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ARTICLE

The Tunisian population history through the Crigler–Najjar type I syndrome

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Crigler–Najjar syndrome type I (CN-I) is a rare and severe metabolic disorder. A recurrent mutation – c.1070A>G in exon 3 – was identified in the Tunisian population, suggesting a founder effect. In 2004, the detection of this mutation in two Kuwaiti Bedouin families has called the Tunisian founder effect in question again. To determine the origin of this mutation, 21 Tunisian and 2 Kuwaiti Bedouin CN-I patients were screened using nine genetic markers. Haplotype analysis confirmed the founder effect hypothesis and dated the appearance of this mutation some 32 generations ago in the Tunisian population. Using the same genetic analysis, the ancestor haplotype was identified in these two families. This result genetically confirms the blending of the Bedouin nomads within today's Tunisian population. After population migration from east to west, this mutation was introduced into the Tunisian population, and then perpetuated, probably because of marriages in isolated communities.
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Introduction

Crigler–Najjar syndrome type I (CN-I, MIM no. 218800) is a rare and severe autosomal disorder (less than $1/10^6$ live births). CN-I is caused by deficiency of the liver enzyme responsible for bilirubin elimination, the uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1; EC 2.4.1.17). Biologically, the disease manifests itself with severe and persistent unconjugated hyperbilirubinemia. Clinically, affected newborns are at high risk for bilirubin-induced brain damage (kernicterus). The UGT1A locus complex on chromosome 2 (located on 2q37.1) encodes nine functional UDP-glucuronosyltransferases type 1A.[1,2] The locus is organized with 13 different exons 1 (responsible for the specificity of the enzyme activity) and 4 common exons. Recently, a new regulation system of the UGT1A1 protein activity was identified with an alternative exon 5.[3] As for the *UGT1A1* gene, about 70 different mutations have been reported since 1992, in the five exons or in splicing sites.[4–6] In our laboratory, the recurrent mutation c.1070A>G responsible for the modification in the peptide sequence p.Gln357Arg has been only observed

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in patients from Tunisia, suggesting a Tunisian founder effect.[7] Recently, this mutation has also been reported in two Kuwaiti Bedouin families, suggesting a wider Arabian founder effect.[8]

The recurrence of a particular mutation may result from three different conditions. First, the mutation occurs in a region of high risk for mutation ('hot spot'), such as CpG dinucleotides.[9] Second, an external event (such as an infectious disease) has exerted a pressure on the population and selected individuals carrying the morbid allele (eg, the mutation responsible for sickle cell anemia was preserved in the African population by conferring on heterozygote patients, a resistance to malaria).[10] Finally, the mutation appeared in one individual and diffused through generations in isolated communities because of consanguineous marriages. In this situation, the chromosomal fragment bearing the mutation is transmitted from a common ancestor to many persons in successive generations. When a founder effect is suspected, several tandem repeats or microsatellites must be analyzed in a restricted genomic region to confirm the single origin (common ancestor) of a recurrent mutation in a population. During meiosis, chromosomal recombination can randomly occur, so that the more recent the mutation is, the more conserved the genetic region around the mutation will be. In these cases, microsatellites around the mutation will disclose the same or at least a similar haplotype in all affected patients.

In this study, genetic markers on both centromeric and telomeric sides of the c.1070A>G mutation in the *UGT1A1* gene were analyzed in 21 Tunisian and 2 Kuwaiti CN-I patients. We have determined that this mutation probably appeared in a Bedouin nomad group and then diffused in Tunisia around eight centuries ago after population migrations from Middle East to Maghreb.

Patients and methods

To determine the origin of the c.1070A>G mutation in the *UGT1A1* gene, 28 healthy unrelated Tunisians (group 1), 21 CN-I Tunisian patients (group 2) and 2 CN-I Kuwaiti patients (group 3) were included. Seven microsatellite markers (centromere-D2S2344, D2S331, D2S1279, D2S2348, D2S2234, D2S2205 and D2S336-telomere) were selected from the Ensembl Genome Project Database. Haplotypes were constructed by amplification of microsatellites with fluorescent primers 5′-labeled in a multiplex PCR except for D2S336 and D2S2205, each amplified in a single PCR. The samples were run on an ABI PRISM 3130 DNA analyzer (Applied Biosystems). The results were analyzed by using Genescan 3.1 analysis software to determine the PCR fragment sizes of PCR products, and the allele sizes were carried out automatically with Genotyper 2.5 analysis software. Single PCRs were performed using the same program for D2S336 and D2S2205. Haplotypes were classified as 1 for the shortest to 20 for the longest.

