

Rapid Molecular Analysis of the *STAT3* Gene in Job Syndrome of Hyper-IgE and Recurrent Infectious Diseases

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With the recent discovery of mutations in the *STAT3* gene in the majority of patients with classic Hyper-IgE syndrome, it is now possible to make a molecular diagnosis in most of these cases. We have developed a PCR-based high-resolution DNA-melting assay to scan selected exons of the *STAT3* gene for mutations responsible for Hyper-IgE syndrome, which is then followed by targeted sequencing. We scanned for mutations in 10 unrelated pedigrees, which include 16 patients with classic Hyper-IgE syndrome. These pedigrees include both sporadic and familial cases and their relatives, and we have found *STAT3* mutations in all affected individuals. High-resolution melting analysis allows a single day turn-around time for mutation scanning and targeted sequencing of the *STAT3* gene, which will greatly facilitate the rapid diagnosis of the Hyper-IgE syndrome, allowing prompt and appropriate therapy, prophylaxis, improved clinical outcome, and accurate genetic counseling. (*J Mol Diagn* 2010, 12:000–000; DOI: 10.2353/jmoldx.2010.090080)

Hyperimmunoglobulin E syndrome (HIES), or Job syndrome (Online Mendelian Inheritance in Man, numbers 147060 and 243700, <http://www.ncbi.nlm.nih.gov/omim/>, last accessed December 13, 2009), was first described in 1966 by Davis et al¹ in two girls with severe eczema, unusual facial features, chronic cutaneous candidiasis, and cold staphylococcal abscesses. The abscesses were described as being “cold” for their lack of the characteristic features of inflammation, such as warmth, redness, tenderness, and fever. In 1972, Buckley et al²

described similar patients who also were noted to have markedly elevated IgE.

Recurrent bacterial and fungal infections are the major cause of morbidity and mortality in HIES.³ The typical patient with HIES also has a history of recurrent pathological fractures, retained primary teeth, hyperextensible joints, deep-set eyes, increased width of the nose, a full lower lip, and thickening of the nose and ears.⁴ Lymphoid malignancies may also be increased in patients with HIES.^{5,6} A systematic study of 30 patients and their families led to the development of a scoring system to aid in the diagnosis of HIES.⁷ Such a scoring system was felt to be necessary because HIES not only lacks pathognomonic markers, but the characteristic manifestations often develop over years, delaying diagnosis. Furthermore, the symptoms and signs often found in HIES are also features of other conditions. For example, elevated serum IgE and severe eczema are found in allergic disorders, as well as other primary immunodeficiency disorders, such as the Wiskott-Aldrich syndrome.⁸ There is no correlation between the clinical phenotype, NIH score, and the serum IgE concentrations, which can rise and fall during the lifetime of patients with HIES, and some do not even have marked elevations of this immunoglobulin, making the name Job syndrome, perhaps, a more appropriate term than the Hyper-IgE syndrome.

Both familial and sporadic cases of HIES have been described.^{9,10} The familial cases show mostly autosomal dominant inheritance, but autosomal recessive cases have also been reported.¹¹ The autosomal recessive cases seem to be an entirely different set of disorders.^{12–14}

Mutations in *STAT3* (signal transducer and activator of transcription 3) have recently been identified by four research groups in both sporadic and familial patients with HIES.^{15–18} All patients with dominant HIES described

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to date are heterozygotes, and the *STAT3* mutations identified are hypomorphic and localized in the DNA-binding, SH2, and transactivation domains. To date, there have been no clear phenotype-genotype correlations described in patients with HIES with various mutations in the *STAT3* gene. All of the specific immunological and somatic defects resulting from the *STAT3* mutations leading to HIES are not known, but deficiency in Th17 cells is part of the problem.^{17,19,20}

Since many common disorders present with elevated serum IgE and recurrent infections, which are also characteristic manifestations of Job syndrome, we have developed a rapid molecular assay to diagnose the disorder. Although mutations can be identified by com-

prehensive sequencing, the time and cost required are substantial. High-resolution DNA-melting analysis is a mutation scanning technique that monitors the change in fluorescence caused by the release of double-stranded DNA-binding dye from DNA as it is denatured by increasing the temperature.^{21,22} Both heterozygous and homozygous variants can be identified. When the change in DNA is heterozygous (as in Job syndrome), two heteroduplexes and two homoduplexes are formed following PCR amplification. Each duplex has a different thermal stability, leading to subtle melting curve differences between wild-type and variant sequences.^{23,24} We have applied this rapid molecular technique to defining the mutations in *STAT3* genes in 10 unrelated HIES pedigrees with 16 patients with classic HIES to facilitate rapid

