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The implications of familial incidental findings from exome sequencing: the NIH Undiagnosed Diseases Program experience

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Purpose: Using exome sequence data from 159 families participating in the National Institutes of Health Undiagnosed Diseases Program, we evaluated the number and inheritance mode of reportable incidental sequence variants.

Methods: Following the American College of Medical Genetics and Genomics recommendations for reporting of incidental findings from next-generation sequencing, we extracted variants in 56 genes from the exome sequence data of 543 subjects and determined the reportable incidental findings for each participant. We also defined variant status as inherited or de novo for those with available parental sequence data.

Results: We identified 14 independent reportable variants in 159 (8.8%) families. For nine families with parental sequence data in our cohort, a parent transmitted the variant to one or more children (nine minor children and four adult children). The remaining five variants occurred in adults for whom parental sequences were unavailable.

Conclusion: Our results are consistent with the expectation that a small percentage of exomes will result in identification of an incidental finding under the American College of Medical Genetics and Genomics recommendations. Additionally, our analysis of family sequence data highlights that genome and exome sequencing of families has unavoidable implications for immediate family members and therefore requires appropriate counseling for the family.

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Key Words: exome sequencing; familial; incidental findings; NIH Undiagnosed Diseases Program; secondary variants

INTRODUCTION

"Incidental findings" are defined as genetic variants with medical or social implications that are discovered during genetic testing for an unrelated indication.[1] On the basis of recent publications,[2] the American College of Medical Genetics and Genomics (ACMG) Working Group on Incidental Findings in Clinical Exome and Genome Sequencing determined that looking for and reporting some incidental findings would probably have medical benefit for patients and their families. The working group therefore recommended reporting incidental findings from a "minimum list" of 56 genes for individuals having clinical exome or genome sequencing.[3] This recommendation has been widely debated and openly challenged.[4]

Although the return of incidental findings represents an important step forward in the use of sequencing for medical benefit,[5] implementing these recommendations requires the development of infrastructure to support evaluation and reporting.[3] Family members other than the proband are often included in diagnostic exome sequencing, and thus this also has implications for unaffected family members. The typical number of reportable variants that will be generated in practice has not been widely studied. One study of 572 subjects, selected for atherosclerosis phenotypes, found that ~1% of exomes may require disclosure of an incidental genetic finding, but the set of genes analyzed in that study did not include all the genes in the ACMG list, and the cohort was nonfamilial.[2] A more recent study found that ~3.4% of European ancestry exomes and 1.2% of African ancestry exomes in the National Heart, Lung, and Blood Institute Exome Sequencing Project bear actionable pathogenic or likely pathogenic incidental findings in 114 genes.[6] More data are needed to assess the possible impact of the ACMG recommendations in a variety of clinical settings. This is an important issue because resources are required to implement the recommendations.

We analyzed research exome sequence data from 543 individuals derived from 159 families. For the recommended 56 genes, this analysis identified 14 independent reportable variants in the exome sequence data of 27 participants. In nine families

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with parental sequence data, a parent transmitted the variant to one or more children. These analyses provide data that may be used to refine strategies for the reporting of incidental findings.

MATERIALS AND METHODS

Subject cohort

Family members gave informed consent or assent to protocol 76-HG-0238, "Diagnosis and Treatment of Patients With Inborn Errors of Metabolism and Other Genetic Disorders," approved by the institutional review board of the National Human Genome Research Institute. The exome sequence data were derived from a 159-family cohort consisting of 543 subjects, with 188 affected subjects, 137 unaffected siblings, and 218 parents. The average and median ages of the 543 subjects at the time of sequencing were 34.0 (SD: 20.8 years) and 37 years, respectively. Some subjects were deceased at the time of sequencing, and for those subjects projected age at time of sequencing was used because it is anticipated that incidental findings will only be sought in living subjects. Self-reported ancestry was white/European (89.1%), black/African American (4.1%), unknown (3.3%), Asian (2.2%), and multiracial (1.3%) (**Supplementary Table S1** online). These families included all those admitted to the National Institutes of Health (NIH) Undiagnosed Diseases Program (UDP) and selected for exome analysis as previously described.[7] The sequencing was performed on a research basis, not in a Clinical Laboratory Improvement Amendments–certified fashion.

Exome sequencing

Genomic DNA was extracted from peripheral whole blood using the Gentra Puregene Blood Kit (Qiagen, Germantown, MD) as per the manufacturer's protocol. The Illumina TruSeq exome capture kit (Illumina, San Diego, CA), which targets ~60 million bases consisting of the Consensus Coding Sequence annotated gene set as well as some structural RNAs, was used. Captured DNA was sequenced on the Illumina HiSeq platform until coverage was sufficient to call high-quality genotypes at 85% or more of targeted bases.