To complete this panel, two other markers were added: the *UGT1A1* TATA box polymorphism and a new polymorphic genetic marker ~77 000 bp upstream from *UGT1A1*. The polymorphism $A(TA)_6TAA$ or $A(TA)_7TAA$ in the promoter of the *UGT1A1* gene was determined by PCR followed by electrophoresis on polyacrylamide gel as described previously.[11] The second genetic marker – called MARK01 – was a CA-repeat sequence whose variability had previously been assayed on 10 French healthy unrelated volunteers. Its physical location was calculated to be 77 440 pb upstream from the mutation c.1070A>G. Primers to amplify this specific region were designed using Primer3 (Primer3 web site: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primer sequences were as follows: forward primer 5′-GGAGCTACTCTTTAGGGATCG-3′; reverse primer 5′-TGTGAGCTTTGACTGTACTAAG-3′. After PCR amplification, fragments were separated by electrophoresis onto a 10% polyacrylamide gel and then were visualized after ethidium bromide staining. As for the other microsatellites studied, haplotypes were classified according to their electrophoretic profile as 1 for the shortest to 4 for the longest.

To determine linkage disequilibrium (LD) score, the appropriate physical-to-genetic distance conversion relation was determined by linear regression analysis (SPSSv8.0 program, SPSS) of genetic *versus* physical map position.[12] Megabase (UniSTS, NCBI) and centimorgan (Marshfield Comprehensive Human Genetic Map) information for 14 markers positioned in a 10-Mb genomic region around *UGT1A1* gene was used (Table 1). Applying

Table 1 Physical and genetic distance of 14 markers on chromosome 2 to determine the appropriate physical-to-genetic ratio in the *UGT1A1* region

Marker	*Physical distance in megabases (Mb)*	*Genetic distance in centimorgans (cM)*
D2S362	229.446	231.27
D2S2297	230.078	232.36
D2S396	230.392	232.90
D2S172	230.881	235.07
D2S2340	231.300	235.07
D2S427	231.914	236.70
D2S2176	233.456	242.17
D2S336	235.441	245.44
D2S1397	236.458	249.22
D2S338	236.900	250.54
D2S345	237.466	251.24
D2S2968	237.743	251.94
D2S125	240.816	260.63
D2S395	240.888	261.34

Physical distances were from UniSTS (NCBI) and genetic distances were from The Marshfield Comprehensive Human Genetic Map. The conversion relation was calculated by linear regression analysis to be $cM = 2.6201 \times Mb\text{-}370.45$ ($R = 0.999$; $R^2 = 0.997$; adjusted $R^2 = 0.997$; $P < 0.0001$).

this relation, genetic distances were calculated in relation to the mutation c.1070A>G for the nine studied genetic markers (Table 2). Kosambi's function was applied to convert genetic distance (cM) into recombination fraction (θ): $\theta = 0.5(e^{(cM/25)}-1)/(e^{(cM/25)}+1)$ (Table 2).[13] Statistical comparison of allele frequencies between disease and normal chromosomes was based on Mantel–Haenszel common odds ratio estimate with alleles classified into two groups: one for the associated allele and all others combined into a single group. The LD was calculated applying the formula $\delta = (p_{11}p_{22}-p_{12}p_{21})/(p_{+1}p_{22})$, where p_{11} is the frequency of the associated allele on disease chromosomes, p_{22} the frequency of the normal alleles on normal chromosomes, p_{12} the frequency of the associated allele on normal chromosomes, p_{21} the frequency of the normal alleles on disease chromosomes and p_{+1} the frequency of disease chromosomes.[13]

The number of generations (g) since the appearance of the mutation was calculated with the formula $g = \ln(\delta)/\ln(\theta-1)$, where δ is the LD and θ the recombination fraction.[14]

Results

Allele frequencies for the nine genetic markers group are presented in Tables 3a and b (for group 1 and group 2, respectively).

Table 2 Linkage disequilibrium between D2S2344 and D2S336 markers and age estimation of the founder *UGT1A1* c.1070A>G mutation

Marker	*Frequency for marker allele*		*LD (δ)*	*Physical distance (pb)*	*Genetic distance (cM)*	*Recombination fraction (θ)*	*Estimated age (generation)*
	Mutation-bearing population	*Normal population*					
D2S2344	19/42	0/56	0.4524	1188369	3.113	0.031087	25
D2S331	33/42	26/56	0.6000	789136	2.067	0.020661	24
D2S1279	25/42	5/56	0.5556	682296	1.787	0.017861	33
D2S2348	34/42	4/56	0.7949	528482	1.383	0.013831	16
D2S2234	39/42	45/56	0.6364	288416	0.755	0.007545	60
D2S2205	27/30	27/48	0.7714	276337	0.723	0.007231	36
MARK01	40/42	20/56	0.9259	77440	0.202	0.002017	38
TATA box	42/42	22/56	1.0000	7702	0.021	0.000210	—
D2S336	24/42	4/56	0.5385	1100526	2.885	0.028815	21