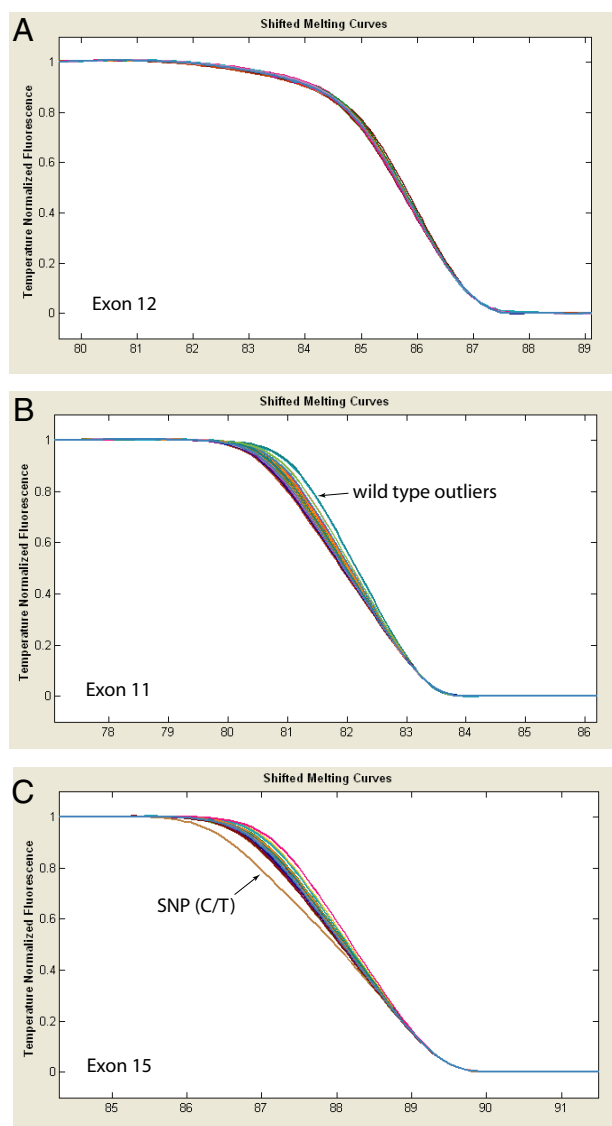


Figure 1. Amplicon melting analysis. Five of the 10 amplicons showed very tight clustering (A), whereas four exons (11, 13, 14, and 16) showed more variation, as shown by exon 11 in B. The genotype of these outliers as determined by sequencing was the same as the main cluster (see the green and blue colored outliers in B as well). In the case of exon 15, however, the one clear outlier from the main cluster was sequenced as a heterozygous C to T polymorphism (c1361T>C, p.L454P; C), which is putatively benign because it was found in an asymptomatic blood donor.

