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Original Article

Clinicopathologic and molecular profiles of Duchenne and Becker muscular dystrophy

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Abstract

Background Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are allelic X-linked recessive diseases caused by mutations in the dystrophin (DMD) gene. To our knowledge, molecular analysis to differentiate between DMD and BMD has never been performed in Indonesia.

Objective To elaborate the clinicopathologic and molecular profiles of DMD/BMD patients in Yogyakarta, Indonesia.

Methods Eighteen muscle biopsy specimens of patients clinically suspected to have DMD/BMD were collected. Possible associations of clinical manifestations, histopathological grading, and immunohistochemistry (IHC) results were analyzed. Polymerase chain reaction (PCR) was performed to identify mutations in exon 52.

Results Positive Gower's sign and high serum creatine kinase (CK) were observed in most patients. The IHC of dystrophin in two female patients suggested that they were manifesting carriers. Of the 16 male patients, 12 showed negative IHC staining, indicating DMD, while 4 patients demonstrated weak expression of dystrophin, indicating BMD. There was a significant association between high CK level and IHC results (P=0.005), indicating higher CK level in DMD patients. Histopathological grading of muscle biopsy was significantly associated with diagnosis of DMD/BMD using IHC (P=0.01), showing more severe tissue damage in DMD patients. None of the subjects had the single exon 52 deletion.

Conclusion This is the first report of a clinicopathologic and molecular profile of DMD/BMD in an Indonesian population. Serum CK level and histopathological grading of muscle biopsy are useful in distinguishing DMD from BMD in settings where an IHC assay is not available. **[Paediatr Indones. 2019;59:257-64; doi: http://dx.doi.org/10.14238/pi59.5.2019. 257-64**].

Keywords: *dystrophin gene; DMD; BMD; CK; immunohistochemistry*

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are recessive, X-linked, hereditary diseases due to mutations in the dystrophin (DMD) gene. Duchenne muscular dystrophy is the most common type of muscular dystrophy, affecting 1/3,500 male births.[1] This disease is characterized by progressive muscle weakness from childhood and loss of ambulation prior to the age of 12 years. Patients generally die due to respiratory or cardiac failure before their third decade of life. BMD has similar clinical features to DMD, however, it has slower disease progression. Patients may able to walk until 16 years of age. BMD patients also have better quality of life and longer life expectancy compared to DMD patients.[1] Early detection of DMD and BMD is possible, even before muscle weakness or clinical manifestations appear, as they are marked by increased serum CK level from the early years of life.[2]

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The dystrophin gene is one of the largest in the human genome, with a size of more than 3 Mb on the X chromosome and 79 exons. Dystrophin codes for a 14kb mRNA which is translated into the dystrophin protein. Dystrophin and other glycoproteins in the cell membrane form a dystrophin-glycoprotein complex (DGC), which has the function of stabilizing muscle fiber membranes. In DMD, dystrophin is completely absent, resulting in progressive muscle weakness which eventualy leads to premature death of the patient. In BMD patients, a partially functional protein is still produced, so the clinical manifestations are less severe compared to those of DMD patients. BMD patients have a higher life expectancy.[3-6] The DMD/BMD screening can be performed by blood serum CK measurements. Gower's sign and waddling gait in males with positive family history could prompt physicians to perform further examinations to diagnose or rule out DMD/BMD.[7]

The DMD/BMD clinical progress can be predicted by the disruption pattern caused by mutation(s) in the dystrophin gene. Mutations may alter the mRNA reading frame to in-frame (partially functional protein), or out-frame (absolutely absent protein).[8] One of the most promising strategies to correct the absence of dystrophin production in DMD is exon-skipping therapy, which involves changing an out-frame mutation to an in-frame one using antisense oligonucleotides or small molecules, so that the patient would have a less severe phenotype, like that of BMD.[9,10]

Immunohistochemical staining of muscle biopsy specimens and genetic analysis to detect mutations in the dystrophin gene are the gold standard tests to diagnose DMD/BMD. However, both of these examinations have not been performed regularly in Indonesia. Thus, robust data regarding clinical characteristics, epidemiology, and molecular profiles of DMD/BMD patients in Indonesia are currently unavailable. Future therapy using 'exon skipping' or 'stop codon read through' strategies will require detailed patient information on molecular and mutational status. Hence, we aimed to determine the clinical, histopathological, and molecular characteristics of DMD/BMD patients in Indonesia by conducting various tests on their muscle biopsy specimens in Sardjito General Hospital, Yogyakarta.

