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Report

Evidence that Griscelli Syndrome with Neurological Involvement Is Caused by Mutations in *RAB27A*, Not *MYO5A*

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Griscelli syndrome (GS), a rare autosomal recessive disorder, is characterized by partial albinism, along with immunologic abnormalities or severe neurological impairment or both. Mutations in one of two different genes on chromosome 15q can cause the different subtypes of GS. Most patients with GS display the hemophagocytic syndrome and have mutations in *RAB27A*, which codes for a small GTPase. Two patients with neurological involvement have mutations in *MYO5A*, which codes for an actin-based molecular motor. The *RAB27A* and *MYO5A* gene products interact with each other and function in vesicle trafficking. We report the molecular basis of GS in a Muslim Arab kindred whose members have extremely variable neurological involvement, along with the hemophagocytic syndrome and immunologic abnormalities. The patients have normal *MYO5A* genes but exhibit a homozygous 67.5-kb deletion that eliminates *RAB27A* mRNA and immunocytofluorescence-detectable protein. We also describe the molecular organization of *RAB27A* and a multiplex polymerase chain reaction assay for the founder deletion in this kindred. Finally, we propose that all patients with GS have *RAB27A* mutations and immunologic abnormalities that sometimes result in secondary neurological involvement. The two patients described elsewhere who have *MYO5A* mutations and neurological complications but no immunologic defects may not have GS but instead may have Elejalde syndrome, a condition characterized by mild hypopigmentation and severe, primary neurological abnormalities.

Griscelli syndrome (GS [MIM 214450]) is a rare autosomal recessive disorder characterized by pigmentary dilution of the skin, due to abnormal melanosomal transport, and by silvery gray hair, due to pigment clumping in hair shafts. Most patients develop the hemophagocytic syndrome, which is characterized by uncontrolled activation of T lymphocytes and macrophages and which leads to death if not treated by bone marrow transplantation (BMT). Some patients show severe neurological impairment early in life, with or without apparent immune abnormalities (Griscelli et al. 1978; Hurvitz et al. 1993; Klein et al. 1994; Menasche et al. 2000).

Recently, GS was reported to consist of two different subtypes, resulting from defects in two separate genes, *MYO5A* and *RAB27A* (Pastural et al. 2000). Myosin 5a, the product of *MYO5A* on chromosome 15q21 (Pastural et al. 1997), is an unconventional myosin motor protein that moves along actin filaments and functions in intracellular vesicle transport (Metha et al. 1999). Among humans with GS, one patient exhibited a missense change in *MYO5A* (Pastural et al. 1997), but this was later shown to be a polymorphism (Lambert et al. 2000). Both remaining reported patients who had GS with mutated *MYO5A* (patients P3 [Pastural et al. 1997] and P8 [Pastural et al. 2000]) exhibited primarily neurological impairment without immune defects.

A second genetic cause of GS was discovered because a large number of patients did not show a mutation in *MYO5A*. Linkage analysis indicated the existence of this second locus in the q21 region of chromosome 15 (Pastural et al. 2000), and physical mapping and mutation analysis identified the *RAB27A* gene, <1.6 cM from *MYO5A* (Menasche et al. 2000). The rab27a protein is a small GTPase that functions in the targeting and the fusion of transport vesicles with their appro-

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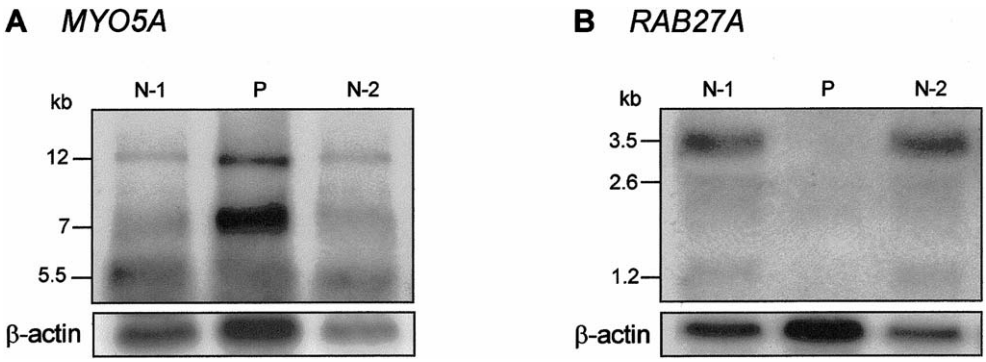

