

Mutation Rates in Humans. I. Overall and Sex-Specific Rates Obtained from a Population Study of Hemophilia B

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Summary

A population-based study of hemophilia B mutations was conducted in the United Kingdom in order to construct a national confidential database of mutations and pedigrees to be used for the provision of carrier and prenatal diagnoses based on mutation detection. This allowed the direct estimate of overall (μ), male (ν), and female (u) mutation rates for hemophilia B. The values obtained per gamete per generation and the 95% confidence intervals are $\mu = 7.73 (6.29-9.12) \times 10^{-6}$; $\nu = 18.8 (14.5-22.9) \times 10^{-6}$; and $u = 2.18 (1.44-3.16) \times 10^{-6}$. The ratio of male-to-female mutation rates is 8.64, with a 95% confidence interval of 5.46–14.5. The higher male rate was not caused by a much higher rate of transition at CpG sites in the male. Attempts to detect evidence of gonadal mosaicism for hemophilia B mutation in suitable families did not detect any instances of ovarian mosaicism in any of 47 available opportunities. This suggests that the risk of a noncarrier mother manifesting as a gonadal mosaic by transmitting the mutation to a second child should be <0.062 .

Introduction

The X-linked recessive disease known as hemophilia B (MIM 30690) or coagulant factor IX deficiency (Giannelli and Green 1996) offers an opportunity to gain important insights into human gene mutation. The clear-cut expression of each hemophilia B gene in the male, the rapid renewal of these genes in the population, and the near-normal life span of even severely affected males

in current populations with good medical care all contribute to the value of this disease for the investigation of the rates of germline mutations.

The factor IX gene is 33.5 kb in length and comprises 8 exons encoding a polypeptide of 454 residues that is produced mainly in the liver and is released into the circulatory system after cleavage of a prepropeptide of 39 amino acids (Anson et al. 1984; Yoshitake et al. 1985).

In countries with long-established national health services, such as the United Kingdom and Sweden, virtually full ascertainment of affected individuals is achieved, since every patient is registered on a national basis at specialist centers responsible for a nationally integrated system of care. This situation, rare in human genetics, greatly facilitates investigations. Thus, we began our population studies on hemophilia B in Sweden and continued in the United Kingdom (Green et al. 1991; Saad et al. 1994). In the latter country, we have created a confidential database of hemophilia B mutations and pedigrees to optimize the genetic service given nationally to families affected by hemophilia B (Saad et al. 1994; P. M. Green, S. Saad, G. Rowley, F. Giannelli, unpublished observations). We now report an analysis of mutation rates in the factor IX gene and discuss its relevance to mutations in the human genome.

Material and Methods

Samples

Blood samples were requested from hemophilia centers in the United Kingdom, from U.K.-resident hemophilia B patients and relatives. Ten milliliters of blood collected in EDTA either was sent directly to Guy's Hospital or was frozen and extracted later.

DNA Preparation, Amplification, and Mutation Analysis

DNA was extracted from peripheral blood cells by standard procedures (Miller et al. 1988). Factor IX exons were amplified by established protocols using primers flanking each exon, as described elsewhere (Green et al. 1989). Radioactive chemical mismatch cleavage was performed as described by Montandon et al. (1989).

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Fluorescent solid-phase chemical cleavage of mismatch was performed with internal fluorescent labeling (fluorescent dUTPs) and biotinylated primers (Rowley et al. 1995). Both the above methods detect and locate any mismatches between the test DNA and control, allowing sequencing to be directed to one of two positions within the PCR product. Direct DNA sequencing was carried out as described elsewhere (Green et al. 1989; Winship 1989) to identify the mutation. Mothers and grandmothers of isolated patients were screened by sequencing the relevant exon for the mutation found in the proband. Carriers of new mutations were screened by both mismatch analysis and sequencing. Origin of mutation in one of the grandparents was established by analysis of intragenic polymorphisms: *Bam*HI, *Dde*I, *Taq*I, *Mnl*I (as described in Green et al. 1992), and *Hha*I (Winship et al. 1989).