Alignment and genotype calling

Reads were mapped to National Center for Biotechnology Information (NCBI) build 37 (hg19) using the Illumina ELAND aligner. When at least one read in a pair mapped to a unique location in the genome, that read and its pair were then aligned with Novoalign (Novocraft, Selangor, Malaysia). These alignments were stored in BAM format and then fed as input to bam2mpg (http://research.nhgri.nih.gov/software/bam2mpg/index.shtml), which called genotypes using a Bayesian algorithm (most probable genotype, or MPG).[8]

Coverage

Using the UCSC Genome Browser's hg19 human genome reference exon annotations for the 56 genes, we identified 1,257 discrete exon regions, including the untranslated regions. We recorded base-by-base coverage (**Supplementary Table S2** online) and calculated the percentage of each exon with 10-, 20-, or 30-fold coverage (**Supplementary Tables S3–S5** online). We also summarized how many exons had at least 90% of their bases covered to at least each of these coverage thresholds (**Table 1**).

Annotations

The variants were annotated using Annovar.[9] Variants and genes listed in Human Gene Mutation Database Professional were added to the annotations. We also used annotations extracted from the **Supplementary Data** online published by Johnston et al.[2] and added annotations for variants listed in ClinVar[10] and locus-specific databases (LSDBs) registered in the Leiden Open Variation Database.[11] For LSDBs not registered in Leiden Open Variation Database, annotations were manually collected from the individual LSDBs and used to annotate the variants on the basis of matching Human Genome Variation Society nomenclature.

Data extraction

Variants within the 56 genes recommended by the ACMG were considered if they had at least one minor allele call with a minimum coverage of 20 and a minimum most probable genotype (mpg)/coverage ratio of 0.5.[12]

Data analysis

The ACMG recommendations state that "known pathogenic" variants in 56 genes (and "expected pathogenic" variants in a subset of those 56) should be reported to subjects sequenced for unrelated clinical reasons. The LSDBs and catalogs of clinically relevant variants, such as Human Gene Mutation Database and ClinVar, catalog variants identified in a gene, together with annotations of each variant as "pathogenic," "probable pathogenic," "variant of unknown significance," "probable nonpathogenic," or "nonpathogenic" (or similar categories). Such annotations can serve as a foundation for determining whether a variant is "known pathogenic."

An accepted standard for determination of variant pathogenicity (with or without consultation of the databases described above) has not emerged, although several have been proposed.[13] Various methods have been proposed to evaluate the likelihood of pathogenicity for variants of unknown significance

Table 1 Summary coverage statistics for exome sequence included in the study

	Threshold		
Exon type	**10×**	**20×**	**30×**
Percentage of exons for which >90% of the subjects had ≥95% coverage of the exon at ≥threshold levels	65.5%	45.4%	23.4%
Percentage of exons for which >90% of the subjects had 100% coverage of the exon at ≥threshold levels	63%	41.6%	20%

in genes associated with disease,[14–16] but we did not use them because they depend on data unavailable to us, i.e., defined penetrance[15,16] or population frequency and phenocopy rate.[14] Additionally, we did not use allele prevalence as supporting criteria because (i) the phenotyping of subjects included in the 1000 Genomes and Exome Sequencing Project cohorts is incomplete[17]; (ii) many of the disorders are of adult-onset type and therefore might not be expressed fully among subjects in the 1000 Genomes and Exome Sequencing Project cohorts[17]; (iii) some disorders have environmentally dependent expressivity (e.g., malignant hyperthermia susceptibility) and therefore might not be expressed fully among subjects in the 1000 Genomes and Exome Sequencing Project cohorts[17]; and (iv) large control cohorts (>10,000) are needed to properly evaluate case–control disparities for rare variants.[13]

Understanding that potential harm is posed both by false-positive and false-negative incidental findings and that variants discovered in sporadic cases may have a high false-positive rate,[18–20] we chose the following criteria for accepting variants as "known pathogenic": (i) designation in at least one variant database as "pathogenic" or "probable pathogenic" and supporting evidence such as experimental assays or segregation with disease or (ii) meeting the criteria for "expected pathogenic" (see below) and a listing in at least one variant database as "pathogenic." This process required review of the literature and required ~320 man-hours from individuals knowledgeable of genetics, experimental methodology, and medicine. Approximately 200 hours were spent intersecting LSDBs with our variant set and flagging variants for further review. The remaining ~120 hours were spent reviewing literature and splice predictions for individual variants under consideration for reporting.

Our minimum acceptable segregation patterns for autosomal dominant disorders were either a confirmed de novo variant in an affected child with two unaffected parents or segregation of the variant to three affected family members in two generations. We judged requiring five informative meioses or positive evidence of linkage as unreasonably stringent criteria[21] and only requiring two affected family members in two generations as too lax a criterion for association of a variant with disease.[18,19] We did not accept clinically identified variants claimed to cause disease as pathogenic without reported functional data or familial segregation.

To define variants as "expected pathogenic," we used previously described criteria.[22] Briefly, these include mutations leading to premature translation termination, loss of a translation termination codon, loss of a translation initiation codon, and alteration of canonical splice donor or acceptor sites.

