the coiled-coil domains of heptad breaks were defined by their interactions with the environment and divided into seven positions within three groups: the core unit (a and d), the outfield unit (b, c, and f), and the medium unit (e and g) (*23*). To determine the coiled coil repeats, we assigned the heptad register along the CC1 region, where Arg162 occupied a surface-exposed position on the coiled-coil dimer. On the register this was in position "g" with a probability of 96.5% that it was in the medium unit (Figure 3C, Table 1). Interactions between a and d, and g and e account for the most structural specificity; for instance, switching the position of specific sidechains in the core unit can cause oligomers to switch into trimeric or tetrameric structures (*24*). Similar to the core unit, the residues in the medium unit (e and g) can also alter the oligomeric state of the protein. Specifically, if they include hydrophilic residues in one of the positions, they build dimers rather than trimers or tetramers (*25*). Therefore, when Arg162, a hydrophilic positively charged residue, is mutated into Cys162, the BICD2 configuration might be converted from dimeric to a different geometric structure. Additionally, a side chain of Arg162 was closely detected with neighboring chains Gln159 and Asp166 in position "d" in the protein model. This interaction could be disrupted when it is mutated into Cys162, resulting in the abolition of these bonds. Furthermore, it could cause changes in the electrostatic surface potential, making a broader hydrophobic unit with two seams of core residues; "g" and "d", "Cys162" and "Gln159", respectively (Figure 3C, 3D). These predicted coiled-coil structure disruptions could lead to dysfunctional interactions between the N-terminal segment of the BICD2 protein and dynein-dynactin. In line with our prediction, a comprehensive set of polar mutations are located in

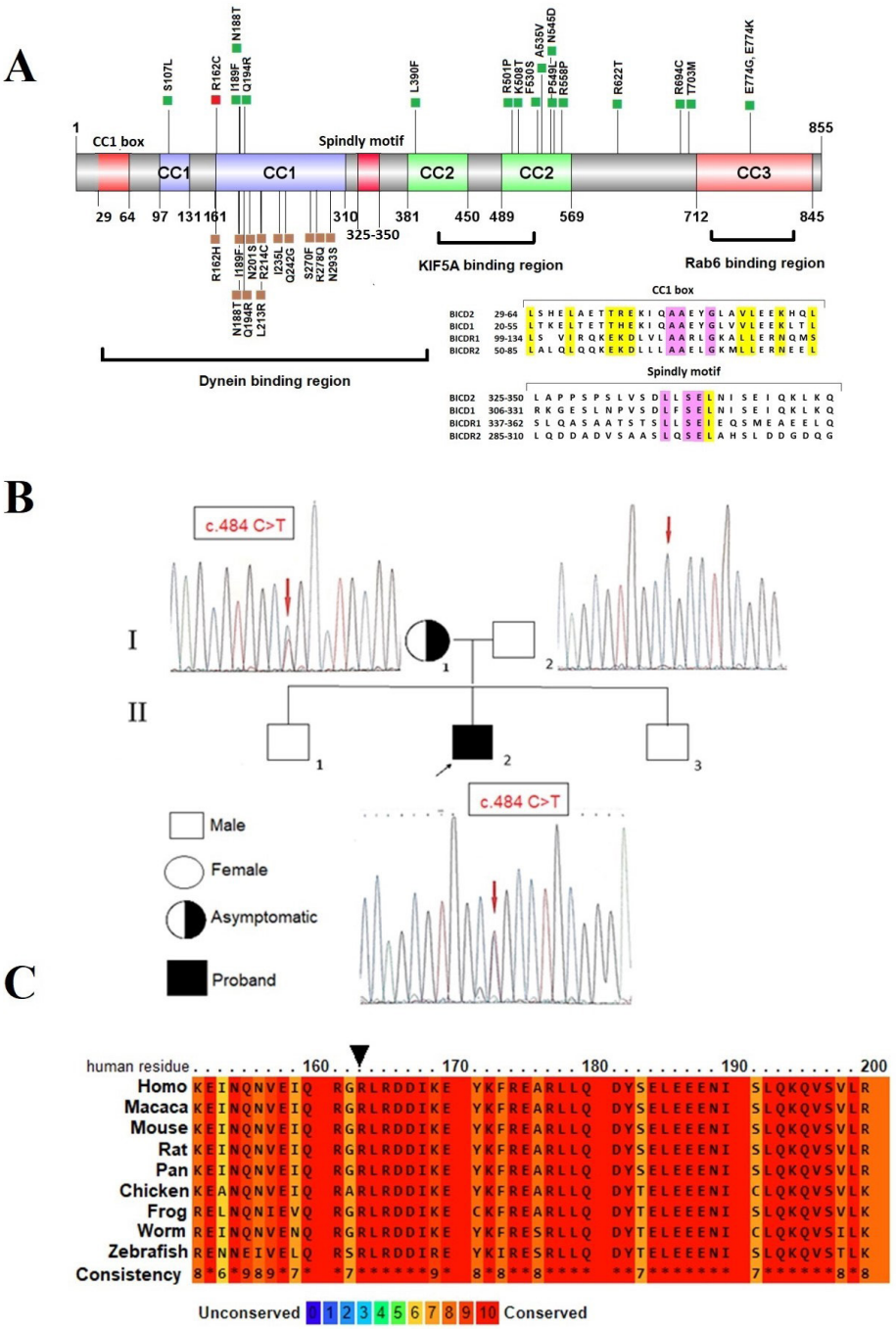

Figure 2. Location, conservation and sequencing of the R162C variant. (A) Green dots represent pathogenic cases, brown dots represent clinical cases, which are reported in the ClinVar database as of June, 2020. The R162C variant is shown in red. BCID2 protein structure, coiled coil domains were identified as CC1a, residues 97-131, CC1b residues 161-310, CC2a residues 381-450, CC2b residues 482-569 and CC3 residues 712-845 (Coiled coil -ExPASy). Sequence alignment of CC1 box (Ala-Ala-x-x-Gly; x-any amino acid) and Spindly motif (Leu-x-Ser-Glu-x; x-any amino acid) of BICD family are shown (*27*). The binding regions of dynein, KIF5A and Rab6 were defined as reported previously (*22,29*). **(B)** Identification of a heterozygous mutation in *BICD2* gene of the parents and son by Sanger sequencing. **(C)** Interspecies alignment of the BICD2 protein harboring the Arg162 residue. The lower panel indicates the conservation score in color code. Black arrow represents the location of Arg162. Aligned species were *Homo sapiens, Macaca mulatta* (rhesus macaque), *Mus musculus* (mouse), *Rattus norvegicus* (rat), *Pan troglodytes* (chimpanzee), *Gallus gallus* (chicken), *Xenopus tropical* (frog), *Thamnophis elegans* (worm) and *Danio rerio* (zebrafish).

positions "e" and "g". This brings a structural diversity to BICD2 and these residues flank the hydrophobic core, resulting in the formation of heterodimers between these positions and the disruption of DDC binding (*26*).

Furthermore, we used protein models (PDB codes 6F1T and 6F3A) to determine the interaction between the Arg162 residue, found on the N-terminus of BICD2, and DDC (*15,16*). Recent studies have shown that other adapters such as BICD family like cargo adapter 1 (BICDR1), Rab6, and HOOK3 are generally unrelated at the sequence level, but they all share regions termed the CC1 box (consensus sequence: Ala-Ala-x-x-Gly;

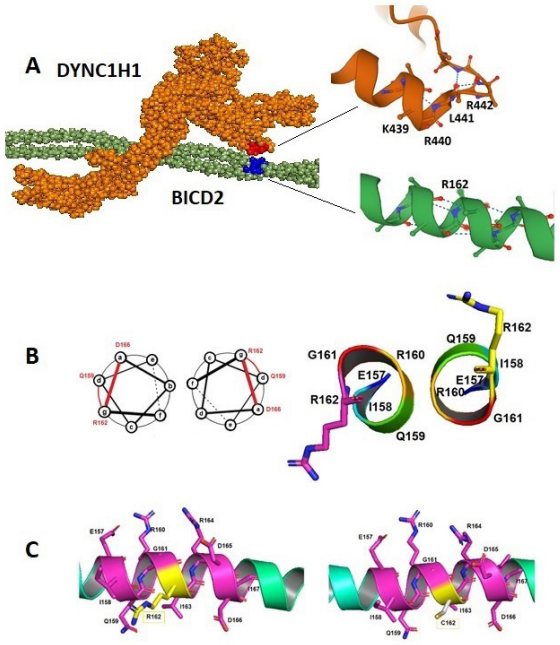

Figure 3. Structural analysis of the position of Arg162 in BICD2. (A) The N terminal part of BICD2 protein is shown as a rope-like structure, highlighted in pink and residues 158-164 of BICD2 spotted as blue dots. DYNC1H1 is represented by a green color and interaction site with BICD2 is colored red. This red site contains residues 436-441 of DYNC1H1 (PDB 6F3A). **(B)** Helical-wheel diagram showing how the heptad repeat *abcdefg* track around helical structure and among them, Arg162 is in the position of "g". **(C)** Representation of the structure of neighboring five residues of Arg162, modelled on the crystal structure from tropomyosin (PDB 1C1G) shown in pink. The Arg162 and Cys162 are shown in yellow.

x-any amino acid) and Spindly motif (Leu-x-Ser-Glu-x; x-any amino acid) to interact with both dynein and dynactin (*27*) (Figure 2A). To date, a high-resolution model of BICD2 has not been reported, and the 6F1T (chain X) structure is the only available high-resolution model of BICDR1's N-terminal region interacting with the DDC. We localized the position of Arg162 of BICD2 to Arg250 of BICDR1 by aligning sequences of the CC1 box and Spindly motif of BICD family proteins (Figure 2A). Arg250 of BICDR1 (PDB code 6F1T) aligned with an amino acid at position 139 of BICD2 (PDB code 6F3A), whereas it would be at residue Arg162 in our research. Arg162 of BICD2 interacted closely with the residues of DYNC1H1 at positions Val438, Lys439, Ar440, and Lys441 (Figure 3A). Residues 441-454 were predicted to be loose strands in the heavy chain domain of DYNC1H1. We found that residues 435-443 of the DYNC1H1 protein interacted with residues 249-256 of BICDR1, enabling the connection of these proteins (Figure 3B). We predicted that these interactions might be necessary for the recruitment of dynein into cargo molecules of BICDR1 or BICD2 to form the DDC. Consistent with our research, high-resolution analysis of DYNC1H1 showed that it has two binding sites with BICDR1. The first site interacts extensively with BICDR1, however the second site is not directly connected, but instead touches the density that packs the coiled coil regions of BICDR1 (*28*). The location of Arg162, which corresponds to Arg250 of BICDR1, was in the second binding site of the DDC, suggesting that

Table 1. Mutations located in CC1 domain of BICD2 associated with spinal muscular atrophy

Residues	SASA%	B/E	Heptad position, (%)	Conserved score	Phenotype	ClinVar №
E101K	68.2	E	*e* (51.8)	10	SMALED2	VCV000541286
S107L	16.8	B	*c* (51.8)	10	SMALED2	VCV000055857
					Distal hereditary motor neuropathy	
L119I	75.7	E	*b* (99.8)	10	SMALED2	VCV000424580
T123M	67.5	E	*f* (99.8)	10	SMALED2	VCV000664075
E124D	44.7	-	*g* (99.8)	9	SMALED2	VCV000654487
N130S	79.7	E	*f* (99.8)	10	SMALED2	VCV000641810
R162H	84.0	E	*g* (96.5)	10	SMALED2	VCV000565772
N188T	22.5	-	*a* (93.1)	10	SMALED2	VCV000055859
I189F	59.1	E	*b* (93.1)	10	Distal hereditary motor neuropathy	VCV000637065
Q194R	68.4	E	*g* (93.1)	10	SMALED2	VCV000617527
S196F	82.5	E	*b* (88.3)	10	SMALED2	VCV000851654
N201S	44.6	-	*d* (92.7)	7	SMALED2	VCV000423894
K213R	62.0	E	*b* (100.0)	8	SMALED2	VCV000453157
R214C	67.7	E	*c* (100.0)	10	SMALED2	VCV000474279
I235L	57.3	E	*c* (100.0)	10	SMALED2	VCV000638377
R238Q	60.4	E	*f* (100.0)	10	SMALED2	VCV000573134
E242G	59.5	E	*c* (100.0)	10	SMALED2	VCV000474280
K254R	26	-	*b* (100.0)	10	Hereditary spastic paraplegia	VCV000424684

SASA% (Solvent accessible surface area %): percentage of each residue that is accessed by solvent was calculated with GetArea program. **B/E**: Buried or exposed to the solvent. Residues are considered if SASA% is less than 20% and to be solvent exposed if SASA% is more than 50%. **Heptad position, (%)**: The heptad sructure was calculated by MARCOIL (MPI BioInformatics toolkit) and the probabilty from 0 to 100%. **Conserved score**: The conserved scoring was performed by PRALINE. The scoring scheme works from 0 for the least conserved alignment position, up to 10 for the most conserved alignment position. Aligned species were *Homo sapiens, Macaca mulatta* (rhesus macaque), *Mus musculus* (mouse), *Rattus norvegicus* (rat), *Pan troglodytes* (chimpanzee), *Gallus gallus* (chicken), *Xenopus tropical* (frog), *Thamnophis elegans* (worm) and *Danio rerio* (zebrafish). **Phenotype**: Spinal muscular atrophy-like phenotypes were reported in ClinVar 2020 by June, 2020.

it would increase the flexibility of DDC.

Previous work in BICDR1 showed that recruitment of two dynein molecules alongside shorter molecules of BICD homology proteins, resulted in faster motility of the DDC, thus increasing its velocity (*28*). Unlike BICD2, BICDR1 recruits two dyneins, moreover, a mutational switch in the position of the N-terminal region of BICD2 is sufficient to recruit a second dynein (*15,16*). BICD2's larger size, and a recruitment site mutation in the DDC could both disrupt the recruitment of the complex or change the electrophysiological bond around important residues, which in turn might cause a more indirect or slower route along the microtubule when compared with BICDR1. Furthermore, the minimal side chain of Cys162 could account for reduced binding to the DDC, because cysteine has a smaller side chain than arginine. In addition, the mother of the proband did not show any of the clinical symptoms of SMALED2. This supports the complex and broader genotype-phenotype correlation of *BICD2* mutations that include both incomplete penetrance and variable expressivity in the next generation.

In conclusion, autosomal dominant SMALED type II is a rare entity with few cases reported in the literature, but it is important for clinicians to be familiar with this disease because of its broad phenotypic expression. Mechanisms leading to variable expressivity and onset of SMALED2 could be explained by alterations in molecular interactions between the domains of BICD2 and the DDC. Thus, suggesting the presence of genetic mutations that act as molecular units of protein structure can alter phenotypic expressivity of the disease.

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**Address correspondence to:*
Munkhtuya Tumurkhuu, Department of Molecular Biology, School of Medicine, International University of Health and Welfare, 4-3 Kozunomori, Narita, Chiba 286-8686, Japan.
E-mail: munkhtuya.t@iuhw.ac.jp, munkhtuya.tumurkhuu@gmail.com

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Brief Report

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Loss of miR-23a cluster in skeletal muscle can suppress bone remodeling

Hui Zhang, Jing Luan, Yazhou Cui, Jinxiang Han*

Biomedical Sciences College & Shandong Medicinal Biotechnology Centre, Shandong First Medical University & Shandong Academy of Medical Sciences, Ji'nan 250062, Shandong, China; Key Lab for Biotech-Drugs of National Health Commission, Ji'nan 250062, Shandong, Shandong, China.

SUMMARY Muscle-bone interaction might regulate bone remodeling in an endocrine manner, but the exact mediators have not been identified. Previous in vitro studies suggest that exosomal miRNAs are a candidate for this interaction. Here we present an in vivo study to show that targeted knockout of a muscle-specific miR-23a cluster including miR-23a, miR-27, and miR-24-2 in skeletal muscle tissues can suppress bone remodeling in mice. The effect of miR-23a cluster seem to not be related to aging, but can worsen the pathological extent of osteoporosis in mice. Our findings suggest that muscle-derived miRNAs may contribute to bone metabolism regulation through exosomes in muscle-bone interaction.

Keywords exosome, microRNA, muscle-bone interaction, bone remodeling

1. Introduction

MicroRNAs (miRNAs) are small endogenous non-coding RNAs that are known to regulate expression of protein-coding genes (*1*). Among them, several miRNAs located adjacently as miRNA clusters, and dysregulation of these clusters has been identified to be involved in multiple pathogenesis events, such as, cancer, heart disease, and metabolic disorders (*2*). Accumulating evidence indicates that miRNAs also act as mediators of intercellular communications (*3*).

Muscle and bone are anatomically and functionally connected organs. Recent studies suggest that besides biomechanical effects, skeletal muscle and bone could regulate the differentiation of each other by secreting factors (*4*). Our previous study indicated that except for secreting proteins, miRNAs cargoing using exsomes also contribute to bone-muscle interaction (*5*). Our *in vitro* data showed that muscle-abundant miR-27a cargoing by myofibroblasts promotes osteoblast differentiation (*5*), and the level of miR-27a in the serum exosome of Amyotrophic Lateral Sclerosis (ALS) patients was significantly lower than that in non-ALS controls (*6*). Here, we examine if miR-27a knockout in muscle can affect bone remodeling *in vivo*. For miR-27a, miR-23a, and miR-24-2 are adjacently located in a miRNA cluster (miR-23a cluster) and are functionally related. Therefore in this study, the effect of miR-23a cluster knockout on bone modeling was investigated.

2. Materials and Methods

2.1. Establishment of muscle-specific miR-23a cluster-knockout mice

The core group breeds miR-23a cluster-knockout mice were constructed following the standard protocol as described previously (*7*). A target region was selected at both ends of miR-23 and miR24-2, and the floxp sites were inserted to achieve three miRNA conditional deletions. The miR-23-cluster-floxp mice were mated with skeletal muscle-specific expression of Ckmm-cre mice to achieve conditional knockout of the miR-23a cluster. The animal care and experimental scheme used in this study was approved and conducted under the guidelines approved by the animal ethics committee of our academy. The KO mouse genotype was confirmed using DNA extracted from the tail tissue by mouse direct polymerase chain reaction (PCR) kits (Bimake, Houston, TX, USA). Primers for genotyping PCR amplification were as follows: CKO-X6-LOXP: forward (5′-GTA GAG GAG GGC TAG GGT GTG-3′) and reverse (5′-TGG GAG CGG AGT GTA GCA C-3′); ckmm-cre: forward (5′-GAC AAA AGG TTT TGC CCT CC-3′) and reverse (5′-AGT TTT TAC TGC CAG ACC GC-3′). The weight and length of the mice were measured from 5 weeks.

2.2. Expression level of miRNAs in muscle tissues with

and without miR-23a cluster knockout

Total RNA was extracted from muscle tissues of wild-type (WT) and knockout mice (KO) (n = 4 of each group) using an animal tissue total RNA extraction kit (Tiangen, Beijing, China). 2μg RNA was used as starting material. Total miRNA was extracted from total RNA using All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia, Guangzhou, China). RT-qPCR was performed on Light Cycler®480 Real-time PCR system (Roche Applied Science, Mannheim, Germany). PCR program was performed as follows: pre-denaturation at 95℃ for 10min, amplification for 40 cycles with denaturation at 95℃ for 10 s, annealing at 60℃ for 20 s and extension at 72℃ for 10 s.

2.3. Establishment of osteoporosis model and micro-CT analysis

Twelve weeks old female KO mice and WT mice were used to establish an osteoporosis model by surgically removing both ovaries (OVX). The female KO mice and their age- and sex-matched WT mice were assigned into 4 groups (n = 10 of each group): WT, WT-OVX, KO, and KO-OVX. After 12 weeks of modeling, the bone density and trabecular bone parameters were measured to determine whether the osteoporosis model was successfully constructed. Bone density and trabecular bone were analyzed using high-resolution in-vivo microcomputed tomography (micro-CT) imaging system (PerkinElmer, MA, USA) according to the manufacturer's protocol. The bone density and trabecular bone data analysis of the femurs was analyzed using Analyze 12.0 software. The bone density standard curve is made based on bone density standards. The captured data were loaded into the BMA module, and the bone and trabecula were separated for BMA parameters determination and cross-section analysis.

2.4. Histological analysis of bone tissues

The femurs of mice from the different groups were harvested. The samples were immersed in 4% paraformaldehyde at 4℃ for 24 h, decalcified and then embedded in paraffin. The paraffin-embedded femur sections were deparaffinized using conventional methods. The femurs were sectioned along the longitudinal axis and stained with Van Gieson (VG) and haematoxylin and eosin (H&E) for analysis of histologic differences.

2.5. Statistics

All the data are expressed as means ± standard deviation and compared using t-tests between groups. $P < 0.05$ was considered statistically significant. All experiments were repeated in triplicate. All statistics were analyzed with Graphpad Prism 8.0.

3. Results and Discussion

The muscle-specifc-miR-23a cluster KO mice were generated by cross-breeding miR-23a cluster-foxed mice with Ckmm-cre mice (Figure 1A-B). To confirm the Ckmm-cre induced miR-23a cluster knockout effect, we found that all the expression of miR-23a, miR-27, miR-24-2 in skeletal muscle tissues was significantly reduced in KO mice compared to their littermate controls (Figure 1C). As seen in Figure 2, muscle-specifc-miR-23a cluster KO mice demonstrated slightly lower body length and weight than WT mice (Figure 2A-B). The micro-CT results show that the KO mice have significantly lower femoral bone mineral density (BMD) than normal mice (Figure 2C), and morphometric parameters except BS/BV notably differs between KO and WT mice, which is consistent with BMD (Figure 2D-H).

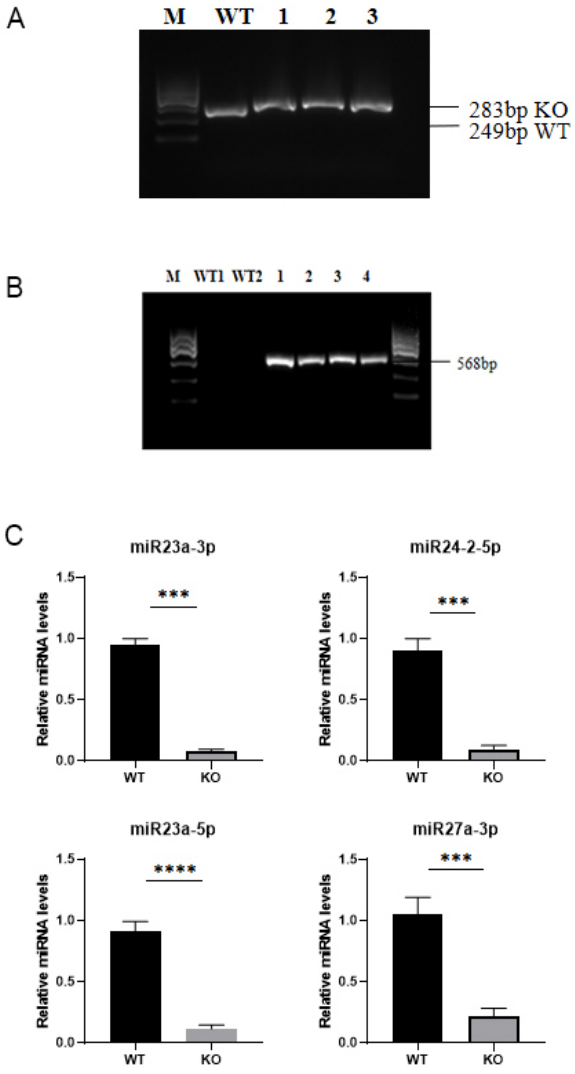

Figure 1. Confirmation of miR-23a cluster knockout in mice. Validation of insertion of flop sites **(A)** and Cas9 **(B)** by PCR. **(C)** Validation of loss of miR-23a cluster components in knockout mice tissues by qRT-PCR. Bar represents the mean ± SD. $^{***}P < 0.001$ and $^{****}P < 0.0001$.

