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Novel mutations in the homogentisate-1,2-dioxygenase gene identified in Slovak patients with alkaptonuria

EDITOR—Alkaptonuria (AKU, McKusick No 203500), a rare autosomal recessive disorder (1:250 000),[1] is a classical example of a specific biochemical lesion leading to degenerative disease. As a result of deficiency of homogentisic acid 1,2-dioxygenase activity (HGO, E.C. 1.13.11.5), AKU patients are unable to degrade homogentisic acid (HGA), an intermediary metabolite in phenylalanine and tyrosine catabolism.[2] Accumulated HGA is excreted into the urine in large amounts, which darkens on standing. Over the years, benzoquinone acetic acid, an oxidation product of HGA, is deposited in connective tissues, causing their pigmentation (ochronosis), which leads to painful and disabling arthropathy of the large joints and spine (ochronotic arthropathy).

AKU was the first disease interpreted in terms of Mendelian inheritance.[3] The *HGO* gene in humans is located on chromosome 3q21-23.[1 4 5] Fernandez-Canon *et al*[5] cloned the human *HGO* gene and by identifying the first loss of function mutations also provided formal proof that AKU results from a defect in this gene. So far, 24 different mutations have been identified in the *HGO* gene in patients from various populations.[5–10]

Notable exceptions to the low prevalence of AKU in all ethnic groups studied are the Dominican Republic and Slovakia (1:19 000).[11 12] Founder effects as the consequence of genetic isolation have been postulated to explain this observation. Here, we present results of mutation screening of the *HGO* gene in 32 AKU chromosomes carried by 17 Slovak AKU patients (in two families, one chromosome was shared by two patients from different generations). All 14 exons of the *HGO* gene were amplified from genomic DNA, using PCR primers and conditions as described by Fernandez-Canon *et al*.[5] PCR products were analysed for the presence of mutations by non-radioactive single strand conformation polymorphism analysis (SSCP).[13] DNA was visualised by silver staining essentially as described by Budowle *et al*.[14] Fragments showing SSCP shifts were sequenced directly using the dye terminator cycle sequencing kit (Perkin Elmer) with *Taq* FS DNA polymerase. Sequences were resolved on an ABI-310 Automatic Analyser.

Table 1 List and frequencies of the mutations identified in 32 AKU chromosomes from Slovak patients. Positions of nucleotide changes are related to the transcription start site as described in Granadino et al.[15] (Human HGO transcript: AF O45167; the ATG initiation codon is located at position c168)

Mutation	*Type*	*Nucleotide change*	*No and % of chromosomes (out of 32)*	*Reference*
IVS 1-1 G→A	Splice site	c183-1G→A	1 (3.125%)	9
S 47 L	Missense	c307 C→T	1 (3.125%)	Present report
IVS 5+1 G→A	Splice site	c509+ 1 G→A	2 (6.25%)	Present report
G 152 fs	Frameshift	c621 ins G	8 (25%)	6
G 161 R	Missense	c648 G→A	8 (25%)	6
P 230 S	Missense	c855 C→T	2 (6.25%)	5
				7
G 270 R	Missense	c975 G→A	4 (12.5%)	Present report
V 300 G	Missense	c1066 T→G	1 (3.125%)	5
				7
P 370 fs	Frameshift	c1273 ins C	5 (15.625%)	Present report

In our patients, we identified nine different mutations (tables 1 and 2). Four of them were novel mutations, two missense (S47L, G270R), a frameshift (P370fs), and a splice site mutation (IVS5+1G→A), increasing the total number of known AKU causing nucleotide changes within the *HGO* gene to 28. The remaining five mutations have been described previously: G161R and G152fs,[6] P230S and V300G,[5 7] and IVS1-1G→A.[9]

Novel mutation S47L is caused by a transition C→T at the second position of codon 47 (fig 1A). This transition abolishes a restriction site for *Mbo*I in exon 3 PCR fragments. The presence of the S47L mutation in our patient was confirmed by *Mbo*I digestion (fig 2A).

Mutation G270R is caused by transition G→A at the first position of codon 270, which creates a novel *Eco*NI restriction site (fig 1C). Therefore, its presence in our patients was confirmed by restriction digestion of exon 11 PCR fragments with *Eco*NI (fig 2B). Glycine at position 270, affected by this mutation, is conserved in man, mouse, and *Aspergillus nidulans* (fig 3).

Splice site mutation IVS5+1G→A affects the donor splice sites of intron 5 (fig 1B). Interestingly, Beltran-Valero de Bernabé *et al*[7] identified in one patient from Holland a transversion G→T affecting the same position of intron 5 as our mutation IVS5+1G→A. This mutation, however, was not identified in our patients.

