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ARTICLE

N219Y, a new frequent mutation among *mut*° forms of methylmalonic acidemia in Caucasian patients

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Mutations in the *MUT* locus encoding for the methylmalonyl-CoA mutase (MCM) apoenzyme are responsible for the *mut* forms of methylmalonic acidemia (MMA). To date, 49 different mutations have been identified in *mut* MMA. Only two frequent mutations have been reported in the Japanese population and in African-Americans. Here we report a new missense mutation N219Y (731 A→T) which we found in five unrelated families of French and Turkish descent. All the patients exhibited a severe *mut*° phenotype and three of them were homozygotes for N219Y. Direct involvement of the mutation in the loss of enzyme activity was demonstrated by mutagenesis and transient expression study. Mapping of the mutation onto a three-dimensional model of human MCM constructed by homology with the *Propionibacterium shermanii* enzyme shows that it lies in a highly conserved secondary structure motif and might suggest impaired folding and/or poor stability compatible with the *mut*° phenotype. Finally, a 1% N219Y carrier frequency was observed in a French anonymous control population. Thus, N219Y is the first frequent *mut* mutation to be reported in the Caucasian population. *European Journal of Human Genetics* (2001) **9**, 577–582.

Keywords: methylmalonic acidemia; methylmalonyl-CoA mutase; mutation; Caucasian

Introduction

Methylmalonic acidemia (MMA; MIM#251000) is an autosomal recessive inherited inborn error of metabolism that results from the functional impairment of methylmalonyl-CoA mutase (MCM, EC 5.4.99.2). MCM is a mitochondrial nuclear-encoded homodimer enzyme responsible for the conversion of L-methylmalonyl-CoA to succinyl-CoA in the propionyl-CoA catabolic pathway toward the Krebs cycle. Each monomer binds one molecule of adenosylcobalamin, a vitamin B_{12} derivative and the cofactor of the enzymatic reaction.

MMA is a heterogeneous condition in which the *mut* forms (incidence estimated 1/30 000 to 1/50 000) are due to MCM apoenzyme deficiency. Studies on fibroblasts from patients with *mut* MMA have distinguished variant forms with undetectable residual MCM activity (designated *mut*°) and forms with hydroxocobalamin-dependent residual activity (designated *mut*$^-$).[1]

In humans, MCM is encoded by a single gene (*MUT*) mapped on chromosome 6 (6p21). *MUT* consists of 13 exons spanning over 35 kb and producing a 2.7 kb mRNA.[2] To date,

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molecular analysis of *mut* MMA has revealed at least 49 different mutations[3–7] demonstrating the highly pleiomorphic nature of this condition. Only two mutations are frequent: the first one (G717V) in African-Americans[8] and the second (E117X) in the Japanese population.[9,10]

A three-dimensional model of the human MCM structure[11] has been deduced from the crystal structure of the corresponding enzyme in *Propionibacterium shermanii*, that shares 65% sequence identity with human MCM.[12] The model divides the molecule into two major domains: (i) a N-terminal eight-stranded β/α barrel including the CoA moiety binding site and the dimer interface, and (ii) a C-terminal α/β domain with five parallel β strands encompassing the 'B_{12}-binding domain'. Availability of this model allows molecular modeling of the potential structural effects of the observed mutations.

Here, we report a novel missense mutation N219Y which, unexpectedly, was found in five independent *mut*$^{\circ}$ Caucasian families. Site-directed mutagenesis and transient expression show that the observed nucleotide variation is indeed the causative mutation of the enzymatic defect. The affected asparagine residue is conserved among species and lies in a highly conserved secondary structure motif. Molecular modelling suggests that substitution by a tyrosine is likely to result in impaired folding and/or poor stability of the MCM molecule, compatible with the *mut*$^{\circ}$ phenotype.