Identification of Independent Mutational Events

Patients having the same mutation were subjected to intragenic haplotype analysis for the most frequent and informative markers (Green et al. 1992). This was generally performed on *Dde*I, *Taq*I, *Mnl*I, and *Hha*I (see above). In families with no extensive history of hemophilia, evidence for independence was also sought by determining the origin of the mutation, because changes occurring in recent generations of nonrelated families are independent.

Statistical Analysis

The rationale for the estimation of mutation rates from population data is explained in the Results section. The confidence intervals for the mutation rates were calculated exactly, where possible (for u , v/u from the number of grandpaternal and grandmaternal mutations), and by simulation otherwise, with the assumption of a binomial distribution for the number of carriers identified. We assumed that the only source of variation was from the proportion of mutation carriers identified. The numbers of families of the specific structure and with information available were assumed to be constants.

Results

Population Analyzed

The United Kingdom's hemophilia B register lists 1,109 patients served by 95 hemophilia centers, which are subdivided into 26 "comprehensive care hemophilia centers" and 69 smaller hemophilia centers. Patients were referred to us from all (except two) comprehensive care centers and from most minor centers. Since the latter share their patients with the comprehensive care centers, minor centers that have not contributed directly may have done so indirectly through their regional compre-

hensive care centers, so the number of noncollaborating minor centers is difficult to assess. We have analyzed 1,111 samples, comprising 540 patients and 571 relatives. These represent 424 independent families affected by hemophilia B. Since the national register does not contain data on family relationships, the total number of hemophilia B families in the United Kingdom is not known. However, from the pedigrees of the patients referred to us, we can arrive at the number of patients represented by the families that we have analyzed. This is 800 patients, 72% of the total, but of course this number is only as precise as the pedigree data allow. The fraction of patients missing is mainly accounted for by centers that elected not to collaborate. A small deficit of mild cases is, however, likely, since these patients have infrequent interaction with centers that act as referral points. To counteract this expected trend, we have specifically asked centers to refer mild patients, and their positive response is demonstrated by the referral, among other patients, of eight individuals with no family history of hemophilia B and with a single, low factor IX assay result that could not be confirmed by subsequent tests. These patients are unresolved diagnostic puzzles, since none showed mutations of the FIX gene, and they are therefore excluded from this study.

Mutation Analysis

The hemophilia B mutation was sought in each of the 424 independent families by examination of the factor IX gene of an index person. This, in 403 families, was an affected male; in 12, an obligatory carrier; and, in 9, a potential carrier. A mutation was found in 398 of the 403 index patients, in 11 of the 12 obligatory heterozygotes, and in 3 of the 9 potential carriers. Of the 412 mutations characterized, 167 were unique, whereas 50 were observed more than once and account for 245 families. Of these families, 135 show mutations that must be of independent origin, because these mutations occur in factor IX genes with different arrangements of polymorphic markers or because they have occurred so recently that they cannot be shared by other families with an identical sequence change. The remaining 110 families show repeats of some of the above mutations. In 19 families, the identical mutation is thought to derive from a common ancestor, as the result of a founder effect that so far appears to be limited to the United Kingdom, whereas in the others, which show repeats of 29 different mutations, positive evidence of independent origin cannot be obtained. The types of mutations observed and their frequency are shown in table 1.

Family Studies

Samples from family members were sought from every kinship that could provide information on mutation

Table 1**Types of Hemophilia B Mutations Observed in the United Kingdom**

LOCATION	NO. OF MUTATIONS IN CATEGORY										
	Promoter	Missense	Nonsense	Splicing Defects	Transitions		Transversions	Frameshifts	In-Frame Deletions/ Insertions	Gross Deletions	Total
					CpG	Other					
In whole gene:											
Independent origin	9	187	39	38				17	7	5	302
Total	13	262	48	58				17	7	7	412
In coding sequence:											
Independent origin					76	80	70	17	7	5	255
Total					143	95	72	17	7	7	341

rates or occurrence of gonadal mosaicism—that is, the families of sporadic patients and the families with a single affected sibship. Every request to the hemophilia centers was accompanied by the appropriate specific motivation and the recommendation to inform us of any inability to provide the requested specimens. Whenever this information was not provided, the sample was requested three times before it was concluded that it was unobtainable.

Of the 211 sporadic patients, 79 had only the mother available for study, and 31 had both the mother and grandmother available. In 10 of the latter three-generation families, the grandfather was also available. Twenty-five nonsporadic families appeared to have a single affected sibship. In five of these families both the mother and the maternal grandmother were available for study; in one the grandfather was also available.

