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Exome Sequencing for Molecular Characterization of Non-*HFE* Hereditary Hemochromatosis

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Abstract

Diagnostic genetic testing for hereditary hemochromatosis is readily available for clinically relevant *HFE* variants (i.e., those that generate the C282Y, H63D and S65C *HFE* polymorphisms); however, genetic testing for other known causes of iron overload, including mutations affecting genes encoding hemojuvelin, transferrin receptor 2, *HAMP*, and ferroportin is not. As an alternative to conventional genetic testing we propose diagnostic use of whole exome sequencing for characterization of non-*HFE* hemochromatosis. To illustrate the effectiveness of whole exome sequencing as a diagnostic tool, we present the case of an 18-year-old female with a probable case of juvenile hemochromatosis, who was referred for specialty care after testing negative for commonly occurring *HFE* variants. Whole exome sequencing offered complete coverage of target genes and is a fast, cost effective diagnostic tool for characterization of non-*HFE* hemochromatosis.

Introduction

Approximately 80% of cases of hereditary hemochromatosis (HH) cases are caused by biallelic mutation of *HFE*, the protein product of which participates in transcriptional control of the gene hepcidin (*HAMP*), the master regulator of iron metabolism.[1-3] Genetic testing for diagnostic purposes is widely available for clinically relevant *HFE* variants (i. e., those that generate the C282Y, H63D and S65C *HFE* polymorphisms); however, genetic testing for other known causes of HH, including mutations affecting genes encoding hemojuvelin (*HJV*), transferrin receptor 2 (*TFR2*), hepcidine (*HAMP*), and ferroportin (*FP* also known as *SLC40A1*) is not readily available for clinical diagnostic purposes. Thus, approximately 20% of patients with clinical and laboratory evidence of HH will have molecularly undefined disease.[2, 4, 5] The combination of increasing availability, improved interpretation (genomic informatics) and decreasing cost has made whole exome sequencing (WES) a

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clinically relevant tool for diagnosis of suspected disease-causing germline mutations.[6] Herein, we use a suspected case of juvenile hemochromatosis (JH) to assess the feasibility of WES as a diagnostic tool for defining the molecular basis of iron overload in a patient with non-*HFE* HH.

Case Report

An eighteen-year-old female with was referred for evaluation of iron overload after previous phlebotomy treatment. Iron studies at the time of initial contact were iron 347 µg/dL, transferrin saturation 100%, and ferritin 2329 ng/ml. The patient had 4 siblings, and iron studies showed that two of the patient's brothers, ages 12 and 16 years old, also had laboratory evidence of iron overload, iron > 290 µg/dl, transferrin saturation 100%, and ferritin >1000 ng/ml. Standard clinical genetic testing of DNA from the proband showed wild-type *HFE*. The early onset of disease in this family suggested JH, however, nucleotide sequence analysis of the three most commonly mutated genes that result in the JH phenotype (*HJV*, *HAMP* and *TRF2*) was unavailable commercially. We therefore undertook to identify the genetic basis of iron overload in the proband by using WES.

Methods

After obtaining informed consent, DNA was isolated observing the guidelines of a protocol approved by the Institutional Review Board of the University of Utah School of Medicine. The DNA was prepared for WES analysis using the Ion Torrent Ampliseq Exome Kit, Life Technologies, (Grand Island, NY) and sequence data was derived using a Life Technologies Ion Torrent Proton analyzer. A Variant Call File was generated using Life Technologies Access Ion Reporter™ Software Targeted analysis of the exome sequencing data was conducted using Genome Browse, a free software program provided by the Golden Helix Corporation (Bozeman, MT). To confirm the validity of the putative disease causing sequence variant identified in *HJV* by WES analysis, PCR primers flanking the area of interest were designed (sequences available on request). The *HJV* gene was amplified and subsequently subjected to the University of Utah Sequencing Core for validation by Sanger sequencing.