Patients and methods

Patients

We have investigated five unrelated patients affected with B12-unresponsive MMA. Patient 1 was the first child of consanguineous Turkish parents. He presented with acute metabolic acidosis at 3 months of age. However poor feeding and mild dehydration had been observed at 4 days of age. It was attributed to sepsis at this time but this was not documented and it could well have been the very first symptoms of the disease. His low protein diet consisted of 1 g/kg/d of protein, ie about 800 mg/d of valine. The patient died at 30 months of age following an acute decompensation. The four other patients were all born to French non consanguineous parents.

Patient 2 is affected with a neonatal onset form. She is presently 4 years old and has experienced six intercurrent decompensations. At 3 years of age, her valine tolerance was 700 mg/d.

Patient 3 is a 10 year-old girl affected with a neonatal form. She has presented with two mild decompensations at 1 and 4 years of age, respectively. Valine tolerance at 3 years of age was 550 mg/d.

Patient 4 presented with a neonatal decompensation. He is presently three and a half years of age and has experienced two mild and one severe intercurrent decompensations. His valine tolerance is 600 mg/d.

Patient 5 is affected with a late onset form. She has experienced only one decompensation at 1 year of age. She is presently 5 years old. Her valine tolerance was 525 mg/d at 3 years of age.

Biochemical phenotype

Fibroblast cultures were obtained for each patient from skin biopsies. Cells were grown in HAM F10 medium with 12% FCS under 5% CO_2. The *mut* MMA phenotype was established by measuring MCM activity as previously described.[7] To discriminate *mut*$^{\circ}$ from *mut*$^{-}$ phenotypes, the propionate incorporation test was performed. Fibroblasts were first cultured for 1 week in a cobalamin-free medium (MEM with 10% FCS under 5% CO_2). Then the incorporation of [^{14}C]-propionate (NEN Life Science Products, Boston, MA, USA) into trichloroacetic acid-precipitable material was measured in cells grown for 18 h in the presence of increasing concentrations of hydroxocobalamin (0, 0.01, 0.1, and 1 μg/mL).[13] Incorporation of [^{3}H]-phenylalanine (ICN Pharmaceuticals France, Orsay, France) was measured simultaneously as a control for cell number and rate of constitutive protein synthesis.

Mutation detection

MCM cDNA was prepared from cultured fibroblasts according to standard procedures.[14] RT–PCR products obtained with the primers described by Ogasawara[9] were directly sequenced on an ABI 310 automatic sequencer (Perkin Elmer, Foster City, CA, USA) using the Dye Terminator method, without the secondary nested-PCR included in the original procedure. To confirm the homozygous state of the N219Y mutation, the sequence of exon 3 was determined on genomic DNA using primers described by Adjalla.[4] Moreover, to confirm the N219Y mutation by a second independent procedure and to screen a control population, a new PCR forward primer with a single base substitution was designed (primer 3mut, see legend to Figure 1) so as to create a *Nde*I restriction site when the mutation is present. Digestion of PCR-amplifed products from genomic DNA was performed according to the manufacturer's instructions (New England Biolabs, Beverly, MA, USA) and the resulting fragments analysed by electrophoresis on a 8% polyacrylamide gel.

Transient expression study

Firstly, a stable *mut*$^{\circ}$ cell line (SCK2) was obtained by transforming MCM-deficient fibroblasts from patient 1 by the pLASwt plasmid (a gift from Prof A Fisher), that contains SV40 DNA encompassing the entire early region with a defective origin of replication.[15] Fibroblasts were transfected by electroporation with a Gene Pulser (Biorad Laboratories, Hercules, CA, USA), using 4.10^6 primary cultured cells, 4 μg of pLASwt plasmid, and a 250 V pulse at a capacitance of 1600 μF. The SCK2 cell line was used for transient expression analysis.