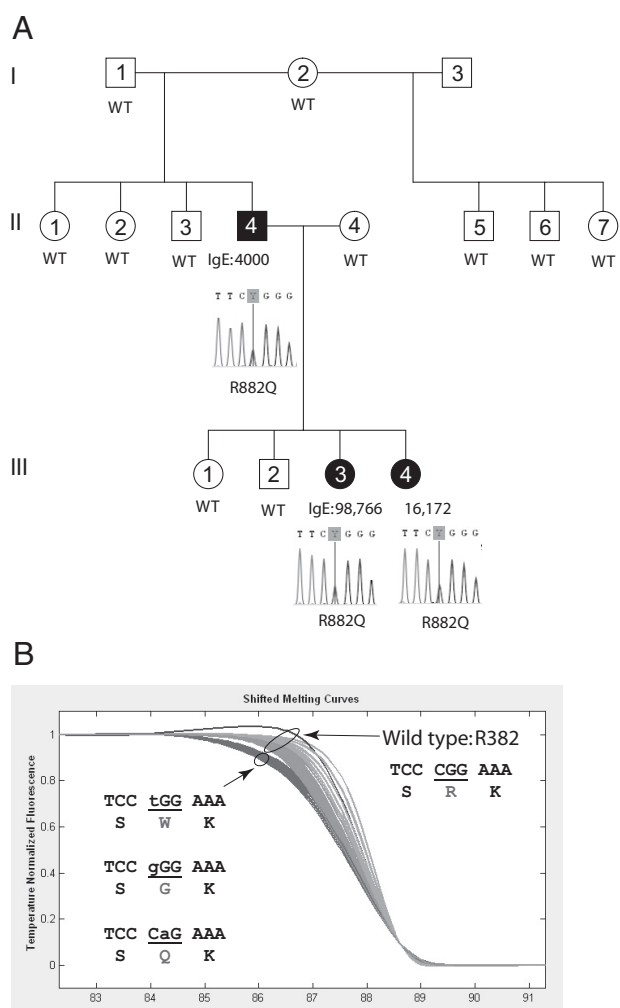


Figure 2. Mutation in exon 13 changing the DNA-binding region. **A:** High resolution melting and sequencing of exon 13 revealed three HIES genotypes in addition to the wild-type (WT). All three exon 13 HIES genotypes change the codon encoding R382. These genotypes are detected as different from wild-type, but were not differentiated. **B:** An example of a *de novo* R382Q mutation that arose in the proband (II/4) and was inherited by two of the proband's four children (III/3 and III/4) is shown. IgE serum levels are shown in IU/ml if known. Family members were tested if available; family members not labeled as WT or HIES were not tested.

diagnosis, therapy, and genetic counseling of families with the disorder.

Materials and Methods

Primers

Primers were designed by using LightScanner primer design software (Idaho Technology, Salt Lake City, UT) and synthesized by the University of Utah DNA core facility. The primers were placed at least 15 nucleotides away from the ends of each exon to include most residues influencing splicing. Twelve exons of the *STAT3* gene encoding the DNA-binding, SH2, and transactivation domains, where the disease-causing mutations have been described,^{15–18} were tested. Exon 21 is amplified in two overlapping amplicons.

PCR Amplification

PCR was performed on MJ Research PTC-200 thermal cyclers (BioRad, Hercules, CA) by using a 96-well plate format. Each 10- μ L PCR includes 0.4 U of KlenTaq1, 88 ng of TaqStart antibody, 1x LCGreen Plus (Idaho Technology), 50 mmol/L Tris (pH 8.3), 1 M betaine, 500 mg/L bovine serum albumin, 1.5 mmol/L Mg²⁺, 0.2 mmol/L of each Deoxyribonucleotide Triphosphate (dNTP), 0.5

μ mol/L primers, and 50 ng genomic DNA. Reaction mixtures were overlaid with 10 μ L of mineral oil. An annealing temperature gradient PCR was used to determine the optimal annealing temperature (64°C). The extension temperature was set to 72°C. During optimization, the amplicons were examined by melting analysis, agarose gels, and were sequenced to ensure the desired product. The PCR amplification starts at 95°C for 10 seconds, followed by 40 cycles of 94°C for 5 seconds, 64°C for 5 seconds, 72°C for 6 seconds, and finished by denaturing the PCR product at 95°C for 5 seconds.

Melting Analysis

After PCR, the plates were heated to 95°C for 30 seconds and then cooled to 15°C for heteroduplex formation. The plates were then centrifuged (1500 $\times g$ for 1 minute) and analyzed in a 96-well LightScanner (Idaho Technology), as previously described.^{21,22} The 96-well plates were heated at 15°C per second, and fluorescence was measured from 65°C to 98°C. Melting curves were analyzed with LightScanner software. Finally, the melting profiles were clustered. Clustering of melting curves for genotype identification was performed manually, assisted by the LightScanner software on the high sensitivity setting. Amplicons within each cluster were sequenced to determine genotype.