Missense variants not previously associated with disease are considered to comprise a class of variants that may or may not cause disease and therefore are not automatically disclosed to the patient.[22] Furthermore, the lack of information regarding these variants in an LSDB, Human Gene Mutation Database, or ClinVar indicates that they are unlikely to be recognized by the medical genetics community as known pathogenic variants. We therefore designated missense variants not present in these databases as nonreportable.

Both alleles of *MUTYH* must be mutated to meet the ACMG reporting recommendations. We therefore selected homozygous nonreference variants and paired compound-heterozygous variants. We deemed a variant pair reportable only if each variant of the pair met the criteria of being listed as "pathogenic" in at least one variant database and having supporting evidence such as experimental assays or segregation with disease.

To count the number of reportable incidental findings per independent exome, one subject per family was selected randomly, and the number of incidental findings in those subjects was counted. We also counted the number of reportable incidental findings in subjects who are currently minors and noted whether the disease associated with the variant in question was of adult-onset or childhood-onset type.

Phenotype correlation

Family and medical history and pertinent laboratory findings were reviewed where available for individuals with a reportable variant.

RESULTS

For the UDP cohort of 543 exome sequence data, there were 5,948 variants in the 56 ACMG-recommended genes (**Figure 1**; see **Supplementary Table S2** online for a complete list of all variants with annotations) when compared with the human reference sequence (NCBI build 37; hg19) (**Table 2**). To select variants of sufficient quality, we limited further analyses to those variants with a minimum coverage of 20 reads and a minimum mpg/coverage ratio of 0.5. Of the 5,928 variants that remained, 4,932 were judged highly unlikely to be reportable under ACMG recommendations because they were not present in LSDBs and were localized to introns outside of the canonical spice sites (67%), resided in 3′-untranslated regions (13%), encoded synonymous amino acid changes (7.5%), or resided in other non–protein-coding regions such as 5′-untranslated regions or the kilobase flanking the gene (6%) (**Figure 1**). Two other classes of variants that we excluded on the basis of absence from LSDBs, predicted functional impact, and per ACMG recommendations[22] were missense variants of unknown significance (6.5%) and variants predicted to affect splicing but outside of the canonical splice sites.

Each of the remaining 996 variants was then annotated with information available from Human Gene Mutation Database, ClinVar, and LSDBs and for the predicted consequence (e.g., frameshift, splicing, and termination). Of these, 250 variants were listed as known pathogenic or probable pathogenic in at least one database or were known to cause a premature translation termination, loss of a translation termination codon, loss of a translation initiation codon, or alteration of canonical splice donor or acceptor site. After reviewing the literature for supporting evidence to justify designating these 250 variants as pathogenic, 3 variants met criteria as "expected pathogenic" and 11 as "known pathogenic" (**Table 3** and

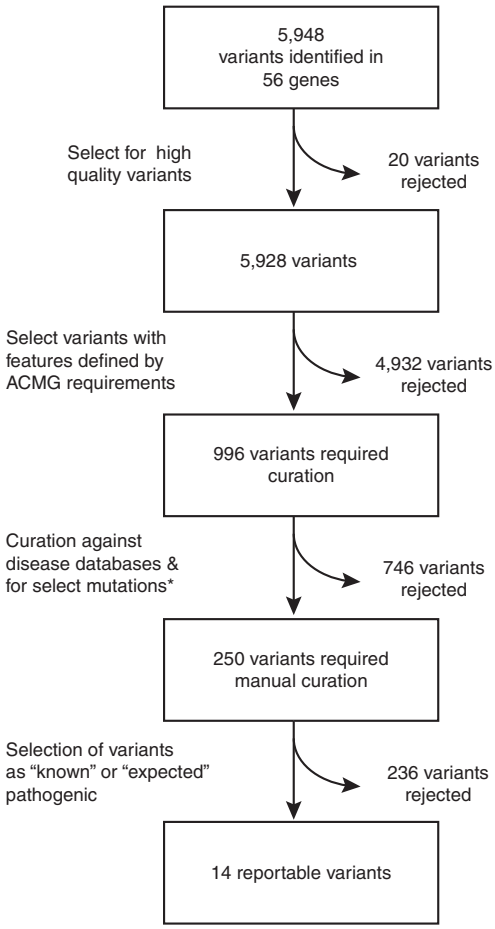

Figure 1 Flow chart summarizing the analysis process of the National Institutes of Health Undiagnosed Diseases Program and observations for the 56 genes recommended for interrogation by the American College of Medical Genetics and Genomics Working Group on Incidental Findings in Clinical Exome and Genome Sequencing. The observations were derived from analysis of exome sequence data derived from a 159-family cohort consisting of 543 subjects with 188 affected subjects, 137 siblings, and 218 parents. *Mutations recommended for reporting as "expected pathogenic" include premature translation termination, loss of a translation termination codon, loss of a translation initiation codon, or alteration of canonical splice donor or acceptor site.

