Am. J. Hum. Genet. 67:1333–1339, 2000

Report

High Frequency of Alkaptonuria in Slovakia: Evidence for the Appearance of Multiple Mutations in *HGO* Involving Different Mutational Hot Spots

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Alkaptonuria (AKU) is an autosomal recessive disorder caused by the deficiency of homogentisate 1,2 dioxygenase (HGO) activity. AKU shows a very low prevalence (1:100,000–250,000) in most ethnic groups. One notable exception is in Slovakia, where the incidence of AKU rises to 1:19,000. This high incidence is difficult to explain by a classical founder effect, because as many as 10 different AKU mutations have been identified in this relatively small country. We have determined the allelic associations of 11 *HGO* intragenic polymorphisms for 44 AKU chromosomes from 20 Slovak pedigrees. These data were compared to the *HGO* haplotype data available in our laboratory for >80 AKU chromosomes from different European and non-European countries. The results show that common European AKU chromosomes have had only a marginal contribution to the Slovak AKU gene pool. Six of the ten Slovak AKU mutations, including the prevalent G152fs, G161R, G270R, and P370fs mutations, most likely originated in Slovakia. Data available for 17 Slovak AKU pedigrees indicate that most of the AKU chromosomes have their origins in a single very small region in the Carpathian mountains, in the northwestern part of the country. Since all six Slovak AKU mutations are associated with *HGO* mutational hot spots, we suggest that an increased mutation rate at the *HGO* gene is responsible for the clustering of AKU mutations in such a small geographical region.

Alkaptonuria (AKU [MIM 203500]), the first human disease to be interpreted as a recessive trait (Garrod 1902), is a rare disorder of the phenylalanine and tyrosine catabolic pathway caused by the deficiency of homogentisate dioxygenase (HGO [E.C.1.13.11.5]) activity (La Du et al. 1958). AKU patients are homozygous or compound heterozygous for loss-of-function mutations in *HGO* (Fernández-Cañón et al. 1996). As a consequence of this defect, AKU patients cannot convert homogentisate to maleylacetoacetate, which results in homogentisic aciduria, ochronosis, and arthritis (La Du et al. 1995). AKU presents a remarkable allelic heterogeneity. In a series of <100 unrelated patients from many different countries, >40 different AKU mutations have been identified (Fernández-Cañón et al. 1996; Gehrig et al. 1997; Beltrán-Valero de Bernabé et al. 1998; Higashino et al. 1998; Ramos et al. 1998; Beltrán-Valero de Bernabé et al. 1999*a*, 1999*b;* Felbor et al. 1999; Müller et al. 1999; Walter et al. 1999; Porfirio et al. 2000; Zatková et al. 2000; authors' unpublished data). The most prevalent mutation in Europe (excluding the Slovak AKU patients) is M368V, which represents ~20% of the AKU chromosomes. Similarly, V300G and P230S each represent ~5% of the European AKU chromosomes. In addition to the AKU mutations, 11 polymorphisms have been encountered within the human *HGO* gene (Granadino et al. 1997; Beltrán-Valero de Bernabé et al. 1998, 1999*a;* this report). The analysis of the haplotypic association of these polymorphic markers in the AKU chromosomes has shown that the three most dif-

Received July 19, 2000; accepted for publication September 13, 2000; electronically published October 2, 2000.

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0002-9297/2000/6705-0032$02.00

fused AKU mutations in Europe—M368V, V300G, and P230S—are not recurrent mutations. Instead, they are probably old mutations that were introduced in Europe with the founder populations and have spread throughout Western Europe with the different migrations (Beltrán Valero de Bernabé et al. 1998).

Slovakia is a country with a notable incidence of alkaptonuria (1:19,000) (Srsen et al. 1978). However, the high frequency of AKU in this small geographical region is difficult to explain. Recently, we and others have demonstrated the existence of many different AKU mutations in Slovakia, suggesting that several independent founders have contributed to the AKU gene pool in this geographical location (Gehring at al. 1997; Müller et al. 1999; Zatková et al. 2000).

