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A Systematic Histopathological Study of Duchenne Muscular Dystrophy Using Semi-Quantitative Image Analysis, Digital Restoration Techniques, and Exploratory Statistical Approaches

(デュシェンヌ型筋ジストロフィー筋生検標本の探索的統合解析による組織病理学研究)

【Background and Objectives】 This dissertation redefines early Duchenne muscular dystrophy (DMD) histopathology by establishing candidate surrogate biomarker that reveals a reproducible breakpoint and aligns muscle tissue change with clinical timelines, drawing on digitally restored archival biopsies and a two-tier analytic framework. DMD is a severe X-linked neuromuscular disease characterized by progressive skeletal muscle deterioration with subsequent cardiopulmonary complications. While therapeutic advances have substantially improved clinical outcomes, major uncertainties persist about the pathophysiology of the early disease stage. Comprehensive descriptions of initial histopathologic alterations remain scarce. In short, this study asks whether microscopic changes can be identified earlier than the traditionally presumed window and whether an alternative, practical histological marker can capture that shift. A prior study suggested a critical histologic phase at ages 6–7, characterized by abrupt increase of connective tissue and implying relative stability beforehand. This notion was met with skepticism because no parallel physiological event has been demonstrated at that age and clinical symptoms usually begin earlier (typically 3–5 years). To resolve this clinicopathological inconsistency, the present study was designed to bridge the gap. Severely aged archival muscle biopsies—lacking clinical records and obtained over four decades ago at the former Yakumo National Hospital (now incorporated into National Hospital Organization Hokkaido Medical Center, Japan)—were analyzed. The aims were to define new histopathologic indicators, clarify early disease mechanisms, and map meaningful clinicopathological associations in early DMD using histology and age at biopsy (*ABx*) alone.

【Materials and Methods】 This retrospective observational study conformed to the Declaration of Helsinki and was approved by the Hokkaido University Hospital institutional review board (No. 024-0310), which has oversight of Hokkaido Medical Center. Owing to the retrospective design, informed consent was waived. Archival quadriceps biopsy slides from steroid-naïve DMD cases diagnosed between 1973 and 1984 were examined. Because clinical documentation was incomplete, preliminary case identification partly relied on contemporaneous recollection. In total, 46 biopsies were evaluated by three pathologists. Frozen and FFPE sections stained with hematoxylin–eosin (H&E), Gomori trichrome (G-T), and Masson trichrome (M-T) underwent digital restoration using look-up tables (LUTs) and Focus Stacking. Actomyosin adenosine triphosphatase (ATPase)-stained slides were excluded from the primary analyses but used for sensitivity analyses. Quantitative morphometry performed in Adobe Photoshop included myofiber size (MFS) metrics (e.g., standard deviation of MFS [*Sd*], coefficient of variation [*Cov*]), myofiber density (*MFD*), myofiber area (*MFA*), and connective/fibrotic area (*CFA*). Preliminary statistics comprised stain-method comparisons, correlation analyses, and multiple regression to predict *ABx*. Model performance was judged by Akaike's information criterion (AIC) and root mean squared error (RMSE) via leave-one-out cross-validation (LOOCV). Residuals were compared across predefined age bands (1–4, 1–6, 6–7, 7–9, 9–11, 11–17 years) using the Kruskal–Wallis test. Group differences were assessed by analysis of variance (ANOVA) or non-parametric tests according to distribution, with effect sizes reported. Logistic regression estimated critical cut-offs near age 6. Segmented regression tested for an age-related inflection in each parameter. Bayesian analyses and Monte Carlo simulations appraised robustness and informed sample size. The primary measure was the *MFD* age-breakpoint; secondary measures were power to detect the breakpoint, *MFD* cut-offs predictive of age, and cross-validation of

conventional indices across breakpoint-defined age strata. Two-sided $p < 0.05$ indicated statistical significance.