Figure 1 Northern blot analysis of *MYO5A* and *RAB27A* expression. Total RNA extracts (20 μg) of two normal individuals (N-1, N-2) and of patient IV with Griscelli syndrome (P) were separated on a formaldehyde gel, were blotted to a nylon membrane, and were hybridized with a [^{32}P]-dCTP–labeled DNA probe to *MYO5A* (A) and to *RAB27A* (B). Both blots were also labeled with a β-actin probe (*lower panels*), which served as a control for the amount of loaded RNA. The patient has abundant *MYO5A* RNA and no *RAB27A* RNA.

priate acceptor membranes. Like other rab proteins, rab27a requires geranylgeranylation of two consensus C-terminal cysteine residues in order to be anchored to membranes. Truncation of the carboxy-terminal part of rab27a would render it inactive (Kinsella and Maltese 1992; Chen et al. 1997; Novick and Zerial 1997; Pfeffer et al. 2001). To date, all patients with GS with mutations in *RAB27A* have developed the hemophagocytic syndrome.

In accordance with the paradigm that patients with GS with neurological involvement have mutations in *MYO5A* and that those with the hemophagocytic syndrome have mutations in *RAB27A* (Pastural et al. 2000; Westbroek et al. 2001), the kindred with GS reported by Hurvitz et al. (1993) was expected to exhibit *MYO5A* mutations. Results of the present study show that members of this kindred have, instead, a large deletion in *RAB27A*. We suggest that the neurological involvement in these patients with GS occurs secondarily to the hemophagocytic syndrome and that patients with primary CNS complications and *MYO5A* mutations have a related disorder, namely, Elejalde syndrome.

The patient described in the present study was part of a highly consanguineous Muslim Arab family, in which Griscelli syndrome was diagnosed (Hurvitz et al. 1993). This family includes four affected children: a brother (patient IV, our proband) and a sister (patient I) in one family and their two female first cousins (patients II and III). All four individuals died in childhood, at ages 11.5 years, 10 mo, 6 years, and 2 years, respectively. All had silvery hair. Skin biopsies revealed normal numbers of melanocytes containing normally sized melanin granules; it was suggested that no melanosomes were transferred to the surrounding keratinocytes (Hurvitz et al. 1993). Patients I, II, and III presented with recurrent vomiting; patient I had an acute febrile illness, and patient III presented with lethargy. All three patients deteriorated neurologically after their initial presentation, as indicated by regression of mental and physical function. The clinical course of patient IV was characterized by periodic episodes of seizures, from the age of 1 age, and by mild cognitive delay. At the age of 2 years 10 mo, patient IV experienced an episode similar to the accelerated phase described by Griscelli et al. (1978) and recovered completely. He was well and had no recurrent infections, and his neurological status was stable until age 8 years. He was not monitored between the ages of 8 years and 10.5 years, after which he was repeatedly admitted to a local hospital because of prolonged fever, severe hepatosplenomegaly, and pancytopenia. His condition deteriorated, and he developed ascites, generalized edema, and jaundice and died at the age of 11.5 years.

Genomic DNA and RNA were isolated from cultured skin fibroblasts of patient IV. The entire coding region of *MYO5A* cDNA (GenBank accession number U90942) of the patient and several normal control individuals was amplified by PCR in eight overlapping fragments (primer sequences available upon request), followed by direct sequencing. This revealed no *MYO5A* cDNA mutations in the patient. We then performed a northern blot, using a *MYO5A* tail region fragment (nt 3735–4628) as the hybridization probe. This blot showed a normal size and amount of each of the three alternatively spliced fibroblast *MYO5A* transcripts (12 kb, 7 kb, and 5.5 kb) in patient IV (fig. 1*A*). The increased intensity of the *MYO5A* bands in patient IV corresponds to the increased β-actin signal, indicating that more of the patient's RNA was loaded on the gel.