To get further insight into the history of the Slovak AKU chromosomes and to provide an explanation for the presence of multiple AKU mutations in Slovakia, we have determined the allelic associations of 11 *HGO* intragenic polymorphisms for 44 AKU Slovak chromosomes. Our sample of AKU Slovak chromosomes includes 29 chromosomes, corresponding to 13 AKU pedigrees that have been reported elsewhere (Zatková et al. 2000), and 15 novel chromosomes from seven patients with AKU who have not been reported. We included in these studies all unrelated pedigrees from Slovakia that we could obtain. Most of the samples were obtained throughout the Research Institute of Rheumatic Diseases of Piešťany, whose activity covers all of Slovakia. AKU mutations were identified in all 44 chromosomes. As many as 10 different AKU mutations were characterized in our sample: IVS1-1G→A, S47L, R58fs, IVS5+1G→A, G152fs, G161R, P230S, G270R, V300G, and P370fs. Interestingly, we identified no novel mutations in the 15 new Slovak AKU chromosomes, supporting the idea that our sample, including 44 AKU chromosomes, is representative of the whole spectrum of AKU mutations in Slovakia. As indicated, these AKU mutations have been reported previously (Fernandez-Cañón et al. 1996; Gehrig et al. 1997; Beltrán-Valero de Bernabé et al. 1999*a,* 1999*b;* Müller et al. 1999; Porfirio et al. 2000; Zatková et al. 2000), and most of them have been demonstrated to be loss-of-function mutations (Fernandez-Cañón et al. 1996; Rodriguez et al. 2000; Titus et al. 2000; authors' unpublished data).

The result of *HGO*-haplotype analysis in the Slovak AKU chromosomes is summarized in figure 1. For comparison, figure 1 also includes the *HGO* haplotypes for all the AKU chromosomes characterized thus far in our laboratory from non-Slovak patients who carry the AKU mutations found in the Slovak patients.

It is noticeable that M368V, the most prevalent AKU mutation in Europe and a relatively frequent AKU mutation in the neighboring countries, has not been encountered in the Slovak patients. Similarly, other AKU mutations carried by patients from different countries like P230S, V300G, R58fs, and IVS1-1G→A are relatively infrequent in Slovakia. Nevertheless, the P230S, V300G, R58fs, and IVS1-1G→A mutations in the Slovak patients are found to be associated with the same *HGO* haplotypes as previously described outside Slovakia, or the differences (as for V300G) can be easily explained by recombination (fig. 1). These data reinforce the concept that IVS1-1G→A, R58fs, P230S, and V300G are relatively old AKU mutations that spread in Europe with different migrations. However, there is a low number of AKU chromosomes carrying these mutations in our sample (7/44), which illustrates that these common European AKU chromosomes have had a marginal contribution to the AKU gene pool in Slovakia.

The data depicted in figure 1 support the idea that six of the AKU mutations found in our Slovak sample probably originated in this geographical location. The IVS5+1G→A mutation is particularly interesting, because it has been found in three Slovak AKU chromosomes associated with two different *HGO* haplotypes (fig. 1). IVS5+1G→A is, therefore, a recurrent mutation in Slovakia. In addition, this finding confirms that c.509+1 is a hot spot of mutation in *HGO*. As noticed by Müller et al. (1999) and Zatková et al. (2000), this same *HGO* nucleotide position has been described earlier to be mutated to a T (IVS5+1G→T) in a Dutch patient with AKU (Beltrán Valero de Bernabé et al. 1998).

G270R is a prevalent mutation in Slovakia that has also been found in an Italian patient (Porfirio et al. 2000). We have compared the *HGO* haplotypes associated with this mutation in the Italian and Slovak patients and found that they differ at both the 3′ and the 5′ ends of the *HGO* gene, suggesting two independent origins for this mutation (fig. 1).

A Slovak origin for the G152fs, G161R, and P370fs mutations is supported both by their high prevalence in Slovakia and by the fact that they are found almost exclusively in this country. G152fs and G161R occasionally have been found outside Slovakia, but we could not exclude a Slovak origin for these few patients with AKU. The *HGO*-haplotype analysis also provides evidence of recombination in the AKU chromosomes carrying the G161R and P370fs mutations, which indicates that some of the Slovak mutations may be relatively old mutations (fig. 1). The S47L mutation is a very rare AKU mutation that has been found only in one Slovak patient.

