A Founder Effect for the *HGD* G360R Mutation in Italy: Implications for a Regional Screening of Alkaptonuria

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Abstract We sought to establish rapid and specific genotyping methods for G360R mutation and for seven tightly linked markers in the homogentisate dioxygenase gene to address the question of whether G360R is a mutational hot spot or the result of a founder effect, as it

has been repeatedly found in alkaptonuric patients from a geographic isolate in Italy.

For G360R and single nucleotide polymorphism genotyping, high-resolution melting analysis was performed. Microsatellites were analysed by multiplex PCR and capillary electrophoresis. To investigate the natural history of the G360R mutation, we genotyped markers in 52 controls and in 8 unrelated patients from the UK and USA, who also segregated the G360R mutation, and calculated its age using DMLE+2.3 software.

A distinct G360R-bearing haplotype was identified in all patients of Caucasian descent. Estimated mutation age was 545 generations (95% credible set, 402–854), suggesting that G360R arose in an ancestor who lived 8,000–10,000 years BC. Archaeological, historical and demographic data support that a G360R carrier has settled the remote valley where present-day population might have a heterozygote frequency of at least 6%.

Given the late health-threatening complications of alkaptonuria and a cure within reach, inhabitants of this isolate would benefit from screening and genetic counselling.

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This paper is dedicated to the memory of Dr. Claudio Castellan

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Introduction

Alkaptonuria (AKU, OMIM 203500) is an autosomal recessive disease caused by mutations of the *HGD* gene encoding homogentisate 1,2-dioxygenase (HGD; EC 1.13.11.5), an enzyme involved in the catabolism of tyrosine (Fernández-Cañón et al. 1996). *HGD* is located on chromosome 3q13.33 (genomic coordinates [GRCh37]: 3:120,347,014–120,401,417) and consists of 14 exons encompassing 2,012 bp (OMIM 607474, coding sequence:

NM_000187.3). Over the past years, the *HGD* mutation spectrum has been described in series of AKU patients from different countries (<http://hgddatabase.cvtisr.sk/>). At this writing, 174 unique *HGD* sequence variants found in 398 individuals carrying a total of 751 variants are reported in the database. In particular, the missense mutation c.1078G>C; p.Gly360Arg (henceforth, G360R) in exon 13 has been detected 13 times in patients from Italy, Australia, the UK, France and the USA (Zatkova et al. 2012). G360R was originally described by Porfirio et al. (2000) who found two independent G360R-bearing AKU chromosomes in two patients from different ends of the Italian peninsula; one patient was a compound heterozygote from Calabria, Southern Italy, whereas the other was the affected son of a consanguineous marriage from South Tyrol, a German-speaking region on the Austrian border. The G360R mutation disrupts a BstNI restriction site, making PCR-RFLP useful in genotyping family members and scanning the general population to rule out this mutation as a coincidental polymorphism segregating in those AKU families (Porfirio et al. 2000). Seven intragenic markers were used to trace the haplotype background of these G360R mutations. Familial segregation provided unequivocal derivation of the haplotypes present on the AKU chromosomes and clearly showed that a single haplotype, closely related to the common A haplogroup (Beltrán-Valero de Bernabé et al. 1998), was harbouring the G360R mutation (Porfirio et al. 2000). Subsequently, another ten compound heterozygous patients were reported to carry G360R (Grasko et al. 2009; Vilboux et al. 2009; Usher et al. 2015). Since G360R takes place in a G run, a sequence motif known as a mutational hot spot in *HGD* (Beltrán-Valero de Bernabé et al. 1999), referral of two large, apparently unrelated, South Tyrolean AKU families, has recently renewed our interest in the origin and spread of G360R mutation among populations of European ancestry.

