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Crossovers within a short DNA sequence indicate a long evolutionary history of the *APRT*J* mutation

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Summary. Adenine phosphoribosyltransferase (APRT) deficiency causing 2,8-dihydroxyadenine urolithiasis and renal failure is present at a high frequency among the Japanese but not other ethnic groups. A special type of mutant allele, designated *APRT*J*, with a nucleotide substitution at codon 136 from ATG (Met) to ACG (Thr) is carried by approximately 79% of all Japanese 2,8-dihydroxyadenine urolithiasis patients. We analyzed mutant alleles of 39 APRT deficient patients using a specific oligonucleotide hybridization method after in vitro amplification of a part of the genomic APRT sequence. We found that 24 had only *APRT*J* alleles. Determination of the haplotypes of 194 APRT alleles from control Japanese subjects and of the 48 different *APRT*J* alleles indicated that normal alleles occur in four major haplotypes, whereas all *APRT*J* alleles occur in only two. These results suggest that all *APRT*J* alleles have a single origin and that this mutant sequence has been maintained for a long period, as calculated from the frequency of the recombinant alleles.

Introduction

Recent studies analyzing genes of various genetic diseases at the molecular level have provided clues as to how such disease-causing genes are generated and maintained. Among such genetic diseases, some are present at high frequencies in some ethnic groups but not others. These diseases have been the focus of special interest since common mutational alleles in different families within each ethnic group often have the same origin. Based on such data, the evolutionary history of each mutation can be studied. In the present investigation, we focussed on one such disease, adenine phosphoribosyltransferase (APRT) deficiency.

There are two known types of homozygous APRT deficiency causing 2,8-dihydroxyadenine urolithiasis and renal failure (type I and type II; Simmonds et al 1989). The type I patients show virtually no APRT activity, whereas the type II patients have a partial deficiency (Simmonds et al. 1989). Although type I patients have been found among both Caucasian and Japanese populations, type II patients have thus far been found only among Japanese (Simmonds et al. 1989; Kamatani et al. 1987b); 79% of all the families with homozygous APRT deficiency were of type II (Kamatani et al. 1987b). In addition, over 70% of all families with 2,8-dihydroxyadenine urolithiasis reported in the literature have been Japanese (Simmonds et al. 1989; Kamatani et al. 1988). In previous studies, a mutant enzyme was found in all type II patients examined; it had the same altered kinetic properties, including a reduced affinity for the substrate 5-phosphoribosyl-1-pyrophosphate (PRPP) (Fujimori et al. 1985). The mutant allele, designated *APRT*J*, coding for this mutant enzyme had a nucleotide substitution at codon 136 (ATG to ACG) (Hidaka et al. 1988, 1989), and the disappearance of Met at position 136 in the amino-acid sequence was accordingly shown (Kamatani et al. 1989).

It is clear that nucleotide substitution at codon 136 (ATG to ACG) is the disease-causing mutation, as opposed to a rare linked polymorphism, since all individuals with type II APRT deficiency synthesize mutant APRT protein (Kamatani et al. 1987b, 1989), and the nucleotide substitution at codon 136 is the only amino-acid-changing substitution seen in the *APRT*J* allele (Hidaka et al. 1988). Additional evidence revealed that the predicted amino-acid substitution is within the putative PRPP binding site of the enzyme (Dush et al. 1985; Hershey and Taylor 1986), and the mutant protein coded for by *APRT*J* has a reduced affinity for PRPP (Fujimori et al. 1985). All other mutant alleles from Japanese APRT deficient patients so far identified synthesized no enzyme protein, and were designated as *APRT*Q0* (Fujimori et al. 1985).

We report here the historical interrelation between the *APRT*J* mutation and very tightly linked polymorphic markers.

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Materials and methods

Diagnosis of APRT deficiency

Diagnosis of APRT deficiency was made by the determination of the enzyme activity in hemolysates and the culture of viable T-cells with 6-methylpurine (Kamatani et al. 1985). Thus, when viable T-cells from patients were resistant to 6-methylpurine, they were diagnosed as homozygotes (both of the two alleles are defective). When the APRT activities determined in the hemolysates were completely deficient, the patients were classified as type I. When they were only partially deficient, the patients were classified as type II (Simmonds et al. 1989).