Next, we attempted to amplify *RAB27A,* using cDNA from patient IV and from normal control subjects as template (forward primer 5′-TGAAGCATTGGTAACTCCAG-3′ and reverse primer 5′-TCTCACTGTGCCATGTATCAA-3′). No PCR product was amplified for the patient (fig. 2). Subsequent northern blot analysis with

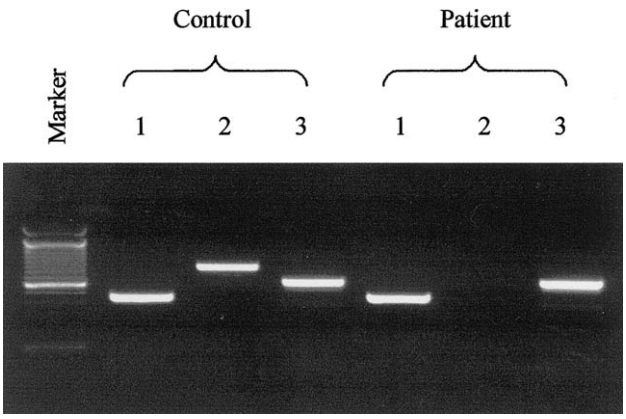

Figure 2 cDNA amplification of *RAB27A* and the adjacent genes, *LOC51187* and *PIGB*. PCR products of portions of *LOC51187* (*lane 1*), *RAB27A* (*lane 2*), and *PIGB* (*lane 3*) obtained by use of cDNA from an unaffected control subject (*left*) and patient IV (*right*). The patient has normal amplification of *LOC51187* and *PIGB* but no signal for *RAB27A*.

a *RAB27A* cDNA hybridization probe (GenBank accession number U38654; nt 155–990) showed no expression of the 3.5-kb major transcript of *RAB27A* in fibroblasts from the patient (fig. 1*B*).

Genomic DNA of the patient and several control individuals was then PCR amplified for each exon of the *RAB27A* gene (GenBank accession number AF443871), using primer sequences for exons 2–6 as reported by Tolmachova et al. (1999). For amplification of the exon 1 region, we used forward primer 5′-GTGCCATATGACAAACAGCTG-3′ and reverse primer 5′-CTCCGTGGTCACTGAAAATGC-3′. All six exons of *RAB27A* (Tolmachova et al. 1999) were successfully amplified by use of control gDNA. However, amplification of the patient's genomic DNA yielded product only for exon 6, while exons 1–5 could not be amplified.

Amplification of the patient's cDNA for genes neighboring *RAB27A* (i.e., *PIGB* and *LOC51187*) yielded the expected bands (fig. 2). For *PIGB* (GenBank accession number NM_004855) amplification, the forward primer was 5′-CGAAAGAGAAAGTCTACCTTGTAC-3′, and the reverse primer was 5′-CAAAGGTGTCCACAGAATGACAGC-3′. For *LOC51187* (GenBank accession number XM_046136) amplification, the forward primer was 5′-CATCTATCCTGGACACGGCATG-3′, and the reverse primer was 5′-GAGCATCTTCCATGTCCACATC-3′. These results pointed to a large deletion extending from its 5′ breakpoint in the region between *PIGB* and exon 1 of *RAB27A* (~49-kb) to its 3′ breakpoint in intron 5 of the *RAB27A* gene (~18 kb).

To identify the exact deletion breakpoints, we first determined the organization of the *RAB27A* gene, including the complete intronic sequences, which were previously unreported. Two overlapping BACs (GenBank accession numbers AC011912 and AC018926) covered the entire *RAB27A* region. Using these BACs and the intron/exon boundaries reported by Tolmachova et al. (1999), we identified the complete *RAB27A* gDNA (submitted to GenBank as accession number AF443871), which spans ~86.6 kb of DNA. We then designed PCR primers to amplify short fragments (~200–300 bp) at intervals of ~3 kb throughout the region of the predicted *RAB27A* deletion, and the breakpoint locations were narrowed until we were able to amplify a fragment encompassing the breakpoints. The sequencing of this fragment revealed a PCR product that lacked 67,536 bp, including all of exons 1–5 and the predicted gene-promoter region. A 6-bp sequence repeat on each end of the deletion exists only once in the affected individual (fig. 3).

To detect the presence or absence of the 67.5-kb deletion (fig. 3), a multiplex PCR amplification assay was designed (fig. 3). We used two primer sets in a standard PCR amplification with an annealing temperature of 58°C. The primer set flanking the deletion resulted in a 656-bp fragment (forward primer 5′-GGACAGTGTATGCTTCCTTTCG-3′ and reverse primer 5′-GGGAGTCCTGCAATTGAATTCAG-3′), and the primer set within the deleted area yielded a 439-bp fragment (forward primer 5′-CTGATGCTTTGAAAGCATTATG-3′ and reverse primer 5′-GCATTGTCAATGGATCCTAC-3′).

