Genomic screening for Duchenne muscular dystrophy: a retrospective study from 10,481 NICU patients based on next generation sequencing data

Tiantian Xiao[1,4*], Bingbing Wu[1*], Yun Cao[1], Renchao Liu[1], Guoqiang Cheng[2], Laishuan Wang[3], Deyi Zhuang[5], Zhengyan Zhao[6], Huijun Wang[1], Wenhao Zhou[1,2]

[1] Department of Neonates, Key Laboratory of Neonatal Diseases, Ministry of Health, Children's Hospital of Fudan University, 201102 Shanghai, China.

[2] Center for Molecular Medicine, Children's Hospital of Fudan University, 201102 Shanghai, China.

[3] Department of Pediatrics, Children's Hospital of Fudan University, Shanghai 201102, China

[4] Department of Neonatology, Chengdu Women's and Children's Central Hospital, School of Medicine, University of Electronic Science and Technology of China, 611731, Chengdu, China

[5] Department of Pediatrics, Xiamen Children's Hospital, 361005, Xiamen, China,

[6] Department of Child Health Care, Children's Hospital, Zhejiang University School of Medicine, 310058, Hangzhou, China

* authors contributed equally: Tiantian Xiao, Bingbing Wu

Corresponding Authors:

Zhengyan Zhao

Department of Child Health Care, Children's Hospital, Zhejiang University School of Medicine, 310058, Hangzhou, China, Email: zhaozy@zju.edu.cn

Huijun Wang

Center for Molecular Medicine, Children's Hospital of Fudan University, 201102 Shanghai, China, 399 Wanyuan Road, Shanghai, China. Email:huijunwang@fudan.edu.cn

Wenhao Zhou

Department of Neonatology, Children's Hospital of Fudan University; Center for Molecular Medicine, Children's Hospital of Fudan University, 201102 Shanghai, China, 399 Wanyuan Road, Shanghai, China. Email: zhouwenhao@fudan.edu.cn.

Abstract

Newborn creatine kinase screening can identify patients at risk for Duchenne muscular dystrophy. However, it is unclear whether the next-generation sequencing-based screening can identify patients early and guide care. Herein, this study investigates clinical utility of next-generation sequencing-based DMD screening. A total of 19 (0.18%, 19/10481) newborns were identified with pathogenic variants of DMD gene, including 4 (21.1%, 4/19) duplications,13 (68.4%,13/19) deletions, and 2 (10.5%, 2/19) nonsense mutations. Six of them were symptomatic after regular follow up. Therapeutic strategies for these patients were modified. Two neonates died, and the remaining 11 newborns were asymptomatic at August 1, 2020. These 13 families were informed the updated genetic report and suggested for further genetic consulting. Genomic screening for DMD would identify patients who might not come to clinical attention prior to disease manifestation. Early targeted intervention of DMD have the positively impact the clinical decision and the potential to improve outcomes.

Keywords

Introduction

Duchenne muscular dystrophy（DMD）is an inherited progressive myopathic disease resulting from mutations in *DMD* gene located on the X chromosome. This gene has 79 exons and 8 promoters, affecting the development of the skeletal and myocardial muscle and brain. At present, an overall incidence is estimated to be 1 case per 5000 live male births according to 10 large DMD newborn screening programs (DBS)(Gatheridge et al., 2016). In clinical setting, patients with DMD are usually not symptomatic till 2 to 5 years old. Therefore, the delay of up to 2 years between the first onset symptoms and the diagnosis are common in the affected patients(Vita & Vita, 2020). DBS has been carried out since 1977. However, because of poor performance of creatine kinase and uncertainty of long-term benefits on

patients, a national wide screening program have never been put in place.

Currently, studies have indicated that improved neuromuscular function, longer life expectancy, and higher quality of life are associated with earlier corticosteroids treatment(McDonald et al., 2018; Schram et al., 2013), advances in cardiac and respiratory care (Passamano et al., 2012; Schram et al., 2013), multidisciplinary care (Passamano et al., 2012), and better family planning (Birnkrant, Bushby, & Bann, 2018), although there is no curative treatment. Moreover, molecular and gene therapies for DMD have been on the horizon. Also, the efficacy of the therapeutic interventions on pre-symptomatic stage in DMD patients need to be proved.