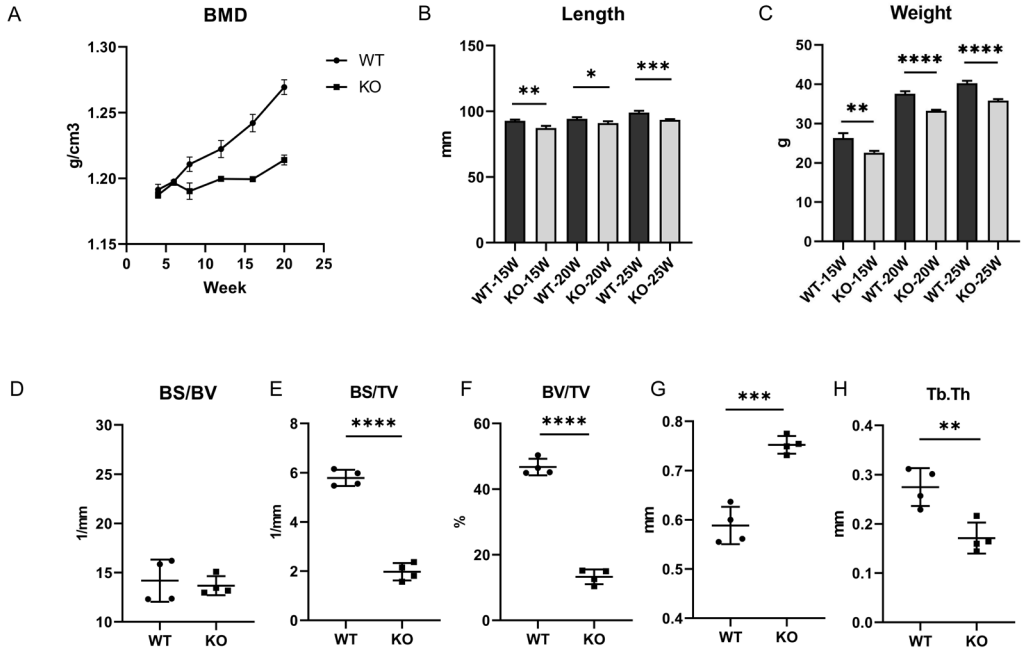

Figure 2. Bone remodeling phenotype of miR-23a cluster knockout mice. (A) Variation of bone mineral density (BMD); **(B-C)** Comparison of body length and weight in wild type and knockout mice; **(D-H)** Comparison of trabecular bone parameters measured by micro-CT in wild type and knockout mice. BS/BV, Bone Surface/Bone Volume; BS/TV, Bone Surface/Tissue Volume; BV/TV, Bone Volume/Tissue Volume; Tb.Sp, Trabecular Separation; Tb.Th, Trabecular Thickness. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

We next examined bone modeling in elder KO mice (60 week), and found the same trends observed in younger mice (Figure 3), however, the suppression seems not to be enhanced by aging. Then, we further confirmed if muscle-specifc-miR-23a cluster KO can affect the pathological extent of osteoporosis. As shown in Figure 4, generally, miR-23a cluster KO in muscle enhanced osteoporosis compared with WT controls. These data support that muscle miR-23a cluster KO could suppress bone modeling in both physiological and pathological conditions. Except for having lower BMD and other morphometric parameters, KO mice also demonstrated enhanced micro-architectural deterioration in micro-CT scanning (Figure 4A) and H&E and VG staining (Figure 4H-I).

The interaction between muscle and skeleton is of great significance to understand the remodeling of bones (*8*). In addition to mechanical forces, secretion between muscles and bones may also play an important regulatory role. miR-23a cluster is highly and specifically expressed in muscles. Wada, *et al*. demonstrated that the ectopic expression of miR-23a can reduce muscle atrophy *in vitro* and *in vivo* (*9*). Numerous studies have reported that miR-23a and miR-27a regulate proteins involved in the atrophy process (*10-12*). A previous study by Lee *et al*. (*13*) found that miR-23a cluster knockout has no obvious phenotypic effect on muscles, but no further observations have been made on bone performance of the model. Different from this previous study, we found

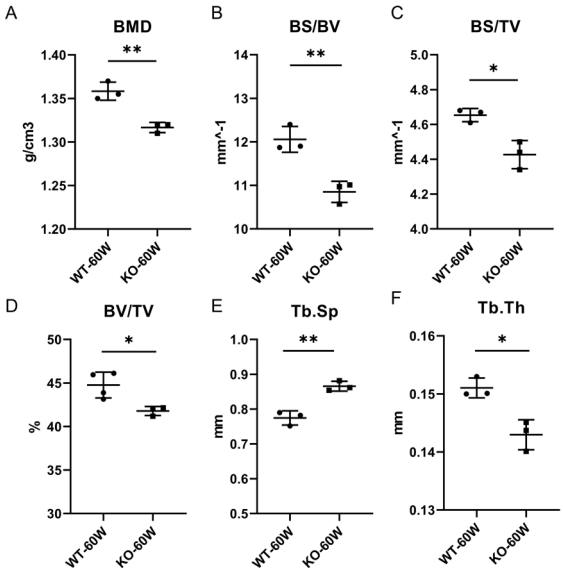

Figure 3. Bone mineral density and trabecular bone parameters in aged miR-23a cluster knockout mice. (A) BMD; **(B)** BS/BV; **(C)** BS/TV; **(D)** BV/TV; **(E)** Tb.Sp; **(F)** Tb.Th. $^{*}P < 0.05$, $^{**}P < 0.01$.

that miR-23a cluster knockout exerts a significant effect on bone remodeling, and knockout can also affect the degree of osteoporosis. Combined with our and other previous findings, there is a possibility that miR-23a secreted by muscles may regulate bone remodeling through exosomes or other means (*14*), but the direct

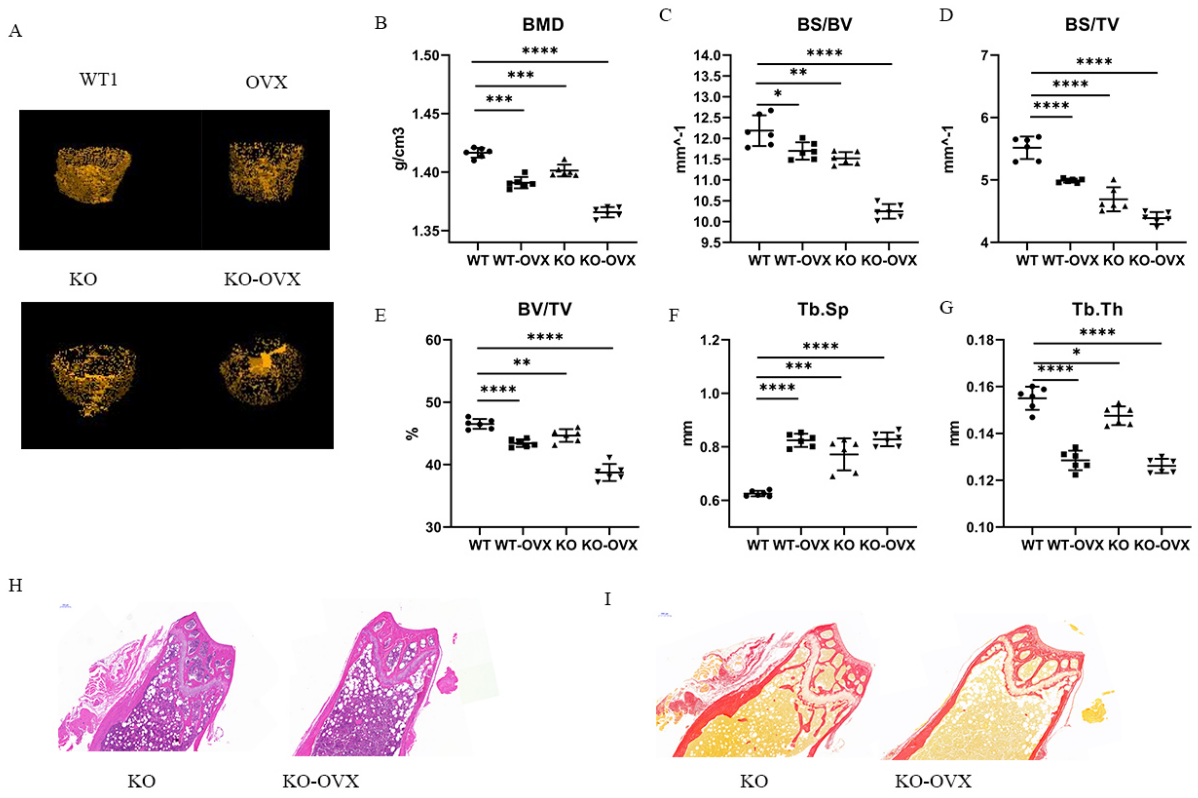

Figure 4. Bone remodeling phenotype of miR-23a cluster knockout in OVX mice model. (A) Trabecular bone construction in different groups; **(B-G)** Comparison of BMD and trabecular bone parameters in different groups; **(H-I)** Van Gieson and haematoxylin and eosin (H&E) in WT and KO OVX mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

muscular factors that cause bone abnormalities also need to be eliminated. Moreover, our data also provide indirect evidence that miRNAs may act in a distant manner. The fact that loss of miR-23a cluster can affect bone modeling also suggests combined miR-23a, miR-27, and miR-24-2 can change differentiation of target cells related to osteogenesis. Although our previous study confirmed exosomal miRNAs from myofibroblasts can target osteoblasts and promote its osteogenesis (*5*), osteoblasts might not be the only target cells. Other bone remodeling related cells may also be candidate targets for muscle exosomes such as osteoclasts. Moreover, the key target genes of miR-24a cluster also need to be identified, and in particular, whether some key osteogenesis regulators exist as co-targets for all three miRNAs.

In conclusion, for the first time, we demonstrate that loss of miRNA cluster in muscle tissues can affect bone remodeling, which is independent of aging, but enhanced under osteoporosis progression. These findings suggest an exosomal cargoing miRNAs regulating mechanism might exist in muscle-bone interaction.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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**Address correspondence to:*
Jinxiang Han, Biomedical Sciences College & Shandong Medicinal Biotechnology Centre, Shandong First Medical University & Shandong Academy of Medical Sciences, Ji'nan 250062, Shandong, China; Key Lab for Biotech-Drugs of National Health Commission, Ji'nan 250062, Shandong, China; Key Lab for Rare & Uncommon Diseases of Shandong Province, Ji'nan 250062, Shandong, China.
E-mail: jxhan9888@aliyun.com

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Case Report

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Clinical manifestation and genetic analysis of familial rare disease genodermatosis xeroderma pigmentosum

Renni Yuniati[1], Nydia Rena Benita Sihombing[2], Donny Nauphar[3], Budi Tiawarman[4], Diah Shinta Kartikasari[1], Meira Dewi[5], Sultana MH Faradz[2,*]

[1] Department Dermatology and Venereology, Faculty of Medicine, Diponegoro University, Semarang, Indonesia/Dr. Kariadi General Hospital Semarang, Indonesia;
[2] Division of Human Genetics, Center for Biomedical Research, Faculty of Medicine, Diponegoro University/Diponegoro National University Hospital, Semarang, Indonesia;
[3] Department of Biomedical Science, Universitas Swadaya Gunung Jati, Cirebon, Indonesia;
[4] Bojonggambir Health Center, Tasik Malaya, Indonesia;
[5] Department of Anatomical Pathology, Faculty of Medicine, Diponegoro University, Semarang, Indonesia/Dr. Kariadi General Hospital Semarang, Indonesia.

SUMMARY Xeroderma pigmentosum (XP) is a rare autosomal recessive disease characterized by hypersensitivity of the skin to ultraviolet radiation and other carcinogenic agents. This ailment is characterized by increased photosensitivity, skin xerosis, early skin aging, actinic keratosis, erythematous lesions, and hyperpigmentation macules. In this serial case report, we presented four cases with XP from two families in Indonesia. Both families were referred from rural referral health centers, and each family has two affected siblings. They had freckle-like pigmentation on the face, trunk, and extremities, which progressed since childhood. One patient of family 2 died because of an infectious disease. Histopathological examination using cytokeratine (CK), CD10, and Ber-EP4 staining from available tissue biopsy of one affected case of family 1 identified basal cell carcinoma (BCC) on the cheek and melanoma on the right eye. Mutation analysis found *ERCC2*, c2047C>T and *XPC*, c1941T>A in the first and second families, respectively. We suppose that this is the first case report of XP in Indonesia that incorporates clinical examination, genetic analysis, and extensive histopathological examination, including immunohistochemistry staining, and a novel pathogenic variant of *XPC* was found in the second family.

Keywords xeroderma pigmentosum, familial, autosomal recessive, photosensitivity, XPC, mutation

1. Introduction

Xeroderma pigmentosum (XP) is characterized by increased photosensitivity, skin xerosis, early skin aging, actinic keratosis, erythematous lesions, and hyperpigmentation macules (*1,2*). Another feature is abnormal lentiginosis (freckle-like pigmentation due to increased numbers of melanocytes) on sun-exposed areas. This is followed by areas of increased or decreased pigmentation, skin aging, and multiple skin cancers if the individuals are not protected from sunlight (*3*). These manifestations are due to cellular hypersensitivity to ultraviolet (UV) radiation resulting from a defect in DNA repair. The mutation that causes XP affects one of eight XP-related genes, including *XPA*, *XPB*, *XPC*, *XPD*, *XPE*, *XPF*, *XPG*, and *XPV* (XP variant), which encodes the *nucleotide excision repair* (NER) mechanism (*4,5*).

XP cases can be found in almost every place in the world with variable prevalence (*6,7*). In the United States, this condition affects one person per one million population (*6,8*). In Europe, this case affects up to 2.3 persons per one million live births. In the Middle East, the prevalence of XP is around 15-20 persons per one million population (*6-8*). Consanguinity itself is highly related to the incidence of XP. Therefore, XP cases are higher in areas where consanguinity is common, including North Africa and the Middle East. Consanguinity is an important factor in autosomal recessive disorders (*8*). Up to 92.8% of XP patients in Libya had consanguinity history (*7*).

XP is a rare autosomal recessive inherited genodermatosis with only a few cases worldwide. Most people are not aware of this condition, which is why XP patients are often neglected and do not receive medical assistance. Seven different genes (labeled A to G), which have deficient excision repair of ultraviolet radiation-

induced DNA damage, are involved. The genes *XPA*, *XPC*, and *XPE* are required for DNA lesion recognition. The *XPB* and *XPD* gene products are helicases mediating local strand unwinding, and *XPF* and *XPG* specify structure-specific endonucleases performing strand incision on either side of the lesion. There exists an additional relatively mild "variant" form of XP caused by a defective ability to convert newly synthesized DNA after UV irradiation.

Advanced molecular techniques using next-generation sequencing (NGS) might help in the diagnosis of XP because of its ability to test multiple XP genes in a single analysis. Here we present two Indonesian families with several children affected by XP in Indonesia.

2. Case Report

2.1. Patients' description

We received four patients from referral physicians from two areas: Salatiga, Central Java, and Tasikmalaya, West Java. These patients were from two unrelated families and had XP. The first family had one son and two daughters, and both of the latter (IV:3 and IV:4) were affected by XP (Figure 1A). The second family had three daughters and two sons, and the third daughter (III:3) and first son (III:5) were affected by XP (Figure 1B). Both families had no history of consanguinity, but they came from an isolated rural area, and none of the parents or other siblings was affected by this disease. The second family had a history of miscarriage, but we did not know whether the miscarriage was due to XP or not. The study was approved by the local IRB (Health Research Ethics Committee, Faculty of Medicine, Diponegoro University, no. 456/EC/KEPK/FK UNDIP/X/2019). Written informed consent was obtained from all parents.

All of these patients were referred to us by rural referral physicians from primary health-care centers with the typical characteristic appearance of XP, including freckle-like pigmentation on the face, trunk, and extremities, which progressed since childhood. Owing to generalized skin lesions and photosensitivity, all patients had dropped out of elementary school. They did not seek medical treatment at the first occurrence of skin lesions, but all of them came to primary health-care centers when they had already developed further abnormalities such as ocular, hearing, and neurological problems.

2.2. Physical examination

In the first family, the oldest daughter (IV:3; 25 years old) was presented to us with freckle-like pigmentation. These pigmentations occurred during childhood and continued to occur on areas that have been more extensively exposed to sunlight, including the face, neck, upper part of the trunk, and extremities. She and her family initially did not seek medical treatment until she developed ocular abnormality and cheek tumor. We have been notified by the physician from the primary health-care center regarding this case and invited them to come to Dr. Kariadi referral hospital. We found uneven, localized brown spots in sun-exposed areas along with cancerous patches on the cheek. The patient also has a prolapsed right eyeball and cataract on the left eye. We found gait disturbance and hearing loss on both ears. After further pathological examination and tests, we confirmed that the ocular abnormality was malignant melanoma, which affects the ocular region, and the tumor on her cheek was confirmed as basal cell carcinoma (BCC). On the basis of the pedigree, we then screened the other siblings of IV:3 and found that her sister (IV:4; 23 years old) had the same uneven, localized brown spots in sun exposed areas. There was a cancerous patch on the cheek similar to patient IV:3, however, now it remains only as a black scar on her right cheek due to previous surgery. The clinical conditions of these patients are shown in Figure 2.

In the second family, the first son (III:5; 25 years old) and the third daughter (III:3; 21 years old) both sought medical attention from the primary health-care center because of a palpebral mass growth. They were referred to our clinic at Diponegoro National University Hospital, Semarang, because of the characteristic freckle-like pigmentation found on these patients. Similar to the first two patients, we found black generalized spots on the face, upper part of the trunk (of individual III:5), and extremities, all of which are directly exposed to

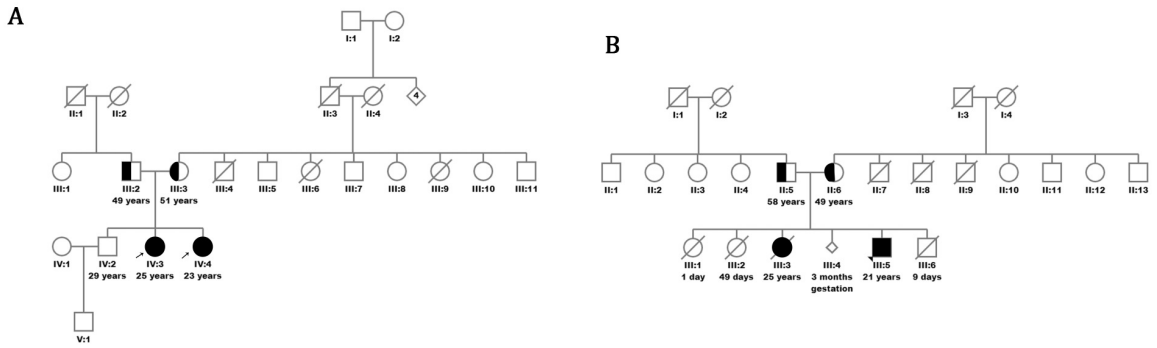

Figure 1. (A) Pedigree of the first family. The last two daughters (IV:3 and IV:4) were affected with *XP*. **(B)** Pedigree of the second family. Individuals III:3 and III:5 (marked in black) were affected with xeroderma pigmentosum.

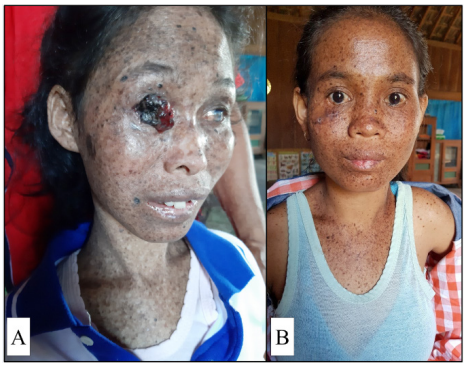

Figure 2. The two daughters of the first family. (A) The first daughter (IV:3), which has the characteristic appearance of XP, with an ocular abnormality confirmed as malignant melanoma and a lesion on the cheek confirmed as basal cell carcinoma. **(B)** The second daughter (IV:4) showed freckle-like pigmentation and cancerous patch on the cheek.

Figure 3. The children of the second family who are affected by XP. (A & B) Note the freckle-like pigmentation and ocular abnormality on both patients. **(C & D)** Close-up of the freckles around the extremities area.

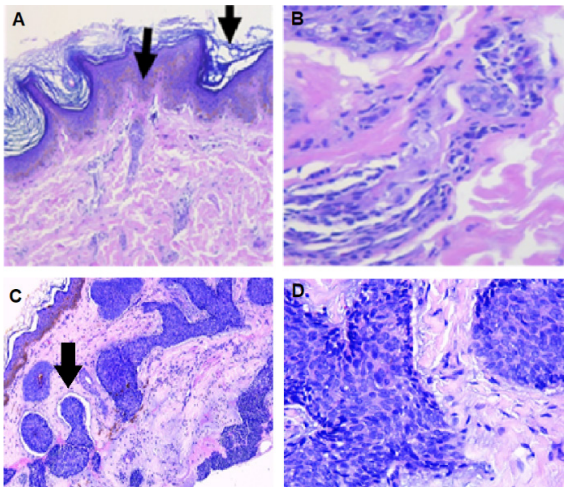

Figure 4. (A) Histopathological image of the macules in HE staining and 100× magnification showing keratinized layered squamous epithelial cells on the edge, epidermal hyperkeratosis and follicular plugging (see short arrow), and increased numbers of melanin pigment (see long arrow). **(B)** The macules showing lymphocytes and perivascular histiocytes (HE 400×). **(C)** Histopathological image of the cheek biopsy sample showing groups of tumor mass with cleft between the tumor mass and adjacent tissue (arrow) HE, 100×. **(D)** Closed-up image of the cells showing round oval nuclei, pleomorphic, hyperchromatic, coarse chromatin, and several prominent nucleoli (400× magnification).

