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Multiple Mutations Are Responsible for the High Frequency of Metachromatic Leukodystrophy in a Small Geographic Area

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Summary

Metachromatic leukodystrophy is a lysosomal storage disorder caused by the deficiency of arylsulfatase A. The disease occurs panethnically, with an estimated frequency of 1/40,000. Metachromatic leukodystrophy was found to be more frequent among Arabs living in two restricted areas in Israel. Ten families with affected children have been found, three in the Jerusalem region and seven in a small area in lower Galilee. Whereas all patients from the Jerusalem region are homozygous for a frequent mutant arylsulfatase A allele, five different mutations were found in the families from lower Galilee. In patients of Muslim Arab origin, we have found a G86→D, a S96→L, and a Q190→H substitution. Two different defective arylsulfatase A alleles, characterized by a T274→M and a R370→W substitution, respectively, have been found among the Christian Arab patients. All mutations were introduced into the wild-type arylsulfatase A cDNA. No enzyme activity could be expressed from the mutagenized cDNAs after transfection into heterologous cells. In all instances, the patients were found to be homozygous for the mutations, and four of the five mutations occurred on different haplotypes. The clustering of this rare lysosomal storage disease in a small geographic area usually suggests a founder effect, so the finding of five different mutations is surprising.

Introduction

Rare hereditary diseases have been reported to be frequent in various populations such as the Old Amish people, the Ashkenazi Jews, the French Canadians, and the Finns (for further references see Zlotogora 1994). This phenomenon is thought to be related to the isolation of small communities, due to geographic conditions, differences in religion, or other reasons. In most cases, the genetic explanation has been a founder effect and/or genetic drift (Diamond and Rotter 1987). This was often supported by genealogical data, which pointed to a common ancestor. However, in some cases, other explanations were proposed, in particular, a selective advantage for heterozygotes. For instance, resistance to malaria, which was demonstrated for sickle cell anemia and thalassemia carriers, explains the high frequency of these disorders in various populations (Flint et al. 1993).

Muslim and Christian Arabs have been living in small villages in the Middle East for centuries, isolated one from the other mainly because of religious differences. In these Arab communities, a high frequency of some rare recessive genetic disorders has been observed, such as Krabbe disease (Zlotogora et al. 1991) or Hurler disease (Bach et al. 1993). We report here on the high frequency of metachromatic leukodystrophy among Arabs in Israel and on the molecular analysis of these patients.

Metachromatic leukodystrophy is an autosomal recessive lysosomal storage disorder caused by the deficiency of arylsulfatase A (E.C.3.1.6.8) (for review see Kolodny 1989). The substrate of the enzyme is the sphingolipid cerebroside sulfate. This sphingolipid can be found ubiquitiously in cell membranes, but it occurs in particularly high concentrations in the myelin sheaths of the nervous system. Deficiency of the enzyme causes accumulation of the substrate. The disease is characterized by a progressive demyelination of the nervous system, which causes a variety of neurologic symptoms in the patients. In a typical case of the late-infantile form, a child shows first symptoms at the age of 18–24 mo. The child loses previously acquired capabilities; develops a spastic quadriplegia, an optic atrophy, and dementia; and finally dies in a decerebrated state. Clinically, the disease is heterogeneous with respect to the age at onset. Depending on the age at onset a late-infantile form starting around the age of 2 years, a juvenile form starting at the age of 3–16 years, and an adult form, which presents beyond the age of 16 years, are distinguished. The late-onset forms may initially show psychiatric symptoms, and the progression of the disease may be slower than in the late-infantile forms. The gene of arylsulfatase A has been cloned (Kreysing et al. 1990), and so far, 29 mutations causing metachromatic leukodystrophy have been characterized (Gieselmann et al., in press). Most of these alleles have been found in only one or a few patients. However, two alleles were found to be frequent