With the affordable, fast, and wide-scale implemented NGS in recent years, the NGS-based genetic testing has been paved the way to a range of possibilities in the field of NBS (Adhikari et al., 2020; Bassaganyas et al., 2018; Bodian et al., 2016).Therefore, our study aims to implement genomic screening for DMD in newborns from intensive care unit using the NGS data, then investigate the clinical course of newborns with molecular diagnosis of DMD and the impact of the early positive molecular diagnosis on the clinical decision.

METHODS

The next generation sequencing data from 10,481 participants at Fudan University of Children from June 1, 2016 to June 30, 2020 were reanalyzed by in-house pipeline of genetic analysis. Patients were recruited with the following inclusion criteria: (1) Patients were from neonatal intensive care unit; (2) Postnatal age less than 28 days; (3) Biological parent or guardian's informed consent;(4) NGS were performed. The reanalysis of clinical data including genetic results, clinical features and laboratory results, and follow-up information in clinic from the medical records was performed on the newborns with positive molecular diagnosis. Patients were excluded if the clinical information were absent. In this study, we conducted a follow-up telephone call for newborns with positive molecular diagnosis at August 1, 2020, and informed the parents the updated molecular diagnosis and provide possible genetic consulting.

The study was approved by the local institutional ethics committee at each participating hospital before the study began.

We identified SNVs and CNVs using in-house analytical pipeline (Backenroth et al., 2014; Dong et al., 2020) (Figure S1). SNVs were then validated by Sanger sequencing, while, diagnostic CNVs were confirmed by Multiplex ligation-dependent probe amplification (MLPA).

RESULTS

Figure 1 showed the study algorithm. A total of 10,481 newborns were enrolled, of which 19 (0.18%) newborns with pathogenic *DMD* gene were identified by in-house pipeline. Table 1 described the detail clinical features of 19 neonates. Among them, 4 (4/19, 21.1%) were preterm infants, while 15 (15/19, 78.9%) were term infants. A median birthweight (interquartile range [IQR]) was 2990 (2320-3450) g. Six newborns were symptomatic within six months of age and the scheduled follow-up plans, including neurological, cardiac, CK levels, and other possible assessment plan, were made since neonatal period. No motor dysfunction and other relevance DMD phenotypes were found in the 11 neonates. Two newborns (neonate_DMD6 and neonate_DMD17) died within 28 days. Neonate_DMD6 were died as result of congenital intestinal atresia and multiple organ dysfunction, and neonate_DMD17 were died due to sepsis and omphalocele. These 13 families were informed the updated genetic report and suggested for further genetic consulting at August 1,2020.

We analyzed genotype and phenotype characteristics based on the 19 newborns with pathogenic *DMD* gene. Among them, one (neonate_DMD16) was admitted to NICU due to hypotonia, while the remaining 18 newborns presented unrelated phenotypes with DMD on admission. 15 had incidence finding of elevated CK, while persistent elevated CK were reported in seven cases (neonate_DMD5, neonate_DMD9, neonate_DMD10, neonate_DMD11, neonate_DMD15, neonate_DMD16, neonate_DMD18). Genetic analysis showed that CNV in 17 (89.5%, 17/19) and SNV in two (10.5%, 2/19) newborns were detected by NGS,

respectively (Table 1). Figure S2 showed the results of the MLPA and Sanger sequencing. Among the 17 CNV cases, the most common types were deletions (76.5%, 13/17), while, duplication was detected in 4 (23.5%, 4/17) neonates. Our results showed that 10 deletions mainly occurred in exons 42-50 but three deletions were in exon 10 (neonate_DMD10), exons 3-10 (neonate_DMD9), and exon22-37 (neonate_DMD16), respectively. The multi-exon deletions represented up to 92.3% (12/13). Of 12 neonates with multi-exon deletions, a 3-exon deletion in 42–48 exons (25%, 3/12) was the most common. Of all the exons, exon 46 was the most frequently deleted, followed by exons 47, 45, and 50. Three duplications involving 10 exons or fewer accounted for 75% (3/4) of all the duplications in the proband (neonate_DMD1, neonate_DMD7, neonate_DMD17). Two newborns (neonate_DMD18, neonate_DMD19) were nonsense mutations.

