Am. J. Hum. Genet. 48:552–562, 1991

Diagnosis of Heterozygous States for Adenine Phosphoribosyltransferase Deficiency Based on Detection of In Vivo Somatic Mutants in Blood T Cells: Application to Screening of Heterozygotes

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Summary

An accurate diagnosis of heterozygotes for autosomal recessive disorders with unknown mutations can be difficult. Using a unique phenomenon occurring in vivo, we designed a method for the diagnosis of heterozygotes for adenine phosphoribosyltransferase (APRT) deficiency which makes way for a qualitative distinction between normal and heterozygous subjects. We cultured peripheral blood mononuclear cells with 2,6-diaminopurine, an APRT-dependent cytotoxin, to search for in vivo mutational cells. Fifteen putative heterozygotes examined were found to possess such mutant cells at rather high frequencies; thus, a false negative diagnosis is unlikely. The analysis of genomic DNA in 82 resistant clones from two of the heterozygotes clarified that 64 (78%) had lost the germinally intact alleles. Thirteen members of APRT-deficient families were examined; eight proved to be heterozygotes. Among 425 individuals from two separate residential areas of Japan, two heterozygotes were found. The authenticity of the heterozygosity was validated by two separate methods for the two heterozygotes; hence, a false positive diagnosis can be ruled out. Our data showed a calculated heterozygote frequency of 0.47% (95% confidence limits; 0.05%–1.7%), a value compatible with that (1.2%) calculated from data concerning the incidence of 2,8-dihydroxyadenine urolithiasis. This novel genetic approach for identifying heterozygotes is now being tested to search for other enzyme deficiencies in humans.

Introduction

Homozygous deficiency of a purine salvage enzyme, adenine phosphoribosyltransferase (APRT), causes urolithiasis and renal failure, but heterozygotes are asymptomatic (Kelley et al. 1968; Cartier and Hamet 1974; Van Acker et al. 1977; Simmonds et al. 1989). There are phenotypically two different types of APRT deficiency: a complete deficiency (type I) and a partial deficiency (type II), both causing 2,8-dihydroxyadenine urolithiasis (Simmonds et al. 1989). Each type II patient possesses at least one *APRT*J* allele (Fujimori et al. 1985) having a missense mutation in codon 136 (Hidaka et al. 1988). This mutation causes an amino acid substitution from Met to Thr (Kamatani et al. 1989) within the putative 5-phosphoribosyl-1-pyrophosphate binding sequence (Dush et al. 1985; Hershey and Taylor 1986; Hove-Jensen et al. 1986) and leads to the production of a mutant enzyme with reduced affinity for 5-phosphoribosyl-1-pyrophosphate (Fujimori et al. 1986). Each type I patient possesses two null-type alleles designated *APRT*Q0*, which represent a set of different mutational alleles (Fujimori et al. 1985; Simmonds et al. 1989). Thus far, two mutations have been identified for *APRT*Q0* alleles (Hidaka et al. 1987). The *APRT*J* allele has been found only among the Japanese (Kamatani et al. 1987*b*; Simmonds et al. 1989), and the haplotype

Received June 13, 1990; revision received October 30, 1990.

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0002-9297/91/4803-0016$02.00

analysis indicated that all the alleles were derived from a single mutation (Kamatani et al., in press).

Heterozygous individuals for complete-type deficiency with a genotype of *APRT*1/APRT*Q0* (*APRT*1* represents a normal allele) can usually be identified by detecting individuals with reduced APRT enzyme activities in the hemolysates (approximately 25% of the control value) (Kelley et al. 1968). However, since there is a considerable variation in the erythrocyte APRT activities among normal individuals, the values for normal individuals may overlap the levels in heterozygotes. The risk of identification of heterozygotes having an *APRT*J* allele (*APRT*1/APRT*J*) solely with the enzyme assay is even higher, since the hemolysate enzyme activities in these heterozygotes range from 49%–66% of the normal average value (Kamatani et al. 1987*b*).

We detected 2,6-diaminopurine (DAP)-resistant cells in peripheral blood T cells in four heterozygotes for APRT deficiency (Hakoda et al. 1990). The mutation causing the DAP-resistant phenotype are considered to have occurred in vivo. The frequency of such mutant T cells was high (on the order of 10^{-4}) in each heterozygote examined. We also designed a method to diagnose heterozygotes for APRT deficiency by selecting DAP-resistant B cells after mutagenizing B cell lines obtained from individuals to be tested (Kamatani et al. 1987*a*). The method using B cell lines, however, was laborious and the time to the diagnosis was extended. In addition, inducing mutations in vitro may make the diagnosis somewhat difficult to interpret.