Secondly, the 731 A→T substitution was introduced by site-directed mutagenesis (USE Mutagenesis Kit – Amersham

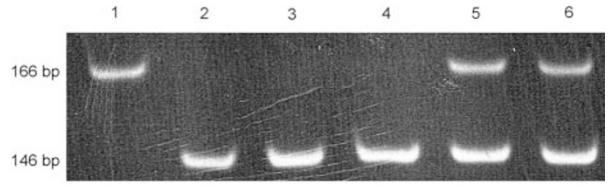

Figure 1 Detection of the 731A→T substitution by *Nde*I restriction. Genomic DNA was PCR-amplified using the 3mut forward primer (5′ ARC TTA CTG GTA CCA TCC CA 3′) localised just upstream from nucleotide 731 in exon 3. This primer contains a single nucleotide substitution (the underlined bold C) so as to create a *Nde*I restriction site when the 731A→T substitution is present. The reverse primer was that described by Adjalla *et al*[4] for this exon. The products of *Nde*I digestion were analysed by electrophoresis on a 8% polyacrylamide gel. Wild-type sequence derived from a normal control (lane 1) gives a 166 bp fragment. DNA from homozygous patients for the 731A→T substitution are cleaved by *Nde*I giving rise to a 146 pb fragment (lanes 2, 3, 4). In heterozygous patients, the two fragments are observed (lanes 5 and 6).

Pharmacia Biotech, Amersham, UK) into an expression vector, pCMVmut (constructed by R Touraine), containing the wild-type MCM cDNA downstream from the CMV promoter. Direct sequencing and enzymatic restriction were used to confirm the presence of the mutation in the recombinant plasmid (pCMVmut731A→T).

SCK2 cells were transfected using the Superfect reagent (Qiagen, Chatsworth, CA, USA) with: (i) the wild-type expression vector (pCMVmut) as a positive control; (ii) a vector containing a stop codon at position 238 (pCMVmutNSCD238) in the beginning of the coding sequence as a negative control; and (iii) pCMVmut731A→T. MCM activity was monitored 48 h after transfection, using the [^{14}C]-propionate incorporation method. All the experiments were carried out three times independently and in duplicate.

Haplotypes

Several microsatellite markers surrounding *MUT* on the short arm of the chromosome 6 were PCR-amplified and analysed on the ABI 310 sequencer. They included: D6S1714, D6S243, D6S465, D6S1669, and D6S1632.

MCM structural modelisation

To construct the three-dimensional structure of human MCM, molecular modelling simulations were performed with the MODELLER 4.0 software[16] using the experimental structure of *P. shermanii* MCM (Protein Data Bank accession code: 1REQ). As notified in Thomä and Leadlay,[11] the very high amino-acid sequence identity between human MCM and the α subunit of the *P. shermanii* enzyme allowed the construction of a 3D model that satisfies spatial constraints.

Results

All the patients' fibroblasts presented with a marked decrease in MCM enzyme activity, ie, less than 10% of the controls (Table 1). As activity is measured with an excess of cofactor (adenosylcobalamin concentration above 100 K_m), this indicates that the defect indeed affects the apoenzyme and not the cofactor and that all the patients belong to the *mut* type. Similarly, propionate incorporation was drastically reduced in all cases and there was no correlation between added exogenous hydroxocobalamin and propionate uptake (Table 1). Altogether these data define a typical *mut*° MMA phenotype.

Comparison of DNA sequences obtained for the patients with the consensus sequence of the human MCM cDNA (Genebank, accession number M65022) revealed a novel single nucleotide substitution in *MUT* exon 3. It consists of an A→T transversion at position 731 in the cDNA. This change was confirmed by sequence determination and restriction analysis of PCR products from genomic DNA (Figure 1). Three patients were homozygotes for the substitution (patients 1–3) and two patients were heterozygotes (patients 4 and 5). As expected, for each homozygous patient, the parents are heterozygotes for the substitution (data not shown). Restriction analysis was also used to screen DNAs from two different control populations for the 731 A→T substitution: in a population of 105 anonymous mentally retarded children and in a control population of 100 anonymous normal children; it was found in the heterozygous state in two individuals from the first population.

For patients 4 and 5, a second sequence modification has been found. It consists in a G→A transition at position 399 and a G→T transition at position 549 in the cDNA, respectively. Interestingly, the 731 A→T substitution was found on chromosomes with various haplotypes. Indeed, except for the Turkish patient who was homozygote for all the studied markers, all the other patients including the two homozygotes for the 731 A→T substitution were heterozygotes for the microsatellite haplotype surrounding *MUT*.

