Resolving an undiagnosed case of Neuromuscular Disorder using predictive mutational profiling

Tsinrong Lee[1], Yuqin Chin[1], Raman Sethi[2], Swati Tomar[2], Stacey Tay[2], Poh San Lai[2]
[1]Hwa Chong Institution [2]Department of Paediatrics, National University of Singapore

Abstract— Accurate diagnosis of Neuromuscular disorders (NMDs) is a challenging task for clinicians. Next Generation Sequencing (NGS) is a promising diagnostic method. Due to the wide gamut of existing predictive tools and lack of tool categorisation from end-user perspectives, we aimed to compare predictive and integrative pathogenicity prediction tools based on a self-designed criterion for end-users. Using Whole Exome Sequencing (WES) data, we prioritized disease-causing variants of an undiagnosed NMD patient by developing a fast, free, in-house mutational profiling pipeline. Our tiered-analysis pipeline entailed Minimum Allele Frequencies, genotype-phenotype correlations, evolutionary conservation and disease-phenotype associations. A unique scoring system was developed to integrate discrepancies between predictive tools. We curated 35 predictive and 11 integrative pathogenicity prediction tools. Of which, five tools- SIFT, PolyPhenV2, MutationTaster, MutationAssessor and FATHMM were ranked superior to others. A total of 212,623 variants were annotated using ANNOVAR and predicted using the above five tools. We ranked two compound heterozygous mutations in TTN gene (p.Thr23012Met, p.Tyr4266Cys) and a missense mutation in KBTBD13 gene (p.Pro247Thr) as strong causative variants in the given proband. Early-onset myopathy with fatal cardiomyopathy, Limb-Girdle Muscular Dystrophy 2J and Nemaline Myopathy 6 were postulated as the probable causative diseases in the undiagnosed patient, in decreasing order of likelihood. Thus, we have presented this diagnostic approach from end-users perspective, with the intention of simplifying the process and making it usable even for those lacking extensive bioinformatics backgrounds. This WES-based pipeline can be successfully adapted in a clinical setting for specific diagnosis of other rare Mendelian disorders.

Neuromuscular Disorder; Predictive mutational profiling; undiagnosed cases

I. BACKGROUND AND PURPOSE OF RESEARCH

Neuromuscular disorders (NMDs) are chronic diseases caused by abnormalities in lower motor neurons or muscle that often lead to disabilities or death. However, NMDs are hard to diagnose by conventional methods. Recently, Next Generation Sequencing (NGS) has emerged as a promising alternative to identify disease-causing variants of Mendelian disorders, which ultimately aids in accurate diagnosis and targeted therapeutics. To analyse NGS data, many *in silico* tools are offered on the Internet. However, navigating the available resources to select the most effective tool for individual use is challenging from the viewpoints of end-users. Thus, our project aimed to compare available *in silico* tools based on a self-designed criterion for end-users. Next, we aimed to design and use a unique, comprehensive pipeline based on selected tools, to prioritize disease-causing variants related to NMD from a set of Whole Exome Sequencing (WES) data. Our project addresses the overall aim of resolving an undiagnosed case of NMD.

II. HYPOTHESIS

We hypothesized that causative variants underlying the Neuromuscular Disorder (NMD) phenotype in the given patient could be detected by a simple, improvised predictive pipeline.

III. MATERIALS AND METHODS

A. NGS Workflow

Sequencing Reads were mapped to Reference Genome hg 19 and variants file was generated.

B. Clinical Presentation of the Patient

The two year old patient presented with increased serum creatinine phosphokinase (CPK) levels (10,000U/l), slow IQ development and dystrophic limb muscle resembling some form of NMD. Clinical diagnosis was not reached despite performance of single gene based molecular tests.