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among Caucasian patients. These alleles are characterized by a splice donor site mutation at the first base of intron 2 (allele 459+1A→G) and an amino acid substitution (P426L) due to a mutation in exon 8 (Polten et al. 1991; Barth et al. 1993). Each of these alleles accounts for ~25% of all mutant alleles among Caucasian patients. Metachromatic leukodystrophy occurs panethnically, with a frequency of 1/40,000 live births (Gustavson and Hagberg 1971). A high frequency (1/75 live births) of metachromatic leukodystrophy has been found in an isolate, the Habbanite Jews, where it is due to a founder effect, and all patients are homozygous for the same metachromatic leukodystrophy allele characterized by a P377L substitution (Zlotogora et al., in press-*a*). This deleterious mutation is found on the background of the so-called arylsulfatase A pseudodeficiency allele, which is characterized by two polymorphisms, one causing the loss of an N-glycosylation site and one causing the loss of a polyadenylation signal (Gieselmann et al. 1989).

Here we report on the clustering of metachromatic leukodystrophy in a small geographic area in Israel. Analysis of the mutations unexpectedly revealed five different disease-causing alleles, so that the clustering cannot be explained by a founder effect.

Subjects, Material, and Methods

Populations in Israel and Patient's Histories

The non-Jewish citizens of Israel include Muslim Arabs (677,000 in 1990) and Christian Arabs (114,700 in 1990) (Central Bureau of Statistics 1991). Most live in small villages of 5,000–15,000 inhabitants. Their largest concentrations are in Jerusalem (146,300 non-Jewish inhabitants) and Nazareth (53,600 non-Jewish inhabitants). The Department of Human Genetics at Hadassah Medical Center is the referral center for the diagnosis of lysosomal storage diseases in Israel. Among the children born to non-Jews between 1975 and 1990, late-infantile metachromatic leukodystrophy was diagnosed in 10 families: 7 Muslim and 3 Christian Arabs. In the same period, the total live births of non-Jews in Israel were ~320,000 Muslim and ~32,000 Christian (Central Bureau of Statistics 1991).

All the patients presented with a disease typical for late-infantile metachromatic leukodystrophy: after a normal development in the 1st year of life, the initial symptoms were stagnation in motor development, followed by further regression. In the 10 families examined, 14 children were diagnosed to be affected with metachromatic leukodystrophy. In six of these children, the diagnosis was confirmed by demonstration of arylsulfatase A deficiency in leukocytes and fibroblasts. In all cases, the parents were first cousins. There are no known relations between the different families. H.R., M., and S. are Christian Arabs, and As., Ab., H., and A.M. are Muslim Arabs. Specifically, H. and A.M. are Bedouins.

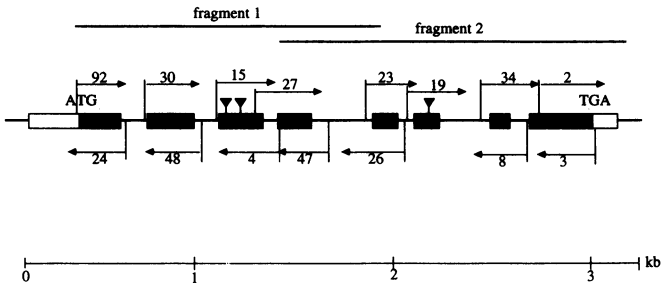

Figure 1 Sequencing strategy and location of the mutations in the arylsulfatase A gene. The structure of the arylsulfatase A gene is shown schematically. Bars represent exons, lines introns. Black parts represent coding sequences, and white parts represent 5′ or 3′ untranslated sequences. Triangles show potential N-glycosylation sites. Numbers indicate oligonucleotides used to prime the sequencing reactions. Sequences of primers are given in table 1. Arrows pointing to the right indicate sequences of the coding strand, and those pointing to the left indicate sequences of the noncoding strand. The thicker lines labeled "fragment 1" and "fragment 2" indicate the two overlapping fragments with which the entire ARSA gene was amplified.

Material

Taq polymerase was from Bethesda Research Laboratories. Oligonucleotides were synthesized on an ABI 346 DNA synthesizer, as described elsewhere (Stein et al. 1989). Restriction enzymes were purchased from United States Biochemical and New England Biolabs, and the dye terminator kit was from Applied Biosystems.

