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Arg–Cys Substitution at Codon 1246 of the Human Myosin Va Gene is not Associated with Griscelli Syndrome

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Myosin Va is an actin-associated motor protein involved in organelle transport such as melanosomes and neuron synaptic vesicles and has always been proposed as the candidate gene for the autosomal recessive Griscelli–Pruniéras syndrome, one of the silvery hair syndromes, which is a lethal disease combining immunodeficiency and neurologic and pigmentary abnormalities. Thus far, two mutations in the myosin Va gene have been described to be associated with this syndrome. One of these mutations was a homozygous mis-sense mutation causing an arginine to cysteine alteration at codon 1246. Because we also found this particular substitution after mutation analysis of a Griscelli patient, we checked its relevance in a control group of 124 unrelated healthy individuals and found it to be present, even in homozygous state, in normal unaffected individuals. It is clear that this arg1246cys substitution is a polymorphism occurring in the human population and not occurring in association with Griscelli syndrome. Distinguishing a polymorphism from a *bona fide* mutation is of uttermost importance and has major ethical implications with regard to prenatal genetic counseling in affected families.* ***Key words: pigmentary disorder/polymorphism/silvery hair syndrome. J Invest Dermatol 114:731–733, 2000***

Myosin Va is a member of the family of unconventional myosins, motor proteins that use energy released through hydrolysis of adenosine triphosphate to move cytoplasmic components along actin filaments. Analysis of organisms carrying mutations in the myosin Va gene revealed that this motor protein is essential in the trafficking and positioning of certain membrane-bound vesicles, such as melanosomes, neuron synaptic vesicles and smooth endoplasmic reticulum (Mermall *et al*, 1998). Griscelli syndrome is a rare, lethal autosomal recessive disorder characterized by early-onset immune and neurologic defects and pigmentary dilution of hair and, to a lesser extent, of skin (Griscelli *et al*, 1978). Hence, the need for genetic advice in affected families is high. Prenatal diagnosis, however, can only be offered if the causal mutation in the affected patient can be identified with certainty. A condition similar to Griscelli syndrome was described in the *dilute* mouse, caused by mutations at the *dilute* locus encoding the myosin Va gene (*MYO5a*) (Mercer *et al*, 1991). In 1997, Pastural and coworkers showed by linkage analysis in three consanguineous and one nonconsanguineous family that the Griscelli syndrome maps to chromosome 15q21 and that it is associated with mutations in the myosin Va gene (Pastural *et al*, 1997) (GenBank accession numbers Y07759 and U90942). In one consanguineous family, mutation analysis of *MYO5a* revealed presence of a homozygous nonsense mutation R779X. In a second patient born to consanguineous parents, a homozygous C to T transition was found at nucleotide 3736, causing a substitution of arginine to cysteine at codon 1246 (R1246C). In a third patient, Pastural and coworkers could not identify any mutations after cDNA sequencing of the complete *MYO5a* coding region. Of the fourth patient no RNA for *MYO5a* cDNA sequencing was available.

We have sequenced the myosin Va complete coding sequence in 2 unrelated Griscelli patients. As intron–exon structure of the *MYO5a* gene is still unknown, mutation analysis was performed at the messenger RNA level starting from cell cultures treated with puromycin, shown to inhibit non-sense-mediated messenger RNA decay (Andreutti-Zaugg *et al*, 1997). In one patient, born to consanguineous parents, we found the same homozygous mis-sense mutation R1246C previously described to be a causal mutation associated with Griscelli syndrome (Pastural *et al*, 1997). This finding prompted us to investigate this alteration according to the recommendations proposed by Cotton and Scriver (1998) in order to verify that this is indeed a causal mutation in Griscelli syndrome. It is clear that, when encountering a mis-sense mutation, one should first verify its effect, e.g., by looking at its prevalence in a larger healthy population, before designating it a phenotype-altering property.

MATERIALS AND METHODS

Human tissue material Human dermal fibroblast cultures and epidermal keratinocyte cultures, respectively, five and six unrelated, healthy individuals were established starting from skin released after abdominoplasty or skin biopsy. Human epidermal melanocyte cultures were derived from neonatal foreskins of seven unrelated healthy neonates. Postprimary cultures of these cell types were obtained and cultured as described previously (Lambert *et al*, 1998). Blood leukocytes of another 106

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Abbreviation: AS-PCR, allele-specific polymerase chain reaction.