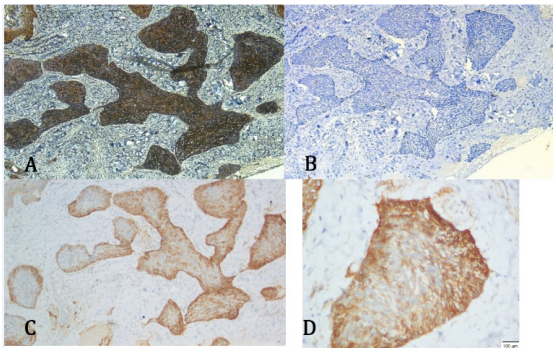

Figure 5. (A) IHC images showing positive pancytokeratin stain on tumor cells (100× magnification). **(B)** Negative HMB45 stain on tumor cells (100×). **(C)** Positive CD10 staining on palisading tumor cells (100×). **(D)** Positive BER-EP4 on tumor cells (400×).

the sun in their daily activities. We found a cancerous patch on the lips and palpebrae of both patients. Both of the patients also had dry eyes and cataract in their eyes, and the daughter (III:3) has blepharitis on her eye. We diagnosed both patients as having XP (Figure 3). However, during the follow-up period, individual III:3 passed away because of an infectious disease. We did not know whether the death was related to the malignancy or not.

2.3. Histopathological investigations

We analyzed a 0.5 cm skin sample taken from the upper right arm of the first daughter of the first family (individual IV:3). On microscopic analysis with HE staining, the tissue is lined with keratinized squamous epithelium, with follicular plugging, spongiosis, and increased melanin pigmentation on some areas of the tissue (Figure 4A). Some of the rete ridges were elongated. The dermal layer consists of fibrocollagenous stroma with scattered lymphocytes and perivascular histiocytes (Figure 4B). Hair follicles, eccrine glands, and sebaceous glands are visible. There were no signs of malignancy in the tissue. On the basis of the pathological anatomy examination, these findings confirmed the XP diagnosis.

Additionally, we analyzed a biopsy sample taken from the patient's right cheek. We found a layered keratinized squamous epithelium with follicular plugging, on microscopic analysis. The dermis layer consists of edematous fibro-collagen connective tissue stroma, with histiocytes and lymphocytes scattered in

the tissue. Skin adnexa and groups of cells with oval round nuclei, pleomorphic, hyperchromatic, coarse chromatins, and several prominent nucleoli were also found (Figure 4C and 4D). These cells are arranged in a palisading pattern on the edge, with a cleft between the tumor mass and adjacent connective tissue stroma, with scattered melanin pigmentation. These descriptions are suggestive of BCC metastasis. Furthermore, analysis of immunohistochemistry (IHC) markers on the sample found positive cytokeratin (CK) on the tumor cells (Figure 5A), negative HMB45 marker on the tumor cells (Figure 5B), positive CD10 (Figure 5C) on the palisading tumor cells, and positive Ber-EP4 stain (Figure 5D) on the tumor cells. These descriptions confirm the diagnosis of BCC.

We found that the lesion in her cheek was a metastasized BCC based on the histopathology examination. Because she also had a similar looking lesion on her palpebra, the BCC most likely metastasized from that region. Her right eye was also surgically resected because of malignancy. Frozen section taken from her right eye and histopathology examination using IHC showed groups of round, oval, and polygonal cells, pleomorphic, hyperchromatic with coarse chromatin, prominent nucleoli and wide eosinophilic cytoplasm. It also demonstrated clear cell boundaries and was scattered with brownish pigment, neutrophils, lymphocytes and histiocytes in fibrous connective tissue and necrotic regions. There were no malignancies found in the optic nerve. These findings confirm the diagnosis of malignant melanoma of the eye. The histopathology examination shows that the cheek lesion is a BCC that metastasized from another site. Because the patient also had a similar looking lesion on her palpebra, the BCC most likely metastasized from that region. The right eye of patient IV:3 was surgically removed because of malignancy. A frozen section taken on her right eye confirmed that the eye lesion is malignant melanoma.

On the laboratory examination of family 2, we found that patient III:5 had serum vitamin D25OH < 4 ng/mL, which is indicative of vitamin D deficiency. Pathological examination of the skin tissues from the second family was not performed because the samples cannot be collected from the inaccessible very rural village.

2.4. Mutation analysis

Exome sequencing was performed for patients from two families at the Genome Diagnostics Laboratory of the Department of Human Genetics, Radboud University Medical Centre, Nijmegen, Netherlands. A pathogenic variant of the *ERCC2* gene (Chr19(GRCh37):g.45855610G>A; NM_000400.3: c.2047C>T, p.(Arg683Trp)) was discovered in the two daughters of the first family, and a nonsense pathogenic variant of the *XPC* gene (NM_004628.4), c.1941T>A, p.(Tyr647*) was identified in patient III:5 of the second family. The variant of the *ERCC2* gene was reported elsewhere (*9*). Meanwhile, the pathogenic variant of *XPC* has not been reported previously, but it is considered a pathogenic variant because it is a nonsense variant. All patients showed homozygous mutation with normal parents. Therefore, we assumed that the inheritance pattern in our patients was autosomal recessive. We summarized the clinical findings, histopathology, and genetic analysis of all patients in Table 1.

2.5. Clinical treatment

We applied sunblock lotion and administered vitamin D3 supplementations to all of the patients. The sunblock lotion, smeared thinly on skin areas exposed to sunlight, is applied twice a day. Sunblock lotion can protect the skin from damage due to sun exposure, thus preventing the appearance and progression of XP lesions. However, supplementation with vitamin D3 is needed to overcome the lack of endogenous vitamin D3 production caused by the sunblock lotion (*2,10*). The progress of their condition was not much better than before treatment because of late treatment and management.

3. Discussion

The genetic mutation that occurs in XP patients causes a defect in the NER mechanism, and UV radiation from sun exposure will cause DNA damage due to the defective repair mechanism, changes in skin cells, and formation of carcinogenic photoproducts, which eventually leads to the formation of malignancies in the skin.

The patients from unrelated families in this serial case report exhibited freckle-like pigmentation on the face, trunk, and extremities since childhood. Patient IV:3, the older sister from the first family, started to show pigmentation of the skin during childhood, which worsened as time progressed. She developed ocular abnormality and malignancy on the cheek. The pigmentation was also worse in areas more frequently exposed to sunlight, such as the face, neck, upper part of the trunk, and extremities. Patient IV:4, the younger sister, also showed similar pigmentation in similar areas as her sister's that started during childhood. Her pigmentation, however, seemed milder, and she had yet to develop more severe complications like her sister. Both patients III:3 and III:5 of the second family exhibited similar symptoms of darker freckle-like pigmentation on the face, neck, upper trunk, and extremities, and complaints of palpebral mass growth.

As XP is a disease caused by the accumulation of damage over time, the milder phenotype of the younger sister may be attributed to several factors, such as age, amount of UV exposure, and the extent of the defect of the DNA repair mechanism. The amount of DNA damage accumulated due to increased photosensitivity is

Table 1. Summary of clinical findings, histopathology, and genetic analysis of all patients

Case/Family	Clinical features				Histopathological Examination	Gene mutation
	Freckle-like pigmentation	Cutaneous malignancy	Ophtalmologic abnormalities	Neurological abnormalities		
Case 1/1 (Patient IV:3)	Uneven brown spots (Localized) in sun-exposed area	Cancerous lumps (patches) on the cheek	Prolapse right eyeball, ectropion. Cataract left eye	Gait disturbance, Hearing loss	Frozen section from right eye and palpebra: melanoma maligna Biopsy sample from right cheek: suggestive of BCC metastasis. Immunohistochemistry of right cheek sample: Cytokeratine positive, Negative HMB45, Positive CD10 on the palisading tumor cells, and Positive Ber-EP4 stain. Skin tissue: Suggestive of Xeroderma Pigmentosum	Pathogenic variant of the *ERCC2* gene (Chr19(GRCh37):g.45855610G>A; NM_000400.3: c.2047C>T, p.(Arg683Trp))
Case 2/1 (Patient IV:4)	Uneven brown spots (Localized) in sun-exposed area	Cancerous lumps (patches) on the cheek	Dry eye	N/A	Skin tissue: Suggestive of Xeroderma Pigmentosum	Pathogenic variant of the *ERCC2* gene (Chr19(GRCh37):g.45855610G>A; NM_000400.3: c.2047C>T, p.(Arg683Trp))
Case 1/2 (Patient III:V)	Black spots evenly (Generalized)	Cancerous patch on the lips and palpebrae	Dry eye, cataract	N/A	N/A Passed away	N/A
Case 2/2 (Patient III:3)	Black spots evenly (Generalized)	Cancerous patch on the lips and palpebrae	Cataract, blepharitis	N/A	Skin tissue: Suggestive of Xeroderma Pigmentosum	Nonsense pathogenic variant of the *XPC* gene (NM_004628.4), c.1941T>A, p.(Tyr647*)

*Remarks: Not all examinations are available for all patients (*eg.* For Vitamin D measurement) due to socioeconomic limitations which hinders the patient to take all laboratory examinations

less at a younger age than at a more advanced age (*11*). The amount of UV exposure also plays an important role in the severity of XP as UV light induces the formation of thymine dimers from covalent linkages between consecutive pyrimidine bases along the nucleotide chain. The amount of thymine dimers increases with increased exposure to UV and coupled with defective DNA damage repair may result in a more severe clinical presentation compared with patients exposed to less UV. The extent of the defect in the NER mechanism may also affect clinical severity.

Mutations affecting the promoter regulatory elements may affect the transcription rate and may have a less severe impact in the defect of the NER mechanism when compared to mutations affecting RNA translation, such as mutations at the initiation codon, mutations causing frameshift, and mutations causing nonsense codons. Meanwhile, in a missense mutation such as that of family 1, nucleotide substitution occurred on the putative nuclear location signal of the *ERCC2* protein, thus limiting the amount of functional protein, which makes it insufficient for DNA repair (*9*). Malignancy of the skin of the palpebral and cheek, as seen in patient IV-3 of family 1 in this case report, might be the result of the defect in repairing genetic changes. In advanced cases, XP may cause malignancies such as BCC, squamous cell carcinoma, and malignant melanoma (*12*).

Approximately 40% to 80% of patients with XP will have ocular problems, which is caused by UV-induced DNA changes on conjunctival, corneal, and palpebral epithelial cells (*13*). One of the early signs of ocular involvement in XP patients is photophobia, and the most frequently found ocular abnormalities caused by XP are conjunctivitis, corneal neovascularization, and dry eyes, up to loss of sight (*14*). Long-term sun exposure can cause neoplasm of eyes and its supporting structures, including epithelioma, squamous cell carcinoma, and melanoma (*15*). Patient IV:3 developed ocular disorders due to XP with histopathological findings of malignant changes of palpebral and periocular skin, malignant melanoma of the eyeball, and ocular manifestations that arise from neurological degenerations. The skin around the eye formed cicatricial changes or skin malignancies that need to be excised. All of the patients in the case series developed ocular abnormality with varying degrees of severity, with one confirmed melanoma both clinically and microscopically.

Around 30% of all patients with XP will experience neurological deficits (*2,3,16*). The neurological deficits and problems that might occur in patients with XP include isolated hyperreflexia, progressive mental retardation, sensorineural deafness, and seizures (*17*). Neurological degeneration may manifest in the eye as a small pupillary size, nystagmus, and strabismus (*2,18*). Neurological deficit in patients with XP mostly occurs in XP-A, XP-B, XP-D, and XP-G patients (*19*). Our patient with XPD from the first family also suffered from hearing and vision loss.

Besides ocular malignancies, patient IV:3 also developed dermal malignancies in the form of BCC. This is a common occurrence in XP patients, due to the high predisposition for developing malignancies. Patients with XP have 10,000-fold risk of developing BCC and 2,000-fold risk of developing melanoma compared to normal people (*18*). Dermatological malignancies in XP patients are difficult to prevent, especially in regions with abundant amounts of sun rays such as in the Middle East and in Asia (*20*).

Advances in molecular techniques such as NGS have massively helped in diagnosing XP faster and in having a higher throughput. For example, exome sequencing for dermatological conditions comprises 619 genes, five of which (*XPA*, *XPC*, *DDB2*, *POLH*, and *ERCC2*) are relevant for XP diagnosis (*2,5,19*). Results from massively parallel sequencing for the XP gene panel revealed an *ERCC2* c.2047C>T mutation, which caused an amino acid change from arginine to tryptophan at position 683 in both daughters from the first family and a nonsense causing mutation at c.1941T>A of the *XPC* gene in the male patient of the second family. The *ERCC2* gene codes the XP group D (XPD) protein, an ATP-dependent DNA helicase that is important in the NER mechanism. A previous *ERCC2* mutation study in Vietnam with a missense mutation (c.2048G>A; p.Arg683Gln) and nonsense mutation (c.1354C>T; p.Gln452*) showed that patients experienced severe sunburn and irritation after unprotected sun exposure, hypopigmentation, dry skin, and skin peeling. Patients also experience ocular damage, photophobia, and dry eyes. The fibroblasts in the Vietnamese cohort showed a defect in the NER pathway (*1*). The defect affects the DNA repair mechanism that occurs in XP, which renders it more sensitive to UVA radiation (*21*). A study of Comorian and Pakistani cohorts revealed that the most common variant of mutation was in the *XPC* c.2251-1G>C mutation, with some patients showing the *POLH/XPV* variant (*22*). The Comorian cohort is unique because of its geographical and historical background. The Comorians live in an isolated area to avoid invasion from other populations, and their people are segregated into different villages based on social status, which leads to a high frequency of consanguineous marriages (*22,23*). Different founder variants are present in specific parts of the world. For *XPA* mutations, there are three different founder variants for India, Japan, and Tunisia. In the Indian population, the mutation that occurs related to the *XPA* gene is c.335_338delTTATinsCATAAGAAA (*11,24*). In Japan, the mutation that occurs in the *XPA* gene is c.390-1G>C, and in Tunisia, the mutation that occurs on the XPA gene is p.Arg228Ter (*11*). In North Africa, the mutation of *XPC* occurs in c.1643_1644delTG, and in an Iraqi Jewish population, the mutation of p.Arg683Gln can be found among xeroderma pigmentosum patients in that

region. *POLH* mutation can be found in a Tunisia/North African population, Japanese population, and Basque/Northern Spain population. *POLH* mutation in Tunisia occurs as the deletion of exon 10; in Japan, it occurs as c.490G>T (splice site variant); p.Ser242Ter; p.Glu306Ter and c.1661delA; and in Basque/Northern Spain, it occurs as c.764+1G>A (*11*). The pathogenic variant found on *XPC* in our patient has not been reported previously. Although mutation analysis of the parents have not been done, we have identified two families with two affected sibs and normal parents, suggesting autosomal recessive inheritance.

Management of XP is based on early diagnosis, follow-up, and minimization of unprotected sun exposure. These are important to reduce or prevent the occurrence of dermatological malignancies in patients with XP, which will improve their quality of life and increase their survival rate. Photoprotection can be achieved using UV-absorbent films on windows, UV-absorbent clothing, sunglasses, and sunscreens. However, all of the patients encountered in this report were not aware of their condition, hence the lack of a photoprotective effort. The rarity of the condition, lack of awareness, and knowledge regarding this condition played important roles in the progress of the disease for all patients. With a prevalence of 1 in 10,000 to 30,000, this condition is rarely seen, and information regarding XP is not widely available to medical care providers managing this disease. Owing to the nature of the cumulative damage, most patients do not have any serious complaints during early life and do not feel the need to check themselves until more complications are present, such as ocular, hearing, and neurological problems.

Another possible reason why the disease is often overlooked is that XP is passed down in an autosomal recessive manner, which sometimes skips generations, causing parents to be unaware of their carrier status and their affected children. Vitamin D supplementation given later during the course of the disease may not be very helpful. Genetic counseling regarding the progressivity of the diseases, pattern of inheritance, and risk of having more children with the same condition was given to the family. Because XP is inherited in an autosomal recessive manner and both families live in an isolated area, further analysis is warranted to evaluate the possibility of a founder mutation.

To the best of our knowledge, this is the first comprehensive clinical and genetic analysis report on patients with XP in Indonesia, with a novel pathogenic variant of *XPC* found in the second family. This case report highlights the importance of a rare disease that is manageable but often overlooked because of its rarity, inheritance pattern, and late onset of severe symptoms. New advances in molecular biology techniques can help diagnosis faster with a higher throughput, but they are still not available in low resource settings.

4. Conclusion

This is the first case report of XP in Indonesia that incorporates clinical examination, genetic analysis, and histopathological examination including immunohistochemistry staining, and a novel pathogenic variant of *XPC*, c1941T>A was found in the second family. Diagnosis of XP has been made using history taking, physical examination, pedigree analysis, and histopathology and mutation analysis using advanced molecular techniques.

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**Address correspondence to:*
Sultana MH Faradz, Division of Human Genetics, Center for Biomedical Research, Faculty of Medicine, Diponegoro University/Diponegoro National University Hospital, Jl. H Soedharto, Tembalang, Semarang 50275, Indonesia
E-mail: sultanafaradz@gmail.com

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Case Report

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Clinical manifestation and genetic analysis of familial rare disease genodermatosis xeroderma pigmentosum

Renni Yuniati[1], Nydia Rena Benita Sihombing[2], Donny Nauphar[3], Budi Tiawarman[4], Diah Shinta Kartikasari[1], Meira Dewi[5], Sultana MH Faradz[2,*]

[1] Department Dermatology and Venereology, Faculty of Medicine, Diponegoro University, Semarang, Indonesia/Dr. Kariadi General Hospital Semarang, Indonesia;
[2] Division of Human Genetics, Center for Biomedical Research, Faculty of Medicine, Diponegoro University/Diponegoro National University Hospital, Semarang, Indonesia;
[3] Department of Biomedical Science, Universitas Swadaya Gunung Jati, Cirebon, Indonesia;
[4] Bojonggambir Health Center, Tasik Malaya, Indonesia;
[5] Department of Anatomical Pathology, Faculty of Medicine, Diponegoro University, Semarang, Indonesia/Dr. Kariadi General Hospital Semarang, Indonesia.

SUMMARY Xeroderma pigmentosum (XP) is a rare autosomal recessive disease characterized by hypersensitivity of the skin to ultraviolet radiation and other carcinogenic agents. This ailment is characterized by increased photosensitivity, skin xerosis, early skin aging, actinic keratosis, erythematous lesions, and hyperpigmentation macules. In this serial case report, we presented four cases with XP from two families in Indonesia. Both families were referred from rural referral health centers, and each family has two affected siblings. They had freckle-like pigmentation on the face, trunk, and extremities, which progressed since childhood. One patient of family 2 died because of an infectious disease. Histopathological examination using cytokeratine (CK), CD10, and Ber-EP4 staining from available tissue biopsy of one affected case of family 1 identified basal cell carcinoma (BCC) on the cheek and melanoma on the right eye. Mutation analysis found *ERCC2*, c2047C>T and *XPC*, c1941T>A in the first and second families, respectively. We suppose that this is the first case report of XP in Indonesia that incorporates clinical examination, genetic analysis, and extensive histopathological examination, including immunohistochemistry staining, and a novel pathogenic variant of *XPC* was found in the second family.

Keywords xeroderma pigmentosum, familial, autosomal recessive, photosensitivity, XPC, mutation

1. Introduction

Xeroderma pigmentosum (XP) is characterized by increased photosensitivity, skin xerosis, early skin aging, actinic keratosis, erythematous lesions, and hyperpigmentation macules (*1,2*). Another feature is abnormal lentiginosis (freckle-like pigmentation due to increased numbers of melanocytes) on sun-exposed areas. This is followed by areas of increased or decreased pigmentation, skin aging, and multiple skin cancers if the individuals are not protected from sunlight (*3*). These manifestations are due to cellular hypersensitivity to ultraviolet (UV) radiation resulting from a defect in DNA repair. The mutation that causes XP affects one of eight XP-related genes, including *XPA*, *XPB*, *XPC*, *XPD*, *XPE*, *XPF*, *XPG*, and *XPV* (XP variant), which encodes the *nucleotide excision repair* (NER) mechanism (*4,5*).

XP cases can be found in almost every place in the world with variable prevalence (*6,7*). In the United States, this condition affects one person per one million population (*6,8*). In Europe, this case affects up to 2.3 persons per one million live births. In the Middle East, the prevalence of XP is around 15-20 persons per one million population (*6-8*). Consanguinity itself is highly related to the incidence of XP. Therefore, XP cases are higher in areas where consanguinity is common, including North Africa and the Middle East. Consanguinity is an important factor in autosomal recessive disorders (*8*). Up to 92.8% of XP patients in Libya had consanguinity history (*7*).

XP is a rare autosomal recessive inherited genodermatosis with only a few cases worldwide. Most people are not aware of this condition, which is why XP patients are often neglected and do not receive medical assistance. Seven different genes (labeled A to G), which have deficient excision repair of ultraviolet radiation-

induced DNA damage, are involved. The genes *XPA*, *XPC*, and *XPE* are required for DNA lesion recognition. The *XPB* and *XPD* gene products are helicases mediating local strand unwinding, and *XPF* and *XPG* specify structure-specific endonucleases performing strand incision on either side of the lesion. There exists an additional relatively mild "variant" form of XP caused by a defective ability to convert newly synthesized DNA after UV irradiation.

Advanced molecular techniques using next-generation sequencing (NGS) might help in the diagnosis of XP because of its ability to test multiple XP genes in a single analysis. Here we present two Indonesian families with several children affected by XP in Indonesia.

2. Case Report

2.1. Patients' description

We received four patients from referral physicians from two areas: Salatiga, Central Java, and Tasikmalaya, West Java. These patients were from two unrelated families and had XP. The first family had one son and two daughters, and both of the latter (IV:3 and IV:4) were affected by XP (Figure 1A). The second family had three daughters and two sons, and the third daughter (III:3) and first son (III:5) were affected by XP (Figure 1B). Both families had no history of consanguinity, but they came from an isolated rural area, and none of the parents or other siblings was affected by this disease. The second family had a history of miscarriage, but we did not know whether the miscarriage was due to XP or not. The study was approved by the local IRB (Health Research Ethics Committee, Faculty of Medicine, Diponegoro University, no. 456/EC/KEPK/FK UNDIP/X/2019). Written informed consent was obtained from all parents.

All of these patients were referred to us by rural referral physicians from primary health-care centers with the typical characteristic appearance of XP, including freckle-like pigmentation on the face, trunk, and extremities, which progressed since childhood. Owing to generalized skin lesions and photosensitivity, all patients had dropped out of elementary school. They did not seek medical treatment at the first occurrence of skin lesions, but all of them came to primary health-care centers when they had already developed further abnormalities such as ocular, hearing, and neurological problems.

2.2. Physical examination

In the first family, the oldest daughter (IV:3; 25 years old) was presented to us with freckle-like pigmentation. These pigmentations occurred during childhood and continued to occur on areas that have been more extensively exposed to sunlight, including the face, neck, upper part of the trunk, and extremities. She and her family initially did not seek medical treatment until she developed ocular abnormality and cheek tumor. We have been notified by the physician from the primary health-care center regarding this case and invited them to come to Dr. Kariadi referral hospital. We found uneven, localized brown spots in sun-exposed areas along with cancerous patches on the cheek. The patient also has a prolapsed right eyeball and cataract on the left eye. We found gait disturbance and hearing loss on both ears. After further pathological examination and tests, we confirmed that the ocular abnormality was malignant melanoma, which affects the ocular region, and the tumor on her cheek was confirmed as basal cell carcinoma (BCC). On the basis of the pedigree, we then screened the other siblings of IV:3 and found that her sister (IV:4; 23 years old) had the same uneven, localized brown spots in sun exposed areas. There was a cancerous patch on the cheek similar to patient IV:3, however, now it remains only as a black scar on her right cheek due to previous surgery. The clinical conditions of these patients are shown in Figure 2.