The *HGO*-A, *HGO*-B, *HGO*-C, *HGO*-D, and *HGO*-E haplogroups were initially defined considering only 7 of the 11 *HGO* polymorphic sites described here: IVS2+35, c.407, *HGO*-3, *HGO*-1, IVS5+25, IVS6+46, and *HGO*-2 (Beltrán Valero de Bernabé et al. 1998). Analysis of these *HGO* haplogroups in 90 individuals from the Slovak population without AKU shows a dis-

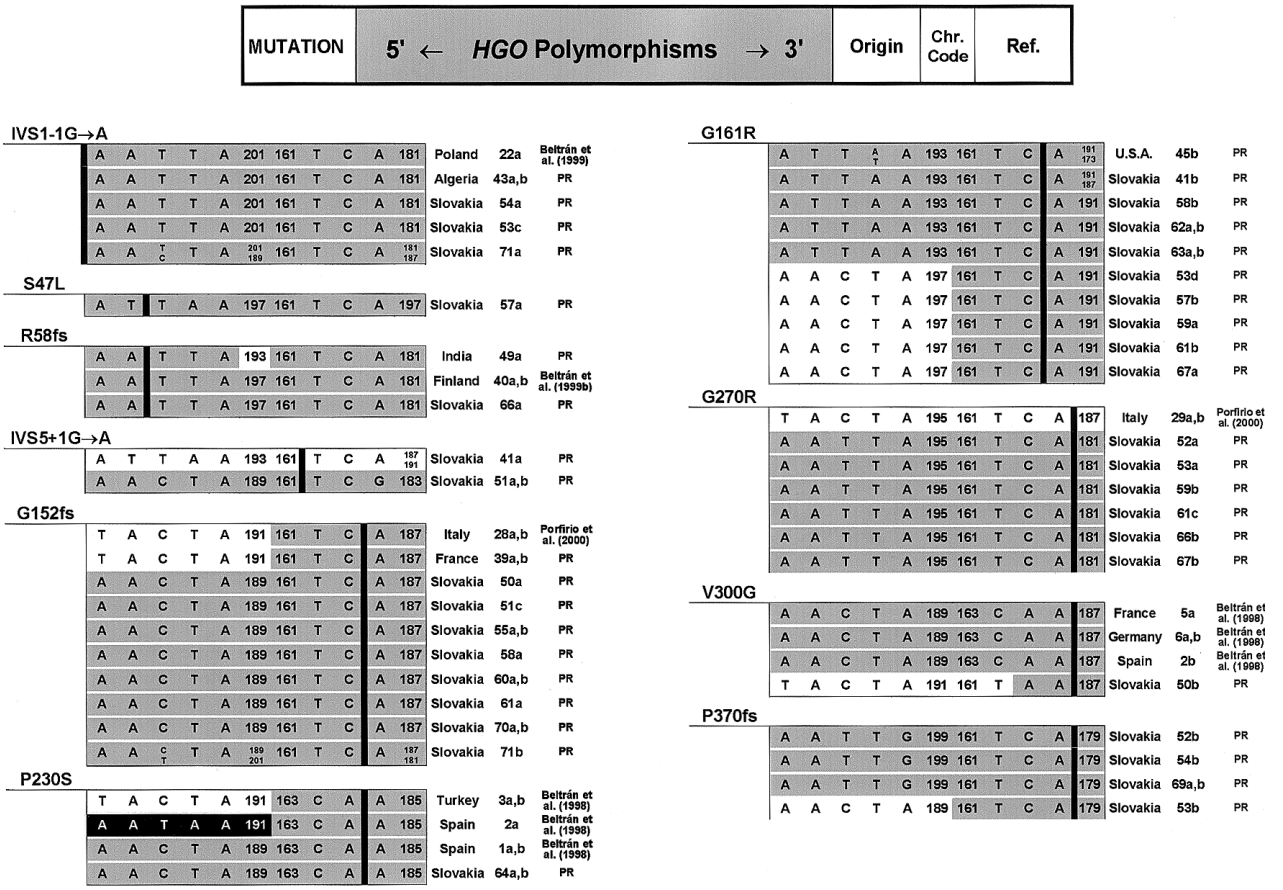

Figure 1 *HGO* haplotypes associated with the AKU mutations. The figure shows the allelic associations of 11 *HGO* intragenic polymorphisms for each of the 44 AKU Slovak chromosomes included in this study. The *HGO* polymorphic loci, ordered from 5′ to 3′, are IVS2+35, IVS2-218, IVS3-112, Ex4 (c407), IVS4+31, *HGO*-3, *HGO*-1, IVS5+25, IVS6+46, IVS11+18, and *HGO*-2. *HGO*-1, *HGO*-2, and *HGO*-3 are (CA)n or (CT)n dinucleotide repeats (Granadino et al. 1997; Beltran Valero de Bernabe et al. 1998). All other polymorphisms are diallelic SNPs (Beltran Valero de Bernabe et al. 1998; 1999*a*). AKU chromosomes are grouped by mutations. Each mutation group also includes the chromosomes described thus far outside Slovakia that carry the same AKU mutation. The chromosomes are identified by the pedigree code number, followed by a, b, c, or d (a and b indicates that the patient is an *HGO* homozygote). A thick vertical bar indicates the position, in the *HGO* haplotype, of each AKU mutation. A grey color code is used to identify the different *HGO* haplotypes. In very few instances, there was no information for the segregation of the alleles and both alleles were included in the haplotype. PR = present report.

tribution that is not significantly different from that described earlier for other populations (table 1).

Analysis of the full *HGO* haplotypes (including the 11 polymorphic sites) in this relatively small sample of the unaffected Slovak populations demonstrated the presence of *HGO* haplotypes that were identical to those associated with the G161R, G270R, G152fs, and IVS5+1G→A mutations. Further analysis of these unaffected individuals demonstrated that none of the individuals who carry the *HGO* haplotype associated with the IVS5+1G→A mutation (three individuals) or the G152fs mutation (two individuals) are AKU heterozygotes. Interestingly, however, one of the two control individuals who carry the *HGO* chromosomes associated with the G161R mutation and one of the two who carry the *HGO* chromosomes associated with the G270R mutation are also carriers of the G161R or G270R AKU mutation, respectively. These data further illustrate the extraordinary frequency (1/90) of AKU alleles in the unaffected Slovak population and demonstrate a coexistence of identical *HGO* haplotypes (including the 11 polymorphic sites) with and without AKU mutations in this population.

