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ORIGINAL PAPER

Identification of fifteen novel mutations in the tissue-nonspecific alkaline phosphatase (*TNSALP*) gene in European patients with severe hypophosphatasia

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Hypophosphatasia is an inherited disorder characterised by defective bone mineralisation and deficiency of serum and tissue liver/bone/kidney alkaline phosphatase (L/B/K ALP) activity. We report the characterisation of tissue-nonspecific alkaline phosphatase (*TNSALP*) gene mutations in a series of 13 European families affected by perinatal, infantile or childhood hypophosphatasia. Eighteen distinct mutations were found, only three of which had been reported previously in North American and Japanese populations. Most of the 15 new mutations were missense mutations, but we also found two mutations affecting donor splice sites and a nonsense mutation. A missense mutation in the last codon of the putative signal peptide probably affects the final maturation of the protein. Despite extensive sequencing of the gene and its promotor region, only one mutation was identified in two cases, one of which was compatible with a possible dominant effect of certain mutations and the putative role of polymorphisms of the *TNSALP* gene. In 12 of the 13 tested families, genetic diagnosis was possible by characterisation of the mutations or by use of polymorphisms as genetic markers. Hypophosphatasia diagnosis was assigned in two families where clinical, laboratory and radiographic data were unclear and prenatal diagnosis was performed in one case. The results also show that severe hypophosphatasia is due to a very large spectrum of mutations in European populations with no prevalent mutation and that

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genetic diagnosis of the disease must be performed by extensive analysis of the gene.

Keywords: hypophosphatasia; alkaline phosphatase; mutations

Introduction

Hypophosphatasia is an inherited disorder characterised by defective bone mineralisation and a deficiency of serum and tissue liver/bone/kidney alkaline phosphatase (L/B/K ALP) activity. The disease is highly variable in its clinical expression, which ranges from stillbirth without mineralised bone to pathologic fractures developing only late in adulthood.[1] Depending on the age at diagnosis, four clinical forms are currently recognised: perinatal, infantile, childhood and adult. Severe forms of the disease (perinatal and infantile) are transmitted as an autosomal recessive trait while the mode of inheritance of clinically more mild childhood hypophosphatasia or adult hypophosphatasia remains uncertain, and both autosomal recessive and autosomal dominant transmissions have been suggested.[2] Heterozygous carriers of the severe forms are usually clinically normal but often show modestly reduced serum ALP activity and increased urinary phosphoethanol-amine.[3]

In at least some cases of severe and mild clinical forms, the disease is due to mutations in the tissue-nonspecific alkaline phosphatase (*TNSALP*) gene. This gene, localised on chromosome 1p36.1–34,[4] consists of 12 exons distributed over 50 kb.[5] To date, mutations have been found on the *TNSALP* gene in North American and Japanese patients only.[6–12] Some of these mutations were found in families with lethal hypophosphatasia, allowing the prenatal diagnosis of the disease in them.[13,14]

We report here the characterisation of 18 mutations in the *TNSALP* gene in European patients, 15 of which have not been previously described, and the use of the characterisation of these mutations in prenatal and postnatal diagnosis of the disease. Our results show that severe hypophosphatasia in European patients may be due to a very large spectrum of mutations in the *TNSALP* gene.

Subjects and Methods

Patients

The families presented here were given adequate information and were volunteers for the study. Nineteen cases (four foetuses, three patients and 12 parents) were analysed, corresponding to 26 distinct chromosomes carrying a possible mutation in the *TNSALP* gene. In three cases, the probands were infants with the infantile and childhood forms of the disease; in the other cases, the probands were affected with the perinatal form. When available, the alkaline phosphatase values are shown Table 1.

Clinical, laboratory and pedigree data concerning family Pa6 had been previously reported.[15] In two cases (Na1 and Mu1) radiographic and clinical features were not clear enough to allow a diagnosis and the cases were referred to our laboratory to examine the possibility of severe hypophosphatasia. In the other cases, diagnosis of hypophosphatasia was suggested on the basis of alkaline phosphatase levels, radiographic features or autopsy examination.