In the second family, the first son (III:5; 25 years old) and the third daughter (III:3; 21 years old) both sought medical attention from the primary health-care center because of a palpebral mass growth. They were referred to our clinic at Diponegoro National University Hospital, Semarang, because of the characteristic freckle-like pigmentation found on these patients. Similar to the first two patients, we found black generalized spots on the face, upper part of the trunk (of individual III:5), and extremities, all of which are directly exposed to

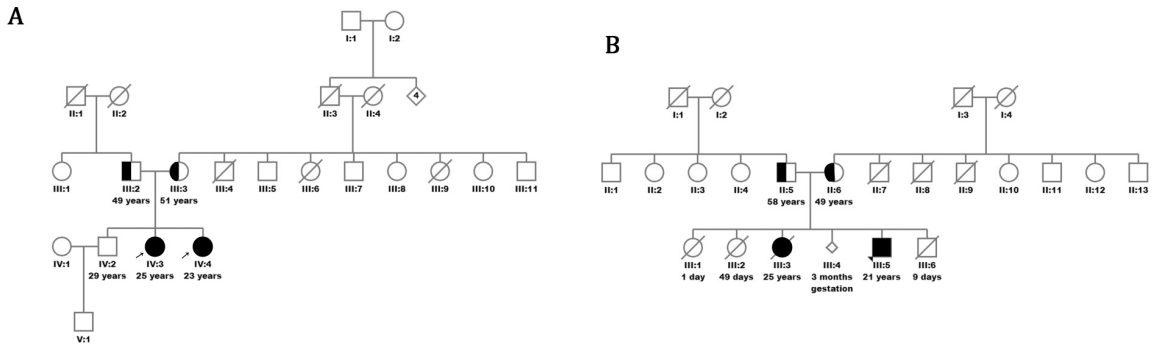

Figure 1. (A) Pedigree of the first family. The last two daughters (IV:3 and IV:4) were affected with *XP*. **(B)** Pedigree of the second family. Individuals III:3 and III:5 (marked in black) were affected with xeroderma pigmentosum.

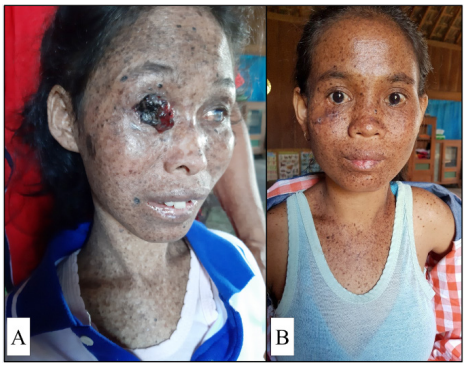

Figure 2. The two daughters of the first family. (A) The first daughter (IV:3), which has the characteristic appearance of XP, with an ocular abnormality confirmed as malignant melanoma and a lesion on the cheek confirmed as basal cell carcinoma. **(B)** The second daughter (IV:4) showed freckle-like pigmentation and cancerous patch on the cheek.

Figure 3. The children of the second family who are affected by XP. (A & B) Note the freckle-like pigmentation and ocular abnormality on both patients. **(C & D)** Close-up of the freckles around the extremities area.

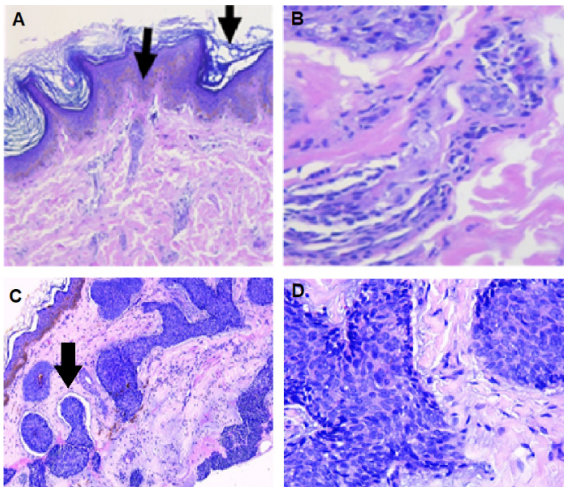

Figure 4. (A) Histopathological image of the macules in HE staining and 100× magnification showing keratinized layered squamous epithelial cells on the edge, epidermal hyperkeratosis and follicular plugging (see short arrow), and increased numbers of melanin pigment (see long arrow). **(B)** The macules showing lymphocytes and perivascular histiocytes (HE 400×). **(C)** Histopathological image of the cheek biopsy sample showing groups of tumor mass with cleft between the tumor mass and adjacent tissue (arrow) HE, 100×. **(D)** Closed-up image of the cells showing round oval nuclei, pleomorphic, hyperchromatic, coarse chromatin, and several prominent nucleoli (400× magnification).

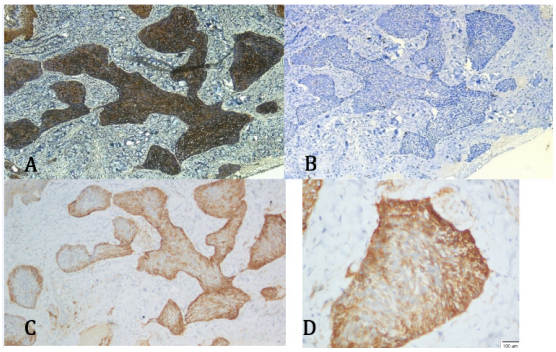

Figure 5. (A) IHC images showing positive pancytokeratin stain on tumor cells (100× magnification). **(B)** Negative HMB45 stain on tumor cells (100×). **(C)** Positive CD10 staining on palisading tumor cells (100×). **(D)** Positive BER-EP4 on tumor cells (400×).

the sun in their daily activities. We found a cancerous patch on the lips and palpebrae of both patients. Both of the patients also had dry eyes and cataract in their eyes, and the daughter (III:3) has blepharitis on her eye. We diagnosed both patients as having XP (Figure 3). However, during the follow-up period, individual III:3 passed away because of an infectious disease. We did not know whether the death was related to the malignancy or not.

2.3. Histopathological investigations

We analyzed a 0.5 cm skin sample taken from the upper right arm of the first daughter of the first family (individual IV:3). On microscopic analysis with HE staining, the tissue is lined with keratinized squamous epithelium, with follicular plugging, spongiosis, and increased melanin pigmentation on some areas of the tissue (Figure 4A). Some of the rete ridges were elongated. The dermal layer consists of fibrocollagenous stroma with scattered lymphocytes and perivascular histiocytes (Figure 4B). Hair follicles, eccrine glands, and sebaceous glands are visible. There were no signs of malignancy in the tissue. On the basis of the pathological anatomy examination, these findings confirmed the XP diagnosis.

Additionally, we analyzed a biopsy sample taken from the patient's right cheek. We found a layered keratinized squamous epithelium with follicular plugging, on microscopic analysis. The dermis layer consists of edematous fibro-collagen connective tissue stroma, with histiocytes and lymphocytes scattered in

the tissue. Skin adnexa and groups of cells with oval round nuclei, pleomorphic, hyperchromatic, coarse chromatins, and several prominent nucleoli were also found (Figure 4C and 4D). These cells are arranged in a palisading pattern on the edge, with a cleft between the tumor mass and adjacent connective tissue stroma, with scattered melanin pigmentation. These descriptions are suggestive of BCC metastasis. Furthermore, analysis of immunohistochemistry (IHC) markers on the sample found positive cytokeratin (CK) on the tumor cells (Figure 5A), negative HMB45 marker on the tumor cells (Figure 5B), positive CD10 (Figure 5C) on the palisading tumor cells, and positive Ber-EP4 stain (Figure 5D) on the tumor cells. These descriptions confirm the diagnosis of BCC.

We found that the lesion in her cheek was a metastasized BCC based on the histopathology examination. Because she also had a similar looking lesion on her palpebra, the BCC most likely metastasized from that region. Her right eye was also surgically resected because of malignancy. Frozen section taken from her right eye and histopathology examination using IHC showed groups of round, oval, and polygonal cells, pleomorphic, hyperchromatic with coarse chromatin, prominent nucleoli and wide eosinophilic cytoplasm. It also demonstrated clear cell boundaries and was scattered with brownish pigment, neutrophils, lymphocytes and histiocytes in fibrous connective tissue and necrotic regions. There were no malignancies found in the optic nerve. These findings confirm the diagnosis of malignant melanoma of the eye. The histopathology examination shows that the cheek lesion is a BCC that metastasized from another site. Because the patient also had a similar looking lesion on her palpebra, the BCC most likely metastasized from that region. The right eye of patient IV:3 was surgically removed because of malignancy. A frozen section taken on her right eye confirmed that the eye lesion is malignant melanoma.

On the laboratory examination of family 2, we found that patient III:5 had serum vitamin D25OH < 4 ng/mL, which is indicative of vitamin D deficiency. Pathological examination of the skin tissues from the second family was not performed because the samples cannot be collected from the inaccessible very rural village.

2.4. Mutation analysis

Exome sequencing was performed for patients from two families at the Genome Diagnostics Laboratory of the Department of Human Genetics, Radboud University Medical Centre, Nijmegen, Netherlands. A pathogenic variant of the *ERCC2* gene (Chr19(GRCh37):g.45855610G>A; NM_000400.3: c.2047C>T, p.(Arg683Trp)) was discovered in the two daughters of the first family, and a nonsense pathogenic variant of the *XPC* gene (NM_004628.4), c.1941T>A, p.(Tyr647*) was identified in patient III:5 of the second family. The variant of the *ERCC2* gene was reported elsewhere (*9*). Meanwhile, the pathogenic variant of *XPC* has not been reported previously, but it is considered a pathogenic variant because it is a nonsense variant. All patients showed homozygous mutation with normal parents. Therefore, we assumed that the inheritance pattern in our patients was autosomal recessive. We summarized the clinical findings, histopathology, and genetic analysis of all patients in Table 1.

2.5. Clinical treatment

We applied sunblock lotion and administered vitamin D3 supplementations to all of the patients. The sunblock lotion, smeared thinly on skin areas exposed to sunlight, is applied twice a day. Sunblock lotion can protect the skin from damage due to sun exposure, thus preventing the appearance and progression of XP lesions. However, supplementation with vitamin D3 is needed to overcome the lack of endogenous vitamin D3 production caused by the sunblock lotion (*2,10*). The progress of their condition was not much better than before treatment because of late treatment and management.

3. Discussion

The genetic mutation that occurs in XP patients causes a defect in the NER mechanism, and UV radiation from sun exposure will cause DNA damage due to the defective repair mechanism, changes in skin cells, and formation of carcinogenic photoproducts, which eventually leads to the formation of malignancies in the skin.

The patients from unrelated families in this serial case report exhibited freckle-like pigmentation on the face, trunk, and extremities since childhood. Patient IV:3, the older sister from the first family, started to show pigmentation of the skin during childhood, which worsened as time progressed. She developed ocular abnormality and malignancy on the cheek. The pigmentation was also worse in areas more frequently exposed to sunlight, such as the face, neck, upper part of the trunk, and extremities. Patient IV:4, the younger sister, also showed similar pigmentation in similar areas as her sister's that started during childhood. Her pigmentation, however, seemed milder, and she had yet to develop more severe complications like her sister. Both patients III:3 and III:5 of the second family exhibited similar symptoms of darker freckle-like pigmentation on the face, neck, upper trunk, and extremities, and complaints of palpebral mass growth.

As XP is a disease caused by the accumulation of damage over time, the milder phenotype of the younger sister may be attributed to several factors, such as age, amount of UV exposure, and the extent of the defect of the DNA repair mechanism. The amount of DNA damage accumulated due to increased photosensitivity is

Table 1. Summary of clinical findings, histopathology, and genetic analysis of all patients

Case/Family	Clinical features				Histopathological Examination	Gene mutation
	Freckle-like pigmentation	Cutaneous malignancy	Ophtalmologic abnormalities	Neurological abnormalities		
Case 1/1 (Patient IV:3)	Uneven brown spots (Localized) in sun-exposed area	Cancerous lumps (patches) on the cheek	Prolapse right eyeball, ectropion. Cataract left eye	Gait disturbance, Hearing loss	Frozen section from right eye and palpebra: melanoma maligna Biopsy sample from right cheek: suggestive of BCC metastasis. Immunohistochemistry of right cheek sample: Cytokeratine positive, Negative HMB45, Positive CD10 on the palisading tumor cells, and Positive Ber-EP4 stain. Skin tissue: Suggestive of Xeroderma Pigmentosum	Pathogenic variant of the *ERCC2* gene (Chr19(GRCh37):g.45855610G>A; NM_000400.3: c.2047C>T, p.(Arg683Trp))
Case 2/1 (Patient IV:4)	Uneven brown spots (Localized) in sun-exposed area	Cancerous lumps (patches) on the cheek	Dry eye	N/A	Skin tissue: Suggestive of Xeroderma Pigmentosum	Pathogenic variant of the *ERCC2* gene (Chr19(GRCh37):g.45855610G>A; NM_000400.3: c.2047C>T, p.(Arg683Trp))
Case 1/2 (Patient III:V)	Black spots evenly (Generalized)	Cancerous patch on the lips and palpebrae	Dry eye, cataract	N/A	N/A Passed away	N/A
Case 2/2 (Patient III:3)	Black spots evenly (Generalized)	Cancerous patch on the lips and palpebrae	Cataract, blepharitis	N/A	Skin tissue: Suggestive of Xeroderma Pigmentosum	Nonsense pathogenic variant of the *XPC* gene (NM_004628.4), c.1941T>A, p.(Tyr647*)

*Remarks: Not all examinations are available for all patients (*eg.* For Vitamin D measurement) due to socioeconomic limitations which hinders the patient to take all laboratory examinations

less at a younger age than at a more advanced age (*11*). The amount of UV exposure also plays an important role in the severity of XP as UV light induces the formation of thymine dimers from covalent linkages between consecutive pyrimidine bases along the nucleotide chain. The amount of thymine dimers increases with increased exposure to UV and coupled with defective DNA damage repair may result in a more severe clinical presentation compared with patients exposed to less UV. The extent of the defect in the NER mechanism may also affect clinical severity.

Mutations affecting the promoter regulatory elements may affect the transcription rate and may have a less severe impact in the defect of the NER mechanism when compared to mutations affecting RNA translation, such as mutations at the initiation codon, mutations causing frameshift, and mutations causing nonsense codons. Meanwhile, in a missense mutation such as that of family 1, nucleotide substitution occurred on the putative nuclear location signal of the *ERCC2* protein, thus limiting the amount of functional protein, which makes it insufficient for DNA repair (*9*). Malignancy of the skin of the palpebral and cheek, as seen in patient IV-3 of family 1 in this case report, might be the result of the defect in repairing genetic changes. In advanced cases, XP may cause malignancies such as BCC, squamous cell carcinoma, and malignant melanoma (*12*).

Approximately 40% to 80% of patients with XP will have ocular problems, which is caused by UV-induced DNA changes on conjunctival, corneal, and palpebral epithelial cells (*13*). One of the early signs of ocular involvement in XP patients is photophobia, and the most frequently found ocular abnormalities caused by XP are conjunctivitis, corneal neovascularization, and dry eyes, up to loss of sight (*14*). Long-term sun exposure can cause neoplasm of eyes and its supporting structures, including epithelioma, squamous cell carcinoma, and melanoma (*15*). Patient IV:3 developed ocular disorders due to XP with histopathological findings of malignant changes of palpebral and periocular skin, malignant melanoma of the eyeball, and ocular manifestations that arise from neurological degenerations. The skin around the eye formed cicatricial changes or skin malignancies that need to be excised. All of the patients in the case series developed ocular abnormality with varying degrees of severity, with one confirmed melanoma both clinically and microscopically.

Around 30% of all patients with XP will experience neurological deficits (*2,3,16*). The neurological deficits and problems that might occur in patients with XP include isolated hyperreflexia, progressive mental retardation, sensorineural deafness, and seizures (*17*). Neurological degeneration may manifest in the eye as a small pupillary size, nystagmus, and strabismus (*2,18*). Neurological deficit in patients with XP mostly occurs in XP-A, XP-B, XP-D, and XP-G patients (*19*). Our patient with XPD from the first family also suffered from hearing and vision loss.

Besides ocular malignancies, patient IV:3 also developed dermal malignancies in the form of BCC. This is a common occurrence in XP patients, due to the high predisposition for developing malignancies. Patients with XP have 10,000-fold risk of developing BCC and 2,000-fold risk of developing melanoma compared to normal people (*18*). Dermatological malignancies in XP patients are difficult to prevent, especially in regions with abundant amounts of sun rays such as in the Middle East and in Asia (*20*).

Advances in molecular techniques such as NGS have massively helped in diagnosing XP faster and in having a higher throughput. For example, exome sequencing for dermatological conditions comprises 619 genes, five of which (*XPA*, *XPC*, *DDB2*, *POLH*, and *ERCC2*) are relevant for XP diagnosis (*2,5,19*). Results from massively parallel sequencing for the XP gene panel revealed an *ERCC2* c.2047C>T mutation, which caused an amino acid change from arginine to tryptophan at position 683 in both daughters from the first family and a nonsense causing mutation at c.1941T>A of the *XPC* gene in the male patient of the second family. The *ERCC2* gene codes the XP group D (XPD) protein, an ATP-dependent DNA helicase that is important in the NER mechanism. A previous *ERCC2* mutation study in Vietnam with a missense mutation (c.2048G>A; p.Arg683Gln) and nonsense mutation (c.1354C>T; p.Gln452*) showed that patients experienced severe sunburn and irritation after unprotected sun exposure, hypopigmentation, dry skin, and skin peeling. Patients also experience ocular damage, photophobia, and dry eyes. The fibroblasts in the Vietnamese cohort showed a defect in the NER pathway (*1*). The defect affects the DNA repair mechanism that occurs in XP, which renders it more sensitive to UVA radiation (*21*). A study of Comorian and Pakistani cohorts revealed that the most common variant of mutation was in the *XPC* c.2251-1G>C mutation, with some patients showing the *POLH/XPV* variant (*22*). The Comorian cohort is unique because of its geographical and historical background. The Comorians live in an isolated area to avoid invasion from other populations, and their people are segregated into different villages based on social status, which leads to a high frequency of consanguineous marriages (*22,23*). Different founder variants are present in specific parts of the world. For *XPA* mutations, there are three different founder variants for India, Japan, and Tunisia. In the Indian population, the mutation that occurs related to the *XPA* gene is c.335_338delTTATinsCATAAGAAA (*11,24*). In Japan, the mutation that occurs in the *XPA* gene is c.390-1G>C, and in Tunisia, the mutation that occurs on the XPA gene is p.Arg228Ter (*11*). In North Africa, the mutation of *XPC* occurs in c.1643_1644delTG, and in an Iraqi Jewish population, the mutation of p.Arg683Gln can be found among xeroderma pigmentosum patients in that

region. *POLH* mutation can be found in a Tunisia/North African population, Japanese population, and Basque/Northern Spain population. *POLH* mutation in Tunisia occurs as the deletion of exon 10; in Japan, it occurs as c.490G>T (splice site variant); p.Ser242Ter; p.Glu306Ter and c.1661delA; and in Basque/Northern Spain, it occurs as c.764+1G>A (*11*). The pathogenic variant found on *XPC* in our patient has not been reported previously. Although mutation analysis of the parents have not been done, we have identified two families with two affected sibs and normal parents, suggesting autosomal recessive inheritance.

Management of XP is based on early diagnosis, follow-up, and minimization of unprotected sun exposure. These are important to reduce or prevent the occurrence of dermatological malignancies in patients with XP, which will improve their quality of life and increase their survival rate. Photoprotection can be achieved using UV-absorbent films on windows, UV-absorbent clothing, sunglasses, and sunscreens. However, all of the patients encountered in this report were not aware of their condition, hence the lack of a photoprotective effort. The rarity of the condition, lack of awareness, and knowledge regarding this condition played important roles in the progress of the disease for all patients. With a prevalence of 1 in 10,000 to 30,000, this condition is rarely seen, and information regarding XP is not widely available to medical care providers managing this disease. Owing to the nature of the cumulative damage, most patients do not have any serious complaints during early life and do not feel the need to check themselves until more complications are present, such as ocular, hearing, and neurological problems.

Another possible reason why the disease is often overlooked is that XP is passed down in an autosomal recessive manner, which sometimes skips generations, causing parents to be unaware of their carrier status and their affected children. Vitamin D supplementation given later during the course of the disease may not be very helpful. Genetic counseling regarding the progressivity of the diseases, pattern of inheritance, and risk of having more children with the same condition was given to the family. Because XP is inherited in an autosomal recessive manner and both families live in an isolated area, further analysis is warranted to evaluate the possibility of a founder mutation.

To the best of our knowledge, this is the first comprehensive clinical and genetic analysis report on patients with XP in Indonesia, with a novel pathogenic variant of *XPC* found in the second family. This case report highlights the importance of a rare disease that is manageable but often overlooked because of its rarity, inheritance pattern, and late onset of severe symptoms. New advances in molecular biology techniques can help diagnosis faster with a higher throughput, but they are still not available in low resource settings.

4. Conclusion

This is the first case report of XP in Indonesia that incorporates clinical examination, genetic analysis, and histopathological examination including immunohistochemistry staining, and a novel pathogenic variant of *XPC*, c1941T>A was found in the second family. Diagnosis of XP has been made using history taking, physical examination, pedigree analysis, and histopathology and mutation analysis using advanced molecular techniques.

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Address correspondence to:
Sultana MH Faradz, Division of Human Genetics, Center for Biomedical Research, Faculty of Medicine, Diponegoro University/Diponegoro National University Hospital, Jl. H Soedharto, Tembalang, Semarang 50275, Indonesia
E-mail: sultanafaradz@gmail.com

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Case Report

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Rituximab use for refractory anti-HMGCR immune-mediated necrotizing myopathy: A case report

Sonia Gupta[1,*], Amandeep Rakhra[2], Vinay Thallapally[1], Joseph Nahas[1]

[1] Department of Internal Medicine, Creighton University, Omaha, Nebraska, USA;
[2] Department of Rheumatology, Dartmouth-Hitchcock Medical Center, Lebanon, New Hampshire, USA.

SUMMARY Immunosuppression is the cornerstone therapy for anti-3-hydroxy-3-methylglutaryl-coenzyme A reductase (anti-HMGCR) myopathy. Typical immunosuppressants such as corticosteroids, methotrexate, and azathioprine have been used in conjunction with removal of the offending agent, yet the use of rituximab is more limited in this type of myopathy. Reported here is a case of a patient who responded well to rituximab (RTX) after the standard immunosuppressants had failed. This case illustrates the importance of further studies to evaluate the role of RTX in anti-HMGCR myopathy.