Table 1. Patients with Job Syndrome of Hyper-IgE and Recurrent Infections

ID	Sex	IgE, IU/ml	Eczema	Abscess	Pneumonia	Candida infection	Associated findings	STAT3 mutation	Protein domain (Exon no.)	Type
1-1*	Male	4000	+	+	+	+	+	c.1145G>A; p.R382Q	DNA-binding (Exon 13)	<i>de novo</i>
1-2	Female	98,766	+	+	+	+	+	c.1145G>A; p.R382Q	DNA-binding (Exon 13)	Transmitted
1-3	Female	16,172	+	+	+	+	+	c.1145G>A; p.R382Q	DNA-binding (Exon 13)	Transmitted
2-1*	Male	1451	+	+	+	+	+	c.1145G>A; p.R382Q	DNA-binding (Exon 13)	?
3-1	Male	3206	+	+	+	+	+	c.1913A>G; p.E638G	SH2 (Exon 21)	<i>de novo</i>
4-1	Female	9477	+	+	–	+	+	c.1863C>G; p.F621L	SH2 (Exon 20)	?
5-1*	Male	8000	+	+	+	+	+	c.1144C>T; p.R382W	DNA-binding (Exon 13)	?
6-1*	Female	21,700	+	+	+	+	+	c.1144C>G; p.R382G	DNA-binding (Exon 13)	<i>de novo</i>
6-2	Male	334	+	+	+	+	+	c.1144C>G; p.R382G	DNA-binding (Exon 13)	Transmitted
7-1*	Male	8127	+	+	+	+	+	c.2069del30bp; p.690.P699del	Transactivation (Exon 21 [†])	<i>de novo</i>
7-2	Female	1293	+	+	+	+	+	c.2069del30bp; p.690.P699del	Transactivation (Exon 21 [†])	Transmitted
7-3	Female	1909	+	+	+	+	+	c.2069del30bp; p.690.P699del	Transactivation (Exon 21 [†])	Transmitted
8-1*	Male	29,899	+	+	+	+	+	c.1144C>T; p.R382W	DNA-binding (Exon 13)	?
9-1*	Male	38,918	?	?	?	?	?	c.1144C>T; p.R382W	DNA-binding (Exon 13)	?
10-1	Female	15,776	+	+	+	+	+	c.1145G>A; p.R382Q	DNA-binding (Exon 13)	?
10-2	Female	40,009	+	+	+	+	+	c.1145G>A; p.R382Q	DNA-binding (Exon 13)	Transmitted

*STAT3 gene was completely sequenced from these patients¹⁷; reference sequence for mutations is NM.139276.2.

[†]The mutation in family 8 includes the splice acceptor site.¹⁷

Results

Assay Optimization and Melting Data Analysis

High resolution melting analysis was performed on each of 12 exons of 96 random healthy blood donors. Amplification was successful 98.7% of the time; repeated PCR of the failed samples was amplified in all instances. Analysis of the 96 normal samples was performed to determine the number of variants present in a normal population in each of the exons. Typical melting profiles of the exons are shown in Figure 1, A–C. Heterozygous variants can clearly be distinguished from the loose clustering of wild-type samples (Figure 1). There was only one variant (c.1361T>C, p.L454P in exon 15) in our Utah-based, predominantly Caucasian (96%) population (Figure 1C), which is probably benign because it was found in a presumably healthy blood donor. Holland et al¹⁵ tested 158 unaffected control subjects of various ancestral origins (125 white subjects, 15 Hispanic subjects, 4 black subjects, 2 Asian subjects, 1 white Hispanic subject, 1 black Hispanic subject, and 10 persons of unknown ancestry), and Renner et al¹⁷ sequenced 94 unaffected individuals of diverse ethnic background, and both found no variants in *STAT3*.

Gene Scanning of HIES Pedigrees

The *STAT3* gene in 16 patients and 75 unaffected family members from 10 kindreds, representing 91 samples enriched for cases of HIES were analyzed by high resolution melting. The samples were blinded to evaluate the accuracy of the procedure. After PCR amplification and melting analysis, we evaluated representatives of the main cluster (assumed to be wild-type) and all of the outliers, if present, by sequencing. Sequence variants were detected in three exons: 13, 20, and 21.

Melting analysis and sequencing of exon 13 revealed three genotypes in addition to the wild-type (Figure 2, A and B). All three variants changed the R382 codon. R382 is located in the DNA-binding region of *STAT3*. Although all three variants are clearly different from the most common wild-type genotype, they were not differentiated from each other. Matching the genotype of samples to family pedigrees showed that melting analysis correctly identified all affected family members. As an example, one large family is shown in Figure 2, in which a *de novo* R382Q mutation arose in the proband (II/4) and was inherited by two of the proband's four children (III/3 and III/4). There were three families (three patients) with the R382W (C to T), three families (six patients) with the R382Q (G to A), and one family (two patients) with the R382G (C to G) mutation (Table 1).¹⁷ The codon of R382 is the most common mutational hotspot described previously,^{15–18} as well as in our data set. It represents ~40% of all patients with *STAT3* mutant HIES described to date. R382W is the most common variant in the literature. In contrast, R382G is a newly described mutation.