Multiplex PCR screening of patient IV's parents and an aunt who had two affected children (patients I and II) confirmed that all three were carriers of the 67.5-kb deletion (fig. 3). To screen for the presence of the 67.5-kb deletion in the Palestinian population, 86 anonymous samples of Palestinian origin were obtained from the National Laboratory for the Genetics of Israeli Populations at Tel Aviv University. None of these samples revealed the deletion.

Immunocytofluorescence studies were performed to show the presence and distribution of the RAB27A protein in the fibroblasts of patient IV and normal unaffected control subjects (fig. 4). Fibroblasts were grown on glass coverslips and were fixed for 10 min in 2% formaldehyde in PBS. The cells were then incubated for 1 h with mouse monoclonal antibodies to RAB27A (Transduction Laboratories) that were diluted in PBS, 0.2% saponin (wt/vol), and 0.1% BSA (wt/vol) and were washed with PBS. The cells were then incubated for 45 min with FITC-conjugated donkey anti-mouse secondary antibodies (Jackson Immunoresearch), also diluted in PBS/saponin/BSA. After the cells were washed a second time in PBS, coverslips were mounted onto glass slides with Fluoromont G (Southern Biotechnology Associates). Microscopy was performed on a Zeiss Axioscop-20 with a FITC filter (Carl Zeiss, Inc.) Normal fibroblasts showed a distinct punctated distribution of the rab27a