High-resolution melting analysis (HRMA) has significantly hastened mutation scanning processes (Wittwer 2009). The method involves precise monitoring of the change in fluorescence caused by the release of an intercalating DNA dye from a DNA duplex as it is denatured by increasing temperature. Therefore, in the present study, we sought to establish rapid and specific genotyping methods for G360R and seven *HGD* intragenic markers to address the question of whether G360R is a mutational hot spot or the result of a founder effect by analysing two new large AKU families from Italy and eight singleton AKU patients from the UK and USA.

Materials and Methods

Patients and Controls

The Italian subjects involved gave written informed consent in accordance to the Helsinki Declaration, and the study was approved by our institutional review board. The US and UK DNA samples were provided upon material transfer agreements between the respective Institutions.

A total of 40 genomic DNA samples, derived from two German-speaking South Tyrolean families with G360R, were genotyped at seven tightly linked markers spanning a region of 44.2 Kb (see below). All haplotype sets were generated by inheritance from the observed genotypes in the family members. In another eight UK and US families in whom G360R was detected, only the index case was available for genotyping. Haplotypes were manually constructed by assigning the phase of heterozygous positions, whenever possible, with the guidance of known *HGD* haplotypes associated to individual AKU mutations (<http://hgddatabase.cvtisr.sk/>). One hundred and four control chromosomes were also studied. Controls included 17 subjects who were spouses of family members, as well as 35 unrelated individuals from the same geographic area. Haplotype frequencies in the general population were derived using a Bayesian method implemented in PHASE v.2.1 software (Stephens and Scheet 2005).

Markers

Three short tandem repeat (STR) markers, a (CA)_n in intron 4 (HGO-3, D3S4556), a (CT)_n in intron 4 (HGO-1, D3S4496) and a (CA)_n in intron 13 (HGO-2, D3S4497), have been described previously (Granadino et al. 1997; Beltrán-Valero de Bernabé et al. 1998) and were analysed by multiplex PCR with modifications to comply with dye-primer detection. PCR products were subjected to electrophoresis on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Size calling was performed using the GeneMapper software (Applied Biosystems, Monza, Italy). Four single nucleotide polymorphisms (SNPs), IVS2+35T/A (rs2733825), c.240T/A H80Q (rs2255543, exon 4), IVS5+25T/C (rs2551607) and IVS6+46C/A (rs3817627), have also been described (Beltrán-Valero de Bernabé et al. 1998) and were studied by HRMA following primer redesign (sequences and conditions available on request). Genotype assignment was made possible by comparison with known sequenced samples.

Differences in haplotype frequencies between patients and normal subjects were analysed using the χ^2 test. The statistical threshold was set at 0.05.

G360R HRMA

Primers flanking the c.1078G>C substitution were designed using Primer3web v. 4.0.0 software (<http://bioinfo.ut.ee/primer3/>), and their sequences are available upon request. The fluorescent high-resolution DNA melting curves of the resulting 116-bp PCR product were predicted using uMelt software (<https://www.dna.utah.edu/umelt/umelt.html>). Genomic DNA (20 ng) was amplified in a final volume of 20 μ L containing 1.25 μ L of PCR Buffer (Applied Biosystems, Monza, Italy), 1.5 mmol/L of magnesium chloride (Applied Biosystems, Monza, Italy), 300 nmol/L of each primer, 1.5 μ mol/L of SYTO9 Dye (Invitrogen, Basel, Switzerland) and 1.25 U of AmpliTaq Gold Polymerase (Applied Biosystems, Monza, Italy). One-tube PCR and HRMA were performed on Rotor Gene 6000 (Corbett Research, Sydney, Australia). Thermal cycler was set at 95°C for 10 min to allow initial genomic DNA denaturation followed by 35 cycles of 1 min at 95°C, 1 min at 60°C and 1 min at 72°C and by a final extension at 72°C for 15 min. The resulting PCR products underwent HRMA as follows: samples were denaturated at 95°C for 1 min and then rapidly cooled to 40°C for 1 min in order to facilitate heteroduplex formation. Melting curve data were acquired in a wide temperature range (78–88°C) at a ramping rate of 0.1°C/s. Results were analysed as both normalised fluorescence and the derivative function of fluorescence raw data versus temperature (supplementary Figs. 1 and 2, respectively). Since the mutant allele 1078C homozygotes may not be easily distinguishable from wild-type 1078G homozygotes by traditional HRMA methods, a spike-in control DNA approach was used (Liew et al. 2004). Briefly, PCR was performed on both the native samples and 1:1 mixtures of the test samples with a reference 1078G homozygous sample. Homozygous 1078C test samples were converted to heterozygosity, a condition associated with an abnormal melting curve owing to heteroduplex formation.