Keywords anti-HMGCR, immune-mediated necrotizing myopathy, immunosuppressants, rituximab

1. Introduction

Immune-mediated necrotizing myopathy (IMNM) is an autoimmune condition resulting from the direct or indirect injury of myofibers by the immune system. Anti-3-hydroxy-3-methylglutaryl-coenzyme A reductase (anti-HMGCR) myopathy is a subtype of IMNM and was first described in 2010 (*1*). It has been seen both in statin-naïve and statin-exposed patients, with a prevalence of 1-2 cases per million in those who are statin-exposed (*2*). It presents with muscle weakness, myalgia, and elevated serum creatine phosphokinase with a mean age of 55 years. Studies have found that statin-naïve patients were younger than statin-exposed patients (*3*). There are currently no guidelines for the management of anti-HMGCR myopathy. Immunosuppressants are the cornerstone of therapy, but very few studies regarding the use of rituximab (RTX) have been published - all with varying responses (*4*). Described here is a case of anti-HMGCR-associated IMNM refractory to conventional immunosuppressants but responsive to RTX.

2. Case Report

A 61-year-old female with a history of primary hypercholesterolemia, hypothyroidism, and hypertension presented with rapidly progressing proximal upper and lower muscle extremity weakness for seven days. Her symptoms were associated with muscle pain and slowly progressive dysphagia to both solids and liquids. Her medication history included taking pravastatin for two years. A physical examination revealed a grade of 3/5 on the Medical Research Council (MRC) Scale for Muscle Strength in the proximal upper and lower extremities bilaterally. Laboratory tests including a complete blood count, renal function test, and inflammatory markers (erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP)) were normal. Her creatinine kinase level was 15,000 U/L (normal, 26-192 U/L), her myoglobin level was higher than 1,000 (normal, 9-83ng/mL), and aspartate aminotransferase (309 U/L; normal: 10-40 U/L) and alanine aminotransferase (691 U/L; normal: 12-78 U/L) were elevated (Table 1). Differentials were polymyositis, statin-induced myopathy, paraneoplastic myopathy, and anti-signal recognition particle-associated myopathy.

Liver ultrasound was normal. Statin was discontinued and oral prednisone at a dose of 60 mg was started since IMNM was suspected. An initial rheumatologic work-up for autoimmune myopathies was negative including rheumatoid factor, antinuclear antibody, and anti-myositis antibodies including anti Jo1, anti Ro, anti-signal recognition particle, anti-mi-2, acetylcholine receptor antibody, and muscle-specific tyrosine kinase antibody. Pan computed tomography including the chest, abdomen, and pelvis did not reveal evidence of a malignancy. Magnetic resonance imaging of the right lower extremity revealed diffuse patchy muscular atrophy throughout the thigh with extensive patchy muscle edema involving the anterior medial and posterior compartments. The right quadriceps was biopsied, an electromyogram and anti-HMGCR antibodies were ordered, and pending biopsy results the patient was discharged on 60 mg of steroids.

Table 1. Laboratory results from the initial visit and subsequent visits

Laboratory tests	Normal reference values	On admission	28 days On the steroid azathioprine	42 days Azathioprine discontinued and rituximab initiated	56 days	84 days	112 days	168 days	365 days
Erythrocyte Sedimentation Rate	0/15 mm/hour	7							
C-Reactive Protein	≤ 9 mg/L	< 2.90	< 2.90						
Creatinine Kinase	26-192 U/L	15,000	2,040	1,300	989	837	132	114	96
Aspartate Aminotransferase	10-40 U/L	309	86		47	29	20	20	
Alanine Aminotransferase	12-78 U/L	691	447		151	49	38	36	
Total Bilirubin	0-1.5 mg/dL	0.4	0.4		0.6	0.4	0.4	0.3	
Myoglobin	9-83 ng/mL	> 1000							
Thyroid-stimulating Hormone	0.4-5 uIU/mL	2.124			2.330				
Carcinoembryonic Antigen		Negative							
Myositis-specific Antibodies		Negative							
CA-19-9		Negative							
CA 125		Negative							
Rheumatoid Factor	< 15 IU/mL	< 10							
Antinuclear Antibody		Negative							
Aldolase	1.5-8 U/L	135	63.6		6.1				

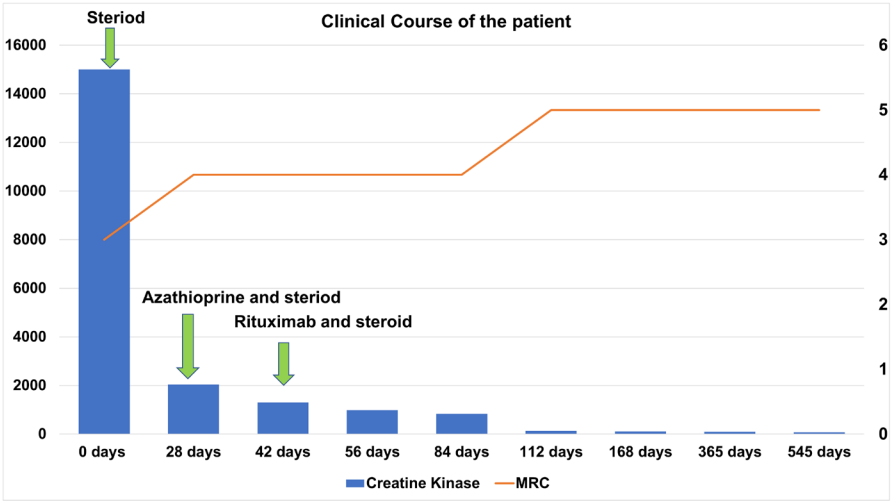

Figure 1. Clinical course after rituximab was initiated and muscle strength recovered to the baseline level after 3 months of rituximab therapy: CK levels and muscle strength on the MRC scale (data points). Less muscle strength in the hip flexors was noted on admission according to the MRC scale.

During her 4-week follow up in the rheumatology clinic, she was still complaining of weakness. Her upper arms and bilateral hip flexors were MHC grade 4/5. Her repeat creatine kinase (CK) level was around 2,000 U/L. The quadriceps biopsy revealed scattered myofiber atrophy, degeneration, regeneration, necrosis, and myophagocytosis suggestive of necrotizing myopathy. Electromyography (EMG) was performed and revealed myositis, and anti-HMG CoA reductase antibodies were positive, with a titer of 99 (normal < 20 units) suggestive of anti-HMGCR-induced myopathy. The patient was subsequently started on azathioprine (AZA) 50 mg daily.

Fifteen days later, patient complained of nausea and did not want to continue taking AZA. She still had weakness in her upper extremities. Her aspartate aminotransferase level was 82 U/L, her alanine aminotransferase level was 269 U/L, and her CK level was 1,300 U/L. The steroid dose was decreased to 30 mg, AZA was stopped, and a plan was formulated to start the patient on an RTX intravenous infusion 1,000 mg/m^2 with a second dose repeated 2 weeks later. Four weeks after RTX was initiated, her strength improved in her shoulders, fingers and toes, and proximal hip flexors. Her RTX dosage was then decreased to 375 mg/mm^2 given once every 3 months. Remission was deemed to have been achieved after six doses of RTX since the patient's CK level and aldolase level were consistently normal and she was asymptomatic. The patient was also started on alirocumab for hyperlipidemia. The patient responded well to RTX within 4 weeks of treatment initiation and went into complete remission within 3 months of initiation of RTX, with normal CK levels and no relapse (Figure 1). She subsequently tolerated her maintenance therapy for one year with no complications. She is currently symptom-free.

3. Discussion

Anti-HMGCR myopathy is a rare subtype of severe necrotizing autoimmune myopathy (*5-7*). There are few prospective studies and clinical trials in the literature to guide therapy for this specific myopathy. The regimen mainly depends on disease severity and a discussion between the rheumatologist and the patient. Prompt discontinuation of statin therapy and immunosuppressants are the initial treatment for statin-induced IMNM. Common immunosuppressive that have been shown to be effective are corticosteroids, intravenous immunoglobulin (IVIG), AZA, methotrexate (MTX), and mycophenolate mofetil (*5,9,10*). MTX or IVIG have been used for refractory cases, with both resulting in improvement (*3,10-12*). The postulated mechanism for anti-HMGCR myopathy includes autoimmunity against HMGCR; since B cells play a big role in autoantibody production, B cell depletion would appear to be an effective treatment strategy for the condition (*2*). Experience with RTX is limited. In 2016, the European Neuromuscular Centre (ENMC) convened a workshop on the definition and treatment of IMNM for anti-HMGCR myopathy. The experts reached the consensus that patients with anti-HMGCR myopathy should be treated with both corticosteroids as well as a second-line agent either immediately or within 1 month of presentation, depending on the severity of disease and the response to steroid treatment. They suggested RTX as a possible alternative and indicated that it could be combined with MTX in severe cases (*13*).

There are very few studies on RTX response in refractory anti-HMGCR IMNM. Landon-Cardinal *et al.* conducted a retrospective study in which 9 out of 46 patients with anti-HMGCR IMNM received RTX after failing to respond to an average of three other immunosuppressants. All patients presented with proximal muscle weakness and a mean CK level 1,602 IU/L prior to RTX. Three out of nine patients on RTX had improved muscle strength and improved MRI findings. The most striking effect of RTX was seen in two statin-naïve younger patients, and the response was seen in the first month after starting the therapy. RTX was also helpful in weaning two patients off of corticosteroids and IVIG medication. All three patients were in remission prior to the last follow up. One patient who received RTX early in the course of the disease did not respond even after five months of treatment but was then successfully treated with CS, IVIG, and AZA (*4*).

Ashton *et al.* (*14*) described a case series of twenty patients with anti-HMGCR myopathy and a mean initial CK level of 7,189 U/L. RTX was initiated in five patients who failed to respond to prednisolone, MTX, AZA, and IVIG. Improvement was seen in two patients treated with RTX. Those two patients were also statin-naive younger patients, so results were similar to those reported by Landon-Cardinal *et al.* (*4*). There are a few other case series in which RTX was used to treat patients with refractory anti-HMGCR but it resulted in no benefit (*10,15*).

The current patient improved slightly with oral steroids and according to tests but did not go into remission. AZA was used for few days and was stopped due to adverse reactions. RTX was ultimately started. The patient started responding within one month of treatment and went into complete remission within 3 months of initiation of RTX, with normal CK levels and a normal MRC grade; the patient has not relapsed.

4. Conclusion

RTX was effective in the current patient with refractory anti-HMGCR myopathy. The patient's response to RTX leads to questions like why statin-naïve patients respond better, why it does not beneficial all the patients if autoimmunity is the cause, and whether RTX can be used in the initial course of disease to minimize that course. In addition to a few cases reported in the literature, the current case highlights the need for a prospective randomized trial to confirm the effectiveness of RTX in anti-HMGCR myopathy.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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**Address correspondence to:*
Sonia Gupta, Department of Internal Medicine, Creighton University, 7710 Mercy Road #3000, Omaha, Nebraska 68124, USA.
E-mail: sgu27944@creighton.edu

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Case Report

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Saccharopinuria accompanied by hyperammonemia and hypercitrullinemia presented with elderly-onset epilepsy, progressive cognitive decline, and gait ataxia

Ryohei Norioka[1,*], Shinsuke Tobisawa[1], Ryusei Nishigori[2], Tomiko Kuhara[3], Masahide Yazaki[4], Masayoshi Nagao[5], Toshihiro Ohura[6], Yasuyuki Takai[7], Asuka Funai[1], Kazuhito Miyamoto[1], Akihiro Kawata[1], Kazushi Takahashi[1]

[1] Department of Neurology, Tokyo Metropolitan Neurological Hospital, Tokyo, Japan;
[2] Department of Neurology, Kyoto University Graduate School of Medicine, Kyoto, Japan;
[3] Japan Clinical Metabolomics Institute, Ishikawa, Japan;
[4] Neurohealth Innovation Division, Institute for Biomedical Sciences, Shinshu University, Matsumoto, Japan;
[5] Department of Pediatrics, Hokkaido Medical Center, Hokkaido, Japan;
[6] Division of Clinical Laboratory, Sendai City Hospital, Sendai, Japan;
[7] Department of Ophthalmology, Juntendo University Urayasu Hospital, Tokyo, Japan.

SUMMARY We report a case of saccharopinuria with hyperammonemia and hypercitrullinemia in a Japanese woman who presented with elderly-onset epilepsy, progressive cognitive decline, and gait ataxia. Blood amino acid analysis revealed an increase in citrulline, cystine, and lysine levels, and urine amino acid analysis showed increased citrulline and cystine levels. Urine metabolomics revealed an increased saccharopine level, leading to the definitive diagnosis of saccharopinuria. In western blots of liver biopsy samples, normal citrin levels were observed, suggesting that adult-onset citrullinemia type 2 (CTLN2) was not present. In addition, decreased argininosuccinate synthetase (ASS) levels were observed, and *ASS1* gene, a causative gene for citrullinemia type 1 (CTLN1), was analyzed, but no gene mutations were found. Because the causes of hypercitrullinemia were not clear, it might be secondary to saccharopinuria. Muscle biopsy findings of the biceps brachii revealed diminished cytochrome c oxidase (COX) activity, mitochondrial abnormalities on electron microscopy and p62-positive structures in immunohistochemical analyses. Saccharopinuria is generally considered a benign metabolic variant, but our case showed elevated lysine and saccharopine levels causing ornithine circuit damage, mitochondrial dysfunction, and autophagy disorders. This may lead to so far unknown neurological disorders.

Keywords saccharopinuria, hyperammonemia, hypercitrullinemia, metabolomics, elderly-onset neurological disorders

1. Introduction

Familial hyperlysinemia is an autosomal recessive disease caused by a defect in the bifunctional alpha-aminoadipic semialdehyde synthase (AASS) protein. AASS includes lysine-ketoglutarate reductase (LKR) and saccharopine dehydrogenase (SD) (*1,2*). A variant of familial hyperlysinemia, saccharopinuria (hyperlysinemia type II), has been described in which only SD activity was undetectable (*3*). While saccharopinuria is generally considered a benign metabolic variant, there are some case reports that describe that saccharopinuria exhibits neurological features such as epilepsy and intellectual impairment, and all of these cases were infant-adolescent cases as far as we know (*4*). On the other hand, hypercitrullinemia is caused by citrullinemia type 1 (CTLN1) with argininosuccinate synthetase (ASS) deficiency and adult-onset citrullinemia type 2 (CTLN2) with citrin deficiency (*5,6*). CTLN1 and CTLN2 can cause neurological symptoms such as epilepsy and consciousness disturbance.

Herein, we report a rare case of saccharopinuria complicated with hyperammonemia and hypercitrullinemia presenting epilepsy, progressive cognitive decline, and gait ataxia.

2. Case Report

A 70-year-old woman was admitted to our hospital with a chief complaint of generalized convulsion (Figure 1).

Her medical history revealed that she had at least two episodes of emergency transport due to decreased level of consciousness for unknown reasons from around 60 years of age. In addition, she reported a history of progressive ataxic gait. Her past medical history described no particular illness. Her parents were not consanguineous and none of her family members had symptoms similar to hers. She had no specific food preferences.

She had been admitted to our hospital for the first time at 69 years of age to determine the cause of the intermittent decline in consciousness level. She was relatively short (height 134 cm) and clinically obese (weight 58.0 kg, body mass index 32.3). Neurological examination performed when her consciousness was clear revealed cognitive decline (Wechsler Adult Intelligence Scale-III, 68), ataxic gait, and deep sensory deficit in her lower limbs. Flapping tremor was not detected. In blood tests, hyperammonemia (75 μg/dL) and abnormal glucose tolerance (HbA1c, 6.5%) were observed. Metabolic disorder was suspected owing to hyperammonemia. Further blood amino acid analysis showed increased levels of citrulline (206.2 nmol/mL, normal range 17.9-48 nmol/mL), cystine (188.5 nmol/mL, normal range 4.7-34.8 nmol/mL), and lysine (1,170.4 nmol/mL, normal range 125.7-281.9 nmol/mL). In addition, urine amino acid analysis showed increased levels of citrulline (396.5 μmol/g·cre, normal range 2-41 μmol/g·cre) and cystine (9,546.4 μmol/g·cre, normal range 13-76 μmol/g·cre). Brain T2-weighted magnetic resonance imaging (MRI) revealed high intensities in the bilateral middle cerebellar peduncles and bilateral precentral gyrus (Figure 2). Based on these results, CTLN2 was suspected, as were fragile X syndrome, mitochondrial disorders, and spinocerebellar ataxias (SCA).

All-exon sequencing of the *SLC25A13* gene, which is a causative gene for CTLN2, was performed, but no gene mutations were found. Genetic tests for fragile X syndrome (*FMR1*), mitochondrial disorders (A1555G,

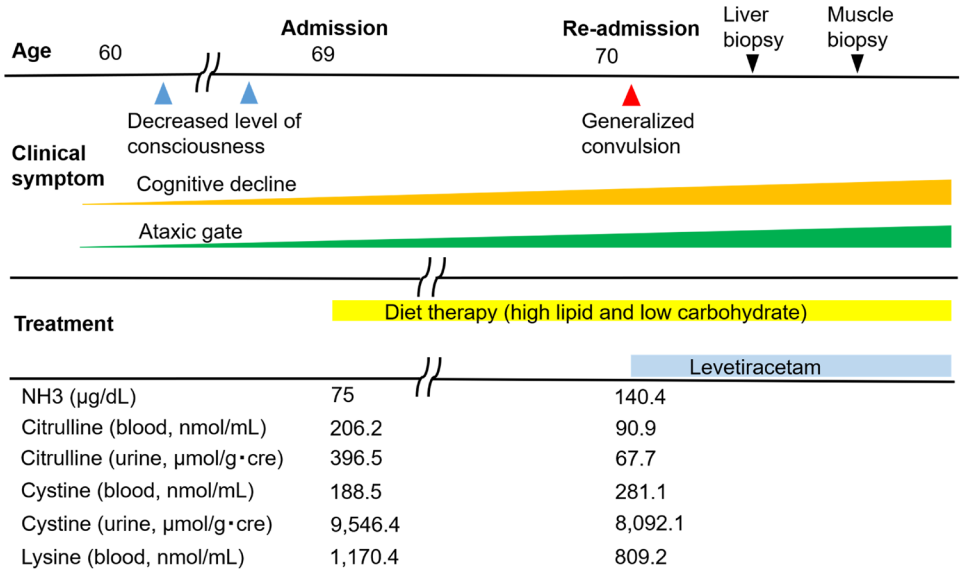

Figure 1. Clinical course of an elderly woman with saccharopinuria accompanied by hyperammonemia and hypercitrullinemia. Since around 60 years of age, the patient had at least two episodes of emergency transport owing to an unexplained decreased level of consciousness. At 69 years of age, she was admitted to our hospital for the first time to identify the cause of the unexplained decreased level of consciousness. At 70 years of age, she was readmitted due to seizures. She was diagnosed with saccharopinuria by metabolomics.

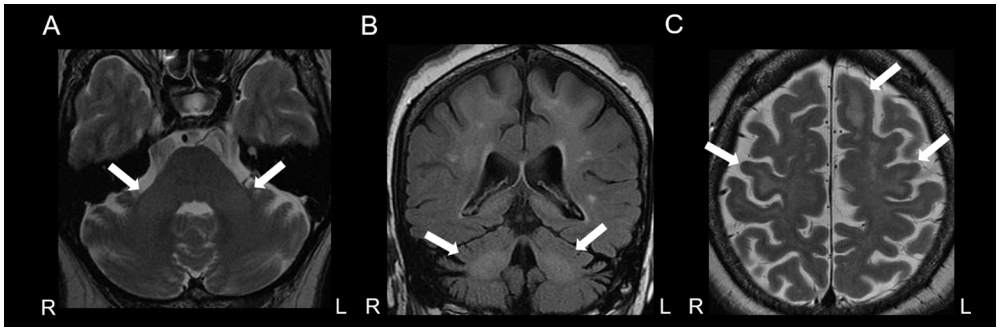

Figure 2. Brain MRI findings for an elderly woman with saccharopinuria accompanied by hyperammonemia and hypercitrullinemia. (A-C) Brain T2-weighed MRI and fluid-attenuated inversion recovery imaging show high-intensity lesions in the bilateral middle cerebellar peduncles and bilateral precentral gyrus (white arrows). MRI, magnetic resonance imaging.

T3271C, A8344G, T8363A, T8993G/C, T9176C, G11778A, G13513A, and A3243G), and common SCA in the Japanese population (SCA1, SCA2, SCA3, SCA4, SCA6, SCA7, SCA8, SCA12, SCA17, SCA31 and dentatorubral-pallidoluysian atrophy) also yielded negative results. Without a definitive diagnosis, the only clinical support that could be provided was moderate dietary guidance (high lipid and low carbohydrate).

At 70 years of age, she was readmitted owing to the development of generalized convulsions. Her cognitive decline had progressed since the last hospitalization. As with the first hospitalization, blood tests showed hyperammonemia (140.4 μg/dL), and blood amino acid analysis showed increased levels of citrulline (90.9 nmol/mL), cystine (281.1 nmol/mL), and lysine (809.2 nmol/mL). In addition, urine amino acid analysis showed increased levels of citrulline (67.7 μmol/g·cre) and cystine (8,092.1 μmol/g·cre). Urine organic acid analysis showed no increase in orotic acid level and uracil excretion, and blood amino acid analysis showed that arginine was within the normal limits. Further testing to determine the cause of her hyperammonemia was conducted, but no indication of gastrointestinal diseases, such as portal venous circulation shunts and cirrhosis, was discovered. Although a liver biopsy was performed, citrin was detected, which suggested that CTLN2 was not present. However, we observed a decrease in ASS (Figure 3). All-exon sequencing of the *ASS1* gene, which is a causative gene for CTLN1, was performed, but no gene mutations were found.

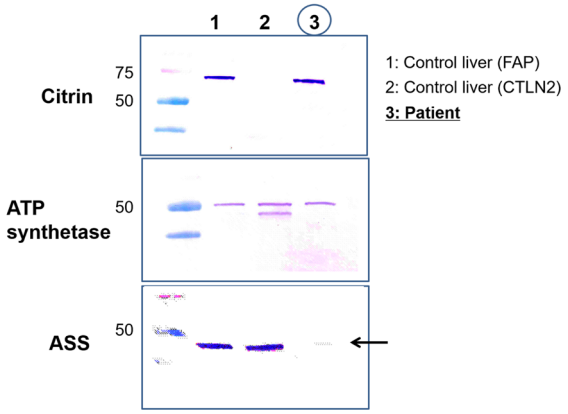

Figure 3. Western blot of liver biopsy. 1 = control liver (FAP), 2 = control liver (CTLN2), 3 = our patient. In our patient, citrin was detected, suggesting the absence of CTLN2. However, a decrease in ASS was observed (a black arrow). FAP, familial amyloid polyneuropathy; CTLN2, adult-onset citrullinemia type 2; ASS, argininosuccinate synthetase; ATP, adenosine triphosphate.

A muscle biopsy sample of the biceps brachii was taken, and structures positive for periodic acid Schiff (PAS) staining were found in the muscle fiber cytoplasm. The nicotinamide adenine dinucleotide dehydrogenase-tetrazolium reductase (NADH-TR) stained sections showed defective areas with a moth-eaten appearance, as well as an irregular intramyofibrillar network. The cytochrome c oxidase (COX)-stained sections showed diminished COX activity. Structures positive for p62 staining were found in the muscle fiber cytoplasm. Electron microscopy showed marked mitochondrial proliferation of different sizes (Figure 4). Filter paper blood screening for Pompe disease yielded normal acid alpha-glucosidase (GAA) activity.