Table 2 Variants analyzed

Type of variant	Number of variants
Total variants in ACMG-recommended genes	5,948[a]
Variants meeting minimum quality standards	5,928
Variants rejected for absence from databases and for mutation properties	4,932
Intronic	3,300
Exonic synonymous	700
3′ UTR	655
5′ UTR	100
5′ Flanking	40
3′ Flanking	49
Noncanonical splice	4
3′ UTR ncRNA	78
5′ UTR ncRNA	6
Variants requiring curation	996
Variants requiring manual curation	250
Variants designated reportable	14

[a]Multiallelic variants were counted as a single variant in the numbers listed in this study, but in Table 3 and in **Supplementary Table S2** online, they are provided as individual allelic variants.

ACMG, American College of Medical Genetics and Genomics; ncRNA, noncoding RNA; UTR, untranslated region.

Figure 1c). These 14 variants were present in 27 subjects from 14 families. No reportable variant was observed in more than one family. Thus, 5.0% (27/543) of the exomes in our cohort had a finding that would result in disclosure under the ACMG recommendations.

To determine how many of the variants arose de novo as opposed to being inherited, we analyzed the parental sequences in 9 of the 14 families where parental sequences were available. For all nine families (nine minor children and four adult children), one parent transmitted the variant to one or more children. The remaining five variants were identified in an adult for whom parental sequence was not available.

We identified a reportable incidental finding in nine minor subjects in our cohort. For these nine subjects, five had incidental findings associated with adult-onset conditions, and four had incidental findings associated with childhood-onset conditions.

A review of family and personal medical history revealed pertinent medical findings in only two cases. An adult subject with an *SCN5A* mutation had a history of exercise-induced fatigue and a first-degree relative with an unspecified early-onset cardiac condition; this relative was not enrolled in our study and, therefore, we could not evaluate segregation of the variant or verify phenotypic relevance. Another adult subject had an *APOB* mutation with a normal lipid profile: serum cholesterol = 161 mg/dl (normal: <200 mg/dl), low-density lipoprotein = 93 mg/dl (normal: <100 mg/dl), and high-density lipoprotein = 56 mg/dl (high risk: <40 mg/dl, low risk: ≥60 mg/dl).

DISCUSSION

By analysis of exome sequence data from 543 individuals distributed among 159 families, we clarify the reporting burden for the recommendations of the ACMG Working Group on Incidental Findings in Clinical Exome and Genome Sequencing.[3] We discovered 14 reportable variants for 27 individuals in 14 families. Therefore, 8.8% of families enrolled for exome sequencing under the NIH UDP protocol had incidental findings requiring disclosure if the sequencing had been performed by a Clinical Laboratory Improvement Amendments–certified laboratory.

Compared with the 1% rate of reportable incidental findings observed for 23 of the 56 genes analyzed by Johnston et al.[2] and the rate of 1.2–3.4% for 114 genes analyzed by Dorschner et al.,[6] we found a higher rate of reportable incidental findings. This increased rate of reportable incidental findings could arise for several reasons, including (i) increased coverage and quality of sequencing of the exome, (ii) differences in variant selection,

Table 3 Reportable variants detected in the NIH UDP exome cohort

Gene	Disease	Chromosome	Variant: Genome	Variant: Complementary DNA	Variant: Protein	ClinVar accession number	dbSNP: rsID	dbSNP: Minor allele frequency	Number of variant chromosomes[a]	Rationale
TP53	Pediatric adrenocortical carcinoma (ACC)	17	7574017C>T	NM_000546.5: c.1010G>A	p.R337H	SCV000115376	rs121912664	NA	2	Meets criteria for known pathogenic variant because a functional assay has shown reduced function at physiological pH.[30] Although the variant is associated with pediatric ACC rather than Li–Fraumeni syndrome, the diseases are related and similarly amenable to medical intervention. Indeed, recent use of neonatal screening for this allele in southern Brazil has demonstrated utility, with authors stating, "Without screening and surveillance, only 50% of children with ACTs survive, and many require intensive, toxic chemotherapy."[26]
SCN5A	Long QT syndrome type 3; Brugada syndrome type 1	3	38592513C>T	NM_000335.4: c.5347G>A	p.E1783K	SCV000115377	rs137854601	NA	1	Meets known pathogenic criteria, with electrophysiological and patch-clamp experiments demonstrating negative inactivation shift and enhanced flecainide block in one study,[31] in addition to segregation with disease and small but prolonged inward current during long depolarizations in another study.[32]
SCN5A	Long QT syndrome type 3; Brugada syndrome type 1	3	38616876C>T	NM_000335.4: c.3575G>A	p.R1192Q	SCV000115378	rs41261344	0.012	3	Meets known pathogenic criteria because the variant (identified in subjects with long QT or Brugada syndrome) has been shown to produce late inactivating current relative to wild-type channels.[33] Subsequent reports have identified the variant in 6% of a small sample of Han Chinese people; the authors of this most recent paper suggest it may still be causal but with reduced penetrance because 1 of 9 carriers did have prolonged QT_c and another 1 of 9 carriers had an intermediate-range QT_c.[34] Recent panels of persons of East Asian ancestry have demonstrated prevalence of this variant varying from 0.2–12.5% (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=rs41261344). Although the upper range of this prevalence certainly casts doubt as to the pathogenicity of the variant, the carriers in these panels were not phenotyped, and this evidence therefore cannot be used to definitively disprove the above-cited functional study.
SCN5A	Lone atrial fibrillation	3	38647498C>T	NM_000335.4: c.1282G>A	p.E428K	SCV000115379	rs199473111	NA	2	Meets known pathogenic criteria by segregation, with lone atrial fibrillation in 3/3 family members.[35] The third family member had atrial fibrillation by history alone, but we give the benefit of the doubt to the authors. Although lone atrial fibrillation is not recognized as a reportable condition for mutations in *SCN5A* by the ACMG, recent studies have found mutations known to cause long QT or Brugada syndrome in families with early-onset lone atrial fibrillation.[35,36] The diseases are related and similarly amenable to medical intervention, so we consider the variant reportable under the spirit of the guidelines.
SCN5A	Sick sinus syndrome	3	38655278G>A	NM_000335.4: c.659C>T	p.T220I	SCV000115380	rs45620037	0.000	1	Meets known pathogenic criteria because patch-clamp experiments have found reduced peak current, delayed recovery from inactivation, and delayed inactivation.[37,38] The variant has been identified in subjects with sick sinus syndrome, although evidence of segregation with disease is thin.[39,40]
PKP2	Arrhythmogenic right ventricular cardiomyopathy	12	32955491C>G	NM_004572.3: c.2146-1G>C		SCV000115381	rs193922674	NA	2	Meets known pathogenic criteria as a splicing mutation that has been identified in >20 subjects with ARVD.[41] Does not segregate perfectly with disease in published reports of two families but segregates with disease in 2/3 subjects in two families.[42]