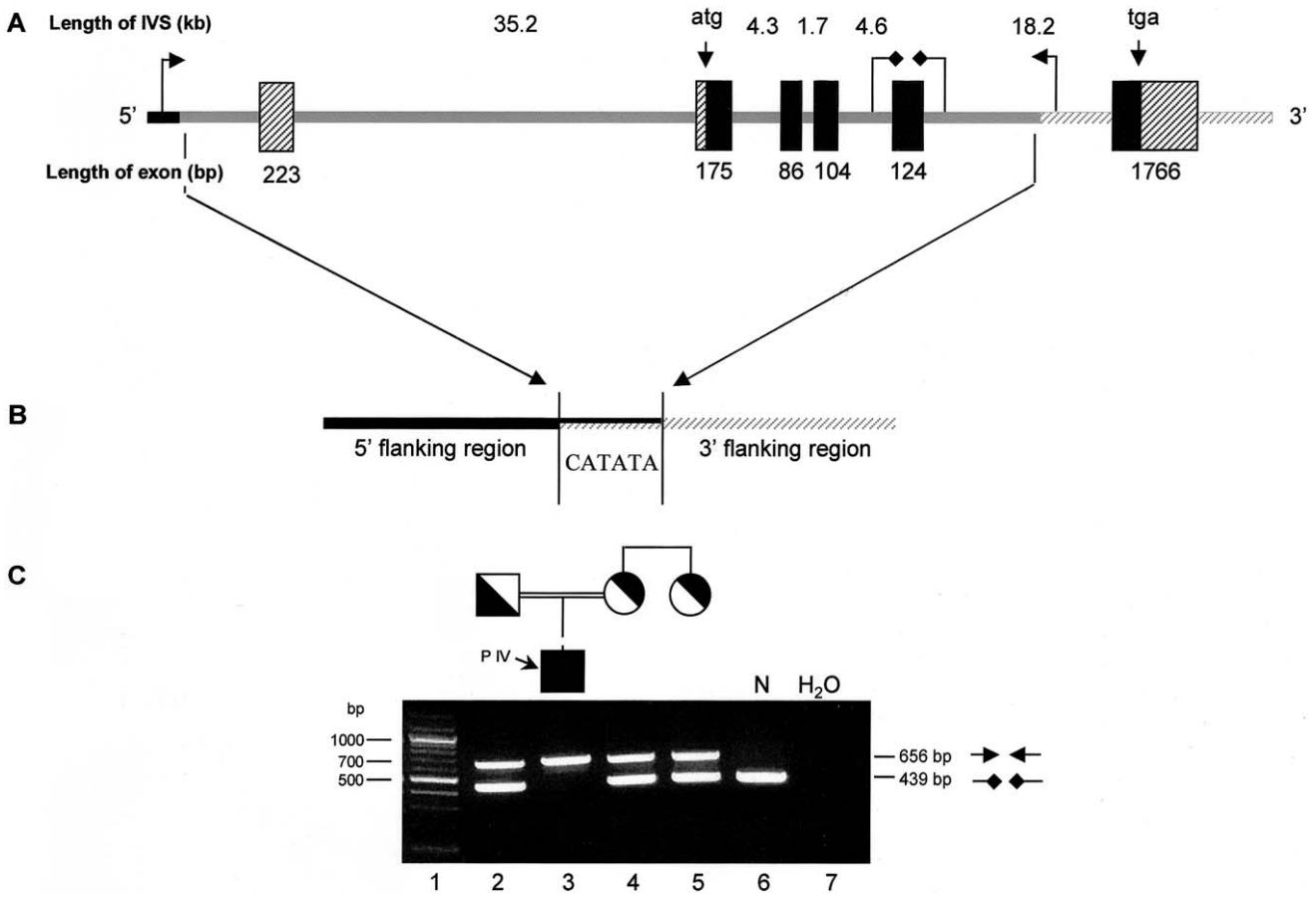

Figure 3 Structure of the *RAB27A* deletion and multiplex PCR amplification assay for its presence or absence. *A,* Schematic basis of the 67,536-bp deletion. The deletion (*shaded line*) contains exons 1–5 (black and crossed boxes), as well as the predicted promoter region, and is flanked by normal sequence. Intron sizes and locations of the start and stop codons are shown above the gene, and exon sizes are shown below. *B,* The deletion breakpoint occurs somewhere within the 6-bp region shared at either end of the deletion. PCR amplification across the deletion yields a 656-bp fragment. Two primers within the deleted area (*diamond-headed arrows*) are used to amplify the DNA of alleles lacking the deletion; the product is a 439-bp fragment. *C,* Multiplex PCR in the affected family. PCR amplification products indicate the presence (656-bp fragment) or absence (439-bp fragment) of the 67,536-bp deletion. *Lane 1,* molecular weight markers; *lane 2,* father of patient IV, heterozygous for the deletion; *lane 3,* patient IV, homozygous for the deletion; *lane 4,* mother of patient IV, heterozygous for the deletion; *lane 5,* aunt of patient IV, heterozygous for the deletion; *lane 6,* normal control, lacking the deletion; *lane 7,* no-DNA control.

protein throughout the cell, similar to the distribution pattern in melanocytes reported by Hume et al. (2001). In contrast, there was no visible rab27a signal in fibroblasts from the patient (fig. 4).

Three different rare, autosomal recessive disorders are characterized by hypopigmentation and silvery hair in infancy or early childhood (table 1). Chediak-Higashi syndrome (CHS [MIM 214500]) presents with variable hypopigmentation of the skin, hair, and eyes, a bleeding diathesis due to platelet-storage-pool deficiency, slowly progressive neurological dysfunction, and severe immunodeficiency (Introne et al. 1999; Ward et al. 2000). CHS cells show giant lysosomes, melanosomes, lytic granules, and azurophilic granules (Kjeldsen et al. 1998; Ward et al. 2000). Platelet-dense granules are absent or reduced in number (Rendu et al. 1983). The gene responsible for CHS, *LYST*, encodes a cytoplasmic protein of ~430 kD (Barbosa et al. 1996; Nagle et al. 1996). Expression studies of *LYST* suggest a role in membrane fusion or fission events (Perou et al. 1997), and the large size of the LYST protein and its cytoplasmic location also argue for a role in vesicle transport. The *beige* mouse is the murine counterpart of CHS (Barbosa et al. 1996; Perou et al. 1996).

Elejalde syndrome (ES [MIM 256710]), also called "neuroectodermal melanolysosomal disease," is characterized by silvery hair, pigment abnormalities, and severe dysfunction of the CNS, with seizures, severe hypotonia, and mental retardation (Elejalde et al. 1979; Sanal et al. 2000). Patients with ES do not manifest the hemophagocytic syndrome or immunologic defects. Large granules of melanin are unevenly distributed in hair shafts, and abnormal melanosomes may occur in melanocytes, along with inclusion bodies in fibroblasts (Duran-McKinster et