C. Comparison of in silico tools

To select *in silico* tools most suitable for our project, all available tools from literature were surveyed. Only regularly maintained and published tools were shortlisted to restrict the scope of analysis. Shortlisted tools were then classified into Predictive or Integrative Software. They were evaluated based on the type of mutation (SNVs/Indels) they predict, input/output formats, pathogenicity score systems, databases used and user-defined advantages and disadvantages observed while using the tool. From this comparison of software, a process for selecting *in silico* tools was generated, specific to the means and purposes of our project. First, tools fulfilling the necessary primary criteria were shortlisted: Able to perform batch analysis on large amounts of data; Ease of accessibility; Produces empirically assessable predictions for likelihood of deleteriousness of variants; Input format accepting output from WES data. Shortlisted tools were ranked based on a formulated secondary criteria and corresponding point system (Table 1) to evaluate tools, by allocating points to tools that fulfil desirable criteria.

TABLE I. TABLE OF SECONDARY CRITERIA FOR TOOL SELECTION

Rank	Criteria	Points	Rationale
1	Can accommodate large input data	5	Large amount of patient data to analyse
2	End user-friendly interface	4	Does not require bioinformatics expertise
3	Fast processing of data	3	Time frame of project makes speed desirable but not crucial
4	Credibility (significant amount of previous reports using them)	2	For credibility of effectiveness of tool. However, difficult to measure and naturally favours earlier-established tools

D. Data Analysis

We identified candidate causative variants following our self-generated pipeline. First, the VCF file obtained from WES was run through ANNOVAR to obtain a list of annotated variants. Next, intronic and synonymous variants were filtered out, as they are unlikely to affect phenotype. Thereafter, known and unknown variants were separated, with reference to the presence or absence of variant data in three population databases—ESP (Exome Sequencing Project), ExAC (The Exome Aggregation Consortium) and 1000 Genomes Project. Only variants with data in any of the databases were considered known. Then, for known variants, variants with minimum allele frequency (MAF) <5% were selected, according to MAF data from ESP, ExAC and the 1000 Genomes Project.

To obtain integrated scores from the predictions of selected tools and account for score discrepancies, we adopted the American College of Medical Genetics and Genomics (ACMG) (Rehm et al. 2013) guidelines to design a unique, holistic scoring system. Since individual score values from predictive tools represent confidence of prediction and not magnitude of effect (Jian et al. 2014), we scored variants according to prediction results. For each variant, a pathogenic or possibly pathogenic prediction from each tool was allocated value '1'. Pathogenic was considered synonymous with predictions of "Deleterious", "Disease causing", "Disease-causing automatic", "High Functional Impact Score (FIS)" and "Medium FIS". Benign predictions were allocated value '0'. Benign was considered synonymous with predictions of "Tolerated", "Polymorphism", "Polymorphism automatic", "Neutral FIS", and "Low FIS". Variants predicted to have "possible" pathogenicity were considered pathogenic, to minimise prematurely eliminating true positives (Walters-Sen et al. 2015). Scores were summed up per variant, and variants with scores ≥2 (out of 5) were shortlisted as deleterious. Variants were categorized by mutation types: nonsense, frameshift, UTR/splice site and missense. Genotype-phenotype correlation analysis was performed. Known variants were shortlisted based on involvement of gene in which the mutations occurs in skeletal muscle disorders and variant functional impact, by accessing OMIM, Pubmed, UniProt and NCBI databases. Analysis of unknown variants was done using available data from aforementioned databases, cell lines and animal model studies. Shortlisted variants were ranked quantitatively based on number of pathogenic predictions by predictive tools and conservation analysis (PhyloP Conserved: >0.850, GERP++ Conserved: >2.00). Associated disease phenotypes were compared with patient phenotype to obtain candidate variants.

IV. RESULTS AND DISCUSSION

A. Table of Predictive Softwares

After comprehensive assessment of available *in silico* tools, 35 predictive and 11 integrative tools for pathogenicity prediction was curated (Appendix 1). From the comparative table of tools, we followed our generated criteria for tool selection, and selected the 5 most effective, accessible and end user-friendly predictive tools for our project: SIFT, Polyphen-2, MutationTaster, MutationAssessor and FATHMM (Table 2). From the analysis of integrative tools, ANNOVAR was found to integrate all five selected predictive tools, and provide separate pathogenicity scores from each tool are provided by ANNOVAR. Hence, ANNOVAR was selected to annotate patient WES data.