Figure 2 showed the nature history of 19 neonates. Neonate_DMD5 born at gestational age of 36 weeks was admitted due to suspected sepsis with incidence finding of elevated CK (5454 to 15852IU/L, range: 0-164IU/L). His electrocardiogram showed sinus rhythm and T wave change. No muscular weakness and family history of DMD were reviewed. Because the pathogenic *DMD* gene was reported, he was arranged to the neurologic specialty clinic for follow-up. At three months of age, he presented pseudohypertrophy of the calf on examination and the persistent elevated CK were reported. Combined with the earlier molecular results of exon 45-50 deletion in *DMD* gene, he had clinical diagnosis with DMD timely and physician discussed the treatment plan with family, including the motor development, cardiac function, and growth development assessment periodically, preventing accidental falling and administering the corticosteroid at 4 years old. Currently, he was 2 years old and no motor and cardiac dysfunction were reported.

Neonate_DMD16 presented hyperbilirubinemia, mild hypotonia and elevated CK (73760IU/L, range: 0-164IU/L) at three days of life. His brother presented difficulty walking up steps and was diagnosed with DMD at 7 years old. Neonate_DMD16 was diagnosed with DMD at 1 month of age due to the persistent elevated CK and early

identification of exon 22-37 deletion in *DMD* gene. The neurological, rehabilitation follow-up plan and additional family support were implemented at one month of age. At present, he was three years old and no abnormality of motor assessment and cardiac functions were found.

Neonate_DMD18 was admitted due to vomiting at 2 days of life. At present, he was three years old without motor dysfunction. However, persistent elevated CK, ranging from 2028 to 19524UI/L, was reported since 2 days of life and one reported nonsense pathogenic variation (exon39:c.5452G>T(p.E1818X)) in the *DMD* gene was detected. During the scheduled clinical assessment, he could not sit alone at 6 months of life, indicating delayed milestones. Family education has been provided, including preventing fractures related to accidental falling, closely monitoring the cardiac function, and the cognitive and global development.

DISCUSSION

Our study revealed that 19 neonates with pathogenic *DMD* gene were identified, suggesting an incidence of 0.18% in our cohort. It was higher compared to that of population-based screening, likely due to the different screening population and methods (Gatheridge et al., 2016). Our study revealed that the relevance phenotype, such as hypotonia and persistent elevated CK, could be manifested since neonatal period. Another study reported that an infant born at 31 weeks presented hypotonia since birth and diagnosed with DMD and X-linked myotubular myopathy at around one month of age (Varma, Mukherjee, Hughes, Sethuraman, & Kamupira, 2020). Our analysis showed that the majority of mutations of *DMD* gene were multi-exon deletions, and nonsense mutation were the most common type of SNVs. This finding aligns with previous studies(Wang et al., 2019) (Aartsma-Rus, Van Deutekom, Fokkema, Van Ommen, & Den Dunnen, 2006; Juan-Mateu et al., 2015; Takeshima et al., 2010).

Thanks to the early molecular diagnosis, 19 patients with molecular diagnosis of DMD were closely monitored to detect symptoms as early as possible. Furthermore,

the neonatal molecular diagnosis of DMD altered the clinical decision, guided physicians to decide the best practices including clinical follow-up plan, additional assessment plan, and provided the timely diagnosis, the type of mutation, early corticosteroid treatment, and possible approach of molecular and gene therapy. Also, the earlier molecular diagnosis would help physicians to recognize the relevance but nonspecific phenotype, and to consider targeted additional investigations, such as metabolic tests, acetylcholine receptor and muscle specific kinase antibodies, muscle biopsy, etc. even when there is a lack of correlation between the positive genetic result and the clinical picture in patients with myopathy disease (Varma et al., 2020). The study from the MD*STARnet* cohort indicated that the earlier onset age of first symptoms was a risk factor for a more rapid progression of muscle weakness (Ciafaloni et al., 2016). Thus, early identification of the relevance phenotype could assistant to predict the prognosis of patients with DMD as well. Currently, all the DMD screening programs used CK level as a first-tier screening marker followed by genetic confirmatory testing (Gatheridge et al., 2016; Mendell et al., 2012). However, the conventional NBS assays may be poor performance because of the variable screening cut-off levels of CK and the lack of consensus on the optimal time to screen. Also, the avoidance of false positive before 2 months old from CK screening is difficult due to the muscle trauma at birth (Vita & Vita, 2020). Importantly, studies revealed that more than 90% parents of affected children and expectance parents supported DBS program, although the studied population is small(Chung et al., 2016; Wood et al., 2014). At present, new therapeutic drugs based on the mutational analysis including Eteplirsen, golodirsen, ataluren, have been approved by FDA, and ataluren can be administered as early as 2 years old(Vita & Vita, 2020). Therefore, these possibilities highlight the importance of identification of DMD as early as possible.

