**Age-Dependent Echocardiographic and Pathologic Findings in a Rat Model with Duchenne Muscular Dystrophy Generated by CRISPR/Cas9 Genome Editing**

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**Summary**

Duchenne muscular dystrophy (DMD) is X-linked recessive myopathy caused by mutations in the dystrophin gene. Although conventional treatments have improved their prognosis, inevitable progressive cardiomyopathy is still the leading cause of death in patients with DMD. To explore novel therapeutic options, a suitable animal model with heart involvement has been warranted.

We have generated a rat model with an out-of-frame mutation in the dystrophin gene using CRISPR/Cas9 genome editing (DMD rats). The aim of this study was to evaluate their cardiac functions and pathologies to provide baseline data for future experiments developing treatment options for DMD.

In comparison with age-matched wild rats, 6-month-old DMD rats showed no significant differences by echocardiographic evaluations. However, 10-month-old DMD rats showed significant deterioration in left ventricular (LV) fractional shortening ($P = 0.024$), and in tissue Doppler peak systolic velocity (Sa) at the LV lateral wall ($P = 0.041$) as well as at the right ventricular (RV) free-wall ($P = 0.004$). These functional findings were consistent with the fibrotic distributions by histological analysis.

Although the cardiac phenotype was milder than anticipated, DMD rats showed similar distributions and progression of heart involvement to those of patients with DMD. This animal may be a useful model with which to develop effective drugs and to understand the underlying mechanisms of progressive heart failure in patients with DMD.

**Key words:** Animal model, Dystrophin, Cardiomyopathy, Echocardiography

Duchenne muscular dystrophy (DMD) is X-linked recessive myopathy caused by out-of-frame mutations in the dystrophin (DMD) gene. Dystrophin is a structural protein stabilizing the plasma membrane and the complete loss of dystrophin protein causes the cytoskeletons of muscle cells to become fragile. As a consequence, patients with DMD suffer from inevitable progressive muscle weakness with systemic multiple complications such as infections, thrombosis, renal disorder, respiratory failure, and progressive heart failure. Although currently limited medications including corticosteroids, angiotensin-converting enzyme inhibitors, and beta-blockers have improved the prognosis, most patients with DMD die by the age of 40 even today and their heart failure is the leading cause of death. There is an urgent need to develop other therapeutic options for heart failure.

To explore novel therapeutic options, several animal models of DMD have been generated. Historically, the most commonly used animal model is mdx mice, which have a nonsense mutation in exon 23 of the DMD gene. However, the phenotype of mdx mice is milder than that of human patients and does not show a similar severity of skeletal muscle damage or cardiomyopathy. In recent years, we have generated a rat model with an out-of-frame mutation in the DMD gene using CRISPR/Cas9 genome editing (DMD rats). Although skeletal muscles in F0 DMD rats showed more severe phenotype than mdx mice, the phenotype of heart function and its progression are

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still unknown. In this study, we aimed to evaluate their cardiac functions and pathologies to provide baseline data for future experiments developing treatment options for DMD.

**Methods**

**Animal model:** We generated a strain of rat with an out-of-frame mutation in the *DMD* gene using a CRISPR/Cas 9 gene-editing system (Figure 1). The DMD rat has mutations in exon3 and exon16 in *DMD* gene (cDNA sequence; exon3del, exon16: c.1949_1950 insT, c.1952_1953 CG > AT). Female rats with a heterozygous mutation in *DMD* gene were mated with adult male wild rats. Male hemizygous DMD rats and male littermate wild rats were used for the experiments. Animals were maintained under controlled environmental conditions: 23°C, 12/12-hour light/dark cycles. Food and water were provided *ad libitum*. All animal experiments performed in this study were in accordance with the Guide for the Care and Use of Laboratory Animals of The University of Tokyo, and were approved by the Institutional Animal Care and Use Committee of The University of Tokyo (P18-125).