Blood and urine amino acid analysis had been performed three times in total, and reproducibility of the tests was confirmed. For the first and second tests, blood

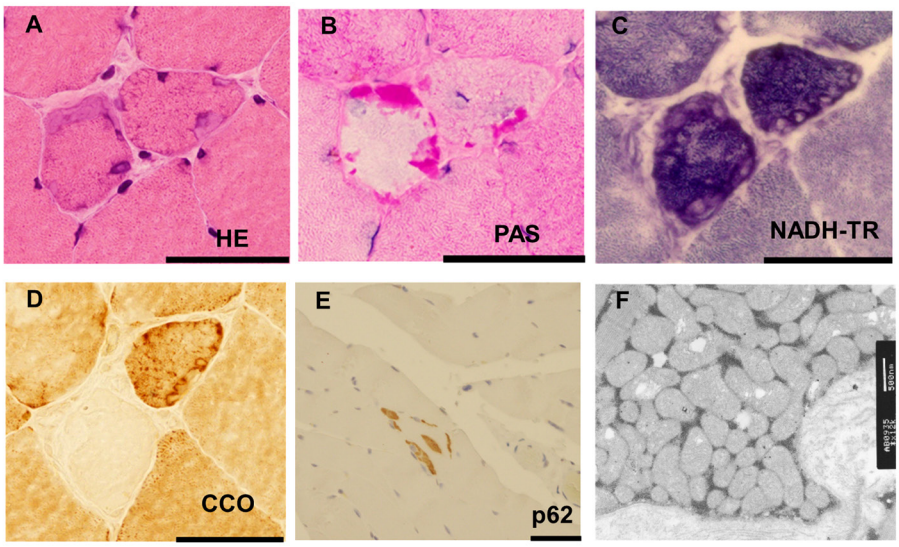

Figure 4. A muscle biopsy of the biceps brachii. (A) HE stain. **(B)** Structures positive for PAS staining were found in the muscle fiber cytoplasm. **(C)** The NADH-TR-stained sections showed defective areas with a moth-eaten appearance, as well as an irregular intramyofibrillar network. **(D)** The COX-stained sections showed diminished COX activity. **(E)** Structures positive for p62 staining were found in the muscle fiber cytoplasm. **(F)** Electron microscopy showed marked mitochondrial proliferation of different sizes. Scale bar 50 μm in (A)-(E). HE, hematoxylin and eosin; PAS, periodic acid Schiff; NADH-TR, nicotinamide adenine dinucleotide dehydrogenase-tetrazolium reductase; COX, cytochrome c oxidase.

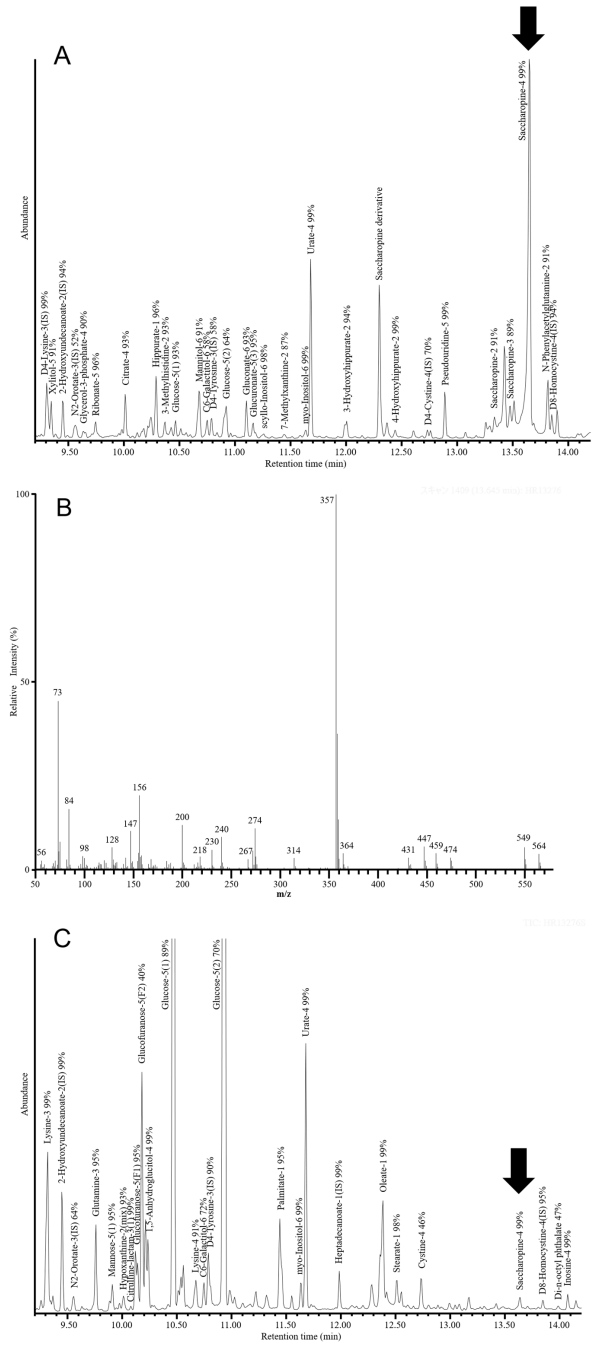

Figure 5. The total ion current chromatogram obtained by GC/MS-based metabolomics of spot urine sample from a 70-year-old female patient. (A) A huge peak was detected at retention time of 13.65 min and the component was identified as saccharopine from the patient sample (a black arrow). **(B)** The mass spectrum of the prominent peak at 13.65 min. **(C)** The total ion current chromatogram obtained by GC/MS-based metabolomics of serum from the same patient. A peak was also detected at retention time of 13.65 min and the mass spectrum of the component was identical to saccharopine (a black arrow). Hyperlysinemia was clearly shown from the peak at 9.314 min (lysine 3-trimethylsilyl derivative). GC, gas chromatography; MS, mass spectrometry.

and urine were analyzed simultaneously. In all cases, citrulline, cystine, and lysine levels were increased in the blood, and citrulline and cystine levels were increased in the urine.

Considering the possibility of hyperlysinemia, metabolomics was performed. Sample preparation and gas chromatography (GC)/ mass spectrometry (MS) measurement were performed as described previously (*7,8*). In Figure 5, the total ion current chromatogram obtained by GC/MS-based metabolomics of spot urine sample from the patient is shown. A huge peak was detected at retention time of 13.65 min (Figure 5A) which is not seen in control subjects or patients with other inborn errors of metabolism. The mass spectrum of this peak is shown in Figure 5B. This component was identified as saccharopine 4-trimethylsilyl derivative because the retention time and the mass spectrum of this peak were the same as those of authentic saccharopine. In Figure 5C, the total ion current chromatogram obtained by GC/MS-based metabolomics of serum from the same patient is shown. Although the component of the peak at 13.65 min had the same mass spectrum of saccharopine 4-trimethylsilyl derivative (as shown in Figure 5B), it was clear that the urine is superior to the serum for the detection of saccharopinuria. Serum lysine was markedly high (+8 standard deviation) suggesting that she had severe hyperlysinemia. Therefore, we diagnosed the patient with saccharopinuria.

3. Discussion

We derived two important findings from the present case. First, elderly-onset epilepsy and progressive cognitive decline and gait ataxia can occur with saccharopinuria accompanied by hyperammonemia and hypercitrullinemia. Second, metabolomics is particularly useful for the diagnosis of saccharopinuria.

Previously, saccharopinuria was thought to present no neurological symptoms, but this issue is now controversial. There are some reports that describe that saccharopinuria may cause neurological symptoms (*4*), which are now corroborated by this report. The patient's complicating hypercitrullinemia might also have affected her symptoms. CTLN2 is an adult-onset hypercitrullinemia, but it was ruled out diagnostically in this case, as citrin was detected and the genetic test yielded negative results. An enzyme deficiency in ASS, the cause of CTLN1, was recognized in our patient. CTLN1 includes an acute neonatal form and a milder late-onset form (*9*). In view of the possibility of milder late-onset CTLN1, genetic analysis of *ASS1* was performed and was negative. In contrast, saccharopinuria has been reported with and without hypercitrullinemia (*10,11*). There are reports that saccharopine and lysine may inhibit normal urea cycle function due to inhibition of ASS and argininosuccinate lyase (ASL), and cause hypercitrullinemia (*12*), as was also the case with our patient. Urea cycle dysfunction may also have led to hyperammonemia because no other cause was identified.

In addition, it had been reported that elevation of saccharopine caused abnormalities in mitochondrial

function (*13,14*), and it had been demonstrated that lysine and saccharopine suppress autophagic-proteolysis through the Akt pathway (*15*). In this case, muscle biopsy findings revealed diminished COX activity, mitochondrial abnormalities on electron microscopy and p62-positive structures in immunohistochemical analyses. It is known that autophagy disorders cause p62 accumulation (*16*). Mitochondrial dysfunction and autophagic failure may have occurred due to saccharopinuria and may be associated with neurological symptoms.

In this case, metabolomics was particularly useful for the diagnosis of saccharopinuria. The patient's epilepsy, cognitive decline, and gait ataxia were accompanied by hyperammonemia, and metabolic disorders were suspected. Amino acid analysis, a liver biopsy and muscle biopsy did not lead to a diagnosis, but the diagnosis of saccharopinuria was soon made by metabolomics. It is important to note, regarding the results of the amino acid analysis, that cystine and saccharopine are indistinguishable chromatographically. Our case also showed elevated levels of cystine in blood and urine, which may have reflected elevated saccharopine levels. For this reason, metabolomics is extremely useful and should be performed at an early stage.

In conclusion, our case findings show that saccharopinuria with hyperammonemia and hypercitrullinemia causes elderly-onset epilepsy, progressive cognitive decline, and gait ataxia, which may be due to ornithine circuit damage, mitochondrial dysfunction, and autophagy disorders.

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**Address correspondence to:*
Ryohei Norioka, Department of Neurology, Tokyo Metropolitan Neurological Hospital, 2-6-1 Musashidai, Fuchu, Tokyo 183-0042, Japan.
E-mail: ryohei.norioka10yctn@gmail.com

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Case Report

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Synchronous occurrence of breast cancer and refractory diffuse large B-cell abdominal lymphoma: Management and review of the literature

Nikolaos S. Salemis[1,2,*]

[1] Breast Cancer Surgery Unit, Army General Hospital, Athens, Greece;
[2] IASO Women's Hospital, Athens, Greece.

SUMMARY The synchronous occurrence of primary breast cancer and lymphoid tissue malignant tumors has been rarely reported in the literature. We present an exceedingly rare case of synchronous breast invasive ductal carcinoma with an abdominal diffuse large B-cell lymphoma (DLBCL). A 78-year-old woman who was diagnosed with a luminal A invasive breast cancer on core biopsy, and complaint of progressively worsening low back pain. An abdominal computed tomography (CT) scan that was performed as part of the preoperative staging showed a large abdominal mass measuring 10.5 × 4.8 × 9.5 cm surrounding the lower part of the abdominal aorta, the right common iliac, right external, right internal iliac, and the left internal iliac arteries. A CT-guided fine-needle aspiration biopsy (FNAB) of the abdominal mass was then performed, to exclude the possibility of being an abdominal tumor metastasis of the known primary breast cancer. Histopathological findings were suggestive of DLBCL. Following a multidisciplinary team discussion, chemotherapy was initiated for DLBCL. The tumor however was refractory to multiple chemotherapy regimens and exhibited a highly aggressive clinical course. The diagnostic evaluation and management of the patient are discussed, along with a review of the relevant literature. This case underscores the fact that the presence of synchronous malignancies may pose both diagnostic and treatment challenges. Accurate staging of both malignancies and multidisciplinary team discussion is of utmost importance to guide an optimal therapeutic approach. Histopathological evaluation is essential for both tumors, for the second malignancy not to be misinterpreted as a secondary deposit of the primary one.

Keywords synchronous, breast, carcinoma, diffuse lymphoma

1. Introduction

Multiple primary malignancies (MPMs) are defined as the presence of two or more primary malignant tumors in an individual patient. Based on the timing of their diagnosis, MPMs are classified as either synchronous or metachronous. Synchronous MPMs are diagnosed at the same time or within a two month-interval, whereas in metachronous MPMs, the second tumor is found more than six months after the diagnosis of the first tumor (*1*). The incidence of MPMs ranges from 0.52% to 11.2% in various series (*2-4*). In a retrospective review of 8,428 autopsies, 1,870 which were performed in cancer patients, Lee *et al.* (*5*) found that 0.8% of all autopsies and 3.6% of all cancer autopsies revealed multiple primary tumors. In another retrospective clinical study, Lv *et al.* (*2*) found a 1% incidence of MPMs among 15,683 patients diagnosed with malignant tumors in seven years. Metachronous tumors are more frequent than synchronous tumors (*3*), with a ratio of 2.7:1 (*6*). MPMs may occur at any age, but 75% of the patients are older than 50 years (*2*). The incidence of MPMs has increased in recent years (*3*). Synchronous occurrence of breast cancer with a lymphoid tissue malignancy is a very rare occurrence. Herein, a case of synchronous invasive ductal breast cancer with a refractory diffuse large B-cell abdominal lymphoma (DLBCL) is described, along with a review of the relevant literature.

2. Case Report

A 78-year-old woman presented to our Breast Clinic with a 2-month history of a left breast mass. Her past medical history was unremarkable, and she had no family history of breast or ovarian cancer.

Physical examination revealed a non-tender ill-

defined firm mass measuring approximately 2.5cm at the 9-o'clock position, 3 cm from the nipple of the left breast. Mammography showed a spiculated left breast mass in the left upper inner quadrant (Figure 1), whereas ultrasonography revealed a hypoechoic irregular mass, highly suspicious for malignancy. A core needle biopsy revealed a luminal A invasive ductal breast carcinoma. During the preoperative staging investigations, the patient had a complaint of progressively worsening low back pain radiating to the left leg. A computed tomography (CT) scan of the abdomen revealed a large soft tissue mass, measuring 10.5 × 4.8 × 9.5 cm surrounding the lower part of the abdominal aorta, the right common iliac, right external, right internal iliac, and the left internal iliac arteries with extension at L4-L5 spinal segment compressing the L4 and L5 nerve roots (Figure 2). The computed tomography scan of the chest and the remainder of the staging investigations were unremarkable.

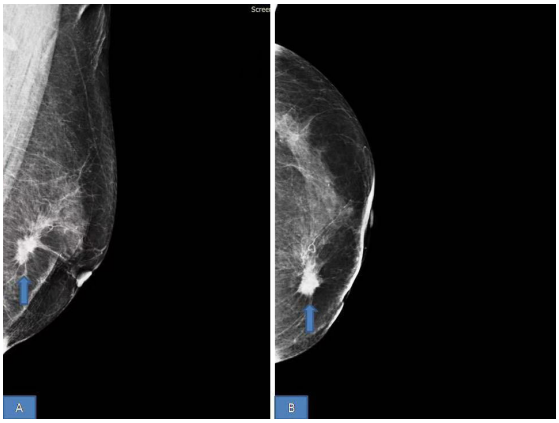

Figure 1. Left mediolateral oblique (A) and craniocaudal (B) mammograms showing a spiculated mass with slight nipple retraction (arrows).

A CT-guided fine-needle aspiration biopsy (FNAB) of the abdominal mass was then performed, which showed a diffuse large B-cell lymphoma (Figure 3). The patient underwent wide local excision of the breast mass with sentinel node biopsy. The two sentinel nodes contained macrometastases, and an axillary lymph node dissection was then performed. The histological examination revealed an invasive grade II ductal breast carcinoma measuring 2.6 × 2.1 × 1 cm according to the modified Bloom-Richardson grading system (Figure 4). On immunohistochemical analysis, the tumor cells were positive for Estrogen and Progesterone receptors, whereas the expression of Her2 was negative, and the ki-67 proliferation index was 8-10%. Two of the thirteen removed axillary lymph nodes contained metastases.

Staging investigations showed that the DLBCL was confined exclusively to the abdominal cavity. The patient was categorized into the high-intermediate risk group according to the International Prognostic Index (IPI) and revised IPI (R-IPI) categories. Soon after, treatment was initiated with the R-CHOP regimen (rituximab 375

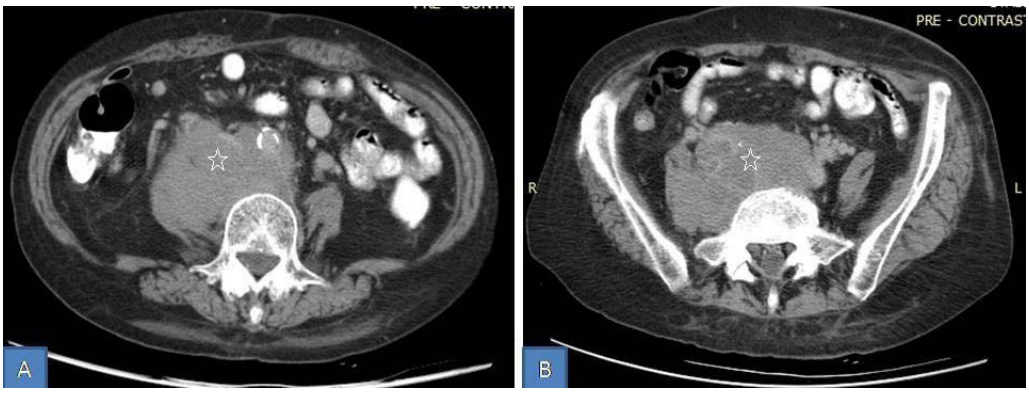

Figure 2. Abdomen CT scan showing a large mass measuring 10.5 × 4.8 × 9.5 cm extending to the pelvis (stars).

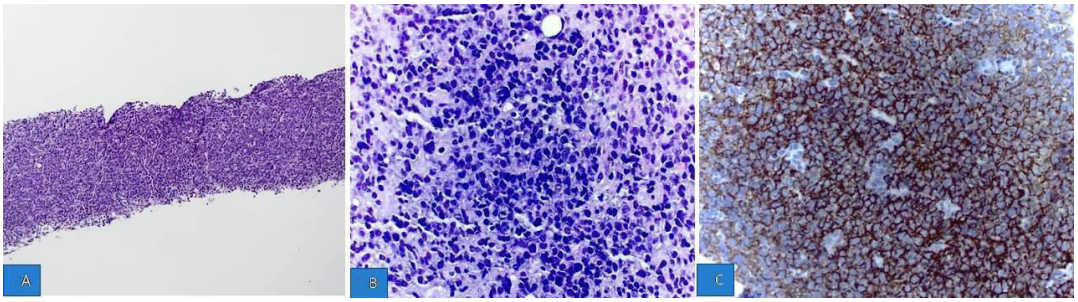

Figure 3. Histopathological findings of DLBCL. (A,B) Tumor cells are of medium and large size with irregular or oval intensely colored nuclei and one or more prominent nucleoli. A. (H-E ×100), B (H-E ×400). (C) On immunohistochemical analysis, the tumor cells were strongly and diffusely positive for CD 20 (×200).

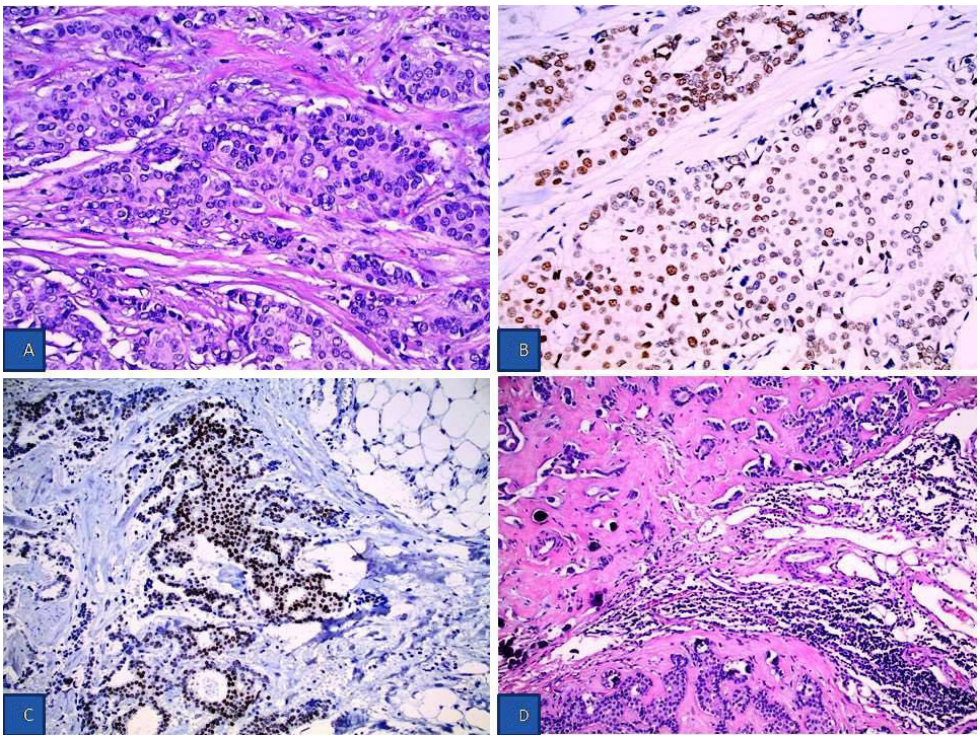

Figure 4. Histopathological findings of invasive ductal breast carcinoma. (A) Tumor cells are medium in size with weakly eosinophilic cytoplasm and include oval slightly irregular nuclei with granular chromatin small nucleus and moderate pleomorphism and atypia (H-E ×400). **(B,C)** On immunohistochemical analysis, the tumor cells stained positive for ER (H-E ×400) and PR (H-E ×200). (D) Lymph node with metastatic breast carcinoma (H-E ×200).

mg/m^2, vincristine 1.4 mg/m^2, doxorubicin 50 mg/m^2, cyclophosphamide 750 mg/m^2, and prednisolone 100 mg orally). Despite a partial remission due to the initial therapy, the tumor rapidly relapsed, and the patient received rituximab in combination with gemcitabine and vinorelbine chemotherapy (rituximab 375 mg/m^2, gemcitabine 880 mg/m^2, vinorelbine 25 mg/m^2). Despite chemotherapy, the disease progressed causing severe neurological symptoms that led to the inability to walk. Radiotherapy was then administered and resulted in improvement in neurological symptoms. Unfortunately, the intraabdominal disease continued to progress, and the patient started salvage therapy with the DICE (dexamethasone, ifosfamide, cisplatin, etoposide) regimen but again with no response. She died of progressive disease 11 months after the initial diagnosis.

3. Discussion

MPMs were first described as a clinical entity by Billroth in 1889 (*7*). However, Warren and Gates in 1962 (*8*) proposed the following specific criteria for the diagnosis of MPMs. First, the presence of clear histological evidence for malignancy in both tumors, second the tumors must be histologically distinct and third, the second tumor cannot be a metastatic tumor of the first (*8*).