At the protein level, the 731 A→T, 399 G→A, 549 G→T substitutions result respectively in the replacement of an asparagine residue by a tyrosine at position 219 (N219Y), an

Table 1 Patients' fibroblasts biochemical phenotypes

Patient	MCM enzymatic activity[a]	[^{14}C]-propionate incorporation[b] Without OHcbl	OHcbl 1 μg/ml	Biochemical phenotype
1	30	4.5	4.5	*mut*°
2	<10	4.3	4.3	*mut*°
3	10	0.9	0.9	*mut*°
4	111	3.4	3.4	*mut*°
5	46	2.1	2.0	*mut*°
Controls	1800±600, *n*=12	80±20, *n*=10		

[a]pmol/mg protein/min; [b]pmol succinate/pmol phenylalanine

arginine residue by a histidine at position 108 (R108H) and a glycine residue by a valine at position 158 (G158V). All this residues are conserved from bacteria to mouse and man, thus, although the R108H and G158V substitutions have not been studied functionally, it is likely that they represent the second deleterious mutation in patients 4 and 5.

In the MCM primary structure, Asn219 is located in an amino-acid stretch that shows a particularly high level of homology between species (Figure 2). Mapping of the N219Y substitution on the three-dimensional model of human MCM[11] shows that Asn219 is located in the fourth β-strand of the N-terminal eight-stranded β/α barrel, ie, a specific

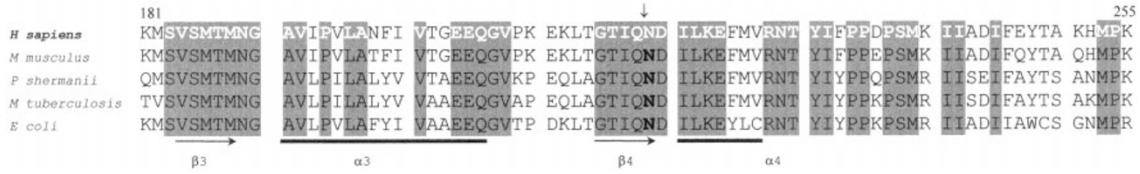

Figure 2 Partial alignment of methylmalonyl-CoA mutase amino acid sequence around asparagine 219 in various species. Sequences span amino acids 181 – 255 in *Homo sapiens, Mus musculus, Propionibacterium shermanii, Mycobacterium tuberculosis and Escherichia coli* enzymes (Swiss Prot accession numbers P22033, P16332, P11653, P71774 and P27253, respectively). Horizontal bars represent alpha helices and horizontal arrows beta-strands in the *P. shermanii* 3D structure. Position of asparagine 219 is indicated in bold and by a vertical arrow.