Our study has several limitations. First, the NGS data used for DMD screening was performed on the patients from a monocentric hospital, which the generalizability may be limited. Second, the dynamic changes of CK level were absent because the patients did not follow up in clinic consistently. Third, our study ended at August 1,

2020. Most of patients were less than 4 years old and asymptomatic. Therefore, it will be essential to continue tracking these families to assess the long-term benefits.

In conclusion, DMD can be diagnosed genetically as early as neonatal period based on the reanalysis of NGS data. Identification of pathogenic *DMD* gene in neonatal period, even if of uncertain clinical significance at the time of testing, can provide an opportunity for further clinical investigation and reorient the care of DMD. Thus, NGS-based newborn screening for DMD has the potential to improve the outcomes of affected patients, but further clinical trials are pressingly demanded.

CONFLICT OF INTEREST
All the authors declare that they have no conflict of interests.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from Fudan University of Children but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available.

REFERENCES
Aartsma-Rus, A., Van Deutekom, J. C. T., Fokkema, I. F., Van Ommen, G. J. B., & Den Dunnen, J. T. (2006). Entries in the Leiden Duchenne muscular dystrophy mutation database: An overview of mutation types and paradoxical cases that confirm the reading-frame rule. *Muscle & Nerve, 34*(2), 135-144. doi:10.1002/ mus.20586

Adhikari, A. N., Gallagher, R. C., Wang, Y., Currier, R. J., Amatuni, G., Bassaganyas,

L., . . . Brenner, S. E. (2020). The role of exome sequencing in newborn screening for inborn errors of metabolism. *Nat Med*. doi:10.1038/s41591-020-0966-5

Backenroth, D., Homsy, J., Murillo, L. R., Glessner, J., Lin, E., Brueckner, M., . . . Shen, Y. (2014). CANOES: detecting rare copy number variants from whole exome sequencing data. *Nucleic Acids Res, 42*(12), e97. doi:10.1093/nar/gku345

Bassaganyas, L., Freedman, G., Vaka, D., Wan, E., Lao, R., Chen, F., . . . Kwok, P. Y. (2018). Whole exome and whole genome sequencing with dried blood spot DNA without whole genome amplification. *Human Mutation, 39*(1), 167-171. doi:10.1002/humu.23356

Birnkrant, D. J., Bushby, K., & Bann, C. M. (2018). Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and neuromuscular, rehabilitation, endocrine, and gastrointestinal and nutritional management (vol 17, pg 251, 2018). *Lancet Neurology, 17*(6), 495-495. doi:10.1016/S1474-4422(18)30125-X

Bodian, D. L., Klein, E., Iyer, R. K., Wong, W. S., Kothiyal, P., Stauffer, D., . . . Solomon, B. D. (2016). Utility of whole-genome sequencing for detection of newborn screening disorders in a population cohort of 1,696 neonates. *Genet Med, 18*(3), 221-230. doi:10.1038/gim.2015.111

Chung, J., Smith, A. L., Hughes, S. C., Niizawa, G., Abdel-Hamid, H. Z., Naylor, E. W., . . . Clemens, P. R. (2016). Twenty-year follow-up of newborn screening for patients with muscular dystrophy. *Muscle & Nerve, 53*(4), 570-578. doi:10.1002/mus.24880

Ciafaloni, E., Kumar, A., Liu, K., Pandya, S., Westfield, C., Fox, D. J., . . . McDermott, M. P. (2016). Age at onset of first signs or symptoms predicts age at loss of ambulation in Duchenne and Becker Muscular Dystrophy: Data from the MD STARnet. *Journal of Pediatric Rehabilitation Medicine, 9*(1), 5-11. doi:10.3233/Prm-160361

Dong, X. R., Liu, B., Yang, L., Wang, H. J., Wu, B. B., Liu, R. C., . . . Lu, Y. L.