[a]Number of variant chromosomes in the UDP data set. All individuals were heterozygous or hemizygous for the variant.

ACC, adrenocortical carcinoma; ACCF/AHA, American College of Cardiology Foundation/American Heart Association; ACMG, American College of Medical Genetics and Genomics; ACT, adrenocortical tumor; ARVD/ARVC, arrhythmogenic right ventricular cardiodysplasia/cardiomyopathy; APOB, apolipoprotein B; BIC, National Human Genome Research Institute Breast Cancer Information Database; dbSNP, Single-Nucleotide Polymorphism Database; EP, expected pathogenic; ERT, enzyme replacement therapy; ESP, Exome Sequencing Project; α-Gal A, α-galactosidase A, HGMD, Human Gene Mutation Database; KP, known pathogenic; LF-like, Li–Fraumeni-like; LOVD, Leiden Open Variation Database; LSDB, locus-specific database; NISC, National Institutes of Health Intramural Sequencing Center; NIH UDP, National Institutes of Health Undiagnosed Diseases Program; UMD, Universal Mutation Database.

Table 3 Continued on next page

Table 3 Continued

Gene	Disease	Chromosome	Variant: Genome	Variant: Complementary DNA	Variant: Protein	ClinVar accession number	dbSNP: rsID	dbSNP: Minor allele frequency	Number of variant chromosomes[a]	Rationale
MYL3	Hypertrophic cardiomyopathy (HCM)	3	46902238C>T	NM_000258.2: c.235G>A	p.V79I	SCV000115382			1	Meets known pathogenic criteria because the variant segregates with disease in 4/6 postadolescent carriers in one family.[43] HCM often displays onset during adolescence, thus carriers younger than 18 years of age would not necessarily be expected to display the phenotype.[44] In this study, three family members demonstrate a borderline phenotype, but the findings are consistent with an HCM spectrum of disease (*T*-wave inversions, left-axis deviation, angulated septum, and diastolic dysfunction). These findings are compatible with a scenario in which only a portion of the left ventricular septum is hypertrophied, or during early emergence of clinical disease, both possibilities recognized by the 2011 ACCF/AHA Guideline for the Diagnosis and Treatment of Hypertrophic Cardiomyopathy.[45]
GLA	Fabry disease	X	100656740C>T	NM_000169.2: c.427G>A	p.A143T	SCV000115383	rs104894845	NA	4	Meets known pathogenic criteria because the variant has been shown to produce low but residual (36% wild-type) α-Gal A activity in a transfection assay.[46] Earlier interpretations of these findings were that this represented a late-onset variant with a nonclassical phenotype,[46,47] but a recent study has called into question whether this variant is pathogenic at all.[48] Although the recent arguments are compelling, some patients with this allele are on ERT[48]; we therefore feel that clinical navigation of this complex medical research is best conducted between the carrier subjects in our cohort and their physicians and that reporting the variant as an incidental finding is not precluded by recent publications arguing against the variant's pathogenicity.
DSP	Arrhythmogenic right ventricular cardiomyopathy	6	7583973C>T	NM_004415.2: c.6478C>T	p.R2160X	SCV000115384			2	Meets expected pathogenic criteria as a stop-gain mutation. Not present in LSDBs.
CACNA1S	Malignant hyperthermia susceptibility	1	201020165T>A	NM_000069.2: c.4060A>T	p.T1354S	SCV000115385			2	Meets known pathogenic criteria because the variant segregated with in vitro contracture test in 7/9 carriers, with the remaining 2/9 equivocal on the contracture test.[49] A tenth carrier in the family was not biopsied.[49] The same study also used patch-clamp experiments to demonstrate accelerated inward Ca^{2+} current and increased sensitization of ryanodine receptor 1 under caffeine exposure in a transfection model.[49]
BRCA2	Breast and ovarian cancer susceptibility	13	32914529A>T	NM_000059.3: c.6037A>T	p.K2013X	SCV000115386	rs80358840	NA	1	Meets known pathogenic criteria as a stop-gain mutation observed in affected subjects. Submitted by two subjects in Sharing Clinical Reports[50]; also identified in a German study in one individual.[51]
BRCA2	Breast and ovarian cancer susceptibility	13	32929240delAC	NM_000059.3: c.7251_7252del	p.His2417 Glnfs*3	SCV000115387			3	Meets expected pathogenic criteria as a frameshift mutation. Not present in LOVD, BIC, or UMD, but frameshift mutations in this region in BIC are listed as clinically relevant.
BRCA1	Breast and ovarian cancer susceptibility	17	41197713insG	NM_007294.3: c.5578dup	p.His1860 Profs*20	SCV000115388			1	Meets expected pathogenic criteria as a frameshift mutation. Although it is very near the end of the coding sequence, many frameshift mutations in these exons are cited in BIC as pathogenic.
APOB	Familial hypercholesterolemia	2	21229161G>A	NM_000384.2: c.10579C>T	p.R3527W	SCV000115389			2	Meets known pathogenic criteria because functional evidence supports reduced low-density lipoprotein binding.[28,52–54] The effects of this variant are thought to be milder than a Gln substitution at the same codon.[28,52–54]