Sanger Sequencing

Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Crawley, UK). Direct DNA sequencing of purified PCR products was performed using dideoxynucleotide termination chemistry. Sequencing reactions were purified using the Dye Ex 2.0 Spin Kit (Qiagen, Crawley, UK), and samples were run on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Dating the G360R Mutation

An attempt was made to estimate G360R mutation age by DMLE+2.3 software (Reeve and Rannala 2002). Parameter settings were as follows. The required map distances between markers and mutation site were taken from NG_011957.1 genomic reference sequence. The proportion of mutation-carrying chromosomes sampled was estimated based on the assumptions that (1) AKU has a worldwide prevalence of 1–4 in one million and hence the overall frequency of disease-causing alleles at the *HGD* locus is 0.001–0.002 (the geometric mean 0.0014 was used for computation); (2) G360R was found 13 times among 751 entries and hence represents 1.7% of all, Western- and publication-biased, AKU variants (<http://hgddatabase.cvtisr.sk/>); and (3) the European and US populations currently embrace 507 million (<http://ec.europa.eu/eurostat/>) and 319 million (<http://census.gov>) residents, respectively. This leads to 19,659 ($0.0014 \times 0.017 \times 826,000,000$) G360R mutation-carrying chromosomes in the reference population. We have analysed ten independent G360R chromosomes, so the desired proportion is 0.0005087 (10/19,659). The growth rate per generation (^{gen}r) was set at 0.0093 and was calculated according to the formula $^{gen}r = \ln(P_t/P_0)/g$, where P_t is the present-day reference population size (Europe and the USA), P_0 is the estimate of the human population size 10,000 years BC (1–10 millions according to several sources (e.g. Livi-Bacci 2012); the geometric mean 3,162,304 was used for computation) and g is the number of generations between these two time points, assuming 20 years per generation. Other DMLE parameter settings remained at their default values.

Results

Haplotype associations between alleles at all seven polymorphic sites within the *HGD* gene were tentatively considered in the 104 control chromosomes. However, as it could be expected, the haplotypes generated by PHASE were most often uncertain due to both the small sample size and the high level of variation at D3S4497 and D3S4556. Thus, we restricted the analysis of the control chromosomes to the haplotype associations at the four SNPs and the dimorphic D3S4496. Figure 1 shows the maximum parsimony relationships among the seven 5-loci haplotypes (rs2733825, rs2255543, D3S4496, rs2551607, rs3817627) of which our population is composed.

HRMA and, for comparison, Sanger sequencing were used to detect G360R in the 40 members of the two new AKU pedigrees. Table 1 shows molecular confirmation of the homozygous status in 7 patients and that of obligate

Fig. 1 Maximum parsimony network showing the seven 5-loci *HGD* haplotypes found in a sample of 52 normal South Tyrolean subjects. Nodes are proportional to the haplotype frequencies (estimated numbers within each node) and are labelled with an alphanumerical string formed by haplotype name and composition. All branches imply a single substitution (e.g. A1 and A2 differ at position 2 where A and T, respectively, are present), with the exception of B1-E which requires two events (161T as opposite to 163C at positions 3 and 4, respectively)

heterozygosity in 12 patients' sons as well as in the patients' parents of only one family, the other couple being unavailable. Among the eight unaffected patients' sibs, we found six G360R carriers. Moreover, among 11 sons of these carriers, another six carriers were identified. Notably, a G360R homozygote was also detected, pointing to the presence of another "independent" obligate carrier among the partners of family members.

