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Molecular diagnostic testing for congenital disorders of glycosylation (CDG): Detection rate for single gene testing and next generation sequencing panel testing

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ABSTRACT

Congenital disorders of glycosylation (CDG) are comprised of over 60 disorders with the majority of defects residing within the N-glycosylation pathway. Approximately 20% of patients do not survive beyond five years of age due to widespread organ dysfunction. A diagnosis of CDG is based on abnormal glycosylation of transferrin but this method cannot identify the specific gene defect. For many individuals diagnosed with CDG the gene defect remains unknown. To improve the molecular diagnosis of CDG we developed molecular testing for 25 CDG genes including single gene testing and next generation sequencing (NGS) panel testing. From March 2010 through November 2012, a total of 94 samples were referred for single gene testing and 68 samples were referred for NGS panel testing. Disease causing mutations were identified in 24 patients resulting in a molecular diagnosis rate of 14.8%. Coverage of the 24 CDG genes using panel testing and whole exome sequencing (WES) was compared and it was determined that many exons of these genes were not adequately covered using a WES approach and a panel approach may be the preferred first option for CDG patients. A collaborative effort between physicians, researchers and diagnostic laboratories will be very important as NGS testing using panels and exome becomes more widespread. This technology will ultimately improve the molecular diagnosis of patients with CDG in hard to solve cases.

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1. Introduction

Glycosylation is the process of adding glycans (sugars) to proteins and lipids [1]. This post translational modification is essential for the proper functioning of many systems within the body. Deficient glycosylation results in a group of disorders known as congenital disorders of glycosylation (CDG). CDG is a group of over 60 different disorders with the majority of defects residing within the N-linked glycosylation biosynthesis pathway [1]. CDG is characterized by multi organ dysfunction with significant morbidity and mortality occurring within the first five years of life [1]. Several CDGs are characterized by distinguishing phenotypes such as cutis laxa. There are also other clinical features that are shared across many CDGs including developmental delay, seizures, intellectual disability, hypotonia and microcephaly. Therefore, it can be difficult to determine the gene defect based on phenotype alone [2]. The most common gene defect is *PMM2* in which more than 600 individuals have been identified [3]. However, for the majority of gene defects only a few families have been identified. The phenotypic spectrum is not fully known for many of the genes associated with CDG and the long term outcome of these patients is unknown.

Biochemical testing of serum transferrin using isoelectric focusing (IEF) or matrix assisted laser desorption-time of flight mass spectrometry (MALDI-TOF MS) is widely used as a diagnostic indicator of CDG [4]. Transferrin is an iron binding protein synthesized and metabolized mainly in the liver that contains two disialobiantennary glycans [4]. Deficient glycosylation results in distinct patterns of transferrin. This analysis can indicate whether the defect resides in the biosynthesis or transfer of glycans to proteins (Type I) or whether the defect resides in the modification of glycans after they are attached to protein (Type II) [5]. However, this analysis cannot pinpoint the specific gene defect. For a more in depth analysis of glycan structures, N-glycans and O-glycans can be analyzed by using MALDI-TOF MS [6]. This in depth analysis can indicate the location within the pathway the defect may reside and can provide candidate genes for molecular testing.

Many individuals diagnosed with CDG based on either biochemical testing and/or clinical phenotype lack a molecular diagnosis and the gene defect remains unknown. Molecular testing for single genes may not be the best option for these individuals if there is no indication of which gene may be defective. In these cases a gene by gene approach could lengthen the time to diagnosis and is not cost

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effective. An alternative to a single gene approach is to screen many genes simultaneously using next generation sequencing (NGS) technology. NGS is used by molecular diagnostic laboratories to sequence panels of genes providing a comprehensive approach to testing. Gene panels are useful when multiple genes are involved in a particular disorder or when there is a lot of phenotypic overlap between different disorders. Panels are also more cost effective and results can be obtained more rapidly than a traditional gene by gene approach. Genetic counseling for families can also be improved and treatment or therapy can begin earlier to improve the patient's quality of life [7,8]. Another recent advancement in the clinical diagnostic laboratory is the use of whole exome sequencing (WES) for identifying the gene defect in patients. WES is a viable option when all previous testing for the patient is negative and there are no additional candidate genes to test.

Currently, there are many different panels offered by molecular diagnostic laboratories including cancer panels, autosomal recessive panels and X linked disorder panels and this will continue to increase in the future [9,10]. In 2010, Emory Genetics Laboratory (EGL) launched NGS targeted gene panels for CDG. Here, we report our experience with molecular testing for CDG in the diagnostic laboratory using single gene testing and NGS targeted gene panels. Coverage of NGS panels versus WES and classification of variants detected are also discussed.

2. Materials and methods

All molecular diagnostic testing was performed at EGL, which is a Clinical Laboratory Improvement Amendments (CLIA) and College of American Pathologists (CAP) accredited laboratory.