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unrelated individuals were isolated using an RNeasy Blood Mini Kit according to the manufacturer's instructions (Qiagen, Westburg, the Netherlands). All control individuals were of Caucasian origin.

Reverse transcription–allele-specific polymerase chain reaction (AS-PCR) Total RNA of the 124 healthy individuals was isolated for each cell type using Tri reagent (Sigma, Bornem, Belgium) according to the manufacturer's instructions. About 2 μg of RNA was reverse transcribed using Superscript II RNase H^- reverse transcriptase (Gibco BRL, Merelleke, Belgium) and random hexamers (Pharmacia, Roosendaal, the Netherlands). For the AS-PCR a primer pair was designed so that the reverse primer contains a 3′OH-mismatch to the wild-type allele, i.e., a thymidine instead of a cytosine at position 3736 of the human myosin Va cDNA sequence (GenBank accession number Y07759). The forward primer P1: 5′-TGGACACAAGAGAACAGACTCC-3′ and the reverse primer P2: 5′-GCTGCTCCATGAGGACACA-3′, encompassing nucleotides 3325–3755 in the myosin Va coding sequence, were both used in a concentration of 0.3 μM, with 1 U Platinum Taq DNA polymerase (Gibco BRL), 10 × PCR buffer II, 1.5 mM magnesium chloride (Perkin Elmer, Mieunverkerk a/d Yassel, the Netherlands), and 200 μM each dNTP (Pharmacia). The amplification program used was: 30 cycles of 1 min denaturation at 95°C, 30 s at 65°C, and 1 min extension at 72°C with an MJ Research PTC-150 Mini-Cycler. The presence of the amplificon of 431 bp was evaluated on a 1.5% ethidium bromide-stained agarose gel.

cDNA sequence analysis The cDNA samples revealing a positive signal in the AS-PCR assay were subjected to 40 cycles of PCR using primers P3: 5′-TCATCATTCAGAAGCGAGTC-3′ and P4: 5′-TTTTTGGCAAAT-ACTTTCAGTT-3′ encompassing nucleotides 2601–4317 in the C-terminal region of the human myosin V and sequenced with 0.15 μM fluorescein-labeled primer P5 at position 3561: 5′-AGGCCAAGG-AAGAAGAAAG-3′ using the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (Amersham, Roosendaal, the Netherlands) and analyzed on an ALF automated DNA sequencer (Pharmacia).

RESULTS AND DISCUSSION

Starting from the observation that, in a Griscelli patient born to consanguineous parents, we found a homozygous mis-sense mutation (R1246C) at codon 1246, caused by a C to T transition at nucleotide 3736 of the *MYO5a* coding sequence (results not shown), previously described to be associated with Griscelli syndrome (Pastural *et al*, 1997), we investigated 124 control individuals, with no phenotypic features of Griscelli syndrome, for presence/absence of this specific substitution, using an AS-PCR method followed by sequencing. The AS-PCR used is based on allele-specific reverse primer matching, at its 3′-end, to the presumed point mutation C3736T. If the base substitution is present, this allele is readily amplified whereas a wild-type allele is not, or poorly, amplified. The aim was to detect rapidly the presence of a C to T substitution at position 3736 of the myosin Va gene in a substantial number of individuals. In 124 individuals, 22 showed a positive signal of the appropriate length after AS-PCR. Direct sequencing revealed that of these 22 individuals, 20 carried the T-allele in a heterozygous manner and that two individuals were homozygous for the T-allele (**Fig 1**).