Mutation IVS1-1G→A[9] abolishes restriction sites for *Rsa*I, so the presence of this mutation on one AKU chromosome was confirmed by restriction analysis of the corresponding PCR fragment with this enzyme (fig 2A).

A novel P370fs frameshift mutation, caused by a single base insertion c1273insC (fig 1D), brings about a premature translation stop four codons downstream and subsequent

Table 2 Genotypes of all analysed AKU patients from 15 families indicating identified disease causing mutations within the HGO gene

		HGO mutations	
Patient No	*Family code*	*Allele 1*	*Allele 2*
1	ALK1	G152fs	V300G
2	ALK2	IVS5+1G→A	G152fs
3	ALK2	IVS5+1G→A	IVS5+1G→A
4	ALK3	G152fs	P370fs
5	ALK4	G270R	P370fs
6	ALK5	G270R	P370fs
7	ALK6	IVS1-1G→A	P370fs
8	ALK7	G152fs	G152fs
9	ALK8	G161R	P370fs
10	ALK9	S47L	G161R
11	ALK11	G161R	G270R
12	ALK12	G152fs	G152fs
13	ALK13	G152fs	G270R
14	ALK13	G152fs	G161R
15	ALK14	G161R	G161R
16	ALK15	G161R	G161R
17	ALK16	P230S	P230S

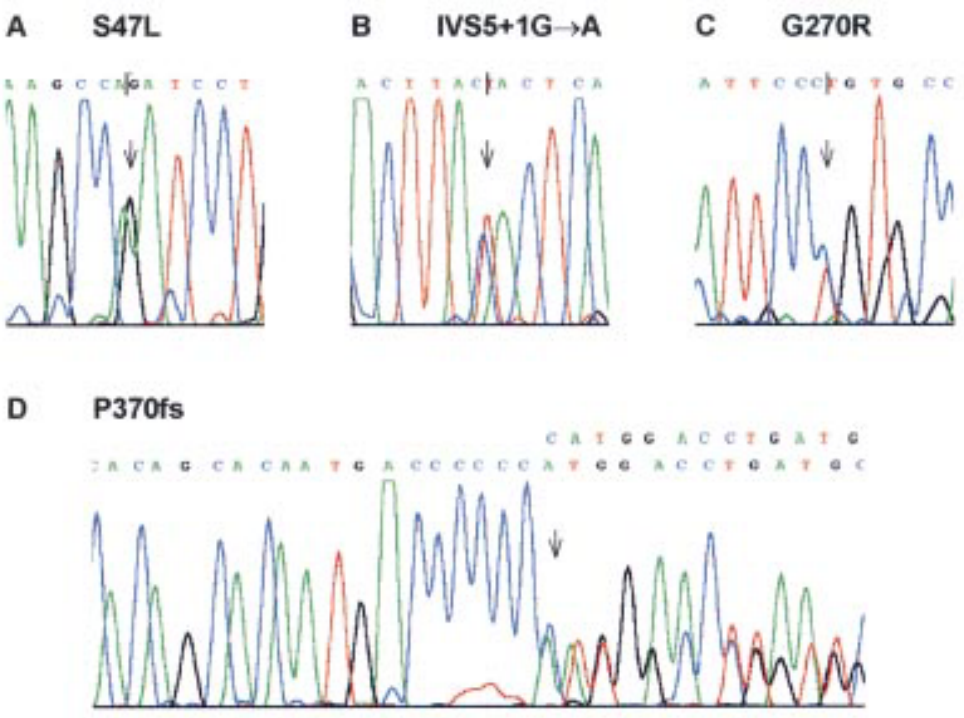

Figure 1 Part of the direct sequencing of exons 3 (A), 5 (B), 11 (C), and 13 (D) in patients heterozygous for mutations S47L, IVS5+1G→A, G270R, and P370fs, respectively (ABI 310, Perkin Elmer). In the case of exons 3, 5, and 11, reverse primers were used.

shortening of translated HGO protein from 445 to 373 amino acids.

The novel mutations were not identified in any of the 50 healthy controls, supporting the evidence that they are disease causing mutations, rather than polymorphisms.

Segregation of all mutations with AKU was confirmed in all families, except for the S47L mutation, where no DNA from family members was available (fig 4). However, serine at position 47 of the HGO protein molecule is conserved in man and mouse (fig 3). In *Aspergillus nidulans,* threonine is found at this site, which, as well as serine, belongs to the group of hydrophilic amino acids with uncharged polar side chains that are usually on the outside of the protein. Conversely, leucine, which is introduced into the HGO protein by transition c307C→T, is an amino acid with non-polar side chains that tend to cluster together on the inside of proteins. This indicates that substitution S47L may influence the HGO protein conformation and therefore also affect its function.