(2020). Clinical exome sequencing as the first-tier test for diagnosing developmental disorders covering both CNV and SNV: a Chinese cohort. *Journal of Medical Genetics, 57*(8), 558-566. doi:10.1136/jmedgenet-2019-106377

Gatheridge, M. A., Kwon, J. M., Mendell, J. M., Scheuerbrandt, G., Moat, S. J., Eyskens, F., . . . Griggs, R. C. (2016). Identifying Non-Duchenne Muscular Dystrophy-Positive and False Negative Results in Prior Duchenne Muscular Dystrophy Newborn Screening Programs: A Review. *JAMA Neurol, 73*(1), 111-116. doi:10.1001/jamaneurol.2015.3537

Juan-Mateu, J., Gonzalez-Quereda, L., Rodriguez, M. J., Baena, M., Verdura, E., Nascimento, A., . . . Gallano, P. (2015). DMD Mutations in 576 Dystrophinopathy Families: A Step Forward in Genotype-Phenotype Correlations. *PLoS One, 10*(8). doi:ARTN e0135189

10.1371/journal.pone.0135189

McDonald, C. M., Henricson, E. K., Abresch, R. T., Duong, T. N., Joyce, N. C., Hu, F. M., . . . Investigators, C. (2018). Long-term effects of glucocorticoids on function, quality of life, and survival in patients with Duchenne muscular dystrophy: a prospective cohort study. *Lancet, 391*(10119), 451-461. doi:10.1016/S0140-6736(17)32160-8

Mendell, J. R., Shilling, C., Leslie, N. D., Flanigan, K. M., al-Dahhak, R., Gastier-Foster, J., . . . Weiss, R. B. (2012). Evidence-based path to newborn screening for Duchenne muscular dystrophy. *Ann Neurol, 71*(3), 304-313. doi:10.1002/ana.23528

Passamano, L., Taglia, A., Palladino, A., Viggiano, E., D'Ambrosio, P., Scutifero, M., . . . Politano, L. (2012). Improvement of survival in Duchenne Muscular Dystrophy: retrospective analysis of 835 patients. *Acta Myol, 31*(2), 121-125.

Schram, G., Fournier, A., Leduc, H., Dahdah, N., Therien, J., Vanasse, M., & Khairy, P. (2013). All-Cause Mortality and Cardiovascular Outcomes With Prophylactic Steroid Therapy in Duchenne Muscular Dystrophy. *Journal of the American College of Cardiology, 61*(9), 948-954.

doi:10.1016/j.jacc.2012.12.008

Takeshima, Y., Yagi, M., Okizuka, Y., Awano, H., Zhang, Z. J., Yamauchi, Y., . . . Matsuo, M. (2010). Mutation spectrum of the dystrophin gene in 442 Duchenne/Becker muscular dystrophy cases from one Japanese referral center. *Journal of Human Genetics, 55*(6), 379-388. doi:10.1038/jhg.2010.49

Varma, U., Mukherjee, D., Hughes, I., Sethuraman, C., & Kamupira, S. (2020). X-Linked Myotubular Myopathy and Duchenne Muscular Dystrophy in a Preterm Infant: A Rare Combination. *Pediatrics, 146*(3). doi:10.1542/peds.2018-2879

Vita, G. L., & Vita, G. (2020). Is it the right time for an infant screening for Duchenne muscular dystrophy? *Neurological Sciences, 41*(7), 1677-1683. doi:10.1007/s10072-020-04307-7

Wang, L., Xu, M., Li, H., He, R. J., Lin, J. F., Zhang, C., & Zhu, Y. L. (2019). Genotypes and Phenotypes of DMD Small Mutations in Chinese Patients With Dystrophinopathies. *Frontiers in Genetics, 10*. doi:ARTN 114
10.3389/fgene.2019.00114

Wood, M. F., Hughes, S. C., Hache, L. P., Naylor, E. W., Abdel-Hamid, H. Z., Barmada, M. M., . . . Clemens, P. R. (2014). Parental attitudes toward newborn screening for Duchenne/Becker muscular dystrophy and spinal muscular atrophy. *Muscle & Nerve, 49*(6), 822-828. doi:10.1002/mus.24100