PCR Amplification of Exons

Primer sequences of the 12 exons are shown in Table 2. They were designed from the intronic sequences obtained from Genbank (accession numbers D87874 to D87888), from reference 12 and from our own results (Mornet E, 1997, unpublished data). These primers allowed analysis of the whole coding sequence. PCR reactions were performed in a final volume of 50 µl with approximately 30 picomoles of each primer, 250 µM of each dATP, dCTP, dGTP and dTTP, 1 unit of Gold Taq DNA polymerase (Perkin-Elmer, Foster City, CA, USA) and 1–3 mM $MgCl_2$ depending on the exon amplified. Reactions were heated at 95°C for 10 min and subjected to 30 cycles of 1 min at 95°C, 1 min at the annealing temperature (Table 2) and 40 s at 72°C.

SSCP (Single Strand Conformation Polymorphism) Analysis

One microlitre of each PCR product was directly analysed on to 12.5% polyacrylamide gels migrated at 15°C with a Phastgel-System (Pharmacia Biotech Inc., Uppsala, Sweden) and the gels were stained with silver nitrate according to the procedure recommended by the manufacturer.

Sequencing Analysis

PCR products were purified and sequenced by the direct sequencing method using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase, FS (Perkin-Elmer Corp., Foster City, CA, USA) and migrated on to an ABI PRISM 310 electrophoresis system (Applied Biosystems, USA).

Analysis of Base Changes in Normal Chromosomes

Mutation A23V was tested by allele-specific PCR. Mutations in exon 12, N153D and 997 + 3C were tested by sequencing.

Mutations R167W, R206W and E274K abolish Hpa II, Hae III and Taq I restriction sites, respectively, and the presence of the mutation was thus tested by use of these enzymes.

Computer-assisted Analysis

Computer-assisted modelling of proteins with missense mutations was performed by using the Chou and Fasman[16] and the

Table 1 Mutations on the TNSALP gene found in the 13 families studied

Family	*Status*	*Geographic origin*	*Alkaline phosphatases*	*Clinical form*	*Mutation*	*Exon*
Pa1	father	France	nd	lethal	R167W	6
	mother		nd		W253X	8
Pa2	father	Sardinia	43 u/l (30–115)	lethal	R433C	12
	mother		27 u/l (30–115)		R433C	12
Pa3	father	France	26 u/l (>40)	lethal	G103R	5
	mother		18 u/l (>40)		658+1A	intron 6
Pa4	father	Greece	normal	lethal	N206W	7
	mother		normal		(–)	(–)
Pa5	patient	France	31 u/l (>100)	childhood	E174K	6
					E274K	9
Pa6	foetus	France	<15 u/l (>100)	lethal	S428P (mat)	12
					Y246H (pat)[a]	7
Na1	foetus	Turkey	8.1 u/l (97)	lethal	N153D	6
					N153D	6
Na2	father	France	0.38 µKat (0.5–1.7)	lethal	G112R	5
	mother		24 u/l (30–110)		G474R	12
Ly1	father	France	28 u/l (35–120)	lethal	997+3C	intron 9
	mother		30 u/l (35–120)		997+3C	intron 9
Ly2	patient	France	40 u/l (>100)	infantile	G58S (pat)	4
					S-1F (mat)	2
Mu1	foetus	Germany	40 u/l (>100)[b]	lethal	A23V (pat)	3
					G456S (mat)	12
Bo1	foetus	Russia	24 u/l (>100)	lethal	G317D	10
					G317D	10
Zu1	patient	Yugoslavia	30 u/l (87–439)	infantile	R433C	12
					R433C	12

The parents of Na1 were first cousins and the parents of Ly1 were from the same village but without known consanguinity. Normal values of alkaline phosphatases are given in brackets. nd = not done. (–) indicates that no mutation was found, despite sequencing of the whole coding sequence. When available, parental origin of mutations in index cases is shown: pat = paternal; mat = maternal.
[a]Previously described as a polymorphism by Henthorn *et al* (1997).
[b]Measured in the parents.