In vitro amplification of the APRT-specific sequence

DNA was extracted from mononuclear cells from blood samples or cells from B cell lines obtained from different subjects. A part of the APRT gene in these samples was amplified in vitro by the polymerase chain reaction (PCR) (Saki et al. 1986) using synthetic primers PN-5 (5'GATGATCTGCTGGCCACTGG3') and PN-9 (5'CAGCTGGAGATGTTGGGCTG'3) (see Fig. 1 for the locations of these sequences in human APRT DNA) and *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, Conn., USA). PN-5 has a nucleotide sequence of 1812 to 1831 of the sense strand of the human genomic APRT sequence ("A" in the initiation codon ATG was counted as position 1), whereas PN-9 anneals to nucleotides 2238 to 2219 of the sense strand. The temperatures and time used for heat-denaturing, annealing and polymerizing steps were 94°C, 65°C and 72°C for 2, 3 and 3 min, respectively. These steps were repeated for 35 cycles. The combination of primers PN-5 and PN-9 succesfully amplified the target 427-bp sequence, which includes the *APRT*J* mutation site located at position 2069. When the amplification was successful, amplified bands could be visualized with ethidium bromide after 3% agarose gel electrophoresis.

Sequence-specific oligonucleotide hybridization

After blotting the amplified DNA on nylon membranes (Biodyne A, PALL Biosupport, N. Y.), dot hybridization was performed using radiolabeled oligonucleotides as probes. Thus, PN-3 (5'CAGGAACCACGAACGCTGC3') for *APRT*J*-specific and PN-4 (5' CAGGAACCATGAACGCTGC3') for normal (non-*APRT*J*) sequences were end-labeled with [γ^{32}P]ATP (6000 Ci/mmol) and T4 polynucleotide kinase, and hybridized with the amplified DNA blotted on the membranes. The methods for the hybridization and wash procedures were as described by Saiki et al. (1986) except that the washing temperature was 53°C. The membranes were then exposed to X-ray films for 8 h.

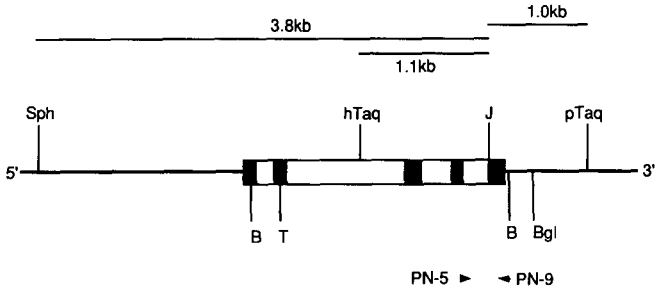

Fig. 1. Map of the human genomic APRT sequence showing physical distances between various restriction sites. *Closed blocks* are coding sequences. *B, T* and *Bgl* indicate non-polymorphic cut sites with *Bam*Hl, *Taq*l and *Bgl*ll, respectively. *PN-5* and *PN-9* indicate the locations of sense and anti-sense sequences respectively, used for the PCR method. *J: APRT*J* mutation site

Determination of haplotypes

The human APRT gene is short with 5 separate coding sequences spreading over 2.2 kb DNA. A highly polymorphic *Taq*I restriction fragment length polymorphism (RFLP) site (h*Taq*) is located within intron 2 of the human APRT gene, whereas a poorly polymorphic *Taq*I RFLP site (p*Taq*) is downstream of the gene (Fig. 1). The *Sph*I RFLP site *(Sph)* is located upstream of the APRT gene. The *Sph* and h*Taq* sites are approximately 3.8 kb and 1.1 kb, respectively, upstream of the *APRT*J* mutation site, which is in exon 5, whereas p*Taq* is about 1.0 kb downstream of the same mutation site (Fig. 1). RFLP sites h*Taq* (Stambrook et al. 1984) and *Sph* (Arrand et al. 1987), both of which are highly polymorphic among Caucasians, have been described. The physical distances between restriction sites, B in exon 1, T, h*Taq*, B in the 3' noncoding region, and the mutation site J (Fig. 1), were calculated from the published sequence (Broderick et al. 1987; Hidaka et al. 1987); others were determined by the double digestion procedure.

For the determination of RFLP patterns, DNA from each individual was digested with either *Sph*I or *Taq*I, and Southern blot analysis was performed by standard procedures using radiolabeled cDNA of human APRT.