Recently, Beltran-Valero de Bernabé *et al*[9] provided the evidence that the CCC triplet or its inverted complement (GGG) are mutational hotspots in the *HGO* gene, because 34.5% (10/29) of *HGO* nucleotide changes identified so far involve these sequence motifs. Data shown in our report further support their finding, since 55.5% (5/9) of the mutations identified in our patients lie within or are adjacent to these triplets. Taking into account the novel mutations found in Slovak patients and one identified by Felbor *et al*,[10] the total number of *HGO* nucleotide variations involving the CCC/GGG motif identified so far can be increased to 38.2% (13/34).

In all 17 analysed Slovak AKU patients, both disease causing mutations were found (table 2). The identification of nine different mutations in this sample was not expected

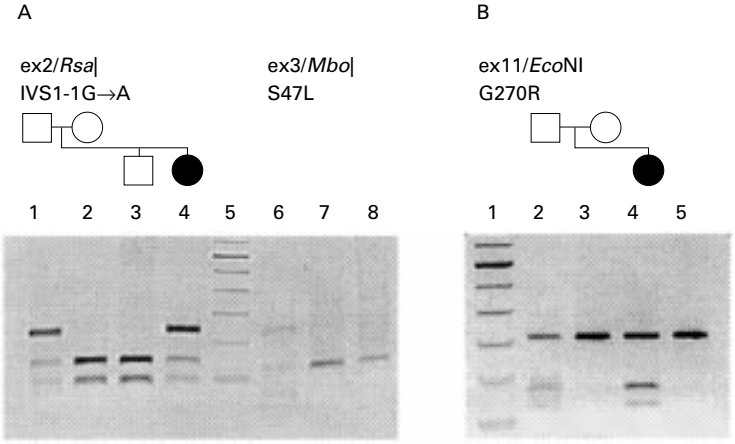

Figure 2 Detection of the (A) IVS1-1G→A, S47L (c307C→T) and (B) G270R (c975G→A) mutations by RsaI, MboI, and EcoNI restriction analysis, respectively. Mutations IVS1-1G→A and S47L abolish restriction sites for corresponding enzymes, while G270R creates a novel site for EcoNI. The figure also shows segregation of mutations IVS1-1G→A in family ALK6 (A) and G270R in family ALK4 (B). In both families, the fathers and affected daughters are heterozygous for screened mutations ((A) lanes 1 and 4, (B) lanes 2 and 4, as well as the patient in (A) lane 6). In (A) lanes 2, 3, 7, and 8 and (B) lanes 3 and 5 are subjects without corresponding mutations. In (A) lane 5 and (B) lane 1 is a 100 bp ladder.

```
HGO         ------MAELKYISGFGNECSSEDPRCPGSLPEGQNNPQVCPYNLYAEQL
MHGO        ------MAELKYISGFGNECASEDPRCPGSLPEGQNNPQVCPYNLYAEQL
HMGA        MPVTEFSFKDPYTYQNGFDSYHESEAIEGALPVGHNSPQKAPYGLYAEKL
                          *    *     *     * ** * * **  ** **** *

                  ↓ HGO S47L
HGO         SGSAFTCPRSTNKRSWLYRILPSVSHKPFESIDEG--HVTHNWDEVDPDP
MHGO        SGSAFTCPRNTNKRSWLYRILPSVSHKPFESIDQG--HVTHNWDEVGPDP
HMGA        SGTAFTAPRHENKQTWVYRILPAAAHENFVEEDASSYHTLSDAKKLQHIP
            ** *** **  **  * *****   *  *   *    *        *

HGO         NQLRWKPFEIPKASQKKVDFVSGLHTLCGAGDIKSNNGLAIHIFLCNTSM
MHGO        NQLRWKPFEIPKASEKKVDFVSGLYTLCGAGDIKSNNGLAVHIFLCNSSM
HMGA        NQLRWDPFDLDET----VDWVHGLHLVAGSGDPTVKQGLGILLYAAGKDM
            ***** **         ** * **    * **     **         *

                          ↓ HGO G161R
HGO         ENRCFYNSDGDFLIVPQKGNLLIYTEFGKMLVQPNEICVIQRGMRFSIDV
MHGO        ENRCFYNSDGDFLIVPQKGKLLIYTEFGKMSLQPNEICVIQRGMRFSVDV
HMGA        GKEAFYSADGDFLIVAQHGVLDIQTELGRLLVRPNEICVIPRGVRYRVTL
                 **  ******* * * * ** *     ******* ** *

                                                ↓ HGO P230S
HGO         FE-ETRGYILEVYGVHFELPDLGPIGANGLANPRDFLIPIAWYEDRQVPG