[a]Number of variant chromosomes in the UDP data set. All individuals were heterozygous or hemizygous for the variant.

ACC, adrenocortical carcinoma; ACCF/AHA, American College of Cardiology Foundation/American Heart Association; ACMG, American College of Medical Genetics and Genomics; ACT, adrenocortical tumor; ARVD/ARVC, arrhythmogenic right ventricular cardiodysplasia/cardiomyopathy; APOB, apolipoprotein B; BIC, National Human Genome Research Institute Breast Cancer Information Database; dbSNP, Single-Nucleotide Polymorphism Database; EP, expected pathogenic; ERT, enzyme replacement therapy; ESP, Exome Sequencing Project; α-Gal A, α-galactosidase A, HGMD, Human Gene Mutation Database; KP, known pathogenic; LF-like, Li–Fraumeni-like; LOVD, Leiden Open Variation Database; LSDB, locus-specific database; NISC, National Institutes of Health Intramural Sequencing Center; NIH UDP, National Institutes of Health Undiagnosed Diseases Program; UMD, Universal Mutation Database.

(iii) differences in the subject cohort, or (iv) higher frequency of reportable variants in the ACMG-recommended genes compared with the previously studied genes.

Regarding the sequence coverage and quality, the study of Johnston et al.[2] analyzed a smaller portion of the exome and aligned the sequences against an earlier version of the human reference genome. These two factors suggest that inclusion of more of the human exome and refinement of the reference genome might increase the number of detectable reportable variants. Testing of this proposal by a detailed analysis of exons—both sequenced and not sequenced—in the two data sets was, however, beyond the scope of this work because we did not have access to the exome sequences studied by Johnston et al.[2] To enable future comparative investigations, we have provided details of coverage for our exome sequence data (**Supplementary Tables S3–S6** online).

Regarding differences in variant selection, the ACMG's estimation of a 1% rate of reportable incidental findings was based on an allele frequency of >0.5% within the cohort and an allele frequency of >0.015% in dbSNP (Single-Nucleotide Polymorphism Database) as exclusionary criteria for a pathogenic designation.[2] We did not use allele frequency as an exclusionary criterion for pathogenicity for two reasons. First, deleterious alleles occasionally exhibit higher prevalence in some populations.[23,24] Second, as discussed above, phenotyping is incomplete in cohorts from which most frequency data are derived.

To classify a variant as reportable, Dorschner et al.[6] required an allelic frequency of less than a predetermined disease-specific maximum prevalence plus various permutations of independently observed segregation with disease. Compared with our study, their criterion was 4 vs. 3 segregations of the variant with disease; however, they did not consider functional assays as evidence for pathogenicity and only considered protein truncation as pathogenic if it occurred in the first 90% of the amino acid sequence. These differences probably contributed to the differences in our rates (5% vs. 1.2–3.4%) of incidental findings. For example, their more stringent segregation requirements and lack of consideration of functional experimental evidence (e.g., patch-clamp results) probably led to their classification of three variants—*CACNA1S* p.T1354S, *SCN5A* p.T220I, and *SCN5A* p.E428K—that we considered "known pathogenic" as "variants of unknown significance."