The linkage disequilibrium (LD) index was calculated according to Delvin and Rish.[13] Genetic distance was determined with the physical-to-genetic conversion relation cM = 2.6201 × Mb-370.45. The Kosambi's function was applied to convert the genetic distance into the recombination fraction.[12] Age of the mutation was estimated using the formula described by Risch *et al*[14]: age = $\ln(\delta)/\ln(1-\theta)$.

Table 3a Allele frequencies for the nine genetic markers in the Tunisian healthy population

Allele	*TATA box*	*MARK01*	*D2S2205*	*D2S2234*	*D2S2348*	*D2S1279*	*D2S331*	*D2S2344*	*D2S336*
TA_6	0.607								
TA_7	0.393								
1		0.036	0.563	0.018	0.018	0.018	0.018	0.018	0.018
2		0.393	0.437	0.124	0.036	0.036	0.125	0.018	0.036
3		0.214		0.018	0.054	0.036	0.089	0.018	0.018
4		0.357		0.018	0.018	0.143	0.036	0.018	0.071
5				0.804	0.071	0.036	0.089	0.000	0.143
6				0.018	0.125	0.071	0.018	0.000	0.071
7					0.018	0.054	0.036	0.036	0.125
8					0.036	0.054	0.125	0.000	0.054
9					0.036	0.089	0.464	0.054	0.000
10					0.054	0.143		0.286	0.107
11					0.018	0.107		0.107	0.036
12					0.036	0.107		0.286	0.107
13					0.304	0.054		0.054	0.054
14					0.018	0.018		0.018	0.000
15					0.036	0.036		0.089	0.036
16					0.018				0.125
17					0.018				
18					0.036				
19					0.036				
20					0.018				

The allele frequencies for nine genetic markers were determined on 28 healthy unrelated Tunisians (ancestor allele frequencies are underlined).

Table 3b Allele frequencies for the nine genetic markers in the Tunisian CN-I population

Allele	*TATA box*	*MARK01*	*D2S2205*	*D2S2234*	*D2S2348*	*D2S1279*	*D2S331*	*D2S2344*	*D2S336*
TA_6	0.000								
TA_7	1.000								
1		0.000	0.900	0.000	0.000	0.000	0.000	0.000	0.000
2		0.000	0.100	0.071	0.000	0.000	0.048	0.000	0.000
3		0.048		0.000	0.000	0.000	0.000	0.000	0.119
4		0.952		0.000	0.119	0.024	0.000	0.024	0.571
5				0.929	0.810	0.000	0.167	0.452	0.143
6				0.000	0.000	0.000	0.000	0.024	0.000
7					0.000	0.024	0.000	0.024	0.024
8					0.000	0.119	0.000	0.048	0.000
9					0.048	0.595	0.786	0.000	0.048
10					0.000	0.119		0.048	0.048
11					0.000	0.095		0.000	0.000
12					0.024	0.000		0.286	0.000
13					0.000	0.024		0.048	0.000
14					0.000	0.000		0.000	0.024
15					0.000	0.000		0.048	0.000
16					0.000				0.024
17					0.000				
18					0.000				
19					0.000				
20					0.000				

The allele frequencies for nine genetic markers were determined on 21 CN-I Tunisian patients (ancestor allele frequencies are underlined).

The polymorphism observed in the CN-I Tunisian population was strongly restricted in comparison with the healthy Tunisian population (Table 2). For example, for D2S2348, only 4 different alleles out of 20 observed in the healthy Tunisian population were identified in the CN-I Tunisian population. In the healthy Tunisian population, frequency of heterozygotes for the $A(TA)_7TAA$ allele was 0.393 and frequency of homozygotes was 0.178. For all Tunisian CN-I patients, the c.1070A>G mutation was associated with the homozygous $A(TA)_7TAA$ mutant allele in the promoter. For MARK01, the allele 4 frequency was 0.952 in the Tunisian CN-I population and only 0.357 in the healthy Tunisian population. The ancestor haplotype (D2S2344: 5, D2S331: 9, D2S1279: 9, D2S2348: 5, D2S2234: 5, D2S2205: 1, MARK01: 4, TATA box: $A(TA)_7TAA$ and D2S336: 4) was observed in three Tunisian CN-I patients and was not observed in the healthy Tunisian population.