Amplification of the Arylsulfatase A Gene

The arylsulfatase A gene was amplified in two overlapping fragments from genomic DNA prepared from cultured fibroblasts. The fragments are indicated in figure 1, and the sequences of the primers are given in table 1. The PCR conditions were 15 s at 94°C, 30 s at 52°C, and 3 min at 70°C for 35 cycles for both fragments. PCR reactions were performed in a total volume of 50 μl, containing 10 mM Tris/Cl pH 8.3, 1 mM $MgCl_2$, 50 mM KCl, 10% dimethylsulfoxide, 0.05% gelatin, 1.25 mM dNTP, and 50 pmol of oligonucleotides. The PCR was carried out in a Perkin Elmer GeneAmp 9600 thermocycler. After this, amplification fragments were passed over a centricon 100 to remove unincorporated oligonucleotides. An aliquot of the PCR was checked on an agarose gel, and the concentration of the DNA fragment was determined by comparison with a lambda *Hin*dIII DNA standard.

Sequence Analysis of the Arylsulfatase A Gene

The entire exonic and parts of the intronic sequence of the arylsulfatase A gene were sequenced with an automatic ABI 373 A DNA sequencer. The sequencing strategy and the sequence of the primers used for the sequencing reactions are shown in figure 1 and table 1, respectively. The sequencing reactions were done with a dye terminator se-

Table 1

Sequence of Oligonucleotides, Used for Sequencing, PCR, and In Vitro Mutagenesis

Primers used for sequencing	Oligonucleotide Number	Position[a]	Oligonucleotide Sequence[b]
Exon:			
1	92/sense	−56→−37	5'GCGCGGAATTCTGCTGGAGCCAAGTAGCCC3'
	24/antisense	246→266	5'TGCAATCCATTGGGAGGGAAA3'
2	30/sense	336→353	5'TGTCTCAGGGACTCTGTG3'
	48/antisense	632→649	5'GGGAGGTGGGAGGGGTGG3'
3	15/sense	685→704	5'ACCTGCCAGCCCAGCCCTCA3'
	4/antisense	1036→1053	5'TGCAAAGCTCTGCCCACT3'
4	27/sense	880→909	5'ATGACCTCATGGCCGACGCCCAGCGCCAGG3'
	47/antisense	1200→1216	5'GCCGCAGCACCCAGCTG3'
5	23/sense	1472→1491	5'GCTCATGAGCGCCTCCTGTG3'
	26/antisense	1624→1653	5'AGGGTTCCAAGGAGAGGGCCTGCGGACTGA3'
6	19/sense	1771→1791	5'CGGAATTCTTGATGGCGAACTGAGTGAC3'
7	34/sense	2039→2055	5'TATGTGCAGTGCTTGGG3'
	8/antisense	2347→2366	5'GTCAGAGAGCTGGAGGCGTG3'
8	2/sense	2304→2323	5'ACCCAGGCTCTGCCCACAGT3'
	3/antisense	2640→2659	5'GCCATCACATGCCCAGGCCA3'
Primers used for PCR			
Fragment:			
1	92/sense	−56→−37	see above sequencing primer exon 1
	94/antisense	1501→1520	5'CGGGACATACGCATGGTCTC3'
2	10/sense	1025→1044	5'TACCCTCAGTTCAGTGGGCA3'
	20/antisense	2797→2817	5'TTCCTCATTCGTACCACAGG3'
Oligonucleotides used for in vitro mutagenesis			
Mutation:			
G86D	157/antisense	397→415	5'GGGTACATGTCCATCCGAA3'
S96L	161/sense	423→448	5'CTGGTGCCCAGCCTCCGGGGGGGCCT3'
Q190H	103/antisense	824→842	5'ACAGGGGGGGTGCGCCTCC3'
T274M	117/antisense	1149→1167	5'ATGACCAGCATCTCTTCAA3'
R370W	104/antisense	2088→2106	5'GAGACTGCCAAGGGCTCTT3'

[a] Refers to the gene nucleotide numbering, with the A of ATG as nucleotide number 1.
[b] Sequences that are underlined are not part of the gene sequence, but introduce restriction sites into the amplified fragments.

quencing kit from ABI, and the reactions were performed according to the protocol supplied by the manufacturer.