To get an insight into the history of the different AKU mutations in Slovakia, we have investigated the geographical origins of the maternal and paternal grandparents of the AKU patients. This information was available for 17 AKU pedigrees, which allowed us to determine a geographical origin for 37 AKU chromosomes, including eight different mutations. Figure 2 summarizes these data and illustrates an extraordinary clustering of the mutations in a very small geographical area in the

Table 1

Most-Representative *HGO* Haplogroups in the Slovak Population without AKU

Haplogroup	Approximate Frequency	Alleles Found To Be Associated with Haplogroup						
		IVS2+35A/T	c407A/T	*HGO*-3[a]	*HGO*-1	IVS5+25T/C	IVS6+46A/C	*HGO*-2[a]
HGO-A	.57	A	T (50%)	193 (48%)	161	T	C	181 (16%)
			A (50%)	189 (14%)				187 (36%)
				195 (14%)				183 (12%)
				197 (14%)				189 (9%)
								191 (8%)
HGO-B	.12	A	T	189 (40%)	161	T	A	177 (35%)
				197 (20%)				175 (30%)
				193 (20%)				
HGO-C	.11	T	T	191 (89%)	161	T	A	179 (61%)
HGO-D	.14	T	T	191 (91%)	161	T	C	…[b]
HGO-E	.06	A	T	189 (50%)	163	C	A	187 (60%)
				191 (30%)				185 (20%)
				193 (20%)				183 (20%)

[a] *HGO*-2 and *HGO*-3 alleles that are found to be predominantly associated with each of the *HGO* haplogroups.
[b] No specific allele was found to be associated with this haplogroup.

Carpathian mountains, the so-called "Kysuce" region, around the city of Cadca. This remarkable situation was first described by Müller et al. (1999), who identified, in this isolated Slovak region, using an independent AKU population sample, seven families carrying five different AKU mutations.

The only information available for the Slovak AKU pedigrees carrying the common European P230S and V300G mutations is that they live in the city of Zilina. However, Müller et al. (1999) found these mutations only in the central and southern parts of Slovakia.

The Kysuce region in Slovakia, where most of the AKU mutations concentrate, is believed to have be populated in the 14th and 15th centuries by Valachian immigrants. Valachians were nomadic tribes of Romanian origin who came to Slovakia from the Balkan countries through western Ukraine. Looking for new pastures, they moved to the north throughout the Carpathian mountains. Among other places, they settled in the valleys of river Kysuca, where they remained isolated until the end of World War II (Srsen 1984). Nowadays, there are populations with Valachian origins in other European countries, such as Romania, Moldavia, Greece, Bulgaria, Hungary, and Albania. However, there is no indication of a high incidence of AKU in these populations, suggesting that the AKU mutations were not introduced into the Kysuce region by this colonization.