Results

Venous blood was collected from 39 patients with 2,8-dihydroxyadenine urolithiasis and from 137 control subjects, all Japanese. All of the 2,8-dihydroxyadenine urolithiasis patients tested were homozygously deficient (both alleles are defective) in APRT, as determined by the resistance of viable T-cells to 6-methylpurine (Kamatani et al. 1985). Among the 39 patients, 9 were type I and 30 were type II, as determined by the enzyme assay.

Use of the PCR procedure and specific oligonucleotide probes clearly showed whether each individual had the *APRT*J* or non-*APRT*J* alleles (or both), except for rare cases in which amplification of the target sequence was unsuccessful. The reason for the unsuccessful amplification was unclear, but repeated experiments almost always amplified the target sequences of the same samples. Absence of hybridization because of the unsuccessful amplification of the specific DNA sequence could be readily distinguished from that caused by the absence of the allele in the individual since, in the former case, the samples hybridized with neither PN-3 nor PN-4, whereas in the latter, the samples hybridized with either. We confirmed that all the 30 type II patients, but none of the 9 type I patients or 97 control subjects tested, carried the *APRT*J* mutation (Fig. 2). Conversely, samples from all control subjects and the 9 type I patients, but not those from 24 out of 30 type II patients, contained non-*APRT*J* alleles (Fig. 2). These observations indicate that none of the type I patients, but all the type II patients, have *APRT*J* alleles. These data also indicate that a small percentage of type II patients (6/30) have another mutation in addition to the *APRT*J* mutation (compound heterozygotes). These samples with both *APRT*J* and non-*APRT*J* mutations were excluded from the present study to avoid confusion.

We subjected DNA samples to Southern blot analysis using a human APRT cDNA probe, the object being to search for RFLPs. As shown in the genomic APRT map in Fig. 1, there are three RFLP sites within and near the

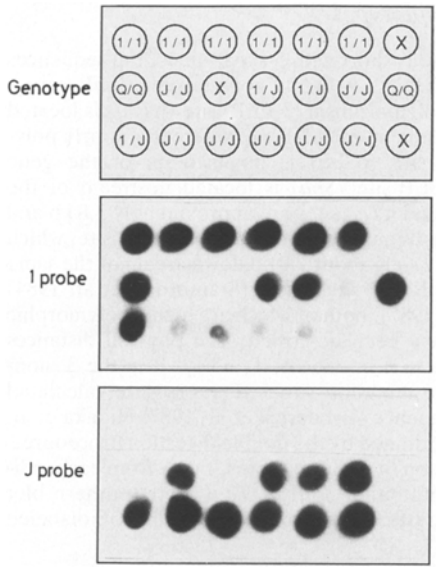

Fig. 2. Sequence-specific hybridization of synthetic DNA probes with *APRT*J* and non-*APRT*J* alleles. After amplification of part of genomic APRT sequences by the PCR procedure, the samples were dot-blotted onto nylon membranes. Probes, PN-4 *(1 probe)* or PN-3 *(J-probe)* were end-labeled and hybridized with the blotted samples. Genotypes are shown in the *top block (1/1: APRT*1/APRT*1, 1/J: APRT*1/APRT*J, J/J: APRT*J/APRT*J, Q/Q: APRT*Q0/APRT*Q0). X* indicates that amplification was unsuccessful for these samples

Table 1. Haplotypes of *APRT* alleles. + indicates that the restriction enzyme was able to cut the RFLP site, − indicates that it failed to cut that site. See Fig. 1 for the location of each RFLP site. *APRT*1* normal allele

RFLP sites			Alleles		
Sph	h*Taq*	p*Taq*	*APRT*1*	*APRT*J*	*APRT*Q0*
+	+	+	42	0	14
−	+	+	42	0	3
+	−	+	40	42	0
−	−	+	68	6	0
−	+	−	1	0	0
+	−	−	1	0	0
Total			194	48	17

human APRT gene, two are highly polymorphic (*Sph* and h*Taq*), the other only poorly polymorphic (p*Taq*). These highly polymorphic RFLPs were present before racial divergence, as both Caucasian (Stambrook et al. 1984; Arrand et al. 1987) and Japanese populations have the same common RFLPs. Since familiy studies were not performed on the control subjects, the haplotype of each allele could be determined only when an individual was heterozygous at less than two RFLP sites. We collected a total of 194 such alleles from 137 normal Japanese subjects (i.e. 40 were not used as they showed heterozygosity at more than one RFLP site) (Table 1). Data from all homozygous APRT deficient individuals were included here since each was heterozygous at, at most, one RFLP site.