Table 2 Sequences and annealing temperatures of primers used in PCR amplification of the 12 exons of the *TNSALP* gene

Exon	*Forward primer*	*Reverse primer*	*Annealing temp.*
1	AAGCCAGATATGTTGACAGA	GCCATTAAAGTTCAACCA	54
2	ATTGCATCTCTGGGCTCCAGGGA	CAGCTTTTAAATACTTTGG	47
3	CTGGAGATAGGAGGCTATCCT	GGTCTCCTAGCTAGTGTCCTG	55
4	AGGAGCACGAGAGACTGAGG	CTGGCTGCTGTCATGTTCAG	51
5	CCTCACGCCCCAGTCCCCAT	GCTGGCCCCTGCTCCCCACT	58
6	GCCAAACCCGCCCCTCCTGC	AATTCATGCCAGCCTGGCCTGAGCCTC	58
7	CAGGAGTCCAGGTTCCAAGC	AGGCCACCTATGCAGCCACAT	55
8	AGGCCTCAGATTTTGATAGC	GGCTTTGTCCCCAGGTGTTGG	51
9	ATTCCCTGAGACACCCCAGC	CAGGGCCGTGTTTCCAGCAG	55
10	TCCCCTCCTCCCTCACCGAGG	TTGCTGGCTCTCCCACCCAC	58
11	TGGGGGGTGGGGACTGTACT	CCCTGTCCCCTCCCAGCCCT	58
12	GGGCATGTGACTCCATCTTTCTCTG	GCTGCCGTGTGGGAAGTTGGCATC	58

Primers amplifying exon 3 (reverse), exon 5 (reverse) and exon 6 (forward) were designed from intronic sequences determined in our laboratory (Mornet E., 1997, unpublished data). Primers amplifying exon 3 (forward) and exons 6,7 and 8 (reverse) were previously described by Orimo *et al*.[12] Other primers were designed from Genbank (accession numbers D87874 to D87888).

Robson [17] secondary structure prediction methods with DNAsis software (Hitachi Software Engineering, Inc.).

Results

The TNSALP *Gene Mutations*

Eighteen distinct mutations were found distributed over the gene (Table 1). Fifteen of them were missense mutations, two affected splicing by abolishing the consensus sequence at the donor site, 648 + 1A and 997 + 3C in introns 6 and 9, respectively, and one was a nonsense mutation, W253X. We also found a mutation (S-1F) in the last codon of the putative signal peptide that is eliminated from the mature protein. Such a mutation may disturb the final maturation of the protein by modifying the recognition site of proteases involved in the final maturation process. Only mutations E174K, R206W and G317D had been previously described,[7,8,10] indicating a strong allelic heterogeneity in severe hypophosphatasia, a result consistent with previous reports from other populations.[7,10]

Six single base-changes resulting in no amino acid change were observed in exons 4, 9, 11 and 12: G201A (V50V), A876C (P275P), G1206C (L385L), G1221A (K390K), T1338C (A429A) and T1542G (A497A). In addition, we found in patient Pa6 the mutation Y246H previously described as a polymorphism.[7] In order to acquire evidence of their true disease-causing nature, 10 mutations were tested in normal unrelated individuals from a French volunteers' panel (Table 3). None of the mutations was found. This suggests that these mutations cannot be frequent polymorphisms and supports evidence of their true disease-causing nature.

Except in the case of mutation R206W that was predicted to abolish a turn between two β-sheets, computer-assisted modelling of mutated proteins did not show any obvious modification in the predicted secondary structure of the protein.