Methods

This observational analytical study had a cross-sectional design. There was no follow-up or intervention for the study subjects. Subjects were patients with clinically-suspected DMD/BMD in Sardjito General Hospital from January 2010 to December 2015, who had undergone muscle biopsy. Muscle biopsy specimens were in formalin-fixed, paraffin-embedded (FFPE) form. To determine the histopathologic grade of dystrophin in the muscle, the FFPE muscle biopsy was sliced and stained with hematoxylin-eosin (HE) for histopathological assay. The samples were also immunostained with dys-2 antibody (*Leica Biosystem*, Newcastle, USA) to detect dystrophin expression, determining whether patient was DMD, BMD or none of them. The pathologist, an expert in muscle histopathology and dystrophin immunostaining, performed the histopathological analysis. Based on histologic appearance, muscle biopsy is categorized into 4 grade. Grade 1 is characterized by retention of fascicular pattern of muscle fibers with no obvious fibrosis and fat infiltration, grade 2 is demonstrated by retention fascicular pattern of muscle fibers with minimal fibrosis and/or fat infiltration, grade 3 is shown by disrupted muscle fibers with marked fibrosis and/or fat infiltration, meanwhile grade 4 is indicated by severe change of muscle fibers with more than 50% fat and fibrosis replacement.[11] Dystrophin immunostaining is interpreted by strong membrane expression of dystrophin in normal control, completely no expression in DMD patients and focally expressed in BMD patients.[12]

The DNA extraction from all FFPE specimens was performed on those showing negative or partially positive immunohistochemical (IHC) results. The DNA extraction was conducted using a GeneJET FFPEDNA extraction kit (*Thermoscientific Carlsba*d, CA, United States). Polymerase chain reaction (PCR) was performed using PCR KAPA Taq ReadyMix (*Kapa Biosystems*, Boston, Massachusetts, United States). The PCR mixtures consisted of KAPA Taq master mix, specific primers of exon 52, and a DNA template in a total volume of 20 ul after addition of H_2O. Exon 52 was amplified using the following specific primer sequences: DMD_52F GTG TTT TGG CTG GTC TCA CA and DMD_52R ATG GAC TGA AAA TCT CAG CAC AAT (expected amplicon size

366bp). DNA was denaturated at 95°C for 3 minutes. Thirty-five PCR cycles were performed as follows: 30 seconds at 93°C (denaturation), 30 seconds at 61.2°C (annealing), and 1 minute at 72°C (elongation). The PCR products were subjected to electrophoresis on a 2% agarose gel, visualized by ethidium bromide fluorescence, and photographed. All specimens were assayed with positive and negative controls.

Research data were analyzed using suitable statistical analyses with *SPSS 23.00* software. Mann-Whitney test was used to analyze for an association between serum CK increase and IHC results. Fisher's exact test was used to analyze for an association between histopathological degree in DMD and BMD patients. This research was approved by the Research Ethics Committee of the Faculty of Medicine, Gadjah Mada University.

Results

From January 2010 to December 2015, 115 patients were clinically diagnosed with DMD/BMD in Sardjito General Hospital. However, only 18 patients had undergone gastrocnemius muscle biopsy to establish the diagnosis histopathologically. The age range of those 18 patients was 3 to 22 years, with mean age of 9.6 years. Among the 18 patients, 2 were female. Demographic and clinical characteristics of subjects are summarized in **Table 1**. All patients in this study had positive Gower's sign during neurological examination. Increased serum CK level was found in 16/18 patients, while 14/18 showed abnormal gait such as waddling or walking on tiptoe. Gastrocnemius muscle pseudohypertrophy was observed in 11/18 patients. Skeletal abnormalities (such as scoliosis, lordosis) and drop foot were seen in 3/18 patients. Cardiomyopathy occurred in 2/18 patients, while mental retardation was obeserved in 1/18 patient. Three patients had positive familial history of muscle weakness. Increased serum CK level was detected in 16/18 patients (CK data for one patient was not available). Subjects' mean CK level was 10,601 U/L, ranging from 190 U/L to 91,746 U/L. Electroneuromyography (ENMG) examination was performed in 11/18 patients, 8/18 of whom were diagnosed with myopathy, and 3/18 of whom were diagnosed with neuropathy.