Finally, in families where the patient's mother was the origin of the mutation, samples were requested from the patient's sisters, and, when one of the grandparents was the origin of mutation, samples were requested from the patient's maternal aunts, because carrier status in such sisters or aunts would reveal gonadal mosaicism.

Direct Estimate of the Overall and Sex-Specific Hemophilia B Mutation Rates

Since our studies are clearly and explicitly population based, we can estimate mutation rates directly by reference to demographic statistics from the United Kingdom for the past 75 years. These should adequately cover our population of hemophilia B patients. Statistical data on the hemophilia patients registered in the United Kingdom were published by Rizza and Spooner in 1983. These authors calculate a median life expectancy of 69.1 years for severely affected hemophiliacs and 72.8 years for normal males. Since our population includes not only severe but also moderate and mild cases that represent 64% of the whole patient population, it is felt that their median life expectancy should

not differ from that of normal males sufficiently to require specific consideration.

As mentioned previously, the families referred to us account for 72% of the current population affected by hemophilia B. However, in 12 families a mutation could not be found, and for 17 the pedigree was not supplied. Therefore, the families with both mutation and pedigree information represent 67.2% of the total population of the United Kingdom. The bulk of our data was obtained by 1995, and at that time the population of the United Kingdom comprised 28.727 million males and 29.878 million females (Office for National Statistics 1997b).

To estimate the mutation rates, it is necessary to determine how many patients and how many patients' mothers are new mutants. Clearly (barring gonadal mosaicism), all patients who are new mutants must be in the sporadic families, and they are considered to be the result of mutation in their mother's germline. We have 211 such families, and in 110 of these the patient's mother was available for carrier tests. Of the testable mothers, 88 (80%) were found to be carriers and 22 were homozygous normal. Thus, extrapolating from this datum, we find that $0.2 \times 211 = 42.2$ is the estimated number of new mutant males in the sample of the United Kingdom's hemophilia B population that we have examined. Since this represents 0.672 of the whole population, the female mutation rate is estimated by $u = 42.2 / (0.672 \times 28,727,000) = 2.186 \times 10^{-6}$ (95% confidence interval = 1.44–3.16).

The patients' mothers who are new mutants must be found both in the sporadic families mentioned above and in those which appear to contain a single affected sibship. In 101 of the sporadic families, the mother was not available, but, on the basis of the proportions of carrier and noncarrier mothers found in the 110 informative families, it can be estimated that $101 \times 88 \div 110 = 80.8$ of the missing mothers should be carriers, giving a total of 168.8 carrier mothers. Our population also contains 25 nonsporadic families in which a single sibship appears to be affected. The patients' mothers in

Table 2
Distribution of Hemophilia B Mutations According to Type and Sex of Origin

SOURCES OF DATA AND SEX OF ORIGIN	NO. OF MUTATIONS				
	Transitions		Transversions	Small Deletions/ Insertions	Total
	CpG	Non-CpG			
Current study:					
Known	12	14	12	4	42
Unknown	68	91	68	28	255
Male	2	5	6	3	16
Female	10	9	6	1	26
Montandon et al. (1992), Kling et al. (1992):					
Male	3	2	1	0	6
Female	1	0	1	0	2
Ketterling et al. (1993):					
Male	3	4	4	1	12
Female	1	3	6	5	15

these sibships should all be carriers, unless they are gonadal mosaics. In total, therefore, our population has 193.8 carrier mothers who should comprise all carriers of a new mutation that was transmitted to at least one son.

To define the number of these carriers that have a new mutation, it is necessary to test the patients' maternal grandparents. This was possible in 35 families (31 sporadic and 4 with a single sibship affected), and in 27 of these families the mother was a new mutant. Of these mothers, 24 belonged to sporadic families and 3 to families with a single affected sibship. Extrapolating from these data, we find that the estimated number of mothers who are new mutants in the hemophilia B population considered in our study is $193.8 \times 27 \div 35 = 149.5$ and that the estimated number in the whole population is $149.5 \div 0.672 = 222.5$.