Although there is an increased risk of developing a second malignancy in women treated for breast cancer, compared with the general population, however, the synchronous occurrence of a non-breast malignancy in breast cancer patients is exceedingly rare (*9*).

Breast cancer is the most frequently diagnosed malignancy after treatment for Hodgkin's Lymphoma, especially in young patients who have undergone radiation therapy. On the other hand, the development of non-Hodgkin lymphoma (NHL) after breast cancer treatment is a very rare occurrence (*10*).

SPMs in patients with breast cancer are rare. In a retrospective review, Sas-Korczyńska *et al.* (*11*) found synchronous malignancies in only 112 (0.009%) patients among 118,952 patients with breast cancer who were treated between 1965 and 2014. The most common type of synchronous primary malignancy was contralateral breast cancer (63.4%), whereas the most frequently observed non-breast synchronous malignancy derived from female genital organs (36.6%). Synchronous lymphatic tissue tumors were found in only 3.6% of the cases (*11*).

In a literature review, Woo *et al.* (*12*) found only 37 cases of synchronous breast cancer with NHL of all types. DLBCL was synchronous with breast cancer in 29.7% of the cases. Other types of concomitant NHL included mantle cell lymphoma (MCL): 8.1%, follicular lymphoma (FL): 27%, marginal zone B-cell lymphoma (MZBL): 5.4%, chronic lymphoid leukemia (CLL): 18.9%, and mucosa-associated lymphoid tissue lymphoma (MALT): 5.4%. The diagnosis of the

concomitant DLBCL was established in all patients after an axillary lymph node biopsy. It is noteworthy that not even a single case of abdominal DLCBL was mentioned in that review. The authors stated that it is not well known if synchronous tumors share a common etiology or each tumor arises independently. In the vast majority of cases (88.9%), the second malignancy had not been detected until initiation of treatment for the first tumor (*12*).

DLBCL is the most common type of Non-Hodgkin Lymphomas (*13,14*). It usually runs an aggressive clinical course characterized by rapidly enlarging lymphadenopathy and constitutional symptoms (*13*). DLBCL can involve extranodal sites such as kidneys, adrenal gland, brain, and bones. The definitive diagnosis is established by excisional lymph node biopsy. Chemoimmunotherapy with R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone) is the most common upfront treatment, resulting in a durable remission in 50-60% of the patients. However, the prognosis is poor for patients who develop disease refractory to up-front treatment (*13*). Papajik *et al.* (*15*) studied 209 NHL patients with F-18FDG PET/CT and found 6 (2.9%) suspicious cases for a second tumor. Only one of these patients had breast cancer. In 83% of the patients, the second malignancy was asymptomatic. The authors suggested that in patients with NHL, the additional imaging of lesions detected at F-18FDG PET/CT may reveal synchronous malignancies (*15*).

In a retrospective analysis of 809 DLBCL patients, Tanba *et al.* (*16*) found 123 (15.2%) cases of multiple malignancies. The majority (76.4%) developed metachronous malignancies, whereas only (23.6%) were diagnosed with synchronous tumors. Stomach cancer was the most frequent second malignancy followed by colorectal cancer, lung cancer, and breast cancer. The overall survival and progression-free survival were significantly shorter in patients having a second malignancy than in those without (*16*).

Synchronous breast cancer and malignant lymphoma is an exceedingly rare occurrence. In most cases, the malignant lymphoma was diagnosed either in the breast or in the ipsilateral axillary lymph nodes (*17-22*). Synchronous breast cancer with abdominal DLBCL is an exceedingly rare clinical entity. We were able to find only one case reported in the English literature (*9*).

The exact pathogenesis of multiple primary malignancies has not been clearly defined (*1,23*). However, it is suggested, that several potential causative factors may contribute, including intrinsic, environmental, genetic, or therapeutic factors (*24*). Intrinsic factors may include susceptibility, immune status, endocrine, and embryonic development. Environmental factors may include lifestyle, prolonged exposure to radiation or pollution. Genetic factors may include genetic mutations, whereas therapeutic factors mainly include chemotherapy and radiation therapy (*23,24*). Subramanian *et al.* (*25*) suggested that the oncogenic Epstein-Barr virus may be the causative factor for the dissemination of both invasive breast carcinoma and lymphomas through the EBNA-3C, which interacting with the Nm23-H1 metastatic suppressor protein reverses its ability to stop breast and lymphoma cell migration. Wiernik *et al.* (*20*) suggested that some patients with both breast cancer and lymphoma may share a viral etiology as a causative factor.

Our patient had synchronous breast cancer with a refractory DLBCL that showed a highly aggressive clinical course refractory to chemotherapy. It has been reported that 50% of DLBCL patients become refractory to or relapse after treatment (*14*).

In 636 patients with refractory DLBCL who were included in the international, multicohort retrospective SCHOLAR-1 study, the objective response rate was 26%, whereas the median overall survival was 6.3 months. Only 20% of patients with refractory DLBCL were alive at two years (*14*).

There is no consensus of prognostic factors for MPMs (*24*). Patients with synchronous malignancies are associated with shorter survival than patients with metachronous tumors (*2*). The presence of synchronous primary malignancies may pose a diagnostic and therapeutic dilemma (*11,12,21*). Axillary lymphadenopathy secondary to NHL can be attributed to metastases of the known breast cancer (*12,19*), whereas the second tumor should not be confused with a progression of the known primary (*17*). Especially in patients with synchronous breast cancer and NHL, the B symptoms of NHL may be mistaken for perimenopausal symptoms (*12*). A biopsy is of utmost importance to obtain a definitive diagnosis of NHL (*10*). Operable synchronous primary malignancies can be surgically treated in a single setting with less morbidity and better survival rates (*1*). The optimal treatment should be discussed in a multidisciplinary team, taking into consideration the prognosis and possibility of a curative or palliative treatment (*11*).

In conclusion, we present an exceedingly rare case of synchronous breast cancer with a refractory abdominal DLBCL and briefly review the relevant literature. The presence of multiple primary malignancies may pose a diagnostic and therapeutic dilemma. A thorough staging investigation of both malignancies and multidisciplinary team discussion is necessary to determine the optimal treatment plan. In our case, which appears to be the second reported in the literature, DLBCL was confined to the abdominal cavity and exhibited a highly aggressive clinical course.

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**Address correspondence to:*
Nikolaos S. Salemis, Breast Cancer Surgery Unit, Army General Hospital, 19 Taxiarhon Street, 19014 Kapandriti, Athens, Greece.
E-mail: nikos.salemis@gmail.com

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Case Report

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New insights on fibrodysplasia ossificans progressiva: discussion of an autoptic case report and brief literature review

Vittorio Bolcato[1], Claudia Carelli[1,*], Silvia Damiana Visonà[1], Marcella Reguzzoni[2], Maja Di Rocco[3], Alessandra Radogna[1], Livio Pietro Tronconi[1,4], Matteo Moretti[1]

[1] Department of Public Health, Experimental and Forensic Medicine, Forensic Science Section "Antonio Fornari", University of Pavia, Pavia, Italy;
[2] Department of Medicine and Surgery, Insubria University, Varese, Italy;
[3] Rare Diseases Unit, Istituto Pediatrico Giannina Gaslini, Genoa, Italy;
[4] IRCCS Fondazione Casimiro Mondino, Pavia, Italy.

SUMMARY Fibrodysplasia ossificans progressiva (FOP) is a rare genetic condition with soft tissue progressive ossification, leading to severe disability. We describe a 27-years-old female affected by FOP who died after a fall. An autopsy was performed. Upper and lower extremities resulted in fixed flexion, with kyphoscoliosis of the spine and chest wall deformity. Moreover, a cranial fracture was pointed out. At histology, atypical abundance of *corpora amylacea* in gray matter was observed. In a sample of macroscopically non-affected muscular tissue, small areas with necrosis of myocytes and hyperplasia of fibroblasts were seen in light microscopy, with intracellular inorganic dystrophic inclusions in transmission electron microscopy. Thyroid gland histology showed diffuse lymphocytic infiltration. Postmortem examination of FOP patients provided precious information about involvement of other tissues, suggesting an initial and widespread inflammatory/dystrophic phase, to be further investigated, because it might reveal new insights about a FOP mutation cascade.

Keywords fibrodysplasia ossificans progressiva, thyroiditis, fibrosis, *corpora amylacea*, transmission electron microscopy

1. Introduction

Fibrodysplasia ossificans progressiva (FOP) is a rare (1/2,000,000 inhabitants) autosomal dominant disorder, characterized by progressive bone formation in soft tissues, due to the mutation of the activin receptor 1/activin receptor-like kinase 2 (ACVR1/ALK2, c.617 G>A, R206H) gene encoding for bone morphogenetic protein type-1 Receptor (BMPR1) (*1-5*). The hyperactivity and widespread dysregulation of the downstream bone morphogenetic protein (BMP) signaling pathway, mainly due to the receptor alteration, causes progressive heterotopic ossification of soft connective tissues (*6,7*). Heterotopic ossification in FOP patients takes place in two phases: inflammation and destruction of connective tissues (phase 1) and bone formation (phase 2). This is further divided into three sub-stages: fibroproliferation and angiogenesis (2A), chondrogenesis (2B), and osteogenesis (2C) (*8*). Moreover, bone morphogenetic proteins, as pleiotropic growth factors, have also important functions in cell proliferation, migration and differentiation. The ossification of muscles, tendons, ligaments and other connective tissues, in association with congenital skeletal malformations, leads to a severe reduction of joint mobility and therefore to severe disability (*9*). Literature data reports a median age of death of 40 years (*3,10*). The most frequently causes of death in FOP patients are thoracic insufficiency syndrome, recurrent respiratory infections or accidental traumas.

The aim of this study is to present postmortem findings in a FOP case, which provides novel insights about the FOP pathological picture. We believe in the importance of forensic pathology also from the point of view of a deeper understanding of rare disease's pathophysiology. Indeed, postmortem data, including gross examination and histology, can add essential information to the clinical picture, providing valuable information potentially helpful in the management and treatment of patients (*11*).

2. Case Report

2.1. Patient's history

This case report describes the postmortem findings

of a 27-year-old female affected by FOP. The patient showed mobility limitations since childhood, but the diagnosis was made only at the age of 23, after she underwent orthopedic surgery for right coxo-femoral joint ossification, On this occasion the subject of a myositis ossificans was brought about. No neurological or thyroidal symptoms were reported. No anatomical nor electrocardiographic anomalies were reported. Brain MRI showed a thin T2- and FLAIR-hyperintense and T1-isointense tissue streak, without contrast enhancement, behind the brain bulb, in the foramina of Luschka and along the ventro-lateral margin of the pontine protuberance; a brainstem dysmorphism characterized by a small posterior protrusion of the *tegmentum pontis* and a very slight FLAIR-hyperintensity in the *nuclei dentati*. A genetic test confirmed the diagnosis, with classical R206H mutation. Radiological findings (CT) showed diffuse muscular calcification (Figure 1).

The patient had severe gait impairment, being able to walk only for short distances, with crutches, and she required her parents' aid in almost the totality of daily activities. The subject was found by her parents at the foot of the stairs, with head injuries and blood spread around. A cardiovascular resuscitation procedure was unsuccessfully carried out by rescuers. Due to the unclear circumstances and the report of a fall, a judicial autopsy was ordered.

2.2. Autopsy

2.2.1. External examination

Rigor mortis was difficult to evaluate due to firm rigidity caused by muscle stiffness and joint fixation. The upper extremities were diffusely and hardly fixed in flexion, abduction and intra-rotation, in particular the left elbow joint, which was fixed in flexion at 80°. There was a major head trauma, with a laceration of the scalp in the vertex region. The skull below was extensively fractured. Periorbital hematoma, as well as bruises in the left forearm, in the back side of the left hand and in the right leg, were also observed. Hand arthrogryposis was seen bilaterally. An old linear scar was seen in the right trochanteric region, due to previous hip orthopedic surgery. There was kyphoscoliosis of the spine and major deformity of the chest wall, similar to *pectus carenatus*. Hallux valgus was observed bilaterally.

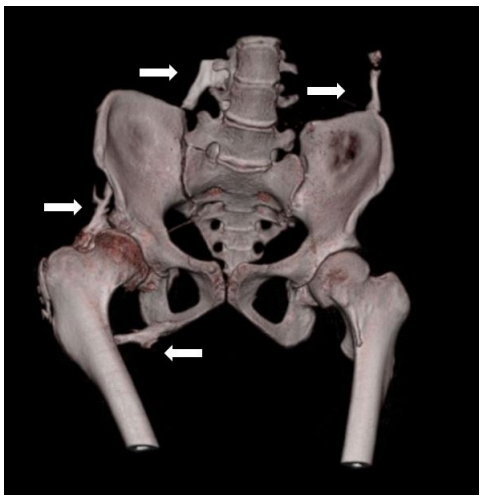

Figure 1. 3D reconstruction of pelvis joint from TC images. White arrows point to diffuse, band-like, ectopic calcifications.

2.2.2. Internal examination

A diastatic median fracture, originating from the vertex and ending next to the *sella turcica*, along the fused metopic suture and across the *anterior fossa cranica*, was observed, together with diffuse subdural and subarachnoid hemorrhage, especially in the cerebellum and around the brainstem. Ogival palate with teeth overcrowding was seen. The thoracic cavity was severely reduced in volume and diverted to the right. The spine was intact, whereas ribs were fractured on both sides (on the right: II, III, IV ribs; on the left: II and III), with hemorrhagic infiltrate. Left pectoralis major muscle's tendon was completely ossified like an accessory rib (0.7 cm in width × 3 cm in length). Both lungs showed some subpleural petechiae, edema and congestion. Heart and liver did not show macroscopic or microscopic alterations. The thyroid gland was macroscopically regular.

2.3. Light microscopy investigation

Tissues were fixed in 10% neutral buffered formalin (formaldehyde solution) for 24 hours. Samples taken from brain, heart, lungs, liver, spleen, kidney, thyroid, ovary, and *quadriceps femoris* muscle were routinely processed and stained with Hematoxylin & Eosin (H&E). Microscopic sections of the brain demonstrated recent cerebral hemorrhage and fields of initial post-mortem autolysis; moreover, in the superficial layer of the cerebral cortex, an abnormally high number of amyloid bodies was observed (Figure 2: A, H&E 200×; B, H&E 400×). Microscopic sections of the lungs showed acute emphysema and endo-alveolar hemorrhage, with areas of edema.

The thyroid gland showed diffuse lymphocytic infiltration, sometimes organized in follicles, and fibrosis (Figure 2: C, H&E 40×; D, H&E 200×). In a histological sample of muscular tissue (right quadriceps femoris), small areas with necrosis of myocytes and hyperplasia of fibroblasts were seen, defining an initial thin fibrosis (Figure 3: A, H&E 100×; B, H&E 200×; C, H&E 200×).

2.4. Transmission Electron Microscopy investigation

To study in depth the muscular histopathologic findings and to investigate possible precocious cellular

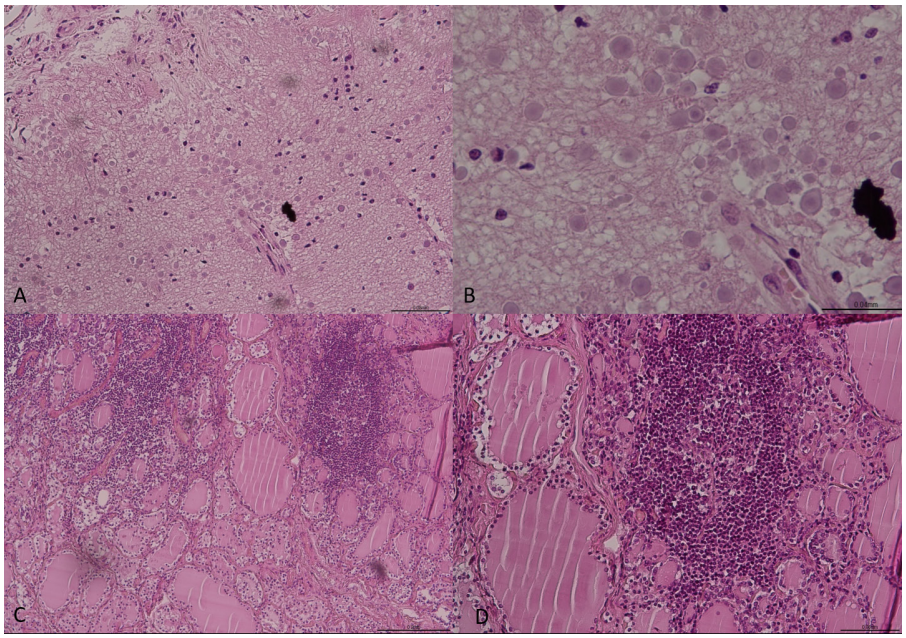

Figure 2. (A) Brain tissue under light microscopy (H&E, 200×): plenty of *corpora amylacea* in the superficial layer of the cerebral cortex. Top left a section of subarachnoid blood vessel; **(B) Brain tissue under light microscopy (H&E, 400×):** plenty of *corpora amylacea* in the superficial layer of the cerebral cortex; **(C) Thyroid under light microscopy (H&E, 40×):** lymphocytic infiltration surrounding follicles, filled with colloid; **(D) Thyroid under light microscopy (H&E, 200×):** detail of the lymphocytic infiltration.

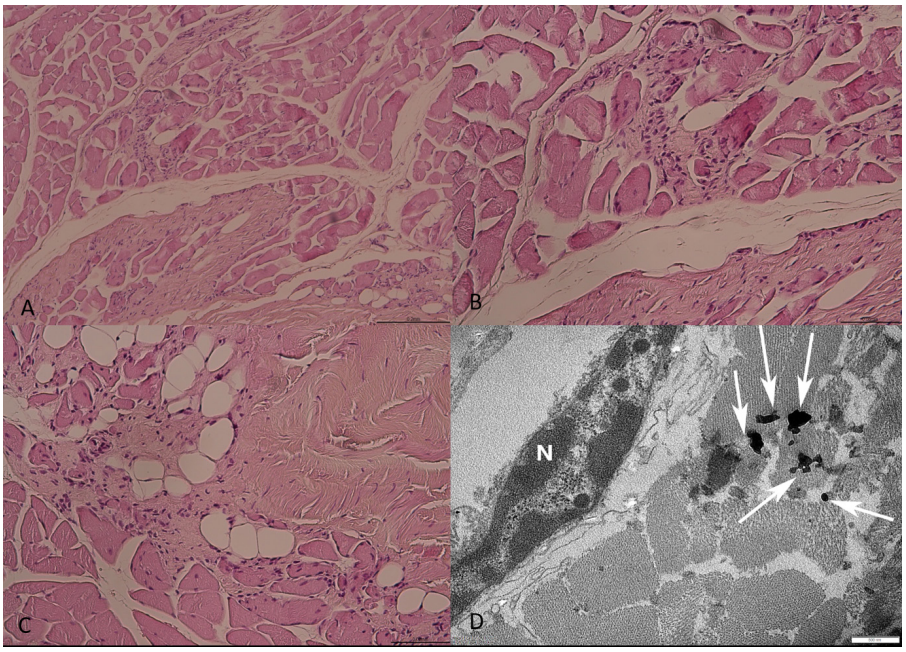

Figure 3. (A) Skeletal muscle (*quadriceps femoris*) under light microscopy (H&E, 100×): field of fibrosis in a contest of myocytes' degeneration. Mild post-mortum dissociation of the tissue; **(B) Skeletal muscle (*quadriceps femoris*) under light microscopy (H&E, 200×):** detail of fibroblast hyperplasia; **(C) skeletal muscle (*quadriceps femoris*) under light microscopy (H&E, 200×):** myocytes' degeneration with fibroblast hyperplasia; **(D) Skeletal muscle (*quadriceps femoris*) transmission electron microscopy in transverse section (18000×):** ultrastructural spatial arrangement of cytoskeletal myofibers, as thick (myosin) and thin (actin) dots. Top left: N for nucleus with nucleoli. In cytoplasm, among myofibers, white arrows point to numerous hyperdense cell's inclusions.

alterations, further ultrastructural analysis was performed on the unharmed quadriceps femoris muscle. Muscular samples were immediately fixed in 4% glutaraldehyde (in 0.1 M Na-cacodylate buffer, pH 7.4) overnight at 4 °C. After post-fixation in 2% osmium tetroxide for 1h, samples were dehydrated in an ethanol series and embedded in resin (Epon 812 mixture). Semi-thin sections were stained with Toluidine blue and observed using an Eclipse 600 microscope (Nikon, Tokyo, Japan) equipped with a TrueChrome II S digital camera system (Tucson Photonics, Fuzhou, China). Ultra-thin sections were stained with uranyl acetate and lead citrate and

observed using a Morgagni 268D TEM, Field Emission Inc, (Philips, Eindhoven, Netherlands) equipped with a Morada digital camera (Olympus, Tokyo, Japan).

In physiological transverse sections the architecture of cytoskeletal proteins and their ultrastructural spatial arrangement are evident and can be seen in Figure 3D (TEM, 18000×) as thick and thin dots, representing thick (myosin) and thin filaments (actin).

In one sample, in transverse section, among myofibers, numerous hyperdense inclusions are seen inside cells (white arrows). Inclusions appear as amorphous, inorganic debris.

3. Discussion

The cause of death of the young woman was identified as a severe head and brain trauma. The fall down the stairs was due to intense and diffuse joint rigidity causing difficulty in walking. The manner of death was classified as accidental.

The first remarkable finding that emerged from the postmortem examination is the abnormally high amount of cerebral amyloid bodies (*corpora amylacea*), absolutely unusual in young individuals. In fact, these amorphous bodies, distributed under the leptomeningeal coat or around blood vessels, are dystrophic inclusions of astrocytes typically found in aged brains, but often also in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, as well as in temporal lobe epilepsy. Similar polyglucosan bodies are found in the nervous tissue of juvenile myoclonic epilepsy, a genetic form of epilepsy called Lafora disease, and in the adult polyglucosan bodies disease (APBD), caused by the alteration of glycogen synthesis and storage (*12-14*). The patient did not show any neurological symptoms, so the clinical significance of the numerous *corpora amylacea* remains unclear. On the contrary, brainstem dysmorphism and FLAIR-hyperintensity in the *nuclei dentati* are consistent with Severino *et al.* findings, suggesting that the ACVR1/ALK2 gene mutation reverberates in the central nervous system (CNS) (*15*). Some other Authors had tried to explain the frequent but non-specific CNS anomalies as focal inflammation and demyelination (*16-18*).

The finding of muscle cell degeneration, with fibroblast hyperplasia, in macroscopically non-affected muscles, may indicate that the disease is in the 1-2A phase (necro-inflammation and fibroblast differentiation) of the endochondral ossification's process. The transmission electron microscopy evaluation reveals the presence of intracellular degenerative inclusions, as hyperdense inorganic dystrophic debris, that could represent foci of early calcification, although not typical of fibroproliferative areas (*19*). Their aspect seems to confirm the light microscopy findings of initial cellular degeneration, in a macroscopically normal muscle.