B. Candidate Variants

WES identified 212623 variants in total, with 7151 variants (MAF <5%) present in exonic, splicing, UTR, upstream or downstream regions in the NMD subject. From the resulting mutations, 1085 were missense, 14 were nonsense, 24 were frameshift, 21 were deletions, 12 were insertions, 72 were unknown and 5923 were returned with NIL results. After analyzing genotype-phenotype correlations of variant with scores ≥2, 18 missense mutations were identified as potential candidate variants, and ranked according to likelihood as predicted by predictive tools and conservation scores from PhyloP and GERP++. From these variants, further comparison between patient phenotype and gene-associated disease phenotypes revealed 3 candidate variants (Table 3). Two compound heterozygous known missense variants in the disease-associated gene TTN were found to be most probable candidate variants. One novel missense variant in the disease-associated gene KBTBD13 was identified as second most probable candidate variant. Based on these candidate genes, the likely disorders underlying the patient clinical condition are, in decreasing order of likelihood, early-onset myopathy with fatal cardiomyopathy, Nemaline Myopathy 6, and Limb-Girdle Muscular Dystrophy Type 2J (Table 3).

1) TTN; Myopathy, early-onset, with fatal cardiomyopathy (AR): Our analysis found 2 rare known missense candidate variants: NM_133378(TTN):c.69035C>T and NM_133378(TTN):c.12797A>G, in conserved regions of the TTN gene. NM_133378(TTN):c.69035C>T was predicted pathogenic by 4 in 5 selected tools. NM_133378(TTN):c.12797A>G was predicted pathogenic by 3 in 5 tools.

TN encodes the protein titin (Chauveau et al. 2014) found in striated muscle, and is important to myofibril assembly, providing passive tension (Forbes et al. 2010), muscle development, elasticity, cell signaling and structure (Chauveau et al. 2014). TTN mutations are linked to multiple muscle disorders including early-onset myopathy with fatal cardiomyopathy (EOMFC) (Carmignac et al. 2007) and limb girdle muscular dystrophy (Huebsch et al. 2005). EOMFC is of particular interest, as it is characterized by early-onset muscle weakness with gradual dystrophic changes in muscle, increased CPK levels and delayed motor development, as well as eventual joint and neck contractures, cardiac dysfunction and death by cardiomyopathy. Our patient presents with slow development, dystrophic muscle and elevated CPK level, which is similar to early symptoms of the disease.

The identified candidate variants in TTN are compound heterozygotes, which supports the mode of disease for early-onset myopathy with fatal cardiomyopathy, which has an autosomal recessive inheritance pattern. In a study on 23 families with congenital Core Myopathies and primary heart disease, compound heterozygous TTN mutations were found causative of Core Myopathies with early onset in a significant number of subjects (Chauveau et al. 2014). Furthermore, both candidate variants in TTN occur in Immunoglobulin I-set domains in the titin protein. These domains make up much of the mass of titin protein (Roberts et al. 2015). They perform structural and regulatory functions in titin, conferring elasticity and participating in binding interactions, ultimately maintaining the structural integrity of the sarcomere (Otey et al. 2009). Hence, mutations in such regions are likely deleterious.

2) KBTBD13; Nemaline Myopathy 6: NM_001101362(KBTBD13):c.739C>A, a novel missense candidate variant in a conserved region of KBTBD13, was identified and predicted pathogenic by 3 in 5 predictive tools. Although the function of KBTBD13 is unknown, a mouse model has shown higher levels of KBTBD13 expression in skeletal muscle (Sambuughin et al. 2010). KBTBD gene family members are involved in transcription regulation, ion channel tetramerization and gating, protein ubiquitination or degradation, and cytoskeleton regulation. KBTBD13 is also reported to be associated with autosomal dominant Nemaline Myopathy 6 (NEM6) (Sambuughin et al. 2010), which is characterised by early onset and muscle weakness, which is similar to the presentation of the patient in this study. A highly similar heterozygous c.742C>A (p.Arg248Ser) mutation in a conserved region of KBTBD13 was reported in an Australian patient with NEM6 (Sambuughin et al. 2010). In both the Australian and our patient, early-onset muscle weakness was observed. The Australian presented with impaired mobility, which we postulate to be common to our patient due to dystrophic muscles. However while an elevated CPK level was detected in our patient, the Australian had a normal CPK level. Even though the identified variant in our proband NM_001101362(KBTBD13):c.739C>A: p.Pro247Thr was found to occur in a region of no known significance, the strong concordance between predictive tools and similarities in variant genotype hints the variant to be a moderately strong causative candidate with NEM6 as the linked disease.