Prenatal Diagnosis of Severe Hypophosphatasia

A prenatal diagnosis was performed in family Na2 in which a first pregnancy was terminated at 35 weeks on the basis of severe hypophosphatasia seen by ultrasound scan and confirmed by low levels of alkaline phosphatase in chorionic villus sampling. Molecular analysis of the parents revealed the mutation G112R in the father and G474R in the mother, and a normal boy from a previous pregnancy was shown to carry none of these mutations. The chorionic villi exhibited only the G474R mutation, suggesting that the foetus was heterozygous and not affected by the disease. Alkaline phosphatase assay showed normal levels in the trophoblast, confirming the molecular diagnosis.

Table 3 Conservation of amino acids affected by the mutations and frequencies of the mutations in the general population

Mutation	*Conservation*	*General population (individuals)*
S–1F	++	not tested
A23V	complete	0/60
G58S	++	not tested
G103R	complete	not tested
G112R	++	not tested
N153D	–	0/36
R167W	complete	0/40
E174K	+	0/57[a]
648+1A		not tested
R206W	++	0/38
W253X	++	not tested
E274K	++	0/42
Y246H	–	24/73[a]
997+3C		0/43
G317D	++	0/74[b]
S428P	–	0/38
R433C	+	0/38
G456S	complete	0/38
G474R	+	0/38

Conservation was tested in 9 distinct ALP proteins from 6 organisms: human TNSALP, human intestine ALP, human placenta ALP, bovine ALP, mouse ALP, cat TNSALP, rat TNSALP, hen TNSALP and *E. coli* ALP (Genbank accession numbers M24438, M31008, J03252, M18443, M61705, U31569, P08289, U19108 and M13345). 'Complete' indicates a position conserved in the 9 peptide sequences; ++ a position conserved in all sequences except *E. coli*; + a position conserved in the *TNSALP* gene products only; and – a position not conserved. The number of tested individuals in the general population ranges from 38 to 74 (76 to 148 tested chromosomes).
[a]Reported and tested in normal chromosomes by Henthorn *et al*[7].
[b]Reported and tested in normal Mennonite chromosomes by Greenberg *et al*[8].

Discussion

By using an SSCP-sequencing strategy, we found 24 of the 26 mutations responsible for severe hypophosphatasia in the families studied. Among the 18 distinct mutations found, 15 had not been previously described. Combined with previous data from Japanese and North American populations,[6–12] 43 *TNSALP* gene mutations have been described to date in a relatively small number of chromosomes analysed. This suggests a strong allelic heterogeneity and no major mutation in severe hypophosphatasia. Thus, genetic diagnosis of the

disease must be performed by extensive analysis of the gene. Except for exon 12, the sizes of the PCR products ranged from 150 to 300 bp, allowing efficient detection of variants by the SSCP methodology. However, in most cases the whole coding sequence had to be analysed by sequencing in order to support the pathogenic nature of the mutation. In our study, we detected 17 electrophoretic variants by SSCP, six of which were shown to be actual mutations (N153D, R167W, N206W, E274K, 997 + 3C and G58S); the others were exonic or intronic polymorphisms.

The pathogenic nature of mutations E174K, R206W and G317D was previously suggested by examining DNA from unrelated normal individuals and patients.[7,8] In addition, mutation R206W was predicted to significantly modify the secondary structure of the protein (not shown). The pathogenic nature of mutations W253X and 648 + 1A did not require confirmation since they result in no complete gene product and affect the second base of the consensus sequence in the donor splice site, respectively. Unless mutagenised cDNAs are expressed in cultured cells, evidence for the disease-causing nature of the other mutations must be obtained indirectly. First, the gene was sequenced completely to ensure that no other mutation was harboured. Secondly all but one mutation (A23V) changed the nature of the biochemical properties of the amino acids. Thirdly except for N153D and S428P, all the mutations described here were found at conserved positions in three *TNSALP* proteins (cat, rat, hen) and mostly in the human intestine, human placenta, bovine and mouse ALP proteins (Table 3). Mutations A23V, G103R, R167W and G456S affect amino acids completely conserved in these species and R167W interacts with substrate phosphate.[18] ALP from *E. coli* only showed nonconservation for most of the mutations. Lastly, screening of a series of individuals from the general population showed that the tested base changes cannot be frequent polymorphisms. It remains possible that they are rare polymorphisms, but such a hypothesis needs to be tested through screening of larger panels of controls from the same geographic origin as the tested patients. Furthermore, when segregation analysis of the mutations was possible (families Pa6, Na1, Na2, Ly2, Mu1 and Bo1), each parent was shown to carry one of the mutations carried by the index case and normal individuals were shown to carry none or only one mutation.