The extended 7-loci haplotypes (rs2733825, rs2255543, D3S4556, D3S4496, rs2551607, rs3817627, D3S4497) were characterised in the 40 members of the two new AKU pedigrees. The G360R mutation in both families was on the same A2-extended *HGD* haplotype A-A-193-161-T-C-197 originally described (Porfirio et al. 2000). Table 2 shows that this haplotype is highly conserved (only HGO-2 changed) on six of eight independent G360R-bearing

chromosomes from the UK and USA. Exceptions mainly come from the US patient of Indian origin, whose G360R-bearing haplotype was different at five of seven loci.

Finding that all but one patient in our cohort shared very similar if not identical allele sequence in the vicinity of the G360R mutation pointed to a common origin. To support this hypothesis, we considered the frequency of the G360R-bearing haplotype in the normal population. The frequency of the 5-loci A2 haplotype in the sample of normal controls proved to be statistically lower than that in the patients, i.e. 20/104 (19%) versus 10/20 (50%), $P < 0.003$. This is consistent with the existence of a founding ancestor for the G360R mutation in the *HGD* gene.

Assuming the existence of a common ancestral mutation event, with the aid of DMLE software, an attempt was made to estimate when this *HGD* G360R mutation occurred. The results of the likelihood analysis of mutation age are very sensitive to the assumptions about the model, particularly the assumptions of a uniform relationship between recombination rate and physical distance and equal mutation rates (Rannala and Bertorelle 2001). To check for these potential flaws, we carried out two separate analyses for SNPs (and the dimorphic D3S4496) and for microsatellites, leading to G360R age estimates of 534 generations (95% credible set, 398–857) and 558 generations (95% credible set, 408–860), respectively (Fig. 2a). Another factor affecting mutation age estimate is the population growth rate (Slatkin and Rannala 2000). In the model, it is assumed that the population has been growing exponentially at the same rate in the past. To explore the effects of variable growth dynamics over time, we ran the programme with both a double ($^{gen}r = 0.0186$) and a half ($^{gen}r = 0.00465$) growth rate setting using the whole set of markers. The most credible *HGD* G360R age set spanned from 276 to 1,686 generations (about 5,500–33,700 years); the older the age, the lower the growth rate considered (Fig. 2b). Using the whole set of flanking markers and the intermediate population growth rate, G360R mutation age was estimated in 545 generations (95% credible set, 402–854), corresponding to 10,900 years.

Discussion

Finding a single mutation responsible for all our South Tyrolean cases prompted us to hypothesise a founder effect in our cohort of patients. A haplotype analysis was consequently performed to check whether our patients had a common G360R-bearing haplotype, since a founder effect is expected to result in a common allele sequence in the vicinity of the mutation due to the existence of a single progenitor. Furthermore, we extended our study to all available G360R-bearing AKU patients worldwide.

Table 1 Assessment by high-resolution melting analysis (HRMA) and by Sanger sequencing of the genotypes at *HGD* c.1078G>C of both the 40 available members and their 17 available spouses from two AKU pedigrees

<i>HGD</i> c.1078G>C		# of members by family	HRMA	Sanger	False positives	False negatives
Obligate CG carriers		12 + 2	14	14	0	0
CC affected		4 + 3	7	7	0	0
Unaffected patients' sibs	GG normals	1 + 1	2	2	0	0
	CG carriers	4 + 2	6	6	0	0
Carriers' sons	GG normals	4 + 0	4	4	0	0
	CG carriers	6 + 0	6	6	0	0
	CC affected	1 + 0	1	1	0	0
Spouses	GG normals	10 + 6	16	16	0	0
	CG carriers	1 + 0	1	1	0	0

Table 2 *HGD* G360R-harboring haplotypes identified in ten independent AKU chromosomes