Table 1. Clinical characteristics of DMD/BMD patients

Characteristics	N	Mean (SD)
Age, years		9.61 (4.15)
0-5	2	
6-10	9	
11-15	6	
16-20	0	
>20	1	
Sex		
Male	16	
Female	2	
Clinical features		
Gower's sign	18	
CK ↑	16	
Abnormal gait	14	
Gastrocnemius pseudohypertrophy	11	
Family history	3	
Bone abnormality	3	
Cardiomyopathy	2	
Mental retardation	1	
CK level, U/L		10,600.5
Myopathy	8	(21,461.6)
Neuropathy	3	
ENMG		
Myopathy & neuropathy	11	
N/A	7	

Muscle biopsy samples were histopathologically assessed using standard HE staining by a trained pathologist (**Figure 1**). The specimens showed variations in muscle fiber diameter size, increased centrally-located nuclei, necrosis, lymphocyte infiltration, fat replacement, and fibrosis in 18/18, 14/18, 13/18, 17/18, 18, and 15/18 patients, respectively (**Table 2**). Grading for muscle damage was performed based on fibrosis and fat infiltration in muscle fibers.11 Four of 18 patients showed a lower degree (grade 2), while 14/18 patients showed higher degrees of grades 3 (8/18 subjects) and 4 (6/18 subjects) (**Table 3**).

Immunohistochemistry (IHC) test results of muscle biopsy specimens are shown in **Figure 2**. Six

Table 2. Histopathological profiles - HE staining (N=18)

Histopathological feature	n
Various diameters of muscle fibers	18
Fibrosis	15
Fat infiltration	18
Increased intralocated nuclei	14
Necrosis	13
Lymphocyte infiltration	17

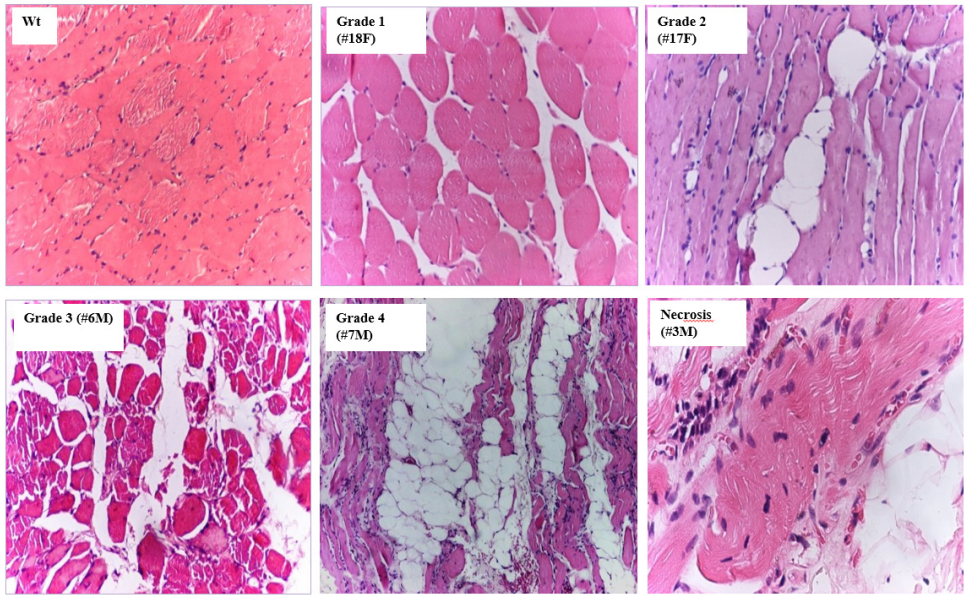

Figure 1. Muscle biopsy grading. Gastrocnemius biopsy with HE staining in wild type (Wt), BMD (#18F, #17F), and DMD (#6M, #7M, #3M) patients. Wt=muscle fibers in fascicular arrangement, uniform muscle fiber diameters, peripherally-located nuclei, and without lymphocyte infiltration, fat infiltration, fibrosis, or necrosis. DMD/BMD=variable muscle fiber diameters, increased centrally-located nuclei, lymphocyte infiltration, fibrosis, and fat infiltration, categorized as grades 1, 2, 3, and 4.

patients expressed the dystrophin protein focally in some muscle fibers, 4 of whom were male, confirming a BMD diagnosis; 2 patients were female, indicating carrier status. The muscle biopsy specimens from the remaining 12 patients showed no dystrophin expression, confirming a DMD diagnosis (**Table 4**). Correlation between histopathological degree and dystrophin IHC. **Table 5** shows that 6/18 patients with grade 4 had DMD and 2/18 had BMD. Among the 8 patients with grade 3, 7/8 had DMD and 1/8 had BMD. Two patients with grade 2 were female, with focal expression of dystrophin in some muscle fibers, suggesting manifestation of carrier status. The

Table 4. Dystrophin immunohistochemistry based on sex (N=18)

Dystrophin IHC	Sex	n	Diagnosis
Negative	Male	12	DMD
	Female	0	(-)
Focal positive	Male	4	BMD
	Female	2	Carrier