**Echocardiography:** Echocardiographic images were acquired under anesthesia using a Vivid E95 digital ultrasound system with a 12 MHz 12S-D probe (GE Healthcare Japan, Tokyo), which is maintained under the guidelines of the Japanese Society of Echocardiography. DMD rats aged 6 and 10 months and age-matched wild rats were first anaesthetized with 5% isoflurane. Next, they were placed in the left lateral decubitus position on a heated platform during echocardiography, and further anesthesia was maintained by 4% isoflurane for wild rats, and by 1%-2% isoflurane for DMD rats. Images of the parasternal long-axis view and the apical 4-chamber view were recorded with careful attention to the minimal ultrasonic scanning depth to obtain a higher frame rate (> 250 frames per second) with higher imaging qualities (Figure 2). The obtained digital raw data was transferred to the EchoPac system Ver. 203 (GE Healthcare Japan) and echocardiographic variables were measured in accordance with the human guideline of the American Society of Echocardiography. From the M-mode parasternal short axis view, left ventricular (LV) end-diastolic diameter, intraventricular septum, and posterior wall thickness were obtained at the timing of end-diastole. In the same view, end-systolic diameter and LV fractional shortening (LVFS) were obtained at the timing of end-systole. From the apical 4-chamber view, right ventricular fractional area change (RVFAC), tricuspid annular plane systolic excursion (TAPSE), transmitral and tricuspid peak E-wave velocity (E), tissue Doppler imaging (TDI) peak velocity at systole (Sa) and early-diastole (Ea) from the septum, and lateral and RV free-wall annulus were obtained. At the end of echocardiography, the left lateral decubitus position was changed to the supine position and the maximum inferior vena cava (IVC) diameter was measured. Heart rate, M-mode, and Doppler variables were measured as the average of consecutive 5 beats.

**Histological analyses:** Paraffin-embedded sections of the heart tissues from 6 and 10 month-old DMD rats and age-matched wild rats were prepared transversely and were subjected to histological evaluations. The sections were stained using Masson's trichrome staining. Photographs were acquired using a fluorescence microscope equipped with a digital camera BX51/DP73 (Olympus, Tokyo).

**Statistical analyses:** Data are expressed as the mean ± standard deviation. Because all echocardiographic vari-
ables were continuous variables, statistical comparisons for age-matched animal groups were conducted using unpaired t-tests (R statistical software Version 4.0.0). P-values less than 0.05 were considered statistically significant.

Results

Echocardiography was successfully evaluated in 6-month-old wild rats \((n = 7)\), DMD rats \((n = 7)\), 10-month-old wild rats \((n = 7)\), and DMD rats \((n = 4)\), respectively. The number of 10-month-old DMD rats was limited to 4 because 1 died before the evaluation date and 2 others died soon after the anesthesia induction.

All echocardiographic results in 6-month-old rats showed that other than LV end-diastolic diameter there are no significant differences between wild rats and DMD rats (Table). Due to progressive systemic muscle atrophy, the body weight of 6-month-old DMD rats \((418 \pm 61 \text{ g})\) was significantly lighter than that of wild rats \((545 \pm 37, \text{P} = 0.003)\).

On the other hand, the results in 10-month-old rats showed significant differences; DMD rats had significantly smaller LV end-diastolic diameter \((\text{P} = 0.001)\), LV fractional shortening \((\text{P} = 0.024)\), transmitral E velocity \((\text{P} = 0.011)\), TDI Sa of lateral LV wall \((\text{P} = 0.041)\), and TDI Sa of RV free-wall \((\text{P} = 0.004)\). The non-significant trend toward a decrease in RV fractional area change was also observed in DMD rats \((\text{P} = 0.057)\). Their weight difference in 10-month-old rats became larger than 6-month-old rats so that the body weight of DMD rats \((368 \pm 92 \text{ g})\) was significantly lighter than that of wild rats \((609 \pm 36 \text{ g}, \text{P} < 0.001)\).

Masson’s trichrome staining of representative heart sections showed that 1) no fibrosis was observed in 6-month-old wild rats, 2) mild transmural fibrosis of RV free-wall and mild epicardial fibrosis in LV lateral wall were observed in 6-month-old DMD rats, and 3) severe transmural fibrosis of RV free-wall and moderate epicardial to transmural fibrosis of global LV myocardium were observed in 10-month-old DMD rats (Figure 3). No fibrosis was observed even in 10-month-old wild rats.

Figure 2. Representative rat echocardiograms. Representative rat echocardiographic images of parasternal long-axis view (A), apical 4-chamber view (B), transmitral E velocities (C), and septal tissue Doppler imaging (D).
Discussion

We generated a rat model of Duchenne muscular dystrophy by CRISPR/Cas9 gene editing and its cardiac phenotype was evaluated for the first time.