In Vitro Mutagenesis

All mutations causing amino acid substitutions were introduced into the normal arylsulfatase A cDNA by in vitro mutagenesis as described elsewhere (Nakamaye and Eckstein 1986). The mutated cDNA was cloned into the expression vector pBEH (Artelt et al. 1988) and was transiently expressed in baby hamster kidney cells. Arylsulfatase A activity and protein in the cell extracts was determined as described elsewhere (Stein et al. 1989). Sequences of oligonucleotides used for in vitro mutagenesis are given in table 1.

Results

Geographic Distribution of Families with Affected Children

The distribution of the patients affected with late-infantile metachromatic leukodystrophy was not random within the different communities. The disease was diagnosed in Arab patients from two small geographic areas: in the Jerusalem region and in lower Galilee. Ten families with children suffering from metachromatic leukodystrophy were identified. Late-infantile metachromatic leukodystrophy was diagnosed in three unrelated Muslim families originating from a small village now included within the Jerusalem municipality. The seven other families with metachromatic leukodystrophy patients (four Muslim and three Christian) originated from seven different villages, which include a total of some 50,000 inhabitants within a small geographic area of ~225 km^2 in lower Galilee (fig. 2).

Sequence Analysis of the ARSA Gene

All DNA samples were initially tested for the presence of already known arylsulfatase A mutations. The three metachromatic leukodystrophy patients from the Jerusa-

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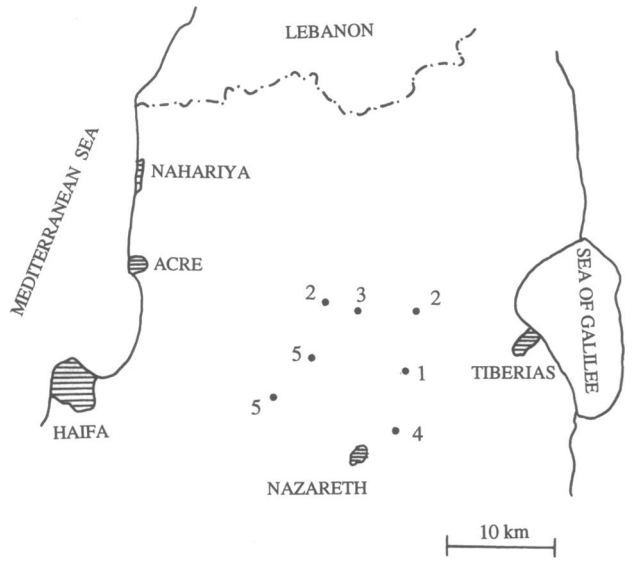

Figure 2 Map of the lower Galilee region in which the patients have been found. The villages in which late infantile metachromatic leukodystrophy was diagnosed are indicated by black dots. The numbers indicate the mutation found in the particular village: 1 = Q190H; 2 = T274M; 3 = G86D; 4 = R370W; and 5 = S96L Hatched regions indicate major cities.

lem region were identified to be homozygous for the 459+1A→G allele. This is one of the two most frequent defective arylsulfatase A alleles among Caucasians and is characterized by a splice donor site mutation at the border of exon/intron 2 (Polten et al. 1991).

None of the known mutations were detected in the other five patients. The arylsulfatase A genes of these patients were amplified in two overlapping fragments and were analyzed according to the sequencing strategy shown in figure 1. DNA analysis was done by cycle sequencing, and sequencing reactions were analyzed on an automatic ABI 373A DNA sequencer. Figure 3 shows the sequences of all patients in the regions where the mutations were found. Patients S. and H.R. are of Christian Arab origin. A C→T transition at position 1158 (gene nucleotide numbering is according to sequence as submitted to the EMBL data bank, accession number X52150; A of ATG is nucleotide number 1) causes a substitution of T274→M in patient S. Another C→T transition, at position 2093, is responsible for the R370→W substitution found in patient H.R. Analyses of the three Muslim patients revealed three different mutations. In patient H., we detected an S96→L substitution. This substitution is caused by the inversion of two nucleotides, changing codon 96 from TCC to CTC. In the other Muslim Arab patient we found a G→C transversion at position 833, which causes the substitution of Q190 by H. Close to this mutation we also found a known W193→C polymorphism, which abolishes a *Bgl*I restriction site (see fig. 3). This polymorphism has so far only been described in the 459+1A→G splice donor site allele mentioned above (Polten et al. 1991). In the third patient of Muslim Arab origin, we found a G86→D substitution owing to a G→A transition at nucleotide 406.