The presence of multiple mutations in a single gene in a population living in a small geographical region has been described elsewhere for other disorders, like Hurler syndrome and metachromatic leukodystrophy (MDL) in Lower Galilee (Bach et al. 1993; Heinisch et al. 1995), and limb-girdle muscular dystrophies (LGMDs) on La Reunion island (Richard et al. 1995). However, in the case of Kysuce, the number of different mutations is remarkably high.

It is difficult to explain the presence of multiple mutations in a specific gene that are restricted to a small geographical area. One hypothesis about the extraordinary allelic heterogeneity of AKU in the Kysuce region involves different founders who immigrated into Slovakia at different times in history and settled close to each other in this region of the Carpathian mountains. It is, however, difficult to imagine how genetic drift or selective pressures (including social pressures) could have driven families with affected children to migrate together and settle in the Kysuce region. As indicated above, the most prevalent and ancient AKU mutations in Europe (M368V, V300G, and P230S) seem to be absent from the critical Kysuce region, which, in turn, clusters a number of AKU mutations found almost exclusively in Slovakia. This peculiar distribution of the AKU mutations and the uniqueness of the Kysuce region in Slovakia, regarding the clustering of AKU mutations, fit best with the idea that a significant number of the Slovak AKU mutations originated in the Kysuce region and that, during recent times, emigrants from the Kysuce region have disseminated these AKU mutations throughout Slovakia. In this regard, it is noticeable that the six AKU mutations that we believe have originated in this region are associated with *HGO* mutational hot spots. IVS5+1G→A was mentioned above and represents a clear *HGO* mutational hot spot. P370fs, G161R, G270R, and G152fs are mutations that involve CCC (or GGG) triplets, a sequence motif that has been shown to be hypermutable in the *HGO* gene (Beltrán Valero de Bernabé et al. 1999*a*). Finally, S47L originates from a C→T change at nucleotide position c.307, within a CpG