Chromosome identifier	Geographic origin	IVS2+35T/A	c.240T/A	HGO-3	HGO-1	IVS5+25T/C	IVS6+46C/A	HGO-2
AKU 030	South Tyrol	A	A	193	161	T	C	197
AKU 031	Calabria	A	A	193	161	T	C	197
AKU 019	UK, Caucasian	A	A	193	161	T	C	179
AKU 121	USA, Hispanic	A	A	193	161	T	C	179
AKU 185	USA, Caucasian	A	A	193	161	T	C	189
AKU 187	USA, Caucasian	A	A	193	161	T	C	179
AKU 188	USA, Caucasian	A	A	193	161	T	C	179
AKU 189	USA, Caucasian	A	A	193	161	T	C	183
AKU 003	USA, Caucasian	A	A	195	161	T	C	189
AKU 186	USA, Indian	T	T	197	161	T	A	179

The estimated age of 10,900 (5,500–33,700) years for G360R would mean that the mutation took place after human populations left Africa (around 100,000 years ago) to spread in the Middle East, South Asia and Europe around 40,000 years ago (Shriner et al. 2014). Although no data are available on sub-Saharan populations (<http://hgddatabase.cvtisr.sk/>), lack of G360R detection among African-American AKU patients would seem to confirm indirectly that this mutation is rare (if not absent) in Africa (Phornphutkul et al. 2002; Vilboux et al. 2009).

If the G360R mutation occurred before the migrant human populations separated to reach Europe or Asia, we could expect to find it in both continents. Although this might be suggested by the presence of G360R in a US patient of Indian ancestry, its occurrence in Asia has never been documented (<http://hgddatabase.cvtisr.sk/>). As mentioned above, G360R reportedly lies in a mutational hot spot. Therefore, its presence in the US-Indian patient may be well interpreted also by this mechanism, which parsimoniously accounts for the detection of a 5-loci C

haplotype harbouring that G360R mutation instead of the 5-loci A2 haplotype found in all other patients.

Numerous archaeological findings (Dondio 1995) witness a diffused human presence in South Tyrol since the Mesolithic (10,000 years ago). The members of the South Tyrolean families reported here come from very small villages scattered along a remote high valley (supplementary Fig. 3). Present-day residents hardly count 9,000 units (<http://demo.istat.it/>). Moreover, the analysis of demographical records shows that the population is very stable and the absolute number of inhabitants has been almost the same across the years (<http://www.istat.it/it/archivio/>). The presence of Italian speakers in the villages has always been very limited as a result of negligible immigration and admixture (<http://www.provinz.bz.it/astat/it/>). The presence of a limited number of surnames also accounts for geographic and cultural isolation and suggests a high endogamy rate. Therefore, not surprisingly, we observed a *HGD* G360R homozygote born from an unaffected patient's sib and an "unrelated" carrier. We cautiously estimate that *HGD*

Fig. 2 Estimated age of G360R mutation using the DMLE software varying either the marker numbers and types with fixed 0.0093 growth rate (a) or the growth rates with the whole set of markers (b). Error bars represent the Bayesian 95% credible interval

G360R carriers might be 6%, meaning that the inhabitants of this valley would benefit from genetic counselling.

Loss-of-function homozygous or compound heterozygous *HGD* mutations cause the inability for AKU patients to metabolise homogentisic acid (HGA). Affected individuals excrete HGA in the urine, which assumes a characteristic dark colour when exposed to air. AKU is a slowly progressive disease with obvious manifestations (ochronosis) occurring after more than three decades. Ochronosis consists of a deposition of brown-black pigment in connective tissues, such as the cartilage, skin and sclerae. Musculoskeletal involvement is the most serious complica-

tion of this condition, leading to a severe and sometimes crippling form of arthropathy (Mannoni et al. 2004). It has been pointed out that if HGA levels are reduced before the onset of overt disease, this might prevent the debilitating progression of AKU (Suwannarat et al. 2005). In fact, the ability of nitisinone to reduce plasma HGA levels and urinary excretion holds promises for treatment (Ranganath et al. 2015).