Analysis of the Five Mutations in the Parents of the Affected Children

The presence of the mutations T274M and Q190H was determined by allele-specific hybridization with mutation-specific oligonucleotides. R370W creates a *Sty*I restriction site and G86D an *Afl*III restriction site. S96L abolishes a *Sma*I restriction site. These tests were used in the five affected children and their parents. In all cases, the parents were found to be carriers of the respective mutation (data not shown). Patient M., who was not analyzed by DNA sequencing, was found to be homozygous for the T274M mutation, and in the seventh family, where no DNA was available from the affected child (patient A.M.), the parents were found to be carriers of the S96L allele.

Expression of Arylsulfatase A cDNAs Encoding Enzymes with the Identified Amino Acid Substitutions

To verify that the amino acid substitutions are not polymorphisms, but are responsible for the defects in the arylsulfatase A genes of the patients, all mutations were introduced into the wild-type arylsulfatase A cDNA by site-directed mutagenesis. The mutated cDNAs were cloned into the eukaryotic expression vector pBEH (Artelt et al. 1988), which was transiently transfected into baby hamster kidney cells. Forty-eight hours after transfection, arylsulfatase A activity and protein were determined in the cell homogenates. Results are summarized in table 2. In no case could arylsulfatase A activity be expressed from the plasmids carrying the arylsulfatase A cDNAs with the base substitutions corresponding to the patients' mutations.

Analysis of the Haplotypes Associated with the Five Mutations

To investigate whether the mutations occur on the same haplotype, fragments 1 and 2 were amplified from genomic DNA and were digested with several enzymes that detect polymorphic sites in the arylsulfatase A gene. *Bgl*I and *Bsr*I detect restriction-enzyme polymorphisms, which are located in the coding sequence. The first is associated with a W193→C substitution and the second with a N350S substitution. Both amino acid substitutions have been shown to be polymorphisms (Gieselmann et al. 1989; Polten et al. 1991). The *Bam*HI polymorphism is found in intron 7. Results of this restriction-enzyme analysis are summarized in table 3. They indicate that the mutations in the Christian Arab patients have most likely occurred on the same haplotype, whereas those of the Muslim patients are found on different arylsulfatase A allele backgrounds.

Discussion

In communities such as those reported here, the high frequency of a hereditary disease is expected to be due to