Melting analysis and sequencing of exon 20 detected one heterozygous mutation in the SH2 domain (not shown). F621L is also a newly described mutation. Melt-

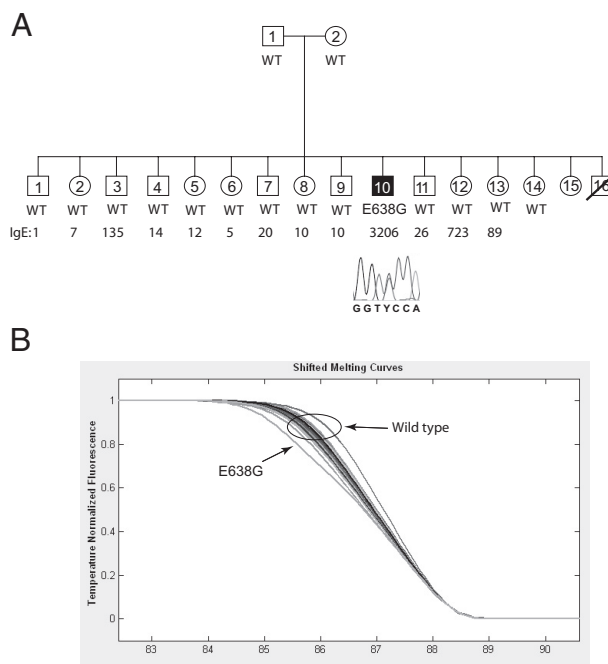


Figure 3. Mutation in exon 21 changing the SH2 region. **A:** This figure shows an example of a mutation changing the SH2 region of *STAT3*. Exon 21 encodes part of the SH2 region of *STAT3* protein. **B:** E638G is a *de novo* mutation in the proband. Family members were tested if available; family members not labeled as WT (wild-type) or HIES were not tested. IgE serum levels are shown in IU/ml if known.

ing analysis and sequencing of exon 21 (tested by two overlapping amplicons, due to its size) detected two genotypes in addition to the wild-type (Table 1).¹⁷ Exon 21 encodes part of the SH2 domain of *STAT3* protein. E638G is a *de novo* mutation in the proband with 13 unaffected siblings who were tested (Figure 3, A and B). A large, 30-bp deletion was found in exon 21, which encodes the beginning of the transactivation domain, in a family with an affected father and two affected daughters (Figure 4, A and B). The in-frame deletion of 10 amino acids occurred *de novo* in the proband (II/4), and was inherited by the two daughters (III/3 and III/4), and was detected in all three patients.

All of the variants in the *STAT3* gene matched a clinically-documented patient with HIES in the family pedigrees. We found no HIES mutations or polymorphisms among the unaffected relatives. There were two unrelated cases, however, where the clinical diagnosis and the molecular testing did not match. The family pedigree of the first patient is shown in Figure 5. The proband (II-3) and her son (III-1; patients 6-1 and 6-2 in Table 1¹⁷) were found to have the same missense mutation (R382G) in exon 13 of *STAT3*. The proband's father possessed some of the signs and symptoms of HIES (numerous abscesses), but not high IgE, thus the clinical diagnosis was uncertain. Genetic testing revealed a wild-type *STAT3* sequence in this individual. In the second family, one brother of the proband (Brother A) was given a clinical diagnosis of HIES, and the proband's daughter was given a diagnosis of HIES as well.¹⁰ Mother and daughter (patients 10-1 and 10-2 in Table 1¹⁷) had the same R382Q mutation; however, "Brother A" had a wild-