Future studies are requested to explain why the pathologic process affected only axial skeletal muscles, sparing other skeletal muscles such as the diaphragm, the tongue and the extraocular muscles; on the other hand, it is well known that smooth muscles are not involved. Some authors had also suggested the role of striated muscle microenvironment, with related specific growth factors, and muscle-restricted stem cells, with a peculiar capacity of BMP-dependent osteogenic differentiation in culture. Wosczyna *et al.* identified and characterized a population of Tie2+PDGFRa+Sca-1+ multipotent mesenchymal progenitors, that reside in the skeletal muscle *interstitium* and represent a significant cell-of-origin for heterotopic ossification in the mouse, contributing to all stages of heterotopic ossification, including the pre-cartilage mesenchyme, and suggesting that the recruitment of Tie2+ (angiopoietin receptor) progenitors in the skeletogenic pathway represent an early key event in the induction of heterotopic bone formation (*20,21*). The analysis of this abnormal evolution of soft tissue could be dramatically significative for the prevention of the rigidity and disability.

Moreover, post-natal FOP flare-ups and the variety of inflammatory cells in the FOP lesions strongly implicate an underlying immunological component. This suggests that FOP pathogenesis is more complex and inflammation-related. In fact, BMP and Activin ligands, that interact with ACVR1 signaling, have critical regulatory functions also in the immune system (*22,23*). A new article of Haviv *et al.*, which saw high levels of IL-1β, proposes even the inclusion of FOP in auto-inflammatory syndromes (*24*). The crosstalk between the morphogenetic and immunological pathways thus regulates normal tissue maintenance and wound healing. This *immunological* hypothesis aligns with the identification, even in the absence of clinical symptoms, of lymphocytic thyroiditis, as in all FOP cases described in the report of Wentworth *et al.* It is not fully understood if the ACVR1 mutation and BMP's altered function directly affect immunity regulation, or if chronic inflammation must be a reactive phenomenon of an unknown thyroid alteration, caused by the mutation cascade itself. Interestingly, BMP pleiotropic signaling is also related to papillary thyroid cancer, found in one FOP patient in Wentworth's report and not in the present case, and that BMP is linked with epithelial-mesenchymal transition and cell regulation (*25-27*), as to say that an altered BMP pathway could induce an anomalous proliferation of thyroid cells, recruiting *editing* T lymphocytes (chronic thyroiditis), and eventually leading to neoplasia.

All the classical features of FOP disease, together with atypical features, reported in the literature, are briefly reviewed in Table 1. Among the unusual findings, neurological and thyroid alterations were mentioned and deserve special attention. For Kaplan *et al.* all the classical and common variable features of FOP, as well as many of the atypical features evaluated in his

Table 1. Summary of the features of FOP disease reported in literature

Features	Chile (*n* = 1) (*Ref. 17*)	Colombia (*n* = 2) (*Ref. 29*)	Spain (*n* = 24) (*Ref. 30*)	China (*n* = 72) (*Ref. 1*)	California (*n* = 3) (*Ref. 3*)	Italy (*n* = 1) Our patient
Genetic variant p.R206H on gene ACVR1	1/1	2/2	14/16	70/72	3/3	1/1
Osteo-skeletal abnormalities						
First toe malformation	1/1	2/2	21/24	70/72	3	1/1
Heterotopic ossification	1/1	2/2	24/24	72/72	3/3	1/1
Scoliosis	1/1	2/2	7/24	NR	3/3	1/1
Ankylosis	1/1	NR	2/24	72/72	3/3	1/1
Micrognatia	1/1	2/2	NR	NR	NR	0
Neuronal abnormalities						
White matter anomalies (at MRI)	1/1	NR	NR	NR	1/3	1/1
CNS cancer	NR	NR	NR	NR	0	0
Corpora amylacea	NR	NR	NR	NR	NR	1/1
Neurological symptoms						
Intellectual disability	1/1	2/2	1/24	NR	NR	0
Epilepsy	NR	NR	NR	NR	NR	0
Migraine	NR	NR	NR	NR	2/3	0
Deafness	NR	0	7/24	12/72	0	0
Thyroid alterations						
Lymphocytic thyroiditis	NR	NR	NR	NR	3/3	1/1
Thyroid papillary cancer	NR	NR	NR	NR	1/3	0
Alopecia	0	0	5/24	NR	NR	0
Teeth anomalies	0	2/2	4/24	NR	NR	1/1
Renal agenesia	1/1	NR	NR	NR	0	0
ECG conduction anomalies	NR	NR	NR	NR	NR	0

NR: not reported; 0: not present.

study, could plausibly be ascribed to dysregulation of the ACVR1 signaling pathway, responsible at the very beginning of a widespread dystrophic/dysmorphic phase (*28*).

4. Conclusion

In conclusion, it appears that the influence of mutated ACVR1 is not restricted to soft tissues, and the effects of alteration in this pathway has to be more thoroughly investigated. Forensic studies could significantly improve the comprehension of the disease physiopathology, identifying precocious tissue alterations and allowing earlier diagnosis with better patient management. Thanks to the new law no. 10/2020, FOP patients' body donation in Italy must be encouraged.

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**Address correspondence to:*
Claudia Carelli, Department of Public Health, Experimental and Forensic Medicine, Forensic Science Section "Antonio Fornari", University of Pavia, via C. Forlanini 12, 27100 Pavia, Italy.
E-mail: claudia.carelli01@gmail.com

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Icatibant promotes patients' behavior modification associated with emergency room visits during an acute attack of hereditary angioedema

Daisuke Honda[1], Isao Ohsawa[1,2,*], Satoshi Mano[1], Hisaki Rinno[1], Yasuhiko Tomino[1,3], Yusuke Suzuki[1]

[1] Department of Nephrology, Juntendo University Faculty of Medicine, Tokyo, Japan;
[2] Nephrology Unit, Internal Medicine, Saiyu Soka Hospital, Saitama, Japan;
[3] Medical Corporation SHOWAKAI, Tokyo, Japan.

SUMMARY Hereditary angioedema due to C1-inhibitor (C1-INH) deficiency (HAE-C1-INH) induces an acute attack of angioedema. In 2018, icatibant available for self-possession and subcutaneous self-administration was licensed for on-demand treatment in addition to intravenous C1-INH administration in Japan. We retrospectively evaluated the percentage of attacks in critical parts at emergency room (ER) visits and the time until visiting ER for C1-INH administration before and after the initial prescription of icatibant. The percentage of attacks in critical parts at ER visits before the prescription was 69.2%, but that was 80.0% when patients visited ER for additional C1-INH administration after the self-administration of icatibant. The time from the onset of an acute attack to visiting ER for the additional treatment after the self-administration of icatibant significantly increased from 6.2 h to 19.2 h ($p < 0.001$). Icatibant, therefore, promoted the patients' behavior modification associated with ER visits for C1-INH administration during an acute attack of HAE-C1-INH.

Keywords C1-inhibitor, emergency room, hereditary angioedema, icatibant, Japan

Hereditary angioedema caused by C1-inhibitor (C1-INH) deficiency (HAE-C1-INH), which induces excess bradykinin production resulting in an unpredictable and recurrent acute attack of angioedema, is a rare autosomal dominant disease. The worldwide prevalence of HAE-C1-INH is 1 case per 50,000 inhabitants (*1-4*). Due to low awareness of the disease, the delay from the initial symptom of the disease to its diagnosis has been reported to be long (mean duration of 13.8-15.6 years in Japan) (*5,6*). Because HAE-C1-INH can be potentially life-threatening when severe edema develops in the upper respiratory tract and can cause unbearable abdominal pain resulting from gastrointestinal edema, the guideline of the World Allergy Organization and the European Academy of Allergy and Clinical Immunology recommends that attacks should be treated as early as possible (*7-9*).

Since 2009, there have been several approved on-demand drugs for self-possession and self-administration which enable early treatment, mainly in the European Union and the United States of America. Patients in these countries can usually select the most appropriate self-administration drug for each acute attack instead of considering whether to visit a healthcare institution for on-demand treatment (*10-15*). In Japan, on the other hand, intravenous administration of plasma-derived human C1-INH concentrate (Berinert P®, CSL Behring, King of Prussia, PA, USA) was approved in 1990 for on-demand treatment. However, self-administration of the drug has not been allowed, and it must be injected only in a healthcare institution such as emergency room (ER) by a healthcare professional. Icatibant (Firazyr®, Takeda Pharmaceutical Company, Tokyo, Japan), licensed in Japan in November 2018, is a selective bradykinin B2 receptor antagonist for subcutaneous self-administration for on-demand treatment. A number of studies and clinical trials have evaluated the medical efficacy of the drug (*14-16*). In the specific Japanese situation, however, evaluating the patients' behavior modification during an acute attack before and after the initial prescription of icatibant is also considered worthwhile from the point of quality of life, because it has not been reported in this aspect. The aim of this study was therefore to retrospectively evaluate the change in the percentage of attacks in critical parts at ER visits and the time from the onset of an acute attack

Table 1. Number of ER visits for C1-INH administration due to an acute attack before and after the initial prescription, and after the self-administration of icatibant

Pt. No.	Age	Sex	Description of icatibant in 2019	Number of ER visits before and after the initial prescription of icatibant			
				Before		After [particularly after the self-administration of icatibant]	
				All attacks	Attacks in critical parts	All attacks	Attacks in critical parts
1	49	F	January	5	0	1 [1]	1 [1]
2	44	F	January	1	0	1 [0]	1 [0]
3	42	F	January	33	25	39 [4]	35 [3]
4	41	M	March	0	0	0 [0]	0 [0]
5	40	F	March	57	39	55 [9]	43 [8]
6	78	M	March	0	0	0 [0]	0 [0]
7	69	F	March	4	2	3 [1]	3 [1]
8	35	F	April	7	6	7 [0]	3 [0]
9	38	F	April	2	2	4 [0]	4 [0]
10	41	F	May	1	1	0 [0]	0 [0]
11	50	F	May	4	3	9 [0]	0 [0]
12	69	M	May	3	3	1 [0]	0 [0]
			Total	117	81 (69.2%)	120 [15]	90 (75.0%) [12 (80.0%)]

Abbreviations: C1-INH, C1-inhibitor; ER, emergency room; Pt. no., patient number; M, male; F, female.

until visiting ER for C1-INH administration during an acute attack before and after the initial prescription, and after the self-administration of icatibant in patients with HAE-C1-INH in Japan.

Our study enrolled 12 patients with HAE-C1-INH (3 males, 9 females; mean age, 49.7 years at their inclusions) who had been prescribed icatibant for the first time between January and May 2019 at the Juntendo University Hospital in Tokyo, Japan. We conducted the study procedures in accordance with the Declaration of Helsinki, and the protocol was approved by the Institutional Review Board of Juntendo University (No. 25-325). Written informed consent was obtained from the patients.

We evaluated the number of ER visits for C1-INH administration due to an acute attack, the percentage of attacks in critical parts at the ER visits, the time from the onset of an acute attack to an ER visit, and the use of icatibant before visiting ER for each 9-month period before and after the initial prescription of icatibant. Because angioedema of the throat, tongue, mouth and neck can potentially lead to suffocation and an abdominal attack can cause severe abdominal pain, we defined angioedema occurring in these parts as attacks in critical parts.

The number of ER visits for C1-INH administration before and after the initial prescription of icatibant was 117 and 120, respectively (Table 1). Within the 120 ER visits after the initial prescription, the number of ER visits for the additional treatment after the self-administration of icatibant was 15.

The percentage of attacks in critical parts at ER visits before and after the initial prescription was 69.2% (81 cases) and 75.0% (90 cases), respectively (Table 1). In particular, ER visits for the additional treatment after the self-administration of icatibant showed a larger

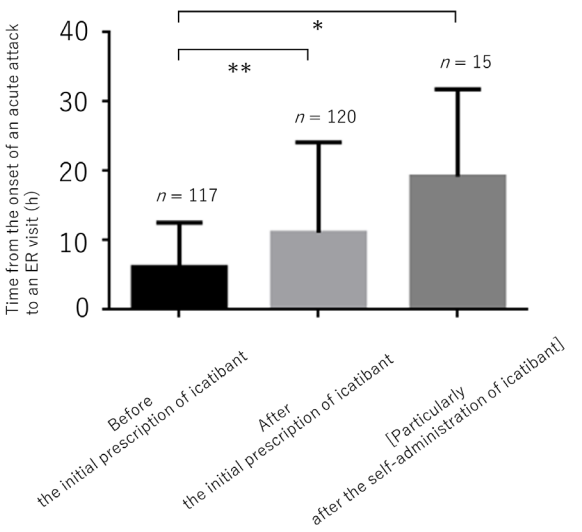

Figure 1. Time from the onset of an acute attack to an emergency room visit. The time from the onset of an acute attack to an ER visit was significantly increased from 6.2 ± 6.3 h to 11.1 ± 13.0 h after the initial prescription of icatibant ($p < 0.05$) and further extended to 19.2 ± 12.1 h after the self-administration of icatibant ($p < 0.001$), when patients needed to visit ER for C1-INH administration due to an acute attack. $^{*}p < 0.001$, $^{**}p < 0.05$. C1-INH, C1-inhibitor; ER, emergency room; h, hour; *n*, number.

percentage of attacks in critical parts (80.0%).

The time from the onset of an acute attack to an ER visit significantly increased from 6.2 ± 6.3 h to 11.1 ± 13.0 h after the initial prescription ($p < 0.05$), and especially 19.2 ± 12.1 h when patients needed to visit ER for the additional treatment after the self-administration of icatibant ($p < 0.001$) (Figure 1).

Early treatment through self-administration during an acute attack of HAE-C1-INH has been reported to reduce the need for an ER visit (*11*). However,

the number of ER visits for C1-INH administration before and after the initial prescription of icatibant did not significantly change in this study. We speculated that the causes were closely associated with the past treatment approach for HAE-C1-INH in Japan. Over many years, patients have depended heavily on the C1-INH administration for on-demand treatment in a healthcare institution and relied deeply on the treatment. Second, many patients in Japan might feel anxious when performing the self-administration, as well as patients in other countries (*17*).

After the initial prescription of icatibant, on the other hand, the percentage of attacks in critical parts at ER visits increased. In particular, the patients who visited ER for additional C1-INH administration after the self-administration of icatibant showed a larger percentage (Table 1). It suggests that patients suffered from acute attacks in more critical parts, when patients needed to visit ER for the additional treatment after the self-administration of icatibant.

In this study, the prescription of icatibant significantly increased the time from the onset of an acute attack to an ER visit (Figure 1). In addition to the efficacy of the drug, the possession itself might affect the time until an ER visit after the initial prescription, because a previous study reported that patients who carried icatibant with them gained confidence in managing their conditions associated with HAE-C1-INH attacks (*17*). Moreover, the time from the onset of an acute attack to an ER visit was further extended when patients needed to visit for additional intravenous C1-INH administration after the self-administration of icatibant (Figure 1). It suggests that the self-administration of icatibant could significantly prolong time to an ER visit, when patients who remained suffering from symptoms even after the self-administration of the drug visited ER for the additional treatment.

Japanese government, unfortunately, had to declare a state of emergency due to the Coronavirus Disease 2019 in April 2020, forcing us to break off data collection at that time, when patients' ER visiting behavior could be considered to be affected, and redefine the study duration for each 9-month period before and after the initial prescription. Furthermore, the number of ER visits due to acute attacks in the enrolled patients varied, but patients 3 and 5 (Table 1) made frequent visits to the ER. However, there was no significant correlation between the number of ER visits and the time from the onset to an ER visit (data not shown). We hope that more patients with HAE-C1-INH will be enrolled in a longer observational study.

In conclusion, when patients with HAE-C1-INH needed to visit ER for C1-INH administration in addition to the self-administration of icatibant due to an acute attack, they suffered from acute attacks in more critical parts, and the time from the onset of an acute attack to an ER visit was significantly extended.

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Address correspondence to:
Isao Ohsawa, Nephrology Unit, Internal Medicine, Saiyu Soka Hospital, 1-7-22 Matsubara, Soka city, Saitama 340-0041, Japan.
E-mail: i.osawa@saiyukai.com

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Letter

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Orphan drugs in different countries and development of new drugs to treat biliary tract cancer

Haihong Cheng[1], Di Zhou[2], Shouhua Wang[2], Jun Ding[3,*], Fei Ma[1,4,*]

[1] Department of Oncology, Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine, Shanghai, China;
[2] Department of General Surgery, Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine, Shanghai, China;
[3] Department of Biliary and Pancreatic Surgery, Shanghai Shuguang Hospital Affiliated to Shanghai University of T.C.M., Shanghai, China;
[4] Shanghai Institute for Pediatric Research, Shanghai, China.

SUMMARY Biliary tract cancer (BTC), which includes cholangiocarcinoma and gallbladder carcinoma, is a rare malignancy. Due to its low incidence, drugs treating these diseases are scarce, so they can be considered orphan drugs. The main treatment choice for BTC is chemotherapy with gemcitabine or cisplatin or combined use of both, but patients fail to significantly benefit from established chemotherapy. Advancements in immunotherapy and targeted therapy will shed light on ways to improve clinical outcomes for patients with BTC. In conjunction, more new drugs will come onto the market. This article compares the conditions for development of orphan drugs in different countries and it describes several types of new drugs that were recently approved to treat BTC.

Keywords biliary tract cancer, orphan drugs, durvalumab, fibroblast growth factor receptor

Biliary tract cancer (BTC), rare but fatal, includes cholangiocarcinoma (CC) and gallbladder carcinoma (GBC). The incidence of CC is about 0.1 cases per 100,000 to 2 per 100,000 annually depending on the geographical region. The highest incidence of GBC is 27 cases per 100,000, which was observed in women in southern Chile (*1*). Due to the low incidence of BTC, there have long been few studies on corresponding drugs to treat this disease. This is because the cost of discovering those drugs cannot be recouped since so few patients have those rare diseases. Countries around the world have introduced different laws to encourage the manufacture and development of new drugs for rare diseases. The United States enacted the Orphan Drug Act in 1983. In the following years, more than 500 orphan drugs were developed. Japan's Ministry of Health has supported the development of orphan medicines since 1985. In 2000, the European Medicines Agency (EMA) enacted orphan drug regulations to encourage the development of orphan drugs. The newly amended (in August 2019) Drug Administration Law of the People's Republic of China also provides a legal basis for the treatment of rare diseases in China (*2*). One chapter of the new Drug Administration Law stipulates that the research, development, and manufacture of drugs in short supply will be encouraged and that priority will be given to the review and approval of these drugs. Despite this, there is still a gap between China and the United States in the treatment of rare diseases. Improving the system of managing orphan drugs will be the first step to narrowing that gap.

Durvalumab (MEDI4736) is a human IgG1 monoclonal antibody targeting the PD-L1 molecule. Binding by durvalumab to PD-L1 prevents the interaction between PD-1 and PD-L1, thus activating cytotoxic T cells and stimulating an immune response to tumor cells. Durvalumab was approved by the US Food and Drug Administration (FDA) to treat urothelial cancer (in 2017) and non-small cell lung cancer (NSCLC) (in 2018) (*3*). The use of durvalumab to treat urothelial cancer was based on the results of a multicenter phase I/II clinical trial (NCT01693562)(3) in which the objective response rate (ORR) was 17.8%. Results of the PACIFIC study are a well-known foundation for use of durvalumab to treat NSCLC. The PACIFIC study was a phase III clinical trial that evaluated the efficacy of durvalumab for maintenance therapy in 713 patients with locally advanced or unresectable NSCLC. Fifty-seven percent of patients receiving durvalumab lived longer than 36 months versus 43.5% of patients receiving a placebo (*4,5*). Durvalumab had a manageable safety profile in treating NSCLC, and it had no detrimental effects on patient-reported outcomes. Another randomized, controlled, open-label, phase III trial (the CASPIAN trial) evaluated the effects of durvalumab with or without tremelimumab in combination with etoposide and either

cisplatin or carboplatin (platinum–etoposide) in patients with extensive-stage small-cell lung cancer (ES-SCLC) (*6*). The CASPIAN trial noted a significantly improved overall survival (OS) in the durvalumab plus platinum–etoposide group, with more patients alive at 12 months and 18 months.

Last year, durvalumab was approved by the FDA as an orphan drug to treat BTC. Before that, a phase I study published results of combination therapy with durvalumab and tremelimumab to treat BTC. The median overall survival (mOS) of patients who received combination immunotherapy was 10.1 months, which was higher than that (8.1 months) of patients treated with durvalumab alone. In addition, promising results announced at the ASCO indicated that patients with advanced BTC who received durvalumab and tremelimumab plus chemotherapy had an mOS of 20.7 months. In addition, two ongoing clinical trials (NCT03482102 and NCT04298008) are assessing the combined administration of durvalumab and tremelimumab or AZD6738 in patients with BTC.

BPI-43487, a small molecule inhibitor of fibroblast growth factor receptor 4 (FGFR4), was approved in December 2020 for clinical use on solid tumors such as hepatocellular carcinoma and CC with upregulated expression of fibroblast growth factor 19 (FGF19). FGFRs are members of the tyrosine kinase family, and mutation or overexpression of FGFRs is found in multiple types of cancer. An inhibitor of FGFR1-3, infigratinib, was also approved for clinical research on treatment of advanced solid tumors including BTC, gastric cancer or adenocarcinoma of the gastroesophageal junction, and other tumors with FGFR mutations. Moreover, clinical trials on FGFR inhibitors such as TAS-120, derazantinib, and erdafitinib for treatment of CC have already yielded encouraging outcomes. A point worth noting is that erdafitinib is the world's first FGFR-targeting drug to be approved. FGFRs have become a hot topic of research. In 2020, pemigatinib, the second FGFR inhibitor that came onto the market, received accelerated approval for treatment of advanced BTC (*7*). The approval of pemigatinib was based on the FIGHT-202 study, which suggested that patients with CC and an FGFR2 fusion gene obtained a considerable benefit from targeted therapy. The mOS of patients receiving pemigatinib was 21.1 months, which was significantly higher than that of other groups (*8,9*). In addition, ongoing clinical trials based on FGFR targeting are also focused on lung cancer, hepatoma, breast cancer, and other types of cancer. In the future, FGFRs could serve as a target for therapies to treat a large variety of tumors.

In recent years, immunotherapy and targeted therapy have achieved remarkable success in the treatment of solid tumors. Increasing numbers of monoclonal drugs and targeted drugs have been approved for clinical use. With support from the government and the great promise of these drugs, increasing numbers of clinical trials are focusing on use of those drug to treat BTC. There are high hopes for improved clinical outcomes as a result of new drug development.

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**Address correspondence to:*
Fei Ma, Department of Oncology, Xinhua Hospital Affiliated to Shanghai Jiaotong University, School of Medicine, No. 1665 Kongjiang Road, Shanghai 200092, China.
E-mail: mafei@xinhuamed.com.cn

Jun Ding, Department of Biliary and Pancreatic Surgery, Shanghai Shuguang Hospital Affiliated to Shanghai University of T.C.M., Shanghai 201203, China.
E-mail: doctording0916@126.com

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World Health Organization. The World Health Report 2008 – primary health care: Now more than ever. *http://www.who.int/whr/2008/whr08_en.pdf* (accessed September 23, 2010).

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Intractable & Rare Diseases Research
Editorial and Head Office
Pearl City Koishikawa 603,
2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan.
E-mail: office@irdrjournal.com