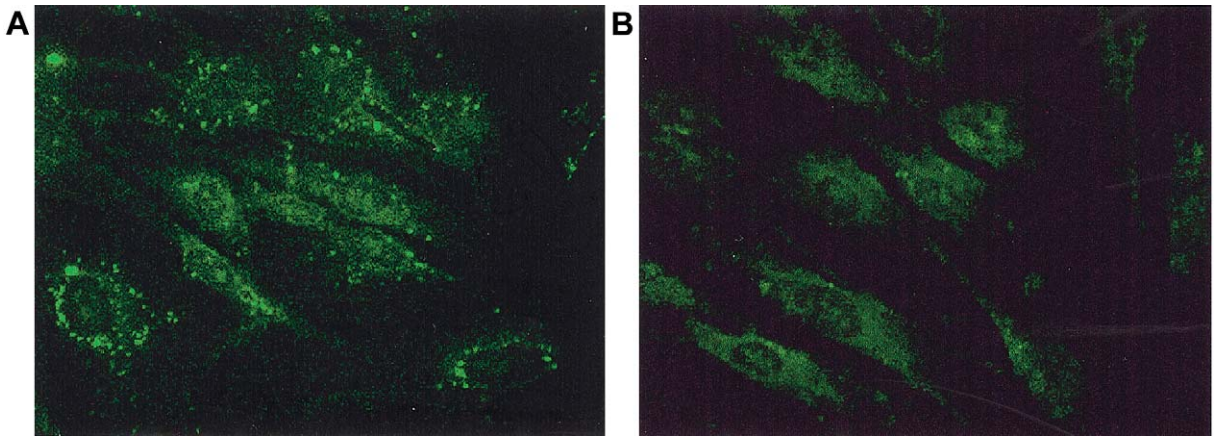

Figure 4 Intracellular rab27a distribution detected by indirect immunocytofluorescence. Normal (*A*) and patient (*B*) fibroblasts were grown on coverslips and were fixed and permeabilized before incubation with rab27a antibodies. FITC-conjugated secondary antibodies were used for fluorescent labeling. Rab27a shows a distinct punctated distribution throughout the normal fibroblasts. The patient's fibroblasts, lacking rab27a, show only background fluorescence.

al. 2000). No gene has been reported to be associated with ES, nor has any mouse model been identified.

Patients with GS exhibit the accelerated phase and immunologic dysfunction seen in CHS but not in ES. In addition, some patients with GS have neurological involvement that shows extremely variable expression and severity. Two genes, *RAB27A* and *MYO5A*, which are separated by only ~3 mB on chromosome 15, have been reported to cause GS. In mice, *Myo5a* mutations correspond with the *dilute* phenotype (Mercer et al. 1991), whereas *Rab27a* mutations cause the *ashen* phenotype (Wilson et al. 2000). These two mouse models, along with *leaden* (which has mutations in *melanophilin* [Matesic et al. 2001]), provide a unique system for studying vesicle transport in mammals. All three mice have a lightened coat color because of defects in pigment granule transport (Swank et al. 1998), and all three are suppressed by the semidominant *dilute-suppressor* (*dsu*) (Moore et al. 1988). Genetic evidence indicates that the gene products of *dilute*, *ashen*, and *leaden* function in the same or overlapping pathways, and molecular and cell-biological studies show that they interact in effecting melanosome trafficking in the distal region of melanocyte dendrites (Wu et al. 2001; Hume et al. 2002; Provance et al. 2002).

It has been reported—and accepted by the medical community—that patients who have GS with immunologic defects have *RAB27A* mutations and that those with neurological involvement have *MYO5A* mutations (Pastural et al. 2000; Westbroek et al. 2001). We present an alternative explanation. Specifically, we propose that all patients with *RAB27A* mutations have GS and that neurological complications in these individuals occur secondarily to lymphocytic and histiocytic infiltration of the brain (Gogus et al. 1995). Patients with *MYO5A* mutations, then, have ES with *primary* neurological involvement, which occurs early in life and in the absence of any signs of the hemophagocytic syndrome.

Several lines of evidence support this thesis. First, the original report of *MYO5A* mutations in GS provides information on only two patients. The first of these, designated patient 2, had an R1246C substitution which later proved to be a polymorphism (Lambert et al. 2000). The second, designated patient 3, was homozygous for a nonsense mutation in *MYO5A,* an observation that predicted a truncated protein lacking 1,087 amino acids. However, this patient was described by the authors as having "a severe neurological disorder, similar to the neuroectodermal melanolysosomal disease described by Elejalde et al." (Pastural et al. 1997, p. 290). There was no history of the hemophagocytic syndrome or of immunologic defects in patient 3, who most likely had ES, not GS.