In this context, we expect that judicious comparison of variant classification may demonstrate that even reasonable parties disagree regarding the benefits and risks of reporting such variants as incidental findings. The ACMG recommendations try to balance the need and ability to return highly beneficial risk information to the patients (true positives) while at the same time limiting the potential harm by not returning false-positive results. The recommendations are written quite conservatively to strike a good balance between these two competing goals. Consequently, the recommendations clearly state that "variants that are previously unreported but are of the type which is expected to cause the disorder, as defined by prior ACMG guidelines, should be reported." The aforementioned guidelines are from the ACMG Recommendations for Standards for Interpretation and Reporting of Sequence Variations: Revisions 2007 (ref. 3) and can be found at https://www.acmg.net/StaticContent/SGs/ACMG_recommendations_for_standards_for.9.pdf. These guidelines state that if a variant is not previously reported to cause the disease, only two paths lead to classification of a variant as reportable. On detecting predicted deleteriousness (stops, indels, and some splice sites) or in case of uncertainty (missense, potential splice site, in-frame indels, single-nucleotide polymorphism association only), the researchers need to collect supporting evidence to favor the deleteriousness of the variant.

Although one might advocate for even stricter criteria, the criteria that we have selected for our study are more stringent than those provided by both the ACMG Recommendations for Reporting of Incidental Findings in Clinical Exome and Genome Sequencing and ACMG Recommendations for Standards for Interpretation and Reporting of Sequence Variations: Revisions 2007. We also acknowledge that the supporting evidence for these uncertain variants will vary in its quality and quantity and that the evidence will never be unequivocal for the simple fact that in light of unequivocal evidence, the variant in question would otherwise have been previously reported as disease causing. These variants and supporting evidence need to be returned to the clinician who ordered the sequencing, and it is the clinician's duty to put these test results in the context of the patient's clinical background. Clinicians do this for other tests, and the clinician's understanding of the test characteristics is more important in the correct interpretation of the test than the test characteristics themselves. A test with high false-positive rate but also with high sensitivity can be quite useful and desirable if used in the correct context with the right information to interpret the results. Our approach is therefore in agreement with the ACMG Recommendations for Reporting of Incidental Findings in Clinical Exome and Genome Sequencing, although until all possible changes in the human genome are annotated with unequivocal evidence to either support or refute the pathogenicity of each variant, there will always be a risk of making a false-positive call. A priori, the sensitivity or specificity of our methods cannot be determined, although higher specificity might be achieved with the use of very demanding requirements with respect to segregation or case–control disparities. The higher rate of incidental findings in our cohort as compared with those of the studies by Johnston et al.[2] and Dorschner et al.[6] highlights a possible limitation of our study in that our criteria may have a high false-positive rate. More research is needed to compare the sensitivity and specificity of different filtering strategies, ideally with long-term follow-up. In any case, incidental findings should be worked up in accordance with the degree of confidence in their deleteriousness, with a conservative approach taken to those variants with a minimum of evidence supporting pathogenicity.

With respect to differences in the study populations, the cohort reported by Johnston et al.[2] was selected for

atherosclerotic phenotypes (including unrelated controls) and was not a familial cohort. The cohort reported by Dorschner et al.[6] was selected from among the National Heart, Lung, and Blood Institute Exome Sequencing Project on the basis of European and African ancestries. Our cohort is largely of European ancestry. Transmission within our cohort increased the number of individuals at risk from 14 to 27. With undiagnosed disorders, there is also the possibility of an antecedent hypermutable disorder; however, no one individual in our cohort had an increased number of reportable variants, and our previous analyses of numbers of exome sequence variants within the UDP families did not identify marked differences from those reported for other cohorts.[25]

Regarding differences in the gene lists used, Johnston et al.[2] analyzed only a subset of the genes recommended by the ACMG Working Group on Incidental Findings in Clinical Exome and Genome Sequencing, i.e., the 23 associated with cancer syndromes. By contrast, the ACMG list also encompasses genes associated with cardiac arrhythmias, myopathies, connective tissue disorders, familial hypercholesterolemia, and malignant hyperthermia susceptibility. Dorschner et al.[6] analyzed 114 genes, including 52 of the 56 genes on the ACMG list.

Another variable in estimating the rate of reportable incidental findings is the thoroughness with which a disease and gene have been studied. In other words, the more individuals who have been identified with a disorder and checked for mutations in a gene, the more disease-causing mutations are likely to have been characterized. Reviewing our data, *SCN5A* (n = 4) and *BRCA2* (n = 2) had the most reportable variants. For *SCN5A,* this may reflect the fact that more variants are entered in databases because (i) both gain- and loss-of-function variants in *SCN5A* can cause disease and (ii) functional testing for pathogenicity is relatively accessible using patch-clamping experiments.