However, the general population of the United Kingdom must also comprise carriers of new hemophilia B mutations who have gone unnoticed because they have not borne an affected son. The number of these carriers is determined by the probability that a carrier has of bearing an affected son. This can be estimated by referring to demographic data on the population of the United Kingdom. Four sets of data are required: (1) the average number of liveborn for mothers of age ≥ 45 years who can be expected to have completed their families, (2) the average number of liveborn children for the cohorts of younger women, (3) the proportion of women of different ages in the population, and (4) the sex ratio of liveborn children. Since most of our data come from England and Wales, we have used the figures on family size for England and Wales, available annually since 1920, and have used the sex ratio of liveborn children observed in 1995 (Office for National Statistics 1997a). This indicates that each woman should have produced,

on average, over all age groups, 0.70864 sons. Hence, a carrier has a $0.5 \times 0.70864 = 0.35432$ chance of producing an affected son, and the estimated frequency of carriers of a new hemophilia B mutation in the population of the United Kingdom is the estimated total number of new carriers in the population: $222.5 / (0.35432 \times 29,878,000) = 21.02 \times 10^{-6}$.

Since the new mutation may have arisen in either of the carrier's parents, 21.02×10^{-6} estimates the sum of the male and female mutation rates, $v + u$. Since u was previously estimated, $v = (21.02 - 2.18) \times 10^{-6} = 18.84 \times 10^{-6}$. It follows that the overall mutation rate $\mu = (v + 2u) / 3 = 7.733 \times 10^{-6}$ (95% confidence interval 6.29–9.12). The rate of male-to-female mutation is $v/u = 8.64$ (95% confidence intervals for v and u are 14.5–22.9 and 1.44–3.16, respectively, and that for v/u is 5.46–14.5).

An independent estimate of the ratio of sex-specific mutation rates (v/u) is the ratio of mothers with new mutations that originated in grandfathers versus those with mutations that originated in grandmothers. In our study, 20 informative families are available: 16 of these showed mutations of grandpaternal origin, and 4 showed mutations of grandmaternal origin. This gives $v/u = 4$ (95% confidence interval 1.258–14.13).

If the sex-specific mutation rates differ according to mutation type, this should be reflected in the distribution of types among mutations originating in males and females (table 2). The 42 mutations of clearly defined origin therefore were divided into small deletions and/or insertions, transitions at CpG sites, transitions not at CpG sites, and transversions, and the distribution of each one of these classes versus all the others among mutations of male or female origin was examined. Fisher's exact test did not reveal a statistically significant difference either when the above data from the United

Kingdom were tested or when our data from the United Kingdom and from Sweden (Kling et al. 1992; Montandon et al. 1992) were added to those of Ketterling et al. (1993). Since the male origin of a hemophilia B mutation can be established only in three-generation families, which are more difficult to obtain than two-generation families, types of mutations predominantly arising in the male should be underrepresented in the group of mutations of known origin, in comparison with the group with undefined origins. However, our data again show no significant difference in the frequency of CpG mutations in the above groups.

Data on Gonadal and Somatic Mosaicism

In hemophilia B, the families that can provide information on gonadal mosaicism are those with a single affected sibship containing at least two patients or a patient and a carrier, those with sibships of sporadic patients born to noncarrier mothers, and those with sibships of patients' mothers who carry new mutations. We tried, therefore, to gather information on these families. The first of the above types of family can be born either to a mother who is a demonstrable carrier or to one with normal somatic cells but mosaic ovaries. In the second type of family, the patient's sisters could be carriers if the mother were a gonadal mosaic, whereas, in the third type of family, the patients' maternal aunts could be carriers if either grandparent were a gonadal mosaic, and patients' uncles could be affected if the grandmother were a gonadal mosaic.

We elected to disregard the families with a single affected sibship containing more than two mutation-carrying individuals, but 49 families with two sibs carrying mutations remained; in 38 of these families, the mother was available for study. All of these mothers were found to be carriers. Twelve sibships born to noncarrier mothers provided a total of 17 informative patient's sibs, and they were all mutation free. In addition, the eight sisters of carriers whose mutation was of paternal origin were mutation free, and so were five brothers and three sisters of patients' mothers whose mutation had arisen in one of her parents. Thus, in none of the opportunities available to us was gonadal mosaicism observed. In particular, we had 47 opportunities to detect ovarian mosaicism (i.e., $38 + 17/2 + 8/[2 \times 8.64] = 47$), without ever succeeding. This suggests that a noncarrier mother should have a risk <6.2% of manifesting gonadal mosaicism by transmission of the mutation to a second child, since 0.062 is the 95% upper exact confidence limit according to the binomial distribution.