MHGO        FE-ETRGYILEVYGVHFELPDLGPIGANGLANPRDFLIPVAWYEDRRVPG
HMGA        PDGPVRGYICELYQGHYQLPELGPIGSNGLANARDFQAPVAAFDDEEGPT
                 **** * *  *  ** ***** ***** ***  * *   *   *

                                          ↓ HGO G270R
HGO         GYTVINKYQGKLFAAKQDVSPFNVVAWHGNYTPYKYNLKNFMVINSVAFD
MHGO        GYTVINKFQGKLFACKQDVSPFNVVTWHGNYTPYKYNLENFMVINAVAFD
HMGA        EYRLYSKFNNHLFSARQDHTPFDIVAWHGNYYPYKYDLGRFNTMGSVSFD
             *    *    **   **  **  * ***** **** *  *     * **

                      ↓ HGO V300G
HGO         HADPSIFTVLTAKSVRPGVAIADFVIFPPRWGVADKTFRPPYYHRNCMSE
MHGO        HADPSIFTVLTAKSLRPGVAIADFVIFPPRWGVADKTFRPPYYHRNCMSE
HMGA        HPDPSIYTVLTGPSDHVGTAIADFVIFPPRWLVAEKTFRPPWYHRNTMSE
            * **** ****  *   * ************ ** ****** **** ***

HGO         FMGLIRGHYEAKQGG-FLPGGGSLHSTMTPHGPDADCFEKASKVKLAPER
MHGO        FMGLIKGHYEAKQGG-FLPGGGSLHSAMTPHGPDADCFEKASKAKLEPER
HMGA        FMGLITGNYDAKTGGGFQPAGASLHNIMSAHGPDMHAFEGASNADLKPTK
            ***** * * ** ** * * * ***  *  ****   ** **   * *

HGO         IADGTMAFMFESSLSLAVTKWGLKASRCLDENY-HKCWEPLKSHFTPNSR
MHGO        IADGTMAFMFESSLSLAVTKWGLKTCSCLDENY-YKCWEPLRSHFTPNSR
HMGA        IGDGSMAFMFESSLMVGVSEWGLKTCQKVQEEYNEHSWQPLKRHFK----
            * ** *********   *  ****      * *    * **  **

HGO         NPAEPN
MHGO        SPTEPK
HMGA        DPRKAQ
             *
```

Figure 3 Comparison of primary structure of homogentisate-1,2-dioxygenase protein from man (HGO, AF000573[15]), mouse (MHGO, U58988[16]), and Aspergillus nidulans (HMGA, U30797[17]) using ClustalX 1.3b. Positions conserved in all three organisms are indicated by (*). Arrows mark sites of identified missense mutations, novel mutations are shown in bold.

because the founder effect had been considered to be the main reason responsible for an increased incidence of AKU in Slovakia. The most frequent mutations, G161R and G152fs (previously identified in two Slovak families by Gehrig *et al*[6]), were present on 50% of 32 screened AKU chromosomes (table 1). So far, these mutations have not been identified in any other screened population. This indicates that they might be specific for Slovakia. The high proportion of these two mutations can be explained by founder effect and subsequent genetic isolation. In addition, however, there must have been at least four other founders contributing to the gene pool of the Slovak AKU population (table 1). Three further mutations were each found on only one AKU chromosome, thus indicating that this mechanism is not the only one responsible for the high incidence of this disease in Slovakia (1:19 000).

Possible common origins of chromosomes carrying the same AKU mutations can be further traced by the analysis of DNA polymorphisms in the *HGO* gene and construction of haplotypes. This work is now in progress.