2.1. Patient information

A total of 162 samples were referred for molecular testing for CDG from March 2010 through November 2012. 94 were referred for single gene testing and 68 were referred for NGS panel testing. Patients ranged in ages from several months old to 65 years of age with the majority being pediatric patients. Clinical information was provided for 80 (50%) patients and 45 (28.3%) patients had previous biochemical testing that was suggestive of CDG based on information included in the requisition form. Whole blood was collected for each patient for molecular testing and DNA was isolated using standard procedures.

2.2. Single gene CDG testing: Individual gene and single exon PCR amplification

Primers were designed for all exons for each of the 25 CDG genes using a bioinformatics in house developed automated primer design script. For details on primer design and PCR amplification refer to [8].

2.3. NGS CDG panels: DNA enrichment, NGS and data analysis

A total of 1.5 μg of genomic DNA from each patient sample was used for DNA enrichment. RainDance PCR amplification (RainDance Technologies, Lexington, MA) was used for DNA enrichment using the same protocol as described in [8]. The Applied Biosystems SOLiD 3 version sequencer (Applied Biosystems, Foster City, CA) was used to sequence the amplified targets through August 2012 and then the MiSeq (Illumina, San Diego, CA) was used beginning in September 2012. Both next generation sequencers were used according to the manufacturer's instructions. NextGene software (SoftGenetics, State College, PA) was used to map the reads to the genes in the RainDance library. The sequence for each gene was obtained from the Hg19 reference genome, except for *ALG9*. For *ALG9*, the sequence in locus NG_009210 was used because of an error in the reference genome. For sequence alignment at least 25 bases and at least 60% of the read had to match the reference. The condensation tool was used to improve the detection of indels for SOLiD. For SOLiD a variant was called if the mutant allele percentage was greater than 15 and the coverage was greater than or equal to 3. For MiSeq a variant was called if the mutant allele percentage was greater than 15 and the coverage was greater than or equal to 10. The data was filtered using an in-house developed bioinformatics pipeline for the diagnostic laboratory. A spreadsheet for further data review and analysis was created as described in [8]. The current criteria for adequate coverage of an exon require a minimum of 20× coverage for all exons and splice sites (±2). Bases ±3 to 10 within the intron require a minimum of 10× coverage. If an exon was found not to have adequate coverage, the exon was Sanger sequenced.

2.4. Confirmatory Sanger sequencing analysis

All variants detected that had the potential to be disease causing were confirmed by Sanger sequencing. Sanger sequencing analysis was performed as previously described in [8].

2.5. Classification of variants

Variants were classified based on a number of databases. Population frequency of the variants was determined by dbSNP and the Exome Variant Server. Human Gene Mutation Database and publications were searched to determine whether the variant has previously been associated with disease, how many families the variant has been detected in and if there was adequate evidence to classify the variant as deleterious.

3. Results

3.1. CDG molecular testing

EGL began offering molecular testing for 25 CDG genes in March 2010 [8]. The 25 genes included: *ALG1*, *ALG2*, *ALG3*, *ALG6*, *ALG8*, *ALG9*, *ALG12*, *ATP6V0A2*, *B4GALT1*, *COG1*, *COG7*, *COG8*, *DOLK*, *DPAGT1*, *DPM1*, *GNE*, *MGAT2*, *MOGS*, *MPDU1*, *MPI*, *PMM2*, *RFT1*, *SLC35A1*, *SLC35C1*, and *TUSC3*. Single gene or NGS panel testing was requested by the referring physician. When only one mutation or variant of unknown clinical significance (VOUS) was identified through sequencing follow up deletion/duplication (del/dup) analysis using Array Comparative Genomic Hybridization (aCGH) was suggested. This comprehensive testing approach could identify the second mutation and provide the patient with a molecular diagnosis.

3.2. Single gene CDG testing

A total of 94 samples were referred for diagnostic single gene testing (Fig. 1). 9 were referred in 2010, 39 in 2011, and 46 from January through November 2012. In addition, seven samples were referred for carrier testing because of a family history of CDG. Of the samples referred for carrier testing three tested positive for the familial mutation. Of the samples referred for diagnostic testing, five had molecular testing for multiple genes. 20 patients were identified with two changes that were previously reported mutations and/or variants likely to be causative of disease (Fig. 1). Seven of these patients had a likely gene defect in *ATP6VOA2*, five of the patients had a likely gene defect in *ALG1*, two patients had a likely gene defect in *SLC35C1* and one patient each had the likely gene defect in *ALG8*, *DPAGT1*, *PMM2*, *SEC23B*, *TUSC3* and *GNE* (Table 1). In addition, two other patients had one mutation in *TUSC3*. One patient had a *TUSC3* whole gene deletion and another patient had a deletion of exons 7 through 10 in *TUSC3*. Sequencing did not identify a second mutation in either of these individuals. Four patients had one detected variant of unknown clinical significance (VOUS) in *ATP6V0A2*, *PMM2*, *B4GALT1*, and

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