At the age of 6 months, most echocardiographic variables showed no significant differences between wild rats and DMD rats. In particular, their LV function such as LV fractional shortening (41 versus 42%) and TDI Sa septum (3.6 versus 3.6 cm/second) were almost the same average values. However, the histological findings of the 6-month-old rats showed mild fibrosis in the RV wall and epicardial LV wall. This mild fibrosis may have resulted in the lack of differences in LV function, but to some extent, a trend toward RV dysfunction (decreased RVFAC, TAPSE, and TDI Sa RV free-wall). These early stages of RV involvement could be because of RV overload due to fibrosis of the diaphragm and respiratory failure. Due to progressive systemic muscle atrophy, the body weight of DMD rats was significantly lighter than that of wild rats.

Rats 6-months old and 10 months old are approximately equivalent to human ages of 18 years and 25 years, respectively. In consideration of the fact that most patients with DMD show mild to moderate LV dysfunction at the age of 18, which progresses to overt LV dysfunction until the age of 25, DMD rats seem to have a milder cardiac phenotype with slower progression than human patients. However, the distribution and the progression of fibrosis seem consistent with patients with DMD in that fibrosis initially localized in the epicardial LV free-wall and RV progresses to the interventricular septum followed by the transmural global walls.

To date, several naturally occurring, or manually generated, animal models for DMD have been established. The most broadly used animal model for DMD is mdx mice. However, they exhibit very limited cardiac involvement, although they have a mild to moderate phenotype in systemic skeletal muscles. The more severe mdx/utrn−/− mice, which lack utrophin (dystrophin homolog working as compensatory protein in mdx mice), show LV dysfunction at the age of 2 months. However, their systemic phenotype is so severe they do not live longer than 3 months of age, which makes it difficult to use them for animal experiments. Another broadly used animal model for DMD is Golden retriever/Beagle dogs with muscular dystrophy. Although their skeletal muscles are severely

Table. Echocardiographic Results of Wild Rats and DMD Rats

| Variables | 6 months | 10 months |
|-----------|----------|-----------|
| Heart rate (bpm) | Wild 306 ± 47 | DMD 316 ± 39 | P-value 0.688 | Wild 328 ± 14 | DMD 339 ± 42 | P-value 0.603 |
| Left Heart Variables | | | | |
| Septal wall thickness (mm) | 1.8 ± 0.5 | 1.6 ± 0.3 | 0.524 | 1.6 ± 0.3 | 1.5 ± 0.2 | 0.523 |
| Posterior wall thickness (mm) | 1.9 ± 0.4 | 1.9 ± 0.3 | 0.994 | 1.7 ± 0.3 | 1.5 ± 0.2 | 0.501 |
| LV end-diastolic diameter (mm) | 8.9 ± 0.6 | 8.2 ± 0.5 | 0.047 | 10.1 ± 0.5 | 7.2 ± 0.8 | 0.001 |
| LV end-systolic diameter (mm) | 5.2 ± 0.7 | 4.7 ± 0.4 | 0.218 | 5.6 ± 0.6 | 4.9 ± 0.8 | 0.146 |
| LV fractional shortening (%) | 41 ± 6 | 42 ± 4 | 0.771 | 44 ± 6 | 33 ± 5 | 0.024 |
| Transmitral E velocity (cm/second) | 94 ± 11 | 99 ± 7 | 0.376 | 115 ± 16 | 83 ± 12 | 0.011 |
| Transmitral E DT (ms) | 54 ± 9 | 47 ± 12 | 0.269 | 44 ± 5 | 43 ± 8 | 0.752 |
| Transmitral E/Ea septum | 17.6 ± 5.2 | 24.2 ± 7.1 | 0.101 | 15.3 ± 3.7 | 16.1 ± 3.1 | 0.733 |
| Transmitral E/Ea lateral | 20.5 ± 3.9 | 23.6 ± 5.4 | 0.304 | 21.7 ± 3.7 | 19.1 ± 1.5 | 0.272 |
| TDI Sa septum (cm/second) | 3.9 ± 1.1 | 4.0 ± 0.9 | 0.955 | 3.6 ± 0.7 | 3.6 ± 0.7 | 0.895 |
| TDI Sa lateral (cm/second) | 5.9 ± 1.9 | 5.4 ± 1.1 | 0.608 | 5.7 ± 0.9 | 4.3 ± 0.7 | 0.041 |
| Right Heart Variables | | | | |
| RV fractional area change (%) | 51 ± 5 | 46 ± 5 | 0.118 | 47 ± 3 | 39 ± 8 | 0.057 |
| TAPSE (mm) | 2.5 ± 0.4 | 2.4 ± 0.4 | 0.725 | 2.4 ± 0.5 | 1.9 ± 0.5 | 0.178 |
| Transtricuspid E velocity (cm/second) | 65 ± 21 | 75 ± 20 | 0.467 | 78 ± 8 | 69 ± 22 | 0.392 |
| Transtricuspid E/Ea RV free-wall | 13.0 ± 10.2 | 15.0 ± 3.7 | 0.673 | 12.3 ± 2.4 | 13.9 ± 4.8 | 0.542 |
| TDI Sa RV free-wall (cm/second) | 5.0 ± 0.9 | 4.2 ± 0.7 | 0.160 | 5.3 ± 0.8 | 3.3 ± 0.7 | 0.004 |
| Maximum IVC diameter (mm) | 2.5 ± 0.6 | 2.4 ± 0.5 | 0.591 | 3.0 ± 0.5 | 2.5 ± 0.3 | 0.284 |