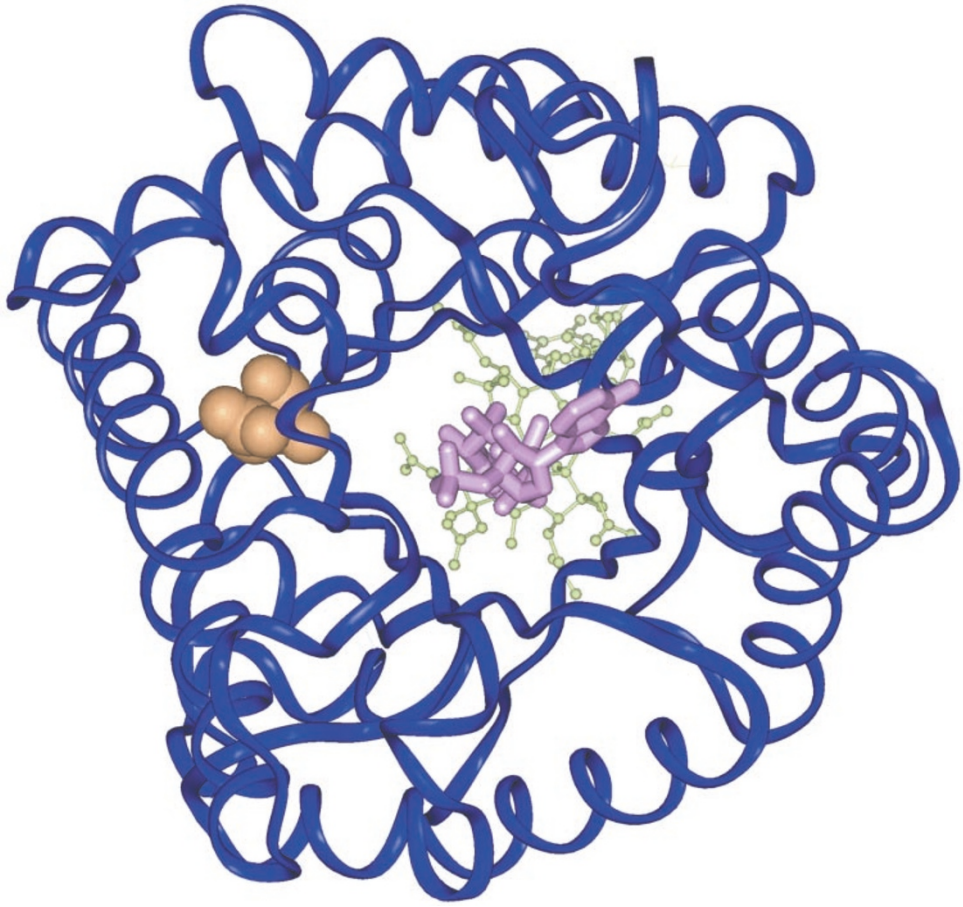

Figure 3 View of the three-dimensional structure of the $(\beta/\alpha)_8$ barrel of the human methylmalonyl-coenzyme A mutase model. The model was built on the basis of the experimental structure of the α chain of the *Propionibacterium shermanii* enzyme (PDB 1REQ). Domains are coloured as follows: blue for the $(\beta/\alpha)_8$ barrel which binds the substrate (desulfo-CoA, as existing in the 1REQ structure, in pink), and green for the cofactor adenosylcobalamin (in the back). Asparagine 195 (corresponding to Asn219 in human) is shown in a CPK representation and is buried between the β-barrel structure and one of the α-helix surrounding this barrel.

secondary structure motif of the molecule (Figure 3). However, it does not appear to have direct interaction with the substrate as its side chain is turned outside of the β-barrel and buried by one of the surrounding α-helices.

To determine if the 731 A→T substitution was indeed responsible for the deficient phenotype, we performed transient expression studies in SCK2, a *mut*$^{\circ}$ cell line immortalised from the fibroblasts of patient 1. As expected, transfection of the wild-type expression vector (pCMVmut) resulted in an increased propionate incorporation by SCK2 cells (Figure 4). In contrast, transfection of the vector containing the 731 A→T substitution (pCMVmut731A→T) did not change propionate incorporation over the background value measured after transfection with pCMVmutNSCD238, a vector in which MCM cDNA contains a null mutation.

Discussion

Here, we report a novel mutation in the *MUT* gene which we observed in five independent Caucasian families with MMA. Insertion of this mutation in a wild-type MCM expression vector abolishes its ability to restore enzyme activity in a MCM-deficient cell line. Thus N219Y is indeed the causative mutation of the patients' *mut*$^{\circ}$ phenotype. Residual MCM activity was very low in the patients' fibroblasts and all the patients were affected with a severe clinical form and a low valine tolerance (<800 mg/d). All experienced acute intercurrent decompensations and one was lethal in one patient. The *mut*$^{\circ}$ phenotype observed in the heterozygous patients suggests that the second mutation involved is also of the *mut*$^{\circ}$ type, although, to our knowledge, the phenotype resulting from the *mut*$^{\circ}$/*mut*$^{-}$ compound heterozygosity has not been precisely characterised.