3) TTN; Muscular dystrophy, limb-girdle, type 2J: Mutations in TTN also cause limb-girdle muscular dystrophy type 2J (LGMD2J). Limb-girdle muscular dystrophies are characterized by progressive proximal pelvic and/or shoulder girdle muscle weakness (Zheng et al. 2015). LGMD2J patients typically present with childhood-onset weakness in all proximal muscles, mild weakness in distal muscles, and normal/elevated CPK levels. This is similar to our NMD patient phenotype. However, muscle weakness in our patient is not restricted to proximal muscles, making LGMD2J a relatively weak candidate disease. In a study on a Chinese family, the mutation c.107788T>C (p.W35930R) in the TTN gene was identified to cause LGMD2J (Zheng et al. 2015). However, our candidate variants occur at different positions and in heterozygous states. While LGMD2J is inherited in an autosomal recessive manner, heterozygous TTN mutations are more likely to cause a less-severe autosomal dominant form of muscular dystrophy, tardive tTbial Muscular Dystrophy (TMD). TMD is characterized by leg muscle weakness and adult onset, which does not correspond with patient data. However due to the heterogeneous nature of LGMD2J symptoms and compound heterozygous state of our identified variants, LGMD2J is still a possible diagnosis.

Predictive tools do not have 100% weighted accuracy (Walters-Sen et al. 2015). Hence, one limitation of our project is the stringent criterion of only considering variants deleterious when predicted pathogenic by 2 in 5 selected tools. This may unwittingly eliminate true positive results. Secondly, variants predicted by ANNOVAR are reported with multiple known accession numbers, thus presenting the single actual accession number per variant with ambiguity. Identification of the correct accession number hence requires manual validation by users across multiple public databases.

While our predictive mutational profiling suggests at least 1 of 3 candidate genes is responsible for NMD in our patient, it alone cannot diagnose the exact NMD with full certainty. First, *in vivo* and *in vitro* functional studies should be conducted to confirm the effect of variants in our candidate genes. Next, segregation analysis of variants in candidate genes must be conducted in the family (Klein et al. 2014). Lastly, genotype correlation and clinical verification is necessary for conclusive diagnoses.

V. Conclusions and Recommendations

Predictive tools SIFT, Polyphen-V2, MutationTaster, MutationAssessor and FATHMM; and the integrative tool ANNOVAR are successful for predicting causative variants in our NMD subject. Our novel variant analysis pipeline has identified 3 strong candidate causative variants in the TTN and KBTBD13 genes of our subject. TTN is related to early onset Myopathy and Limb-Girdle Muscular Dystrophy Type 2J; KBTBD13 is linked to Nemaline Myopathy 6. Future similar

studies with larger sample sizes could be performed to validate and improve the effectiveness of this pipeline.

Our survey of tools focuses on the application aspect, presents findings in ways catered to end-users and provides an intuitive overview of tools applicable to NGS projects for researchers new in this area. This minimizes time surveying tools, making NGS more time-efficient. Our study reveals a strong correlation between high scores given by predictive tool analysis and associated phenotypes, suggesting that predictive software effectively shortlist causal variants. While predictive profiling may not be conclusive in pathogenicity, it reduces efforts required in clinical validation. This NGS approach and variant-selection pipeline would help narrow down candidate genes and disease conditions to provide clinicians with refined data for more accurate diagnoses for such patients.

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APPENDICES

Appendix 1: Table of 35 predictive and 11 integrative in silico tools

The full table of 35 predictive and 11 integrative *in silico* tools for pathogenicity prediction of SNVs and Indels can be freely accessed at this link: http://tinyurl.com/ToolTable.