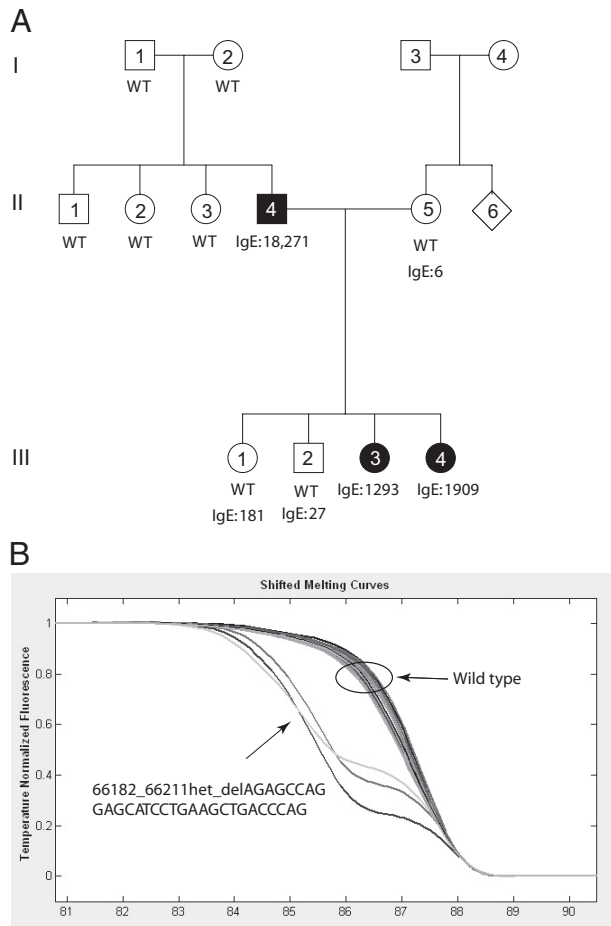


Figure 4. Deletion in the transactivation domain. **A:** A large, 32-bp deletion occurred in the proband (II/4) and was inherited by two of the proband's children (III/3 and III/4). **B:** This large heterozygous deletion was detected in all three patients. Family members were tested if available; family members not labeled as WT (wild-type) or HIES were not tested. IgE serum levels are shown in IU/ml if known.

type *STAT3* sequence despite the elevated IgE and onychomycosis, highlighting the utility of genetic testing. DNA from the parents and grandparents of the proband were not available for testing.¹⁰

Most cases of classic HIES have unaffected parents, suggesting *de novo* mutations. In the reported 10 pedigrees, we demonstrated *de novo* mutations in four families (see Figures 2, 3, and 4), whereas in the other six families we did not have DNA from both parents.

Discussion

In this report, 96 healthy blood donors and 91 DNA samples from HIES families collected over 35 years were scanned for variants by a newly developed melting assay. All of the mutations were hypomorphic and heterozygous, and the inheritance was autosomal dominant in all cases where we had sufficient data to make the determination. The melting assay was 100% sensitive for the identification of *STAT3* mutations in autosomal dominant HIES and along with targeted sequencing of the abnormal exon it can be per-

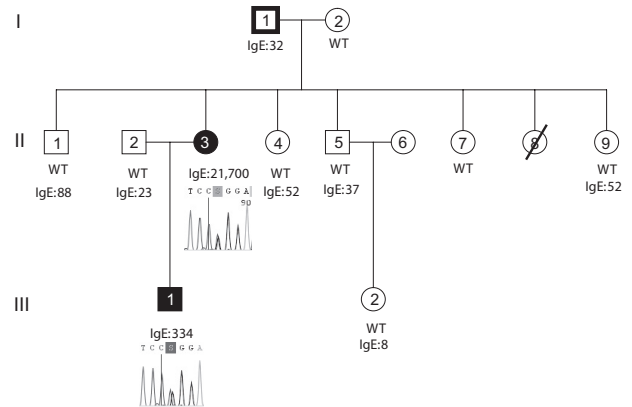


Figure 5. Clinical diagnosis versus genetic testing. The proband of this family pedigree and her son were confirmed to have a missense mutation in exon 13 of *STAT3*. The proband's father (bold square) was suspected to have HIES because he had some signs and symptoms. Genetic testing showed a wild-type (WT) sequence. Family members were tested if a sample was available; family members not labeled as WT or HIES were not tested. IgE serum levels are shown in IU/ml if known.

formed in a single day. These performance characteristics are in line with other studies in which the authors used melting analysis (for reviews on sensitivities and specificities see van der Stoep et al²⁵ and Farrar et al²⁶).