LD calculated with the Delvin and Risch formula decreased from 1.000 to 0.4524 with increasing distance of markers from the mutation, except for D2S2234 and D2S2348. The age of the mutation was estimated to be between 21 and 60 generations (mean 32 generations).

In the two Kuwaiti patients, the c.1070A>G mutation was also associated with the homozygous $A(TA)_7TAA$ mutant allele in the promoter. Their haplotype was identical to the ancestor one.

Discussion

CN-I is a rare genetic disease whose frequency is estimated to be $1/10^6$ births and it affects boys and girls in the same proportion. Founder effects have already been suspected for CN-I in isolated communities such as in France, Portugal or Sardinia.[15,16]

In our laboratory, clinical and/or biochemical CN-I was genetically confirmed for 56 patients. Twenty-five of these patients originated from different part of Tunisia (Tunis, Sfax and Sousse) and 21 were homozygous for the c.1070A>G mutation in exon 3 associated with the $A(TA)_7TAA/A(TA)_7TAA$ polymorphism in the TATA box (three of the other patients were homozygous for the deletion c.396_401delCAACAA associated with the $A(TA)_7TAA/A(TA)_7TAA$ polymorphism and the fourth was homozygous for a large deletion including the promoter and the exon 1). The implication of this mutation in the CN-I phenotype has never been determined by *in vitro* expression studies, but convincing arguments support this relation, particularly the frequency of this mutation in the CN-I Tunisian population. However, the c.1070A>G mutation has always been found associated with the $A(TA)_7TAA/A(TA)_7TAA$ polymorphism, so that it is, to date, impossible to determine whether this mutation totally abolishes UGT1A1 activity by itself or whether its association with the $A(TA)_7TAA/A(TA)_7TAA$ genotype in the TATA box is necessary. In the Tunisian population, the Gilbert allele $A(TA)_7TAA$ frequency was found to be 0.393 and 0.178 for heterozygotes and homozygotes, respectively. These data were comparable to those observed in France (0.385 and 0.17, respectively) or Greece (0.328 and 0.186, respectively).[17,18]

CN-I or II cases have been described in several regions of the Middle East and the Maghreb such as Morocco, Algeria,

Tunisia, Saudi Arabia, Lebanon or Kuwait.[7,8,15,19,20] The high prevalence of this disease, particularly in Saudi Arabia or Tunisia can be explained by a high rate of consanguineous marriages in restricted communities.[19] In the Tunisian population, the large prevalence of the c.1070A>G mutation in exon 3 had suggested an ancestral common origin.[7] The absence of particular susceptibility of the exon 3 to this mutation (only observed in Tunisia and in two Kuwaiti Bedouins families) reinforces the founder effect hypothesis.[7,8] On the other hand, the question of a protective effect of Crigler–Najjar causing alleles at the heterozygote state against oxidative damages such as heart coronary diseases is not clear. Vitek *et al*[21] pointed that patients with mild increase of serum bilirubin could be protected against heart disease. But on the other hand, Bosma *et al*[22] and Gajdos *et al*[23] in two different studies did not find any correlation between mutations in *UGT1A1* gene promoter and protection against heart disease. Moreover an eventual protective effect would be observed after reproductive age. These observations could not explain the high prevalence of Crigler–Najjar syndrome in the Tunisian population.

In this study, genetic analysis allowed us to confirm for the first time the founder effect hypothesis in the Tunisian population. The c.1070A>G mutation appeared some 32 generations ago (nearly eight centuries ago), and its diffusion was strongly limited, probably thanks to consanguineous marriages. The systematic presence of the $A(TA)_7TAA/A(TA)_7TAA$ genotype in the promoter in c.1070A>G mutation carriers suggests that this mutation appeared after the promoter polymorphism, reinforcing the founder effect hypothesis. Genetically, the mutation c.1070A>G and the TATA box are very close (7702 bp or 0.021 cM). In a population of 21 patients, the number of generations necessary to observe one meiotic recombination between these two points is estimated to be 191, spanning about 4000 years.

Unfortunately, the Kuwaiti population studied was too restricted to determine LD, but genetic marker analysis allowed us to identify the ancestor haplotype in the two patients.

Referring to the Bedouin population history, the mutation probably appeared in the Bedouin community and was introduced in the Tunisian population nearly eight centuries ago after human migrations from east to west. Indeed, Bedouin nomads spread out from the Arabian Peninsula into all countries between the Arabic Gulf and the Atlantic. Bedouins arrived in Tunisia very early during the first Arab-Muslim invasions and married the native population, leading to today's population.

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