In 1995, Moore and coworkers (Moore *et al*, 1995) mentioned the same substitution at this particular codon (TGT instead of CGT) in a human myosin heavy chain 12 (*MYH12*) gene sequence, designated myosin V (GenBank accession number S74799), when comparing with the partial myosin V cDNA sequence available at that time (Engle and Kennett, 1994) (GenBank accession number L19401). As they did not specify whether this substitution occurred in a heterozygous or homozygous state, this report does not contradict the possibility that the homozygous substitution as reported by Pastural *et al* (1997) might indeed be associated with the recessive Griscelli syndrome. The fact that we found two of the investigated normal control persons to carry this substitution in a homozygous form, without any phenotypic signs related to Griscelli syndrome, however, is clear evidence that this substitution is not a deleterious mutation, but a polymorphism in the human population. Based on our findings, the frequency p of the 3736C allele is 0.9 (224 C-alleles/248 total alleles) and the frequency q of the 3736T allele 0.1 (24 T alleles/248 total alleles). According to the Hardy–Weinberg equilibrium, the population frequency of the homozygous 3736T allele (q^2) is approximately 1%, which is far above the frequency at which the very rare Griscelli syndrome is seen.

Although this polymorphism does not cause any phenotypic changes in individuals carrying it in a homozygous way, it is interesting to consider whether this Arg–Cys substitution could have an influence on myosin Va structure and function. Northern blotting revealed no change of levels in RNA expression between the homozygous wild-type alleles and the heterozygous and homozygous polymorphic alleles (results not shown). At protein level, the introduction of a cysteine could induce conformational changes by alternative folding of the protein through disulfide bonding. Furthermore, changing arginine into cysteine replaces a large basic amino acid by a small nonpolar amino acid. The polymorphism is located in the second globular domain of the myosin Va tail region (Cheney *et al*, 1993), a region assumed to be involved in cargo binding (Prekeris and Terrian, 1997; Espreafico *et al*, 1998; Costa *et al*, 1999; Huang *et al*, 1999; Tsakralides *et al*, 1999). In particular, the alteration of an arginine to a cysteine has been reported in several disorders [e.g., cystic fibrosis (Casals *et al*, 1997); lethal skeletal dysplasia (Tavormina *et al*, 1995)] to have a deleterious effect. Our findings, however, show that, certainly in the case of a mis-sense mutation, extreme care is needed in the absence of functional studies before a conclusion can be made that the alteration is disease causing, especially when they are presumed to be associated with severe lethal conditions for which uptake and request for prenatal diagnosis are expected to be high. False diagnoses may result if polymorphisms are erroneously reported as *bona fide* mutations. The fact that in five unrelated Griscelli patients investigated so far – three by Pastural *et al* (1997) and two by our group (unpublished results) – only one *bona fide* mutation (R779X) in the *MYO5a* gene has been described, raises the question whether Griscelli syndrome is not a genetically heterogeneous disorder. Another interesting candidate gene is myosin IC as this gene maps

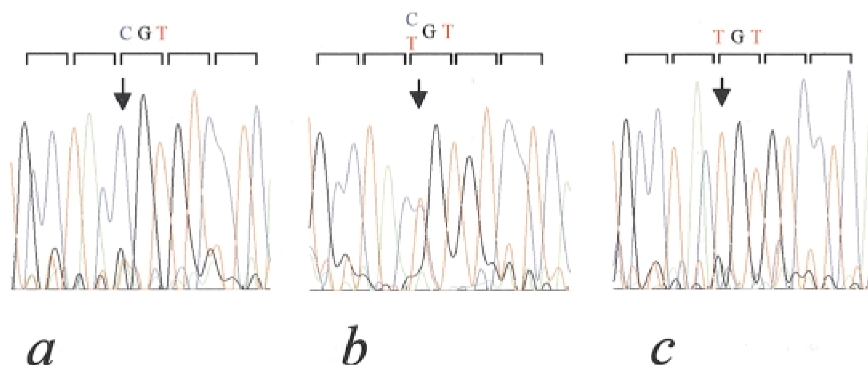

Figure 1. cDNA sequence analysis. Sequence chromatogram of the reverse transcription–PCR amplified myosin Va cDNA fragment encompassing nucleotide 3736 in: (*a*) a normal individual homozygous for the wild-type 3736C allele; (*b*) a normal individual heterozygous for the C to T transition; and (*c*) a normal individual homozygous for the 3736T allele.

to the same region on chromosome 15q21 (Bement *et al*, 1994). Alternatively, more loci for Griscelli may exist and we therefore consider that the ubiquitous and neuroneal kinesin heavy chain genes (Hirokawa, 1998) are interesting functional candidate genes and deserve further investigation.

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