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Refining NGS diagnosis of muscular disorders

In our original publication by Sevy *et al.*,¹ we described a cohort of patients affected with distal myopathy analysed by a large gene panel approach. Given the rapid evolution of genomic diagnostic data and interpretation standards, we now provide the re-evaluation of genetic diagnoses for this cohort. We reported in 2016 a patient (P8 in table 1) carrying a variant in *KBTBD13* which led us to give a probable diagnosis implicating this gene.¹ Based on the initial medical history of the patient, this case was considered as sporadic. Despite efforts to collect further family samples, only the index patient's DNA

was available for analysis at that time. Once further investigation of this family became possible, clinical examination of the patient's mother revealed a similar phenotype as her son, suggesting an autosomal dominant inheritance. Targeted sequencing showed that she did not carry the *KBTBD13* variant, arguing against the initially suggested pathogenic role of this variant. Patient P8 and the patient's mother were then analysed by a newly designed gene panel with improved gene coverage and a larger list of genes using an actualised version of the Gene Table of Neuromuscular Disorder.²

Doing so, we identified the c.1483G>A (p.(Gly495Arg)) variant in the *DNM2* gene (NM_001005361.3) for both of

these patients. Even though this variant is not yet described in the literature, we classified this variant as likely pathogenic according to the American College of Medical Genetics and Genomics (ACMG) criteria³ due to the following reasons. This variant is found at the frequency of 0.004% in the European subpopulation (1/21648 alleles in gnomAD v2.1)¹ and is predicted to affect protein function by several *in silico* prediction tools (UMD-predictor^{s2}, SIFT^{s3} and PolyPhen-2^{s4}). For additional references s1 to s12 (online supplemental file).

Moreover, the muscular biopsy results for patient P8 and his mother revealed the presence of centronuclear abnormalities that are typical for *DNM2* pathogenic

Table 1 Pathogenicity reassessment of each identified variant for Sevy *et al* publication¹ patients with definite, probable and possible diagnoses

| Patient | Gender | Variants with actualised nomenclature (status) | ACMG classification ³ | Modifications since Sevy <i>et al</i> publication ¹ |
|---|--------|---|--|---|
| Patients with definite diagnosis (n=7) | | | | |
| P1 | F | <i>CAPN3</i> (NM_000070.2): c.477C>T (p.(Tyr159Tyr)) (heterozygous) <i>CAPN3</i> (NM_000070.2): exons 7-8-9 deletion (heterozygous) | Pathogenic (class 5) Pathogenic (class 5) | – – |
| P2 | M | <i>TTN</i> (NM_001267550.1): c.95134T>C (p.(Cys31712Arg)) (heterozygous) | Pathogenic (class 5) | – |
| P5 | F | <i>TTN</i> (NM_001267550.1): c.95371G>C (p.(Gly31791Arg)) (heterozygous) | Likely pathogenic (class 4) | Shift from probable to definite molecular diagnosis group and confirmation of this HMERF associated <i>TTN</i> variant initially reported by Uruha <i>et al.</i> ⁴ |
| P13 | F | <i>MYH7</i> (NM_000257.2): c.4850_4852delAGA (p.(Lys1617del)) (heterozygous) + <i>PMP22</i> 1.5 Mb deletion (heterozygous) | Pathogenic (class 5) Pathogenic (class 5) | Molecular diagnosis , initially missed because of low coverage of the <i>MYH7</i> concerned region within the initial NGS gene panel used. Particular phenotype due to a ' double trouble ' situation (associated with a <i>PMP22</i> deletion). |
| P8 | M | <i>DNM2</i> (NM_001005361.3): c.1483G>A (p.(Gly495Arg)) (heterozygous) | Likely pathogenic (class 4) | Molecular diagnosis reassessment leading to the reclassification of the initially retained <i>KBTBD13</i> (NM_001101362.2) c.89G>A variant as likely benign. |
| P3 | F | <i>DYSF</i> (NM_003494.3): c.1168G>A (p.(Asp390Asn)) (heterozygous) <i>DYSF</i> (NM_003494.3): c.5302C>T (p.(Arg1768Trp)) (heterozygous) | Likely pathogenic (class 4) Likely pathogenic (class 4) | See patient P4 for details. See patient P4 for details. |
| P4 | M | <i>DYSF</i> (NM_003494.3): c.1168G>A (p.(Asp390Asn)) (heterozygous) <i>DYSF</i> (NM_003494.3): c.5302C>T (p.(Arg1768Trp)) (heterozygous) | Likely pathogenic (class 4) Likely pathogenic (class 4) | Controversial variant finally considered as likely pathogenic after additional analysis (western blot). P3 and P4 are two siblings with suspected dysferlinopathy. Western blot performed after muscular biopsy for patient P4 revealed the absence of dysferlin consistent with this molecular diagnosis. |
| Patients with probable diagnosis (n=3) | | | | |
| P6 | F | <i>AARS</i> (NM_001605.5): c.1019A>G (p.(Asn340Ser)) (heterozygous) | VUS (class 3) | Additional c.848G>A (p.(Arg283His)) <i>DYNC1H1</i> (NM_001376.4) heterozygous class 3 variant (VUS) identified on complementary NGS gene panel. |
| P7 | F | <i>KLHL9</i> (NM_018847.2): c.953G>A (p.(Arg318Gln)) (heterozygous) | VUS (class 3) | – |
| P9 | M | <i>CAV3</i> (NM_033337.2): c.216C>G (p.(Cys72Trp)) (heterozygous) | VUS (class 3) | Additional controversial c.25381G>A (p.(Glu8461LysHis)) <i>SYNE1</i> (NM_182961.3) heterozygous class 3 variant (VUS) identified on complementary NGS gene panel. |
| Patients with possible diagnosis (n=3) | | | | |
| P10 | F | <i>TTN</i> (NM_001267550.1): c.66527C>T (p.(Ser22176Phe)) (heterozygous) | VUS (class 3) | – |
| P11 | M | <i>TTN</i> (NM_001267550.1): c.20792A>G (p.(Asn6931Ser)) (heterozygous) | VUS (class 3) | – |
| P12 | F | <i>TTN</i> (NM_133379.1): c.13432_13433insA (p.(Thr4478fs)) (heterozygous) | VUS (class 3) | – |