Second, the initial linkage studies that identified *MYO5A* as a candidate GS-causing gene were misleading because of the extremely close proximity of *RAB27A* to *MYO5A* on chromosome 15. In fact, the majority of the patients with GS who were used to identify *MYO5A* as a GS-causing gene actually had *RAB27A* mutations. At the same time, patients with *MYO5A* mutations would not disrupt the linkage analysis, so their diagnosis of GS was not questioned. However, these individuals may, in fact, have had ES.

Third, the only other patient (P8) found to have mutations in *MYO5A* had a clinical presentation consistent with ES and not with GS (Pastural et al. 2000). The child had profound CNS deficits but no immunologic problems and no evidence of hemophagocytic syndrome.

Fourth, the expression patterns of *RAB27A* and *MYO5A* are consistent with the clinical phenotypes of GS and ES, respectively. *RAB27A* is not expressed in

Table 1

Syndromes That Involve Silvery Hair

Characteristic	GS	ES	Chediak-Higashi
Inheritance	Autosomal recessive	Autosomal recessive	Autosomal recessive
Hair color	Silvery/metallic	Silvery/metallic	Silvery/metallic
Age at onset	Infancy/early childhood	Infancy/early childhood	Infancy/early childhood
Skin pigment dilution	+	+	+
Eye pigment dilution	+/−	+/−	+
Accelerated phase	+++	−	++
Immune defects	++	−	+++
Giant granules	−	−	++
Neurological involvement	+	+++	+
No. of patients reported	>40	>10	~200
Gene	*RAB27A*	*MYO5A*?	*LYST*
Mouse model	*dilute*	*ashen*	*beige*
Bleeding diathesis	−	−	+
Treatment	BMT	−	BMT

NOTE.—+ = present; − = absent; +/− = may or may not be present; ++ = a significant feature of disease; and +++ = the primary characteristic of disease.

brain tissue, in keeping with the lack of primary neurological abnormalities in GS. In addition, rab27a-deficient T cells exhibit reduced cytotoxity and cytolytic granule exocytosis required for immune homeostasis (Menasche et al. 2000; Stinchcombe et al. 2001), consistent with the occurrence of the hemophagocytic syndrome of GS. In contrast, some splice products of *MYO5A* are expressed in brain tissue (Huang et al. 1998; Lambert et al. 1998), and those that are expressed in brain tissue may not interact with rab27a (Wu et al. 2002).

Finally, the molecular analysis of patient IV and his family is consistent with our proposal. Because of his neurological involvement, patient IV was expected to have mutations in *MYO5A*. However, the neurological symptoms of the affected kindred varied extensively in age at onset and rate of progression. Furthermore, the hemophagocytic syndrome antedated the neurological complications, so histiocytic and lymphocytic invasion could have caused the CNS involvement. Indeed, the *MYO5A* gene was normal in sequence and expression in our patient, who instead exhibited a large homozygous deletion in *RAB27A*. We eliminated the possibility of a contiguous gene syndrome by showing normal expression of the flanking genes, *PIGB* and *LOC51187*, and we conclude that our patient has genuine GS due to a *RAB27A* mutation.

The identification of the breakpoints of the large deletion present in the family of patient IV has provided a useful tool for genetic counseling of this kindred. In addition, we are now able to screen for the mutation among unaffected individuals. That the mutation was absent in 86 anonymous Palestinian individuals is not surprising. Although a low carrier frequency for the mutation would produce this result, it can also be explained by the marriage practices of the isolated kindreds that constitute the Palestinian Arab world. High rates of consanguinity and the rarity of interfamily marriage prevent the spread of genetic variants through the population, causing unequal distribution of many autosomal recessive disorders (Zlotogora 1997; Zlotogora et al. 2000).

As more patients with GS or ES are studied at the molecular level, the clinical and genetic distinctions between these two entities will be resolved. Because prevention of the hemophagocytic syndrome by BMT may be lifesaving for patients with GS and an unnecessary risk for patients with ES, the distinction is critical. We suggest that patients with *MYO5A* defects not be assigned the diagnosis of GS at this time.

Acknowledgment

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *RAB27A* gDNA [accession number AF443871], *RAB27A* cDNA [accession number U38654], *MYO5A* cDNA [accession number U90942], *PIGB* cDNA [accession number NM_004855], and *LOC51187* cDNA [accession number XM_046136])

Map Viewer, http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/map_search (for *RAB27A, MYO5A, PIGB,* and *LOC51187* location on chromosome 15)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for GS [MIM 214450], ES [MIM 256710], and Chediak-Higashi syndrome [MIM 214500])

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