Appendix 2: Table of raw data obtained from ANNOVAR and data obtained from respective filtration steps

Raw data used in this study can be viewed in detail at this link: http://tinyurl.com/NMDrawdata

Table 2. Selected Predictive in silico Tools

Software	Mutation Category	Input	Score system	Details	Advantages	Disadvantges
SIFT	SNVs	Variant list in text (residue/space-based coordinate system)	□ Deleterious: <0.05 □ Tolerated: >0.05	- Uses multiple sequence alignment (MSA)	Batch query; Fast: 6-7 minutes to run; Web-based; Results deleted after 1 hour	N.A
Polyphen -2	SNVs	Variant list in text (chr number:position ref base/variant base)	□ Probably damaging:≥ 0.957 □ Possibly damaging: 0.453-0.956 □ Benign: ≤ 0.452	-Uses naïve Bayes classifier & empirical rules (phylogenetic information, sequence conservation, structure to model position of amino acid substitution, SWISS-PROT annotation)	Batch query; Web-based	N.A
Mutation Taster	SNVs & Indels	.vcf files	□ 'Disease causing': Probably deleterious □ 'Disease causing automatic': Deleterious □ 'Polymorphism': Probably benign □ 'Polymorphism automatic': Benign	-Uses naïve Bayes classifier -Integrates information from multiple databases and prediction methods (e.g. NNSplice, polyadq, Grantham Matrix, phastCons, phyloP) -Analyses comprise evolutionary conservation, splice-site changes, loss of protein features	Batch query	Only VCF of data from one sample can be used
FATHMM	SNVs	Protein + substitution + dbSNP rs identifiers	□ 'Damaging': Deleterious □ 'Not Damaging': Tolerated □ Lower score indicates higher probability of deleteriousness	-Uses sequence conservation within hidden Markov models (HMMs) with "pathogenicity weights" -Annotates molecular and phenotypic consequences of mutations	N.A	N.A
Mutation Assessor (V2)	SNVs	Query variant + UniProt protein accession/RefSeq protein ID/transcript ID	□ 'High': Altered protein function □ 'Medium': Altered protein function □ 'Neutral': Protein function unaltered □ 'Low': Protein function unaltered	-Uses Multiple Sequence Alignment (MSA) to identify conserved positions -Specificity Score and Conservation Score determine Functional Impact Score -Calculates change in entropy of conserved residues that have functional specificity	N.A	N.A

Table 3: Table of ranked strong candidate variants

VariantRank	Gene	Candidate Variant(s) Genomic Reference(s)	Disease rank	Candidate Disease	Disease Phenotype	Minimum Allele Frequency of Variant		
						ESP	ExAC	1000 Genome
1	TTN	NM_133378:exon275:c.C69035T:p.Thr23012Met NM_133378:exon53:c.A12797G:p.Tyr4266Cys	1	Myopathy, early-onset, with fatal cardio-myopathy	Muscle weakness with onset in neonatal period or early infancy. Dystrophic changes later occurring. Motor development is delayed; independent walking achieved between age 20 months and 4 years. CPK levels may be increased. Joint and neck contractures and spinal rigidity may begin in the first decade, but become more apparent in the second decade. Scoliosis develops after age 11 years. Development of cardiac dysfunction with fast progression between ages 5 and 16. Death occurs between ages 8 and 20 years, typically from cardiac arrhythmia.	unknown 0.000252	0.0017 0.0022	0.00459265 0.00678914
			3	Muscular dystrophy, limb-girdle, type 2J	Childhood onset muscle weakness in arms and legs, particularly in muscles of shoulders, upper arms, pelvic area, and thighs, with dystrophic changes occuring. No cardiomyopathy. Severe disability within 20 years of onset. CPK may be increased.			
2	KBTBD13	NM_001101362:exon1:c.C739A:p.Pro247Thr	2	Nemaline myopathy 6	Childhood onset progressive weakness in proximal muscles. Intolerance to exercise, slow movements with stiff muscles, hypotonia, and depressed/absent deep tendon reflexes. Disease progresses slowly, and motor development is usually normal, but motor responses are slowed. No respiratory or cardiac involvement	unknown	unknown	unknown