【Results】 Paired comparisons showed no meaningful difference in *MFD* between H&E and G-T (Cliff's δ 0.015 [confidence interval (CI) $-0.28, 0.31$]; negligible), with strong concordance showing interchangeability (Pearson's $r = 0.970$, $p < 0.001$); evaluation versus M-T was limited by sample size. Bayesian modeling indicated minimal batch effects. Of the 46 biopsies, 38 (ages 1–16 years) met inclusion criteria. Histology demonstrated an age-associated decline in *MFD* together with hallmark DMD changes—expansion of *CFA*, variability of *Sd* and *Cov*, fatty infiltration, and internal nuclei and/or hypercontracted fibers. Correlation analyses identified *MFD* as the strongest inverse correlate of *ABx* ($\rho = -0.85$). Multiple regression produced robust age-prediction models primarily driven by *MFD* and its interaction with *Sd* (adjusted $R^2 = 0.79$). The all-ages model yielded AIC 53.84 and RMSE 0.49, but accuracy worsened in older subgroups (Kruskal–Wallis $p < 0.01$). A model restricted to patients younger than 7 years improved fit (AIC = 29.12) with similar RMSE (0.47). Across age strata (1–6, $n = 13$; 6–7, $n = 8$; 7–11, $n = 14$), ANOVA showed large between-group differences in *MFD* (Glass's $\Delta -1.75$ [CI $-2.85, -1.25$] for 1–6 vs 6–7; -1.81 [CI $-2.91, -1.35$] for 1–6 vs 7–11). Additional indices (MFS metrics, *MFA*, *CFA*) also differed between 1–6 and ≥ 7 years. Substituting ATPase-derived *MFD* confirmed the same pattern. Logistic regression identified *MFD* thresholds of 546–572 fibers/mm² distinguishing those below versus above age 6. Segmented regression localized a breakpoint at 6.25 years (CI 5.08–7.42). Bayesian inference and Monte Carlo simulations supported this threshold and indicated roughly 80% probability of detecting the breakpoint within ± 1.25 years. Variance analyses within the breakpoint CI confirmed consistency across parameters.

【Discussion】 The results overturn earlier assumptions by demonstrating a pronounced decline in *MFD* before age 6, revealing a previously unrecognized phase of accelerated myofiber loss. Framed clinically, the microscopic inflection point appears to precede functional decline, aligning tissue change with the earlier symptom onset. Methodologically, digital restoration permitted reliable quantification from faded archival material, and cross-stain agreement with negligible batch effects supports the soundness of the derived metrics. Modeling and residual-pattern analyses delineated precise age-linked histopathological transitions, with *MFD* proving more sensitive to early change than conventional indices. A model confined to children under roughly 7 years predicted *ABx* more accurately, implying relative uniformity in early-phase dynamics and rising heterogeneity thereafter as fibroadipogenic remodeling advances. Logistic models yielded presumable *MFD* cut-offs around age 6 (mid-500s fibers/mm²), and segmented models confirmed an inflection near this boundary (approximately 6–6.5 years), reinforcing histopathological coherence. These values align with functional declines often seen at 6–7 years, highlighting a therapeutic window in which microscopic deterioration is underway yet potentially modifiable. Accordingly, *MFD* can anchor the timing of corticosteroids and evaluation of new therapies, and it motivates development of noninvasive surrogates (e.g., magnetic resonance imaging or ultrasound) for longitudinal monitoring. Whether post-6 reductions in *MFD* represent an irreversible shift remains unsettled, underscoring the need for prospective datasets linking histology, imaging, and function. Overall, these findings emphasize the need—and challenge—of building assessment frameworks beyond existing paradigms, defining early-stage DMD histopathologic hallmarks and offering a practical basis for therapeutic planning and trial stratification.

【Conclusions】 *MFD* emerged as a sensitive early-stage indicator in DMD, uncovering a previously unappreciated phase of rapid myofiber loss before age 6 and a reproducible histologic inflection near the 6-year mark. Using *MFD*-based thresholds provides a rationale for optimizing initiation of corticosteroids and assessment of novel agents. Future work should emphasize longitudinal, multicenter validation that integrates digital pathology with imaging and functional endpoints to refine cut-offs and develop clinical surrogates. Together, these elements provide a concise framework—what to measure (*MFD*), when to expect acceleration (~ 6 years), and how to apply it in trials and care. Additionally, the evidence also supports broader, ethical reuse of archival specimens in translational studies, deepening mechanistic insight into DMD pathophysiology and informing timely, data-driven decisions for this debilitating neuromuscular disorder.