As shown in Table 1, normal alleles had four common (+++, −++, +−+ and −−+) and two rare (−+− and +−−) haplotypes. A χ^2 analysis of a 2 × 2 table did not reject the non-association hypothesis ($P > 0.1$) between the two common RFLP sites (*Sph* and h*Taq*), thereby suggesting that these polymorphisms have been present long enough to dissolve the linkage disequilibrium. We then tested samples from the 24 patients with type II APRT deficiency, all of whom possess two *APRT*J* alleles. Among a total of 48 alleles, 42 (87.5%) showed the +−+ haplotype, whereas the remaining 6 (12.5%) showed the −−+ haplotype (Table 1).

One of the 18 *APRT*Q0* alleles showed an aberrant band, as previously reported (Hidaka et al. 1988). In this allele, a major gene alteration can be expected since digestion of this allele with other restriction enzymes such as *Eco*RI and *Bam*HI also showed aberrant bands (data not shown). All the other *APRT*Q0* alleles showed either the +++ (14/17) or −++ (3/17) haplotype, and only these 17 alleles are included in Table 1.

Our data show that there is a strong linkage disequilibrium between the *APRT*J* mutation site and h*Taq*. Thus, although 44% and 56% of the control APRT alleles (without the *APRT*J* mutation) showed h*Taq*+ and h*Taq*− RFLPs, respectively, all the alleles having the *APRT*J* mutation showed a h*Taq*− RFLP, thereby indicating a strong association between the *APRT*J* mutation and the h*Taq*− RFLP. χ^2 analysis indicates that the non-association hypothesis can be rejected ($P < 10^{-7}$). A positive association is also present between the *APRT*J* mutation and *Sph*+ RFLP ($P < 10^{-7}$).

Discussion

It is unlikely that mutant *APRT*J* alleles have multiple origins, for the following reasons. (a) If *APRT*J* alleles were a reflection of the same disease-causing mutation that occurred more than once, then normal alleles with the h*Taq*− RFLP should have been the exclusive targets of the mutations, even though normal alleles have almost equivalent proportions of the h*Taq*− and h*Taq*+ RFLPs. (b) Of all defective APRT alleles among the Japanese, 69% are found to be *APRT*J*, but none of the Caucasian APRT deficient patients had *APRT*J* alleles; hence multiple *APRT*J* alleles are probably not the result of a hot spot for mutations. (c) The presence of a hot spot leading to the *APRT*J* mutation also disagrees with the finding that none of the in vitro new mutations at the APRT locus in human B-cells had characteristics of the *APRT*J* allele (Kamatani et al. 1987a). Therefore, the *APRT*J* alleles from different Japanese families probably derive from a single origin.

If all *APRT*J* alleles from different Japanese families do indeed derive from a single mutational ancestor gene, then these alleles should continue to have the same haplotype, unless additional mutations, crossover or gene conversions have taken place. Since all *APRT*J* alleles tested had either the +−+ or −−+ haplotype, the possibility that recombinations have not occurred be-

tween the *APRT*J* site and h*Taq* cannot be ruled out. In contrast, crossovers between the *APRT*J* mutation site and *Sph*, or mutations at *Sph* should have occurred since the association between the *APRT*J* mutation and the *Sph*+ RFLP is incomplete. When we compare the probability that a crossover occurred between the *APRT*J* mutation site and *Sph* (3.8 kb apart) with the probability that a point mutation destroying the *Sph*I recognition sequence occurred, the former (approximately 3.8×10^{-5} per generation, if the rate of recombination is 10^{-8} per bp per generation; Cox et al. 1985) is much higher than the latter (approximately 6×10^{-8} per generation, if we assume that the rate of point mutation is 10^{-8} per bp per generation; Neel et al. 1986).