Results and Discussion

Review of the WES data showed that coverage of *HFE*, *TFR2*, *HAMP*, *HJV*, *FP* was complete and read depth was generally >20 - fold, except for a low read depth involving exon 3 of *HJV*. Visual inspection of the nucleotide sequence of *HFE*, *HJV*, *HAMP*, *FP*, and *TFR2* exons revealed a homozygous nucleotide substitution, *HFE* 959G>T (rs74315323) that generates the G320V missense mutation in *HJV* that has been previously associated with JH. [7-10] Upon identification it was recommended bi-monthly phlebotomy treatment continue until a serum ferritin level of less than 200 ng/ml is reached, at which point maintenance phlebotomies would occur every 2-3 months to maintain a ferritin level less than 200 ng/ml. These data confirmed that mutations in *HJV* were responsible for the early onset iron overload in this case. PCR-based amplification and Sanger sequence analysis confirmed the homozygous nucleotide substitution in *HJV*.

The purpose of this study was to determine the feasibility of using WES as a method for identifying the molecular basis of iron overload in a patient with clinical and laboratory evidence of HH not attributable to *HFE* sequence variants. Using this approach, the patient was found to be homozygous for the G320V mutation of *HJV* shown to underlie JH. [7-10] Early onset iron overload is characteristic of biallelically mutated *HJV*, and the observation that two of the proband's adolescent siblings had laboratory evidence of hemochromatosis (but two did not) supports the conclusion that mutant *HJV* is the causative molecular abnormality in this family.

The lack of commercially available molecular testing for non-*HFE* HH deprives patients and affected family members of a complete understanding of the basis of their disease, evidenced-based genetic counseling, effective therapy and disease-specific data that may inform prognosis and guide therapy. We elected to test the feasibility of using WES to address this clinically relevant knowledge gap. We deemed this approach preferable to subjecting each potentially causative gene (mutation of at least 3 genes, *HJV*, *HAMP* and *TFR2* could produce the phenotype) to PCR-based nucleotide sequence because of the expensive and labor-intensive nature of such analyses (obstacles that likely contribute to the absence of a commercially available option for molecular testing for non-*HFE* HH). Historically, WES has been considered both too complex technically and analytically and too expensive for clinical use.[11] The degree of complexity is rapidly being minimized by technical advances in both hardware and software that have reduced expense and made data generation rapid and analysis relatively straightforward[12, 13]. For example, in the case described herein, WES was performed at a cost of about \$600.00 and data was generated and interpreted in a day. Another 5 days were required to confirm the presence of the G320V sequence variant by PCR-based Sanger sequencing at a cost of approximately \$40.00. For example, WES could have failed to identify a known causative mutation; but each WES run generates thousands of possible causative genetic variants.[11] Subsequent studies may identify previously unknown disease causing mutations affecting genes that were not scrutinized in the initial data interrogation. The nucleotide sequence of those genes could be analyzed retrospectively from data archived from the original analysis, thereby providing the opportunity to identify the genetic basis of the disease without the need for additional testing. Archived sequence data may also become clinically relevant as sequence variants that affect disease severity (modifier genes) are identified, as evidenced by *HFE* dependent HH modifier gene, *GNPAT*. [14]

The recent marketing authorization by the FDA of the Illumina MiSeqDx, a non-disease-specific sequencing platform, allows any laboratory to test any sequence for any purpose, thus moving next generation sequencing into the mainstream of diagnostic testing.[6] Privacy and reimbursement issue remain to be resolved, but this type of genetic analysis has the potential to provide immediate and long-term clinical benefit.[15] (Figure 1)

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Key Points

Whole exome sequencing is an effective diagnostic tool for molecular characterization of non-HFE hereditary hemochromatosis.

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Figure 1. Proposed algorithm for incorporating WES in the evaluation of patients with suspected HH

Patients are not anemic and have no symptoms suggestive of brain iron accumulation. These features distinguish them from patients with iron-overload due to pathophysiologic mechanisms other than abnormalities in the hepcidin-ferroportin axis such as defects in iron transport and ineffective erythropoiesis. FP mutations can result in either loss of function or gain of function. Those that cause loss of function do not produce high transferrin saturation.

* T-sat >45% for females or >50% for males

† *HJV*, *HAMP*, *TFR2*, FP

§ Archive sequence data for re-evaluation when new molecular mechanisms are identified and when clinically relevant gene modifiers are recognized