Four additional issues arising during our analysis were as follows: (i) defining the level of disease penetrance warranting reporting of a potential disease-causing variant, (ii) determining how to weight variants deposited by clinical laboratories without corroborating evidence of pathogenicity, (iii) the need for clinical correlation, and (iv) obligations to extended family members. Relevant to the first issue, the ACMG recommendations state that variants with "higher" penetrance should be reported, but they leave the determination of "higher" to the clinical laboratory. For example, we identified a *TP53* variant (p.R337H/chr17:g.7574017C>T, see **Table 3**) with 2.5–9.9% penetrance for pediatric adrenocortical carcinoma,[26,27] and newborn screening programs in Brazil have shown that screening for carriers of this mutation reduces morbidity and mortality.[26] This reporting conundrum was not resolved by the relationship of *TP53* to Li–Fraumeni syndrome because this variant has not been associated with Li–Fraumeni syndrome. Consequently, the reporting of a variant is difficult to code bioinformatically and will require human interpretation and possibly clinical consultation.

Regarding delineation of the pathogenicity of variants deposited by clinical laboratories, *BRCA1* and *BRCA2* variants provide an excellent illustration. Although our criteria for pathogenicity are scientifically sound, many *BRCA1* and *BRCA2* variants in public databases lack information on segregation with disease or experimental functional assays. Because variants lacking this information would not be considered pathogenic according to our paradigm, our approach may well underreport the *BRCA1*- and *BRCA2*-associated cancer risks.

Another issue arising from this analysis is that a molecular finding is not a clinical diagnosis. Clinical records are often not available to testing laboratories, although, in some cases, they may substantiate or cast doubt on a variant's pathogenicity. The subject in whom we identified a pathogenic *APOB* mutation (p.R3527W/chr2:g. 21229161G>A), a conclusion supported by functional assays demonstrating reduced low-density lipoprotein receptor binding,[28] had a favorable serum cholesterol and lipoprotein profile. A similar finding was also reported by Andreasen et al.[20] on "causative variants" for cardiomyopathies. This highlights that even conservative standards to determine pathogenicity do not obviate the need for clinical interpretation and correlation.

The last issue is that of obligation to provide potentially helpful medical information to extended family members. For example, the person with an *SCN5A* variant and exercise-induced fatigue had a brother with an unspecified early-onset cardiac condition. If this brother carried the *SCN5A* variant, then this information might be diagnostically and therapeutically useful to him. Possible ethical approaches to notification include encouraging the subject in our cohort to discuss this finding with his brother, with or without provision of counseling to the brother, or direct notification of the brother. The American Medical Association's Code of Medical Ethics endorses encouraging the subject to notify at-risk relatives, with provision of assistance to the subject regarding communication of opportunities for testing and counseling.[29] This serves as a reminder that genetic testing may generate professional ethical obligations extending beyond the subject being tested.

Discussion on whether to inform individuals enrolled under the NIH UDP protocol about the identified variants focused on the delineated and perceived obligations defined by the language of the consent document and the process by which the consent was explained. In conclusion, whether to return or not return the incidental findings was deferred to the choices the individual or guardian had made when completing the written informed consent.

An issue raised by our study was the amount of work needed to determine the variants that are reportable. We found that variants were listed occasionally as mutations or known pathogenic alleles in LSDBs without published evidence of segregation with disease or functional assays to support pathogenicity. Consequently, it is incumbent on the reporting laboratory to assemble and determine the credibility of the evidence used to determine the pathogenicity of a variant. Confounding this is the failure of many LSDBs to provide access to variants in a format that is easily applied to data sets derived from exome and genome sequencing. In contrast, ClinVar provides the required

annotations as readily usable variant call files. Deposition of variants and their clinical significance in ClinVar would improve the efficiency of the recommended analysis.

Our analysis had some limitations. First, the exome sequencing that produced the variants for analysis was a research-grade exercise rather than a clinical-grade investigation, and therefore not all exons in the 56 recommend genes had sufficient sequence coverage to call variants in all individuals. In addition, we did not validate the variants by Sanger sequencing but rather inspected the alignments of short reads using Integrative Genome Viewer, a method that we have found more sensitive than Sanger sequencing. Second, our curation of variants was limited by the availability of annotations in public databases; we expect that the number and quality of these annotations will improve with time, as will the number of reportable variants. This raises the question of whether exome and genome sequence data should be reanalyzed at regular intervals to take into account the increasing information.

In summary, clinical exome and genome sequencing are cost-effective methods for identifying the molecular bases of genetic conditions. These untargeted approaches, however, also uncover genetic variants with medical or social implications unrelated to the indication for testing. In this context, the ACMG Working Group on Incidental Findings in Clinical Exome and Genome Sequencing recently recommended reporting "known pathogenic" and "expected pathogenic" mutations for 56 genes. Approximately 5% of all exomes in the NIH UDP familial cohort and 8.8% of the families in our cohort had a reportable finding. The most time-consuming aspect of fulfilling these recommendations was assembling the evidence for "pathogenicity" or "probable pathogenicity" because no well-curated comprehensive public database is currently available.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

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DISCLOSURE

The authors declare no conflict of interest.

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