The method using T cells and in vivo somatic mutations described in the present report is more likely to be of practical application. The possibilities of false negative and false positive diagnoses had to be evaluated by testing additional heterozygotes and normal individuals. In the present investigations, we used mutant T cells from additional putative heterozygotes as well as from normal individuals, and confirmed that this method makes for a qualitative rather than a quantitative distinction between heterozygotes and normal subjects. We also analyzed DNA in the resistant clones, and found that many had lost the normal alleles. Subsequently, this method was used to diagnose heterozygotes in family members with APRT deficiency and to screen large numbers of individuals.

Material and Methods

Individuals Studied

Fifteen individuals who were parents or children of the patients with homozygous APRT deficiency were studied as putative heterozygotes. Nine siblings, one grandmother, one aunt, and two nephews of the patients were also studied to detect heterozygotes. The methods used to determine the genotype for each homozygote have been described elsewhere (Kamatani et al., in press). To estimate the frequency of disease-related genes for APRT deficiency in the general population, blood samples were obtained from 225 individuals randomly selected from outpatients seen at the Institute of Rheumatology, Tokyo Women's Medical College, Tokyo, and from 208 individuals selected from outpatients being followed at Chikugo City Hospital, Fukuoka, Japan. Thus, 433 individuals were studied.

Selection of DAP-resistant T Cells

For the cloning of DAP-resistant T cells, peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque density centrifugation from two putative heterozygotes. DAP-resistant T cells were cloned as described elsewhere (Hakoda et al. 1990). Thus, PBMCs were inoculated into 96–well microtiter plates at a density of 2×10^4 cells/well. X-irradiated Raji cells were also added at 10^4 cells/well as the feeder cells. The cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 1% human male serum, 0.7 μg phytohemagglutinin/ml (Difco, Detroit), 0.5 ng recombinant human interleukin 2/ml (Takeda, Tokyo) and 100 μM DAP (Sigma, St. Louis) for 2 wk. Lymphocyte colonies proliferating in the wells were transferred to larger 10 × 10-mm wells with feeder cells and were expanded to more than 3×10^6 cells (Hakoda et al. 1989*b*).

For the diagnosis of heterozygotes, we slightly modified the methods described above. Thus, PBMCs were inoculated into the wells of 96-well microtiter plates with round bottoms (Costar, Cambridge, MA) at a concentration of 10^5 cells/well. X-irradiated (10,000 rad) Raji cells were also inoculated into the wells at 10^4 cells/well. The cells were cultured in 200 μl/well in medium containing recombinant interleukin 2, phytohemagglutinin, and DAP. Each well was observed by using an inverted microscope on day 7 and day 10 after initiation of the culture.

Southern Blotting

DNA was extracted from mutant T cell clones as described elsewhere (Hakoda et al. 1989*b*), digested with *Taq*I (Toyobo, Japan) or *Sph*I (Toyobo) as recommended by the supplier, electrophoresed on 1.0%

agarose gels (type 1, Sigma) and transferred to nylon membranes (Biodyne A, PALL Biosupport, New York). The membranes were hybridized to cDNA for human APRT (a gift from Y. Hidaka, University of Michigan) labeled with [^{32}P]dCTP, using a random primer method. Autoradiography was done at −80°C for 7–14 d.

Measurement of APRT Enzyme Activity in T Cell Colonies

APRT activities in the cell extracts of nonselected and DAP-selected T cell colonies were determined as described elsewhere, using [8 − ^{14}C] adenine (Hakoda et al. 1990).

Amplification of a Part of the APRT Gene by the PCR Followed by Allele-specific Oligonucleotide Hybridization on Dot-blotted Samples