DMD indicates Duchenne muscular dystrophy; DT, deceleration time; E, peak E-wave velocity; Ea, peak velocity at early-diastole; IVC, inferior vena cava; LV, left ventricular; RV, right ventricular; Sa, peak velocity at systole; TAPSE, tricuspid annular plane systolic excursion; and TDI, tissue Doppler imaging.
involved, their cardiac dysfunction is relatively mild even at the age of 70 months. Consequently, our DMD rats can be a suitable animal model with which to develop therapeutic options for DMD, considering the relatively easy maintenance, easy breeding, and their cardiac involvements.

The present study has a major limitation that the number of 10-month-old DMD rats was limited for adequate statistical analyses. The risks of respiratory failure and sudden death increase with aging. As we lost 3 of the 7 DMD rats for experiments, some important differences might have been underestimated. Although the number was limited, our current results can provide baseline data for further studies developing drugs, understanding mechanisms, comparing their cardiac effects, and screening their side effects.

**Figure 3.** Representative rat heart sections of Masson’s trichrome staining. No fibrosis was observed in a 6-month-old wild rat (A). Relatively mild epicardial fibrosis was observed in a 6-month-old DMD rat (B). More severe transmural fibrosis was observed in a 10-month-old DMD rat (C).

**Conclusion**

We have generated a novel rat model of DMD by CRISPR/Cas9 genome editing. The DMD rats showed progressive cardiac fibrosis and overt dysfunction. Although the cardiac phenotype was milder than anticipated, DMD rats showed similar distributions and progression of heart involvement to those of patients with DMD. This animal can be a useful model with which to develop drugs and to understand the underlying mechanisms of progressive heart failure in patients with DMD.

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Disclosure

Conflicts of interest: All authors declare that there are no conflicts of interest regarding this study.