In the course of our mutation studies, we also had a chance of detecting somatic mosaicism, because our mutation-screening procedure detects mutant DNA even when it represents merely 10% of the total (Verpy et

al. 1994; A. J. Montandon, personal communication), whereas the sequencing subsequently applied to characterize the mutation detects mutant DNA only when it represents $\geq 30\%$ of the total. Discrepancies between the results of screening and sequencing were not found in any one of the 30 individuals who were carrying a new mutation and who were tested by both these methods.

Discussion

Overall and Sex-Specific Mutation Rates in Hemophilia B

Perfect data for population genetic studies are very difficult to obtain. We have mentioned the positive features of hemophilia B in this regard, such as the full registration of patients in the United Kingdom, and their near-normal median life expectancy, and to this we could add the ease and specificity of clinical diagnosis (Rizza 1997). Unfortunately, our study did not achieve 100% referral, but we feel that the sample examined is representative of the whole because our deficit is mainly due to lack of participation by specific centers, rather than selective referral from centers as a whole. Since its onset, our work was aimed at studying all families with the disease in the United Kingdom, and all centers were aware and were frequently reminded of this fact. Since large families may be shared by several centers, whereas sporadic families usually are not, there is the possibility of overrepresentation of large families; however, this is tempered by the fact that mutational information is particularly useful for counseling the families of sporadic patients, and therefore physicians tend to make an extra effort to obtain a sample from the isolated patient available in such families. Failure to recognize multiple reporting of the same family is unlikely in our study, because each pedigree was used to find matches in the whole pedigree database, and families with identical mutations were investigated as thoroughly as possible, to establish whether their mutations were of independent origin.

In the period between the present and the year of birth of the oldest hemophilia B patient in the United Kingdom, the mean age at death and the median life expectancy have changed for the 36% of patients who have severe disease (Rizza and Spooner 1983; Larsson 1985). This is due to the gradual introduction of more effective treatment that has led to near-normal life expectancy since as far back as 20 years ago. Currently infections resulting from the use of therapeutic blood products are a cause of concern, but they have so far had modest effects on the hemophilia B population of the United Kingdom. The remaining 64% of patients with moderate to mild disease have had near-normal life expectancy even when therapy was less effective than it was 20 years

ago. It is therefore reasonable to assume that the structures of the hemophilia B and normal populations in the United Kingdom are not markedly different.

Our data on hemophilia B derived from a sample that is very likely to be representative of the whole population and that is also the largest sample examined so far. These data therefore provide an excellent opportunity to obtain a direct estimate of mutation rates. This we have done with the help of appropriate demographic data, and the following estimates were obtained for the overall (μ), female (u) and male (v) mutation rates, which are, respectively, $\mu = 7.73 \times 10^{-6}$ (95% confidence interval 6.29–9.12); $u = 2.18 \times 10^{-6}$ (95% confidence interval 1.44–3.16); and $v = 18.82 \times 10^{-6}$ (95% confidence interval 14.5–22.9). This gives a ratio of male-to-female mutation rates of 8.64 (95% confidence interval 5.46–14.5), which is compatible with the independent estimate of the ratio of sex-specific mutation rates obtained by dividing the number of carriers of new mutations whose defective gene is of paternal origin by those whose hemophilia B gene is of maternal origin ($v/u = 4$ [95% confidence interval 1.26–14.1]). Furthermore, the first direct estimate of the ratio of sex-specific mutation rates for hemophilia B obtained on the much smaller Swedish sample ($v/u \sim 11$) lies in the confidence interval of the estimate for the United Kingdom (Montandon et al. 1992).