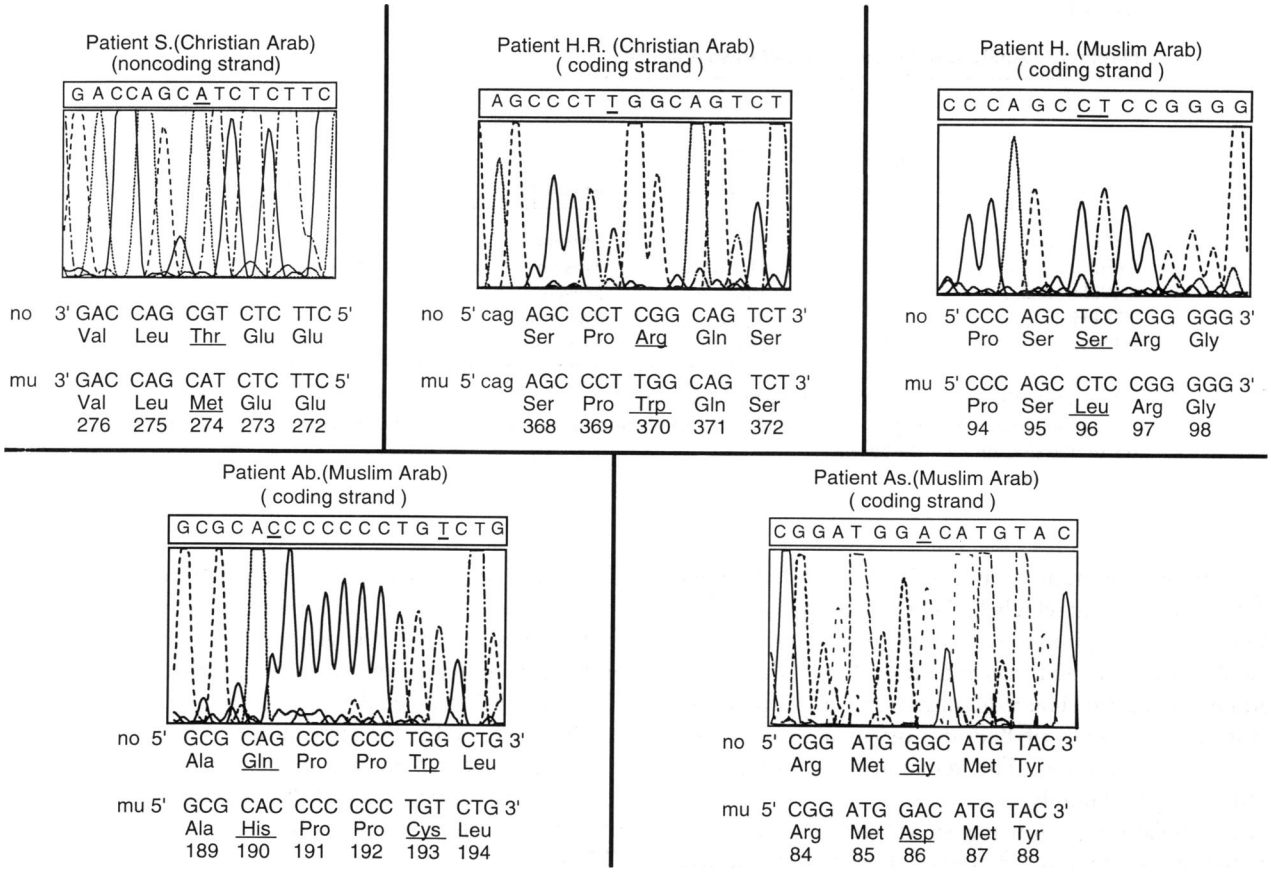

Figure 3 Mutations in the arylsulfatase A gene. The figure shows the sequence analysis of the automatic ABI DNA sequencer for the regions of the gene in which the mutations have been found. Initials and ethnic origin of patients are given at the tops of the graphs. The sequence panels show the mutant sequence. For patients H.R., H., Ab., and As. the coding strand is shown; the noncoding strand is shown for patient S. The nucleotide and amino acid sequences of the normal and the mutant allele are shown below. The codon in which the mutation occurred is underlined.

Table 2

Expression of Mutant Arylsulfatase A

Transfected Vector Substituted Residue[a]	Arylsulfatase A Activity (mU/mg protein)	
	Total	Minus Endogenous Activity
pBEH	1.85	...
pBEH Wt	11.62	9.77
pBEH G86D	1.8	0
pBEH S96L	1.54	0
pBEH Q190H	1.90	.05
pBEH T274M	1.82	0
pBEH R370W	1.93	.08

[a] pBEH indicates the eukaryotic expression vector, and the designation is followed by the amino acid substitution, which has been introduced into the arylsulfatase A cDNA of that vector. Wt = the wild-type cDNA.

a founder effect. This is the case for late-infantile metachromatic leukodystrophy in the Jerusalem region, since three patients were homozygous for the same mutation, 459+1G→A, which has been reported to be the most frequent mutation causing late-infantile metachromatic leukodystrophy among Caucasians (Polten et al. 1991; Barth et al. 1993). The high frequency of the disorder in the area is most probably due to the propagation of the mutation from one founder (Zlotogora et al., in press-*b*). On the other hand, the high frequency of late-infantile metachromatic leukodystrophy in Galilee was due not to a founder effect but to the presence of five different mutations clustering within a small region of ~225 km^2. Mutations T274M and S96L were each found in two families originating from different villages (fig. 2). T274M was found in two different Christian Arab families with no known relation between them. However, a recent report from Australia (Harvey et al. 1993) describes six Australian metachromatic leukodystrophy patients of Lebanese origin who were homozygotes for the same mutation. The Christian community in Galilee has always been in contact with