References

1. Ryder S, Leadley RM, Armstrong N, et al. The burden, epidemiology, costs and treatment for Duchenne muscular dystrophy: an evidence review. Orphanet J Rare Dis 2017; 12: 79.
2. Mercouri E, Muntoni F. Muscular dystrophies. Lancet 2013; 381: 845-60.
3. Kimura K, Morita H, Nakamura A, Takenaka K, Daimon M. Therapeutic strategy for heart failure in becker muscular dystrophy. Int Heart J 2016; 57: 527-9.
4. Kimura K, Morita H, Daimon M, et al. Prognostic impact of venous thromboembolism in patients with Duchenne muscular dystrophy: Prospective multicenter 5-year cohort study. Int J Cardiol 2015; 191: 178-80.
5. Kimura K, Morita H, Daimon M, et al. Utility of cystatin C for estimating glomerular filtration rate in patients with muscular dystrophy. Int Heart J 2016; 57: 386-8.
6. Eagle M, Baudouin SV, Chandler C, Giddings DR, Bullock R, Bushby K. Survival in Duchenne muscular dystrophy: improvements in life expectancy since 1967 and the impact of home nocturnal ventilation. Neuromuscul Disord 2002; 12: 926-9.
7. Seguchi O, Kuroda K, Fujita T, et al. Heart transplantation ameliorates ambulation capacity in patients with muscular dystrophy: An analysis of 9 cases. Circ J 2019; 83: 684-6.
8. Grady RM, Teng H, Nichol MC, Cunningham JC, Wilkinson RS, Sanes JR. Skeletal and cardiac myopathies in mice lacking utrophin and dystrophin: a model for Duchenne muscular dystrophy. Cell 1997; 90: 729-38.
9. Nakamura K, Fujii W, Tsuibo M, et al. Generation of muscular dystrophy model rats with a CRISPR/Cas system. Sci Rep 2014; 4: 5635.
10. Daimon M, Akaishi M, Asanuma T, et al. Guideline from Japanese Society of Echocardiography: 2018 focused update incorporated into Guidance for the Management and Maintenance of Echocardiography Equipment. J Echocardiogr 2018; 16: 1-5.
11. Kimura K, Daimon M, Morita H, et al. Evaluation of right ventricle by speckle tracking and conventional echocardiography in rats with right ventricular heart failure. Int Heart J 2015; 56: 349-53.
12. Mitchell C, Rahko PS, Blauwet LA, et al. Guidelines for Performing a Comprehensive Transthoracic Echocardiographic Examination in Adults: Recommendations from the American Society of Echocardiography. J Am Soc Echocardiogr 2019; 32: 1-64.
13. Sengupta P. The Laboratory Rat: Relating Its Age With Human’s. Int J Prev Med 2013; 4: 624-30.
14. Tandon A, Villa CR, Hor KN, et al. Myocardial fibrosis burden predicts left ventricular ejection fraction and is associated with age and steroid treatment duration in duchenne muscular dystrophy. J Am Heart Assoc 2015; 4: e001338.
15. Kimura K, Takenaka K, Ebihara A, et al. Prognostic impact of left ventricular noncompaction in patients with Duchenne/Becker muscular dystrophy—prospective multicenter cohort study. Int J Cardiol 2013; 168: 1900-4.
16. Goldberg SJ, Feldman L, Reinecke C, Stern LZ, Sahn DJ, Allen HD. Echocardiographic determination of contraction and relaxation measurements of the left ventricular wall in normal subjects and patients with muscular dystrophy. Circulation 1980; 62: 1061-9.
17. Kovick RB, Fogelman AM, Abbasi AD, Peter JB, Pearce ML. Echocardiographic evaluation of posterior left ventricular wall motion in muscular dystrophy. Circulation 1975; 52: 447-54.
18. Mori K, Hayabuchi Y, Inoue M, et al. Myocardial strain imaging for early detection of cardiac involvement in patients with Duchenne’s progressive muscular dystrophy. Echocardiography 2007; 24: 598-608.
19. Frankel KA, Rosser RJ. The pathology of the heart in progressive muscular dystrophy: epimyocardial fibrosis. Hum Pathol 1976; 7: 375-86.
20. Meyers TA, Townsend D. Early right ventricular fibrosis and reduction in biventricular cardiac reserve in the dystrophin-deficient mdx heart. Am J Physiol Heart Circ Physiol 2015; 308: H303-15.
21. Fayssoil A, Renault G, Guerchet N, Marchiol-Fournigault C, Faugeron JF, Richard I. Cardiac characterization of mdx mice using high-resolution Doppler echocardiography. J Med Ultrason 2013; 32: 757-61.
22. Verhaert IEC, van Duijn RJM, den Adel B, et al. Assessment of cardiac function in three mouse dystrophinopathies by magnetic resonance imaging. Neuromuscul Disord 2012; 22: 418-26.
23. Deconinck AE, Rafael JA, Skinner JA, et al. Utrophin-dystrophin-deficient mice as a model for Duchenne muscular dystrophy. Cell 1997; 90: 717-27.
24. Guo LJ, Soslow JH, BETTS AK, et al. Natural History of Cardiomyopathy in Adult Dogs With Golden Retriever Muscular Dystrophy. J Am Heart Assoc 2019; 8: e012443.
25. Yugeta N, Urasawa N, Fujii Y, et al. The burden, epidemiology, costs and treatment for Duchenne muscular dystrophy. J Int Heart J 2020; 1284.