Ketterling et al. (1993), who also report a high ratio of male-to-female mutation rates in hemophilia B, have suggested that this is mainly due to an excess of CpG transitions in the male gonad. We were unable to confirm this suggestion, since our data do not show any significant difference when the four mutation types (transitions at CpG sites, transitions at non-CpG sites, transversions, and small deletions/insertions) are compared according to sex of origin. Furthermore, the combined data on mutations of known origin from Ketterling et al. (1993), the Swedish sample that we examined elsewhere (Kling et al. 1992; Montandon et al. 1992), and the present study also show no significant differences regarding the above four classes of small mutations. Finally, our accompanying paper (Giannelli et al. 1999 [in this issue]) shows data, on the divergence of human and chimpanzee sequences from the Y and X chromosomes, that are also inconsistent with the contentions of Ketterling et al. (1993), because they do not reveal a much greater proportion of CpG transitions in the Y than in the X chromosome sequences when these transitions are considered in relation to the other transitions, as would be required if the conclusions of Ketterling et al. (1993) were true.

Ketterling et al. (1993) consider the excess of transition at CpG sites in the male to be an important argument in favor of their tenet that the pattern of hemophilia B mutations negates a significant role of en-

vironmental factors in germline mutagenesis, because, in their view, the higher methylation in the male germline would be the cause of such a high male-to-female ratio. Furthermore, they also report an excess of transversion at CpG sites, which they ascribe to the instability of methylated cytosines (Ketterling et al. 1994). However, Chen et al. (1998) report that cytosine methylation enhances guanine alkylation by mutagens; the latter observation by Ketterling et al. (1994) thus could also be construed as evidence of environmental mutagenesis. Our study, however, does not show an excess of CpG transversion (see accompanying paper Giannelli et al. 1999 [in this issue]), but data on this type of mutation are still too scanty to allow us to arrive at reliable conclusions.

A high male-to-female ratio has been reported for other diseases with a mutational spectrum similar to that of hemophilia B. Thus, for example, the study of 126 sporadic families with severe hemophilia A (MIM 30670), by Becker et al. (1996), suggests a ratio of 5–10 for point mutations and a ratio >10 for the inversions that cause almost half of all severe cases of this X-linked recessive disease (Lakich et al. 1993; Naylor et al. 1993). An even higher ratio of male-to-female mutation rates—52—was claimed by Tuchman et al. (1995) for the semidominant X-linked disorder ornithine transcarbamylase deficiency (MIM 31125). That study, however, was based on only 43 mutants collected in different countries.

Gonadal Mosaicism for Hemophilia B Mutations

A question of concern to the genetic counselor is whether mutations occur in such a way as to lead frequently to gonadal mosaicism and hence to significant recurrence risk for “noncarrier” parents. Work on Duchenne muscular dystrophy (DMD [MIM 31020]) has highlighted this problem, since noncarrier mothers of DMD patients were found to have a 20% risk of transmitting the DMD mutation to a second child whenever they transmitted the haplotype present in their sporadic affected sons (van Essen et al. 1992). However, most DMD mutations are gross deletions, and data on DMD may be irrelevant to diseases with a different mutational spectrum, such as hemophilia B. Gonadal and somatic mosaicism have been reported for hemophilia B mutations, but these observations are essentially anecdotal and are difficult to relate to population risks (Taylor et al. 1991; Sommer et al. 1995). Furthermore, a variety of technical and clerical errors may result in apparent mosaicism and may contribute to positive reports. Therefore it would be important to obtain broad-based population data. We have attempted to do so. Our efforts have been rewarded by a moderate number of potentially informative events, and in none of these events

was mosaicism observed. This seems to suggest that gonadal mosaicism for hemophilia B mutations is less common than in DMD, since the probability of a noncarrier mother manifesting as an ovarian mosaic should be <0.062 .

In conclusion, using the population data from the United Kingdom we obtain the best direct estimates so far of the overall and sex-specific mutation rates for hemophilia B. Our data clearly contradict the claim that the higher mutation rate observed in males is essentially due to transitions at CpG sites and thus change the perspective on the causes of the high ratio of sex-specific mutation rates. Finally, the data collected on gonadal mosaicism suggest that this phenomenon is not likely to be a major cause of problems in the genetic service for hemophilia B.

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Electronic-Database Information

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

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim> (for hemophilia A [MIM 30670], hemophilia B [MIM 30690], Duchenne muscular dystrophy [MIM 31020], and ornithine transcarbamylase deficiency [MIM 31125])

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