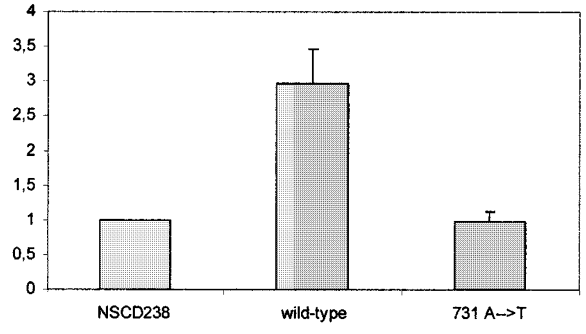

Figure 4 Transient expression of MCM activity in the SCK2 *mut*$^{\circ}$ cell line. MCM activity was estimated by the [^{14}C]-propionate incorporation test during the transient phase of gene expression, 48 h after transfection of MCM cDNA containing vectors in SCK2 cells. pCMVmut is wild-type expression vector, pCMVmut731A→T the vector in which the 731 A→T substitution has been introduced and pCMVmutNSCD238 a null expression vector. Results are expressed related to the basal activity obtained with pCMVmutNSCD238. The results shown are the mean and SD of three independent experiments performed in duplicate.

Previous analysis of natural *mut*$^{\circ}$ mutations on a homology model for human MCM has identified two types of mutations. Most of them affect a residue directly involved in catalysis, but modelling predicts that some mutations act rather by interfering with protein folding.[11] Our modelling analysis of the N219Y mutation suggests that it belongs to this second type. Indeed, substitution of Asn219 by a Tyr residue might severely influence the overall MCM conformation in different ways. First, as this residue is located in a regular secondary structure motif (end of a beta-strand within the beta-barrel), it might result in an impaired folding process of this motif and/or in a poor structure stability. Second and most importantly, Asn219 has PHI/PSI angle values typical of left-handed helix, a conformation almost exclusively occupied by glycine and asparagine residues. Substitution by a tyrosine should therefore inhibit the formation of such particular conformation. Finally, the much bulkier and hydrophobic Tyr side chain might hamper the positioning of the adjacent helix (due to a steric clash) and lead to the disruption of the whole N-terminal beta/alpha barrel domain of the MCM molecule. The *mut*$^{-}$ mutations concern residues mainly located in the B_{12}-binding domain. Few belong to the eight-stranded beta/alpha barrel domain but lie at the interface between these two domains that form a crevice which accommodates the adenosylcobalamin molecule. These mutations would alter the adenosylcobalamin binding.[7]

Genetic heterogeneity in *mut* MMA is high. Since the cloning of the *MUT* gene in 1988 by Ledley *et al*,[17] at least 49 different mutations have been identified. All were reported in only one or two patients except for two (G717V and E117X) which are found in specific ethnic groups. G717V was found in four African-American and one Ghanaian MMA patients with the *mut*$^{-}$ phenotype, suggesting that this mutation might be ancient and of African origin.[8] E117X is a *mut*$^{\circ}$ mutation and has been found with a high prevalence (24% of the alleles tested) in a group of Japanese MMA patients.[9,10] To our knowledge, no frequent *mut* mutation has been described to date in the Caucasian population. In our *mut* population (19 patients), the N219Y mutation has been found in 21% of the alleles screened. The carrier frequency is even higher (57%) among our seven *mut*$^{\circ}$ patients. Four of the families in which we observed the N219Y mutation were of French descent. Screening an anonymous population of mentally retarded children living in France unexpectedly showed a 2% frequency of N219Y carriers but none were found in a similarly sized-group of normal children. As there is no reason to expect that carriership for N219Y leads to mental retardation, we can consider a global carrier frequency around 1%. This would suggest that N219Y is a frequent mutation in the French population and that the incidence of MMA in the French population is underestimated.

The finding of N219Y in a Turkish family indicates that N219Y is also present in Caucasian populations from the

Eastern part of the Mediterranean Basin. This could suggest an ancient origin to this mutation. Alternatively, the fact of finding the mutation on chromosome of different haplotypes may suggest independent origins to the mutation. Finer mapping of polymorphic markers around the mutation will be necessary to solve this issue.

Acknowledgments

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