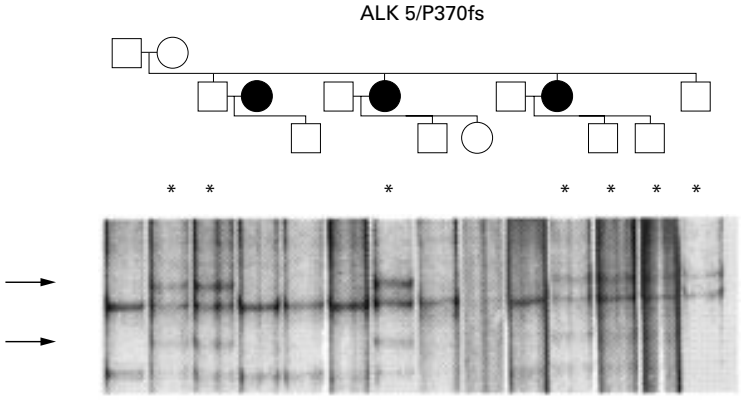

Figure 4 Non-radioactive SSCP analysis of exon 13 indicating segregation of mutation P370fs in family ALK5. Arrows mark the SSCP shifts corresponding to this mutation. The presence of the mutation in heterozygous state is indicated by (*). Patients were also heterozygous for mutation G270R (exon 11) (table 2).

Links in electronic databases involve: primary structure of HGO from man (HGO, AF00573,[15]), mouse (MHGO, U58988,[16]), and *Aspergillus nidulans* (HMGA, U30797[17]), and human HGO transcript (AF045167[5 15]).

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Clinical and molecular correlates of somatic mosaicism in neurofibromatosis 2

EDITOR—Neurofibromatosis 2 (NF2) is an autosomal dominant disorder that is characterised by benign nervous system tumours (such as vestibular schwannomas (VSs), intracranial meningiomas, and spinal tumours) and other abnormalities.[1] Somatic mosaicism (the presence of a mutation, deletion, or chromosomal abnormality in a subpopulation of somatic cells) is thought to be relatively common in NF2, affecting perhaps 15% of sporadic cases.[2 3]

There can be considerable clinical variability in mosaics because somatic mutation can occur at different stages of the postzygotic cell lineage. Evans *et al*[2] reported the degree of mosaicism for five NF2 patients. Two patients with an estimated <10% of peripheral lymphocytes with *NF2* mutations had ages of onset of symptoms of 41-48 years, while three patients with an estimated 21-44% of affected cells had ages of onset of symptoms of 21-28 years. This is consistent with a relationship between degree of mosaicism and disease severity, although there are too few patients to draw firm conclusions.

Few NF2 somatic mosaics have been reported, and clinical and molecular differences between mosaics and sporadic non-mosaic NF2 patients have not been quantified. To examine this question, we compared somatic mosaic and sporadic non-mosaic NF2 patients selected from NF2 populations in the United Kingdom (341 patients)[4 5] and Germany (118 patients).[6 7] The study groups included 13 previously identified somatic mosaics[2 3] and 86 sporadic non-mosaic NF2 patients, all of whom had head and spine gadolinium enhanced magnetic resonance imaging. Sporadic NF2 patients were defined as non-mosaic if they had identified germline *NF2* mutations with normal strength gel bands. In theory, somatic mosaic patients could have near normal band strength, but in practice band strength will be reduced when <50% of peripheral lymphocytes have *NF2* mutations.[2] Clinical data were not available in sufficient detail to determine if the distribution of lesions was non-uniform, which could result from somatic mosaicism.

The covariates that were examined were age at onset of symptoms, age at diagnosis, number of VSs (none or unilateral versus bilateral), presence and number of intracranial meningiomas, presence of spinal tumours, and germline *NF2* mutation type (frameshift or nonsense versus other identified mutations). In univariate analyses, the two tailed *t* test was used for continuous variables and the two tailed Fisher exact test for binary variables. Multiple logistic regression was then used to examine the association between somatic mosaic status and covariates that differed between groups; interaction terms of age with number of tumours were also considered.

The characteristics of somatic mosaics and sporadic non-mosaics are compared in table 1. Age at onset of

Table 1 Characteristics of somatic mosaic and sporadic non-mosaic NF2 patients

Characteristic	*Somatic mosaic*	*Sporadic non-mosaic*	*p*
No of patients	13	86	
Age at onset of symptoms, years, mean (SE)	26.5 (2.4)	18.7 (1.1)	0.012
Age at diagnosis, years, mean (SE)	33.6 (2.7)	24.7 (1.3)	0.013
Germline mutation type (%)			0.055
Frameshift or nonsense	92.3	62.8	
Other	7.7	37.2	
Vestibular schwannomas (%)			0.391
None or unilateral	30.0	12.9	
Bilateral	70.0	87.1	
Spinal tumours (%)			0.733
Absent	30.8	24.4	
Present	69.2	75.6	
Intracranial meningiomas (%)			0.773
Absent	46.2	41.9	
Present	53.8	58.1	
Intracranial meningiomas, number, mean (SE)	1.5 (1.0)	1.5 (0.2)	0.993