It is likely that not all *STAT3* mutations that cause HIES are known. Because mutation scanning relies on the melting profile of the PCR amplicon, new mutations are easily identified. When a novel melting profile is observed, the targeted exon is sequenced. Both known and previously unknown mutations in the DNA-binding, SH2, and transactivation domains of *STAT3* have been detected in our study.

Most patients with classic HIES have mutations in the *STAT3* gene. Minegishi et al¹⁶ identified mutations in *STAT3* in 8 of 15 sporadic HIES cases (53.3%). In contrast, Holland et al¹⁵ found mutations in all 50 sporadic and familial patients studied with HIES, and Renner et al,¹⁷ including our group, failed to find a mutation in only 1 of 38 patients from 35 families. Jiao et al¹⁸ investigated 12 patients from 9 families and found mutations in *STAT3* of all HIES cases. This wide variation in the number of patients with HIES without a mutation in *STAT3* (from 0% to 46.7%) suggests differences in the diagnostic criteria used by the clinicians or in the ethnic background. The finding that, at least in some cases of autosomal recessive HIES, the gene-defect may be in *Tyrosine Kinase 2* (*TYK2*),¹² but that in the majority if not all of autosomal dominant HIES cases the defect is in *STAT3*, strongly suggests that the *TYK2*-*STAT3* signaling pathway is the key to the pathogenesis of HIES because *TYK2* can phosphorylate and activate *STAT3*. The assay presented here is expected to identify *STAT3* mutations in more than 90% of patients who have been given a clinical diagnosis of classic HIES, and can easily be expanded, if necessary, to include new mutations in other *STAT3* exons.

DNA variant scanning by high-resolution melting is a rapid and cost effective method. The PCR amplification for high-resolution melting requires only standard unlabeled PCR primers and a fluorescent dye that detects heteroduplexes and can be included in the PCR mixture

before amplification. It takes about 2 hours after DNA purification to perform PCR amplification and high-resolution melting. High-resolution melting is an in-tube method that requires no opening of the tube or plate after PCR amplification, greatly simplifying the testing process and preventing amplicon contamination with rapid turnaround times. The cost of scanning one sample as described here is estimated to be \$29, including disposables, PCR reagents, and the LCGreen Plus dye. The estimated reagent cost of full bi-directional sequencing of the same sample at our facility is \$165 (12 PCR products, sequenced in both directions, \$6.90 for each sequencing reaction). Therefore, the major time and cost component of the method described here is reflexive sequencing. Bi-directional sequencing of an exon costs \$13.80. The amount of sequencing required is determined by the number of variant melting curves detected. As long as there are fewer than nine sequencing reactions required per sample, our assay is cost effective. We encountered four to six variant melting curves caused by wild-type outliers at most (Figure 1) per 96-well plate that translate into less than one potentially avoidable additional sequencing reaction per patient. The ordering pattern of the test is the other determining factor for the amount of reflexive sequencing required. If genetic testing is only ordered for patients with a high NIH score,¹⁵ then most samples will be positive and require reflexive sequencing, thus increasing the cost by two sequencing reactions (bi-directional sequencing of an exon, \$13.80) and lengthening the time required. However, if the test is also used for screening and differential diagnosis in addition to confirmation of clinically documented cases, the majority of the samples will be negative and can be returned rapidly without reflexive sequencing in a time and cost-effective manner. Our experience with similar tests supports the second scenario because most cases of HIES are born to unaffected parents, and clinicians have to maintain a high level of suspicion to diagnose primary immunodeficiencies based on repeated or unusual infection. HIES lacks specific symptoms and signs and is difficult to diagnose with currently available screening assays. High resolution melting analysis of the *STAT3* gene provides rapid diagnosis of this potentially fatal disorder, and thus, facilitates both prophylaxis and symptomatic therapy to prevent further morbidity and mortality and genetic counseling of these patients and their families. The rapidity of the high-resolution melting analysis followed by targeted sequencing allows detection of *STAT3* mutations as a clinically useful diagnostic test for this primary immunodeficiency syndrome characterized by diverse manifestations.

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lected the pedigrees and DNA samples. Ralph J. Faville Jr, Paul G. Quie, Hans D. Ochs, and Ralph J. Wedgwood identified and cared for patients, provided clinical information, enrolled patients, and collected DNA samples for the study.

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