Table1 Genotype and phenotype of 19 neonates with pathogenic *DMD* variations

No.	Neonatal period		Blood CK level at last follow-up (IU/L)	Diagnostic age	Current age	Phenotype at last follow-up	Family History	DMD Variant
	Main clinical phenotype	Blood CK level (IU/L)						
Neonate_DMD1	Term infants, Hyperbilirubinemia,	222	/	/	4 years	No motor dysfunction	No	exon1-7 dup
Neonate_DMD2	Term infants, Pneumonia, Cardiomegaly, Suspected Myocarditis	12070	/	/	3 years	No motor dysfunction and cardiac dysfunction	No	exon 46-47 del
Neonate_DMD3	Term infants, Hyperbilirubinemia Umbilical hernia, CMV infection	2519	/	/	3 years	No motor dysfunction	No	exon 45-47 del
Neonate_DMD4	Term infants, poor feeding	/	/	/	3 years	No motor dysfunction	No	exon46-47 del
Neonate_DMD5	Late-preterm, Sepsis Suspected Muscular dystrophy	5454	15852	3 months	2 years	pseudohypertrophy of the calf, Persistent elevated CK	No	exon 45-50 del
Neonate_DMD6	Preterm, Small for gestational age Sepsis, Congenital intestinal atresia Multiple organ dysfunction	139	/	/	/	Died within 28 days of life	No	exon 48-51 del
Neonate_DMD7	Preterm, Extremely	247	/	/	9 months	No motor dysfunction	No	exon 1-2 dup

	low birth weight Patent ductus arteriosus							
Neonate_DMD8	Term, Pneumonia	elevated	/	/	1 year	No motor dysfunction	No	exon 3-10 del
Neonate_DMD9	Term, Urinary tract infection	50000	3090	/	1 year	No motor dysfunction	No	exon 31-43 dup
Neonate_DMD10	Term, Congenital laryngomalacia	2010	2320	4 months	1 year	Persistent elevated CK without motor dysfunction	No	exon 10 del
Neonate_DMD11	Vomiting, Urinary tract infection Polycythemia Tachycardia	66439	2510	2 months	10 months	pseudohypertrophy of the calf without cardiac dysfunction, Persistent elevated CK	Yes*	exon 45-50 del
Neonate_DMD12	Term, Asphyxia, Respiratory distress, Hyperbilirubinemia, Congenital laryngomalacia	302	/	/	9 months	No motor dysfunction	No	exon 48-51 del
Neonate_DMD13	Term, Vomiting, Urinary tract infection, Hyperbilirubinemia	119	/	/	8 months	No motor dysfunction	No	exon 42-44 del
Neonate_DMD14	Term, Suspected Muscular dystrophy	elevated	/	/	8 months	No motor dysfunction	Yes***	exon 46-48 del
Neonate_DMD15	Term, Hyperbilirubinemia,	11660	9600	/	9 months	No motor dysfunction	No	exon 46-50 del

	Cholangiectasis Suspected Myocarditis							
Neonate_DMD16	Term, Hypotonia, Hyperbilirubinemia Suspected Muscular dystrophy	73760	22300	1 month	3 years	Hypotonia Persistent elevated CK	Yes**	exon22-37 del
Neonate_DMD17	Late-preterm, Omphalocele, Sepsis, Encephalopathy	60	/	/	/	Died within 28 days of life	No	exon 1-7 dup
Neonate_DMD18	Term, Vomiting, Asphyxia required resuscitation, Abnormal liver function, Suspected Muscular dystrophy	19524	4858	6 months	3 years	Muscular Weakness Persistent elevated CK	No	exon39: c.5452G>T (p.Glu1818Ter)
Neonate_DMD19	Term, Hyperbilirubinemia, Abnormal liver function, Suspected Muscular dystrophy	elevated	9076	1 month	3 years	Persistent elevated CK	No	exon44: c.6408G>A (p.Trp2136Ter)

Figure Titles and Legends

Figure 1. The study algorithm of next-generation sequencing data-based DMD screening

Figure 2. Trajectories of clinical natural course of 19 newborns with molecular diagnosis of DMD

CK: Serum creatine kinase; AST: aspartate transaminase