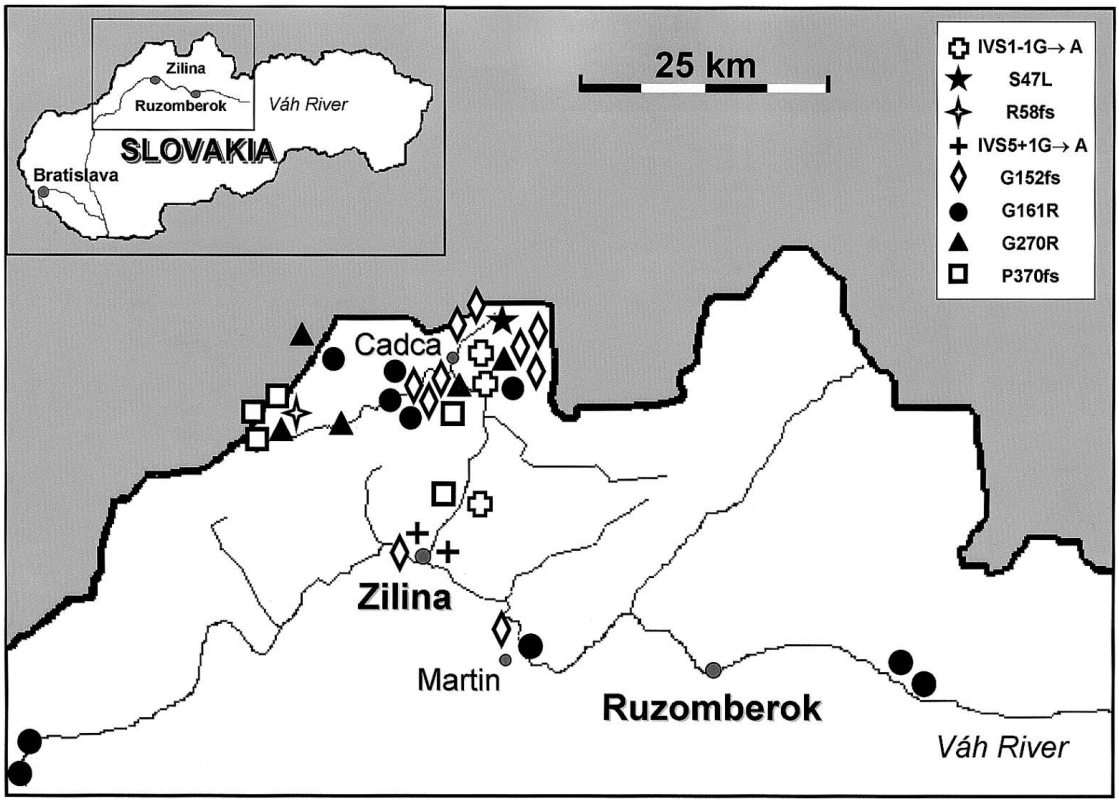

Figure 2 Geographical distribution of the Slovak AKU mutations The figure shows the geographical locations for 37 of the 44 Slovak AKU chromosomes included in this report. No family data relative to geographical origins were available for the remaining 7 AKU chromosomes. The code used for the mutations is depicted in the right inset.

dinucleotide that is predicted to be a mutational hot spot by the MUTPRED program (Cooper and Krawczak 1990).

Both the molecular mechanisms that could be responsible for the hypermutation process at CCC (or GGG) triplets (or at nucleotide c.509+1) and the reasons why this process appears to be restricted to the *HGO* gene are unknown. High mutation rate could result from both a sequence motif prone to mutation and some interference with the nucleotide-repair machinery. Similarly, differences between genes for the occurrence of mutations at specific short sequence motifs are perhaps a consequence of structural (sequence modification, chromatin organization, etc.) and functional (transcription rate, etc.) features of the individual genes. The observation that several AKU mutations associated with this mutational hot spot have originated in a small geographical area in Slovakia may offer an opportunity to analyze these possibilities. In this regard, it would be interesting to determine whether particular diets or living conditions may have exposed the inhabitants in this region of Slovakia to chemical or physical agents with the ability to induce specific mutations in specific human genes.

In conclusion, in this report, we provide new data that allow us to formulate novel hypotheses to explain the incidence of AKU in Slovakia. We show that various factors have probably contributed to the AKU gene pool in Slovakia. Some mutations—such as P230S, V300G, R58fs, and IVS1-1G→A—are shared by different populations and were probably introduced to Slovakia with the founder populations that spread throughout Europe. However, these mutations represent only 16% of the Slovak AKU chromosomes. The most prevalent mutations in Slovakia (G152fs, G161R, G270R, and P370fs) are relatively old mutations that most likely originated at a single and very small geographical location, the Kysuce region, in the northern part of Slovakia. These mutations probably spread from this region to other regions of Slovakia, as suggested by the radiation patterns observed for two of these mutations, G161R and G152fs (see fig. 2 and also Müller et al. [1999]). Interestingly, the G152fs, G161R, G270R, and P370fs mutations all involve CCC (or GGG) triplets, a sequence

motif that behaves as a mutational hot spot in the *HGO* gene (Beltrán Valero de Bernabé et al. 1999*a*). On the basis of these results, we postulate that the remarkable allelic heterogeneity of AKU in Slovakia was a consequence of an increased mutation rate at the *HGO* gene in the Kysuce region. This increased mutation rate involved different mutational hot spots. Subsequent genetic drift and isolation preserved and increased the frequency of these mutations and led to the high frequency of alkaptonuria in Slovakia.

Acknowledgments

We thank the families with AKU and all the clinicians, particularly Drs. F. Cisarík, M. Lukaèoviè, N. Mišovicová, J. Kršiaková, A. Kanabová, and Prof. J. Rovenský, D.Sc., for their collaboration and donation of blood (DNA) samples. We would also like to thank I. Szomolayová and L. Gulliksen for their excellent technical assistance and contribution to this work. This research was supported by the Fundación Jose Antonio de Castro, the Spanish Comisión Interministerial de Ciencia y Tecnología (SAF99/0013), and the Comunidad de Madrid (08.6/0015/1997). In addition, this study is based on work supported by the Fundación Conchita Rábago de Jiménez Díaz, under a grant awarded to D.B.-V.de B., and partially by HESP grant number G/004/00/61010, awarded to M.Z. by the Open Society Foundation, Bratislava.

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

AKU database, http://www.cib.csic.es/~akudb/index.htm (for published and unpublished data of mutations and polymorphisms in the *HGO* gene)

Entrez, http://www.ncbi.nlm.nih.gov/Entrez (for genomic sequences of *HGO* and its transcript; accession numbers AF000573 and AF045167, respectively)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for AKU [MIM 203500])

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