variants causing centronuclear myopathy type 1 with autosomal dominant inheritance (OMIM, #160150). Therefore, using the ACMG guidelines³ we re-reclassified the initially implicated *KBTBD13* variant (NM_001101362.2), c.89G>A (p.(Gly30Asp)), as likely benign and established the molecular diagnosis of centronuclear myopathy type 1 with autosomal dominant inheritance (OMIM, #160150) due to the likely pathogenic *DNM2* variant, c.1483G>A (p.(Gly495Arg)).

These findings prompted us to re-evaluate other distal myopathy patients in our initial cohort.¹ Additional segregation data allowed us to re-assign patient P5 into the definite diagnosis group. Indeed, the ACMG³ class 4, c.95371G>C (p.(Gly31791Arg)), *TTN* variant (NM_001267550.1) found for this patient presenting with a typical form of hereditary myopathy with early respiratory failure (HMERF), was also identified in the affected daughter showing early clinical signs of this same muscular disorder. Additional familial investigations revealed that patient P5's mother was also clinically affected and died prematurely. Therefore, this additional information enables us to confirm the likely pathogenic effect of this *TTN* variant associated with HMERF.⁴

In our original study, a certain number of patients from the distal myopathy cohort remained undiagnosed. Additional genetic analysis allowed establishing the molecular diagnosis for one additional patient (P13) after identifying a pathogenic, c.4850_4852delAGA (p.(Lys1617del)) heterozygous *MYH7* variant (NM_000257.2).⁵⁵ Interestingly, this patient was also diagnosed with the well-known 1.5 Mb deletion encompassing the peripheral myelin protein 22 (*PMP22*) gene (NM_000304.2) on chromosome 17p11.2–12.⁵⁶

In the original study, patients P3 and P4 were given a 'probable' diagnosis since at that time there were not enough data to classify one of the identified variants as pathogenic. Since then, the c.1168G>A (p.(Asp390Asn)) *DYSF* variant (NM_003494.3) has been identified and classified as pathogenic or likely pathogenic in three patients (ClinVar database ID: 282410).⁵⁷ Moreover, western blot on muscle biopsy from patient P4 showed a complete absence of dysferlin, confirming the definite diagnosis of dysferlinopathy.

All results are summarised in table 1.

The pathogenicity classifications were also updated for these variants in the Leiden Open Variation Database (LOVD).⁵⁸

Overall, this diagnosis re-evaluation enabled us to reassess or confirm the molecular diagnosis of five patients (P3, P4, P5, P8 and P13 in table 1) and three additional affected family relatives of our distal myopathy patient's cohort initially reported in 2016.¹

Our reanalysis of the original cohort highlights several key difficulties associated with genetic analysis by next-generation sequencing. For example, the presence of pathogenic variants associated with two different diseases ('double trouble') can make phenotypic correlations extremely challenging, thus possibly leading to erroneous initial diagnosis.⁵⁹

A thorough clinical evaluation supported by complementary analyses such as muscle biopsy, western blot and muscle MRI are key for establishing a correct molecular diagnosis. Our study also highlights the importance of re-evaluating the available evidence to update the pathogenicity classification. Indeed, several variants with uncertain pathogenicity at the time of our initial study can now be confidently classified as pathogenic or likely pathogenic due to additional segregation data, complementary analysis or additional reports of patients carrying the same variant. This re-evaluation of pathogenicity is rarely done for variants published before extensive population frequency data became available⁶¹ and before the guidelines for variant classification became widely used.³ We believe this is a gap that must be remedied, as we now know that there are many examples of erroneous pathogenicity classifications of variants in the scientific literature and different databases.^{5,510,511} It is in that end that international curation efforts such as those initiated by ClinGen are currently underway to establish gene-specific interpretation guidelines and to curate a certain number of variants in databases. However, these efforts are not intended to correct all of the outdated classifications currently present in the literature and variant databases.^{57,58,512}

Therefore, we emphasise the importance for authors to re-evaluate the pathogenicity of variants in their published cohorts and to work with the curators of variant databases to update the information for the re-classified variants. In our case, as the original study was published before the ACMG guidelines³ became available, we re-evaluated the identified variants using these recommendations and updated the data in the LOVD database.⁵⁸

In summary, our new study highlights several common challenges in genomic diagnostics and draws attention to the

critical need of re-evaluating variant interpretations in previously published cohorts. This is of importance as the scientific literature, especially regarding the pathogenicity of genetic variants, has a fundamental role for the interpretation of these genetic variants and the potentially resulting molecular diagnosis.

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