Table 3

Haplotype Analysis of Mutant Arylsulfatase A Alleles

Mutation	Map Number[a]	Patient	Religion	Polymorphic Restriction Sites[b]		
				*Bgl*II	*Bam*HI	*Bsr*I
G86D	3	As.	Muslim	+	−	−
S96L	5	H., A.M.	Muslim	...[c]	−	+
Q190H	4	Ab.	Muslim	−	+	−
T274M	2	S., M.	Christian	+	+	−
R370W	1	H.R.	Christian	+	+	−

[a] Refers to the location of the mutations as shown in fig. 2.

[b] Arylsulfatase A haplotypes were defined by three polymorphic restriction sites. + = Presence of the particular restriction site; and − = the absence.

[c] Unknown.

the Lebanese community, and, indeed, one of the members of the families in which T274M was found came from Lebanon at the beginning of the century, supporting the possibility that T274M was introduced into the Galilee region by migration. The S96L mutation was found in two Muslim Arab families of Bedouin origin with no known relation between them. The remaining mutations were found in only one family.

The population in non-Jewish villages is divided into large families known as "Hamula." Traditionally, in marriages within the Hamula, first cousins are preferred (Freundlich and Hino 1984; Jaber et al. 1992). Intermarriages of individuals from different villages are rare and are almost nonexistent between individuals from different religions. Most families have many children, often eight or more, and, therefore, although in late-infantile metachromatic leukodystrophy selection eliminates the homozygotes, the large number of children increases the number of carriers in each generation. In such communities, a recessive mutation may be found in homozygosity already in one of the great-grandchildren of the individual in whom the mutation first occurred. The presence of only one or two families with members homozygous for one mutation suggests that in each case the mutation first occurred recently. Furthermore, the lack of compound heterozygotes supports the assumption that the mutant alleles have not been present in that area for a time sufficient to allow spreading in the population. Such a spreading of mutant alleles from different centers of origin, with the concomitant appearance of genetic compounds, for instance, has been reported for Tay-Sachs disease, which is caused by the deficiency of the lysosomal enzyme β-hexosaminidase A. This disease is frequent among the French Canadians. Most of the patients show a 7.6-kb deletion in the β-hexosaminidase A gene (75% of the alleles) (Hechtman et al. 1990). It seems that the center of origin of this mutation is the Gaspé region in Canada. Most of the other French Canadian patients were compound heterozygotes for the deletion and an intron 7 splice site mutation. This second mutation has a different geographic center of diffusion, and the founder seems to originate from Charlevoix in Canada (Hechtman et al. 1992; Zlotogora 1993).

The reason(s) for the appearance of multiple mutations in a single gene in a population living in a small geographic region is unknown. Some of the mutations may have been introduced into the region by the migration of carriers (T274M). Other mutations may have occurred at random and may have been identified only because of the high degree of consanguinity in the population. However, if the high degree of consanguinity were the only reason, one would have expected to observe a random distribution of the different mutations all over the country. The particular geographic distribution of late-infantile metachromatic leukodystrophy, as reported here, argues against this possibility. Alternatively, it may be postulated that a particular arylsulfatase A allele frequent in that region may be prone to a high spontaneous mutation rate. Since four of the five mutations have occurred on a different haplotype, this cannot be the case. It is also conceivable that the cause is an environmental factor. In that case, other genes should also be involved. In fact, a similar phenomenon was observed in Hurler syndrome (Bach et al. 1993). Three different mutations have been characterized among Hurler patients originating from the very same region in Galilee. Each of the mutations was found in one or few families, and all patients were homozygous. It may be that this phenomenon is particular to genes related to lysosomal storage diseases, and a possible explanation may be a selective advantage for carriers (Zlotogora et al. 1988). However, since our interest in lysosomal storage diseases make the ascertainment biased, it may be that this in fact represents a general phenomenon.

To resolve these questions, we are planning a systematic study of all hereditary disorders and malignancies found in

the region. Molecular studies of the genes involved should help to determine the cause of the observations reported here. These findings will help us to understand the reasons that have caused genetic disorders to be frequent in various populations.

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