Table 3. Histopathological grading (N=18)

	Grading	n
Grade 1	Retention fascicular pattern of muscle fibers with no obvious fibrosis and fat infiltration	0
Grade 2	Retention fascicular pattern of muscle fibers with fibrosis and/or fat infiltration	4
Grade 3	Disrupted muscle fibers with marked fibrosis and/or fat infiltration	8
Grade 4	Severe change of muscle fibers with more than 50% fat and fibrosis replacement	6

Table 5. Histopathological grading and dystrophin IHC result (n=18)

Histopathological grading	BMD	DMD	Carrier
Grade 1			
Grade 2	2		2
Grade 3	1	7	
Grade 4	1	5	

Table 6. Association between histopathological degree and diagnosis of DMD/BMD (N=16)

Histopathological grading	BMD	DMD	P value
Grade 1 and 2	2	0	0.01
Grade 3 and 4	2	12	

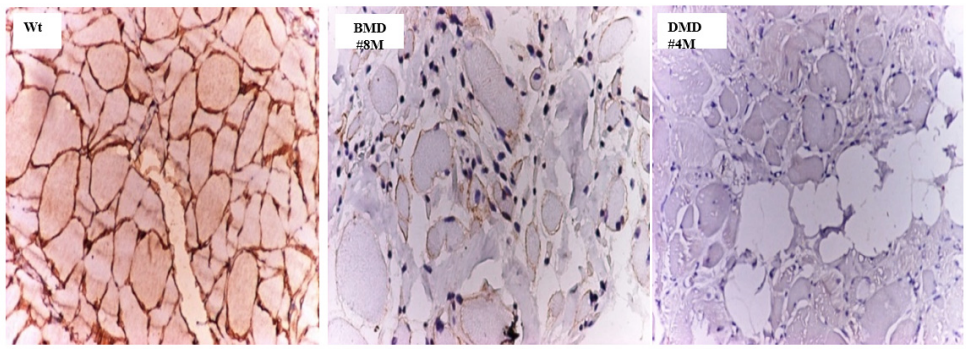

Figure 2. Dystrophin immunostaining (IHC). Dys-2 immunostaining was performed in wild type (Wt), BMD (#8M), and DMD (#4M) patients. Wt=dystrophin was strongly expressed in muscle fiber membranes, giving a spider web appearance. BMD=dystrophin was focally expressed in some muscle fibers due to inframe mutation in the dystrophin gene. DMD=dystrophin expression was completely absent due to outframe mutation in the dystrophin gene.[12]

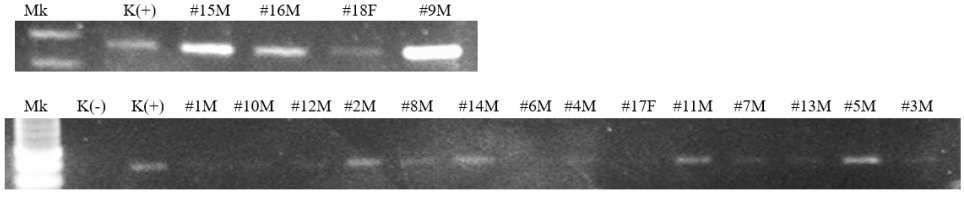

Figure 3. Gel electrophoresis of the PCR analysis. Exon 52 deletions were not observed in all DMD/BMD patients, since PCR amplification of exon 52 was detected in all specimens. (Mk= marker, K= control).

mean age of DMD patients based on dystrophin IHC results was 8.5 years, while the mean age of BMD patients was 11.4 years. However, this age difference was not statistically significant (P=0.18). Mann-Whitney test revealed that serum CK level was significantly higher in DMD than in BMD patients, based on IHC results (P=0.005). Fisher's exact test showed that histopathological degree was significantly associated with a diagnosis of DMD/BMD using IHC (P=0.01, **Table 6**), as patients with grades 3 and 4 were more likely to be diagnosed with DMD in IHC test, while grades 1 and 2 were more likely to be diagnosed with BMD. Mutation analysis (**Figure 3**) showed that the exon 52 deletion was not found in any of our patients. This analysis was confirmed by comparing all the samples to wild type (positive control) amplification.

Discussion

A DMD/BMD diagnosis is established based on genetic analysis or dystrophin staining of muscle biopsy specimens.[7,13] There were 115 patients clinically diagnosed with DMD/ BMD in Sardjito General Hospital, Yogyakarta in year 2010-2015. However, only 18 patients had undergone gastrocnemius muscle biopsy to establish the diagnosis histopathologically. Genetic analysis was not performed. In Indonesia, diagnosis of the disease is still based on clinical manifestations, which cannot be used to differentiate DMD from BMD or other muscular dystrophies.