In family Pa4, we did not find any mutation in the mother, despite sequencing the whole coding sequence. Since the father was shown to carry R206W, it is likely that the index case (an aborted foetus from which no sample was available) was affected by severe hypophosphatasia. Thus, the mother very probably harbours a mutation in the *TNSALP* gene. Analysis of exon 1 and of the promoter region showed no abnormality in the sequence, and two heterozygous polymorphisms in intron 7 (793-31T) and exon 11 (G1206C) suggest absence of a large deletion of the *TNSALP* gene, but a smaller deletion involving a part of the gene cannot be ruled out. It is also possible that the missing mutation might be localised in an intronic region. This may also be the case in family Pa6 where we found only one mutation (S428P) and a polymorphism (Y246H) previously described with a frequency of 33% in the North American population.[7] In this latter family, however, another hypothesis is that the paternally transmitted Y246H polymorphism may have an effect on the pathogenesis when combined with S428P, as has been suggested in the case of the mutation D361V.[19] Corroborating this hypothesis, the mother had a significantly low level of alkaline phosphatase (10 μ/l *vs* 70–100 in controls) and showed premature loss of milk and adult teeth; her 30-year-old brother, unfortunately not tested in this study, was shown to be affected with infantile hypophosphatasia diagnosed on the basis of low levels of alkaline phosphatase (< 3.5 μ/l *vs* > 100 in controls) and skeletal features.[15] Since the probability that the brother was homozygous for *TNSALP* deleterious alleles is very low (approximately 1/300 on the basis of a disease prevalence of 1/100 000), this strongly suggests that he carried S428P, and that this mutation was more severely expressed, perhaps because combined with Y246H as in the index case.

Finally, in 12 of the 13 families studied, prenatal or postnatal diagnosis of severe hypophosphatasia was possible by looking for the characterised mutations or, in the case of family Pa6, by using the paternal Y246H polymorphism as genetic marker of the disease together with the maternal S428P mutation. In only one case (family Pa4) was genotyping not possible because only one mutation was found and no sample from the index case was available.

In homozygotes R433C, N153D, 997 + 3C and G317D, it makes sense to associate the mutation with the severe perinatal form of the disease since these four cases were prenatally detected by typical signs of lethal hypophosphatasia. The previously described mutation G317D[8] was also found in patients affected with the perinatal (lethal form) of the disease. Correlation

between the clinical form of the disease and mutations remains difficult in compound heterozygotes, as it is not possible to know the effect of each mutation, except for W253X, which necessarily results in absence of enzymatic activity. Interestingly, the previously described E174K mutation[7] was found in a patient with the perinatal severe form of the disease but also in four affected children and two patients with the adult form. We found this mutation associated with E274K in a child showing clinical symptoms of the childhood form of the disease. This may suggest that E174K results in moderately severe disease, perhaps because of residual enzymatic activity produced by the mutated gene.

In conclusion, we have characterised the *TNSALP* gene mutations in 13 families affected by severe hypophosphatasia. Among the 15 novel mutations we discovered, two affect donor splice sites, one affects the final mutation of the protein, and one is a nonsense mutation, three kinds of mutations that have not been previously described in hypophosphatasia patients. As in the Japanese and North American populations, severe hypophosphatasia in European patients displays marked allelic heterogeneity, and genetic diagnosis of the disease must therefore be based on extensive sequencing of the gene. It remains possible that some polymorphisms may have a role in the disease when they are associated with genuine mutations.

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