It would thus seem that a local programme to identify asymptomatic persons having AKU deserves consideration since all fundamentals of screening are met (Bonita et al. 2006). From its nature, a screening must have the potential

for decreasing morbidity in high-prevalence groups through its capacity to detect a disease at a stage when cure or control is possible. Biochemical evaluation (exposure of urine to alkali) of at-risk population is reliable and feasible at very low cost (Valmikinathan and Verghese 1966). The test is reasonably sensitive and specific with high predictive values. Moreover, biochemical screening can easily be followed by genetic testing with the HRMA procedure described here.

The observation that a high proportion of families with *HGD* alterations from South Tyrol may harbour a founder mutation has significant impact in health community policies, since a role for heterozygous mutations of *HGD* as one of the liability genetic determinants in complex traits of rheumatologic interest has been envisaged (Mistry et al. 2013; Gallagher et al. 2015). Biochemical screening could be undertaken as a first step before direct molecular analysis for G360R, at least in individuals of ascertained South Tyrolean ancestry. The identification of a large set of families carrying an identical AKU mutation would also provide unique opportunities to study the effects of other genetic and environmental factors on penetrance and disease phenotype.

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Synopsis

Inhabitants of a geographic isolate, where a founder mutation of the homogentisate dioxygenase gene was characterised, would benefit from screening and genetic counselling for alkaptonuria.

Compliance with Ethics Guidelines

Conflict of Interest

All authors, Berardino Porfirio, Roberta Sestini, Greta Gorelli, Miriam Cordovana, Alessandro Mannoni, Jeanette L. Usher, Wendy J. Introne, William A Gahl and Thierry Vilboux, declare that they have no conflict of interest.

Informed Consent

The Italian subjects involved gave written informed consent in accordance to the Helsinki Declaration, and the study was approved by our institutional review board. Proof that informed consent was obtained will be available upon request. The US and UK DNA samples were provided upon material transfer agreements between the respective Institutions.

Animal Rights

This article does not contain any studies with animal subjects performed by the any of the authors.

Details of the Contributions of Individual Authors

BP planned, conducted and reported the work described in the article with the substantial contribution of RS, GG, MC, JLU and TV for data gathering, analysis and interpretation and of AM, WJI and WAG for revising the manuscript critically for important intellectual content.

BP serves as guarantor for the article, accepts full responsibility for the work and/or the conduct of the study, had access to the data and controlled the decision to publish.

Competing Interest

None.

Electronic-Database Information

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

<http://bioinfo.ut.ee/primer3/> (for guidance in the identification of PCR primers)

<http://census.gov/> (for current US population census data)

<http://demo.istat.it/> (for current population census of the present-study South Tyrolean valley)

<http://ec.europa.eu/eurostat/> (for current European population census data)

<http://enzyme.expasy.org/> (the enzyme nomenclature database for HGD [EC 1.13.11.5])

<http://hgddatabase.cvtisr.sk/> (a Leiden Open Variation Database [LOVD] v.2.0 on *HGD* mutations and polymorphisms curated by A. Zatkova)

<http://stephenslab.uchicago.edu/phase/download.html> (to get PHASE v.2.1, a programme for reconstructing haplotypes from population data)

<http://www.dmle.org/> (to get the DMLE+ v2.3 linkage disequilibrium mapping software)

<http://www.istat.it/it/archivio/> (for historical population censuses of the present-study South Tyrolean valley)

<http://www.ncbi.nlm.nih.gov/nuccore/> (for genomic sequences of *HGD* and its transcript, accession numbers NG_011957.1 and NM_000187.3, respectively)

<http://www.provinz.bz.it/astat/it/> (for data on language usage, migration rates, marriages in the present-study South Tyrolean valley)

<https://www.dna.utah.edu/umelt/umelt.html> (for HRMA pattern prediction)

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/omim/> (for AKU [OMIM 203500] an *HGD* [OMIM 607474])

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