There were 2 female patients with muscle weakness among our 18 subjects. Since females have two X chromosomes, theoretically they are carriers of the dystrophin gene mutation, and generally have no clinical manifestations. However, some manifesting carriers have been reported previously, with mild to severe clinical features.[14-20] Clinically, these two female subjects showed muscle weakness with positive

Gower's sign, but CK level was slightly elevated. One girl had a family history of muscle weakness. Both subjects had histopathological grade 2 muscle damage and IHC-demonstrated focal dystrophin expression in some muscle fibers. Explanations on how carriers may show clinical symptoms include (1) mutations in both alleles of the Xp21-chromosome,16 (2) loss of one X chromosome, e.g., in Turner syndrome,21 (3) abnormal X chromosome, e.g., deletion, duplication, or translocation,[21] and (4) extremely skewed X chromosome inactivation.[22]

Subjects' mean age was 9.6 years; the oldest patient (22 years) was diagnosed with BMD based on IHC results. In agreement with previous studies, DMD generally has an early onset and severe phenotype, while BMD has a late onset with milder phenotype and longer life expectancy.[1,23] All subjects had positive Gower's sign, and most had high serum CK level, both of which are valuable clinical markers in suspecting DMD/BMD.[7,13] Other symptoms such as abnormal gait, muscle weakness, pseudohypertrophy, skeletal abnormality, cardiomyopathy or mental retardation should also be carefully observed to support the diagnosis of DMD/ BMD.[8, 24-26]

The supporting ENMG examination revealed that 8/18 patients had muscle abnormality or myopathy. Electrodiagnostic studies have an important role in evaluating patients suspected of having myopathy. This examination can be used as an alternative diagnostic tool in confirming muscle abnormality, narrowing the differential diagnosis and identifying the best muscle biopsy location. Needle electromyography is the most informative tool to assess spontaneous muscle activity and motor unit action.[27]

In DMD/BMD patients, dystrophin gene mutations cause dystrophin loss, affecting DGC complex function in stabilizing cell membrane, which makes cells susceptibile to injury. Inflammatory mediators are attracted to injury sites, causing continuous chronic inflammation, necrosis, and replacement of muscle fibers with fat tissue and fibrosis.[23] Muscle abnormalities appear as varied sizes of muscle fibers, increased centrally-located nuclei, necrosis, lymphocyte infiltration, fibrosis, and fat replacement (pseudohypertrophy).[11,28,29]

In our study, patients diagnosed with DMD by IHC showed higher serum CK levels compared to BMD patients. This finding was in agreement with previous studies showing that more severe muscle damage occurred in DMD patients compared to BMD patients. The DMD patients also had higher CK levels with early onset of age 1-6 years (peak of 3-5 years), with a 0.18 U/L/year rate of decline. The BMD patients had later onset compared to DMD patients, generally at 10-15 years of age, with a slower rate of decline of 0.06 U/L/year. These reports indicated that the loss of muscle mass was more progressive in DMD patients.[2,30]

In our study, histopathological grading was significantly correlated with diagnosis of DMD/BMD using IHC. Patients with grades 3 and 4 were more likely to be diagnosed with DMD by IHC test, while patients with grades 1 and 2 were more likely to be diagnosed with BMD. To our knowledge, studies of this type have not been performed in Indonesia. Histopathological grading of muscle damage was introduced by Kinali *et al.*[11] to compare severity of muscle damage in DMD patients with magnetic resonance imaging (MRI) results. Our study shows that histopathological grading using the method introduced by Kinali *et al*. can be used to distingush DMD from BMD. This result may help many hospitals without IHC-staining facilities to diagnose DMD/ BMD.

Genetic analysis of DMD gene exon 52 showed no exon deletion in any of the 18 subjects. Takeshima *et al.*[1] reported that deletion of exon 52 is the most common single exon deletion. The small sample size of our study may be the reason for this discrepancy. Analysis on other exons should be performed to identify possible mutations in other regions of the dystrophin gene. Further study using a larger sample size is also needed to understand the characteristics of this disease in Indonesian populations.

In conclusion, this is the first report of clinicopathological and molecular profiles of DMD/ BMD in Indonesian population. The IHC and genetic analysis are standard tools to diagnose DMD and BMD. Serum CK level and histopathological grading of muscle biopsy are useful in distinguising DMD from BMD in setting where IHC analysis is not available. In this study, single deletion of exon 52 was not found in small size of Indonesian population.

Conflict of Interest

None declared.

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