In the case of *PI Z* alleles, which cause an α1-antitrypsin deficiency in Caucasians, only 1/52 of the *PI Z* alleles reflected a possible crossover between two RFLP sites (about 9 kb apart) near the *PI Z* mutation site (Cox et al. 1985), clearly indicating that they are of a single origin. Assuming that there is one meiotic crossover per 10^8 base pairs per generation, the authors calculated that 216 generations, or 6480 years, would be required to generate 1.9% crossovers. Assuming that all *APRT*J* alleles have a single origin, we calculated, by the method of Bodmer (1972), how long it would take for the linkage disequilibrium (Δ) to decrease to the current level. We assumed that, at generation 0, all *APRT*J* alleles had a *Sph*+ RFLP, and the frequency of *APRT*J* alleles *(p)* and that of *Sph*+ alleles (83/194 = 0.428) in the population have not changed since generation 0. At generation 0, Δ was $p - 0.428p = 0.572p$ (Δ_1), but now (generation *n*), it is $p(42/48 - 0.428) = 0.447p$ (Δ_2). Therefore, if the recombination fraction (θ) between the *APRT*J* mutation site and *Sph* (3.8 kb apart) is 3.8×10^{-5}/generation, then: $(1-\theta)^n = (1-3.8 \times 10^{-5})^n = (\Delta_2/\Delta_1) = 0.447p/0.572p = 0.781$. Therefore, the number of generations *(n)* required for Δ to decrease from $0.572p$ to $0.447p$ will be: $n = 6505$

Thus, according to this method, the age of the *APRT*J* mutation is calculated to be 6505 generations or 130092 years (one generation was assumed to be 20 years).

The *APRT*J* mutation calculated here is surprisingly old; it is much older than the ages of the β^s-globin mutation (1000–2000 years; Bunn and Forget 1986) and the *PI Z* mutation (6480 years). In the case of the *PI Z* mutation, the distance between the polymorphic marker and the mutation site was longer and the percentage of the recombinant alleles was smaller than in the *APRT*J* mutation. Another piece of evidence suggesting the old age of the *APRT*J* mutation is a nucleotide difference found in intron 2 between two different *APRT*J* alleles (Hidaka et al. 1988).

There are several inaccurate values in the calculation of the age of the *APRT*J* mutation. For example, the rate of recombination between *Sph* and the *APRT*J* mutation site may be higher than the average. Indeed, 3–30 fold and 24 fold higher than the average rate of crossovers have been described near the β-globin gene (Chakravarti et al. 1984) and the insulin gene (Chakravarti et al.1986), respectively. If the crossover rates were 3 fold and 30 fold higher than the average, then the calculated age of the *APRT*J* mutation would be 43360 years (2168 generations) and 4340 years (217 generations), respectively.

Although the procedures for calculating the ages of mutations from linkage disequilibrium are not likely to lead to accurate values, it does nevertheless seem that the *APRT*J* mutation has a long history. Indeed, the validity of the data presented here can be tested by analyzing mutational APRT alleles in other ethnic groups, especially in Asians. Since the detection of heterozygotes for the *APRT*J* mutation is not difficult, such investigations are feasible.

The presence of the same mutations in many separate families has been noted in other clinical entities. Thus, in hemoglobinopathies (Weatherall et al. 1989), thalassemias (Weatherall et al. 1989), Gauscher's disease (Tsuji et al. 1987) and phenylketonuria (DiLella et al. 1987), the same mutant genes in each ethnic group often have the same haplotype, and hence may derive from a single mutation. In addition, recent investigations have shown that about 69% of the cystic fibrosis genes among Caucasians have a single mutation, and that these mutant genes may have a single origin (Riordan et al. 1989). Since cystic fibrosis is present widely in Europe and is in about 5% of the normal Caucasian population, this special mutant gene is also likely to have an ancient origin. At least in the case of sickle cell anemia, compensating advantages play an important role (Weatherall et al. 1989), but in many other diseases, it is controversial whether the compensating advantage, a founder effect or random genetic drift (Ohta 1973) is responsible for amplification and retention of disease-causing genes. With regard to *APRT*J*, evidence for or against compensating advantage is not available. To examine various hypotheses regarding the roles of deleterious genes in evolution, it is important to expand our knowledge of the historical fate of disease-causing genes in general. Historical documents have been useful in such cases, including Tay-Sachs gene(s) among the antecedents of modern Ashkenazi Jewry (Petersen et al. 1983) and porphyria variegata among Afrikaners in South Africa (Diamond and Rotter et al. 1987). However, such a strategy is of limited value, especially when mutant genes have persisted for long periods of time. A more practical method is to investigate the histories of disease-causing mutations, based on molecular biological data. Our data suggest that some disease-causing genes persist in some populations for unexpectedly long periods.

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