The techniques were similar to the methods stated in our previous paper (Kamatani et al. in press), except that different primers were used for the amplification. DNA was extracted from PBMCs from each individual or from each clone. A part of the APRT gene in these samples was amplified in vitro by the PCR (Saiki et al. 1986), using synthetic primers PN-13 (5′-CCGAGTCACTCCTGTCACTTA-3′) and PN-14 (5′-GGATCCAGCTGGAGATGTTGGGCT-3′), and *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) PN-13 has a nucleotide sequence of 1317 to 1337 of the sense strand of the human genomic APRT sequence ("A" in the initiation codon ATG was counted as position 1), while PN-14 anneals to nucleotides 2243 to 2220 of the sense strand. The temperatures and time used for heat denaturing, annealing, and polymerizing steps were 94°, 65°, and 72°C for 2, 3, and 3 min. respectively. These steps were repeated for 35 cycles. The combination of primers PN-13 and PN-14 successfully amplified the target 904-bp sequence, which includes the *APRT*J* mutation site located at position 2069. When the amplification was successful, the bands could be visualized with ethidium bromide after 3% agarose gel electrophoresis. AFter blotting the amplified DNA on nylon membranes (Biodyne A, PALL Biosupport, New York), dot hybridization was done using radiolabeled oligonucleotides as probes. Thus, PN-3 (5′-CAGGAACCACGAACGCTGC-3′) for *APRT*J*-specific and PN-4 (5′-CAGGAACCATGAACGCTGC-3′) for normal (non-*APRT*J*) sequences were end labeled with [γ-^{32}P]ATP (6,000 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 elsewhere (Saiki et al. 1986), except that the temperature for the latter was 53°C. The membranes were then exposed to X-ray films for 8 h.

Results

Detection of DAP-resistant Colonies in Peripheral Blood of Putative Heterozygotes

As shown in table 1, DAP-resistant T cells were readily identified in the culture of PBMCs from all the

Table 1

Detection of DAP-resistant T Cells from Probable Heterozygotes

Family	Probable Heterozygote	Genotype	Total Wells	Positive Wells	% Positive Wells
1.....	E.T.	*APRT*1/APRT*J*	24	12	50
	S.T.	*APRT*1/APRT*J*	24	19	79
2.....	M.A.	*APRT*1/APRT*J*	24	22	92
	K.A.	*APRT*1/APRT*J*	24	15	63
3.....	H.T.	*APRT*1/APRT*J*	24	18	75
4.....	Y.I.	*APRT*1/APRT*J*	47	30	64
5.....	M.K.	*APRT*1/APRT*J*	48	14	29
	K.K.	*APRT*1/APRT*J*	48	19	40
	S.S.	*APRT*1/APRT*J*	48	25	52
6.....	A.S.	*APRT*1/APRT*J*	21	18	86
7.....	Y.T.	*APRT*1/APRT*Q0*	28	5	18
	M.T.	*APRT*1/APRT*Q0*	41	12	29
8.....	Y.S.	*APRT*1/APRT*Q0*	24	8	33
9.....	K.K.	*APRT*1/APRT*J*	18	10	56
	K.K.	*APRT*1/APRT*Q0*	26	6	23

Table 2

Detection of DAP-resistant T Cells from Control Subjects

Individual	Total Wells	Positive Wells
M.H.	5,350	0
N.K.	1,440	0
Y.S.	920	0
N.M.	1,180	0
T.I.	720	0
M.H.	1,180	0
M.I.	950	0

15 putative heterozygotes. The difference between the wells with totally dead cells and those with proliferating resistant cells was clear under an inverted microscope, as described in previous papers (Albertini et al. 1982; Morley et al. 1983; Hakoda et al. 1990). For each heterozygote, the cells were cultured in more than 18 wells, each containing 10^5 PBMCs. At least one positive well containing DAP-resistant T cells was observed among the initial 10 wells examined, except for one individual. Even for the latter individual, five wells were found positive for resistant lymphocyte colonies among 10 wells subsequently examined. As 20 wells (a total of 2×10^6 mononuclear cells) were sufficient for the detection of DAP-resistant T cells in the peripheral blood from heterozygotes, 2 ml of blood is sufficient to detect mutant T cells in most cases when the average recovery rate of blood mononuclear cells is taken into account. In contrast, no positive wells with proliferative DAP-resistant T cells were observed in the culture of PBMCs from seven control subjects in 11,700 wells (1.17×10^9 cells) (table 2).

Analysis of DNA in Mutant Clones from Two Heterozygotes

Two heterozygotes with two different germinal mutations at the *aprt* locus were chosen because their genomic DNA showed a heterozygous RFLP pattern. One of them (H1) had a mutant allele, *APRT*J*. The other heterozygote studied (H2) has a germinally mutated allele, *APRT*Q0*. Although the precise mutation of this allele has not been defined, the family study clearly indicated that this germinally defective allele codes for a complete APRT deficiency (Fujimori et al. 1985). Southern blot analysis of the genomic DNA indicated that both H1 and H2 had a genotype of 2.1 kb/2.8 kb for the common *Taq*I RFLP (fig. 1*A* and *B*) (Stambrook et al. 1984). The family study confirmed

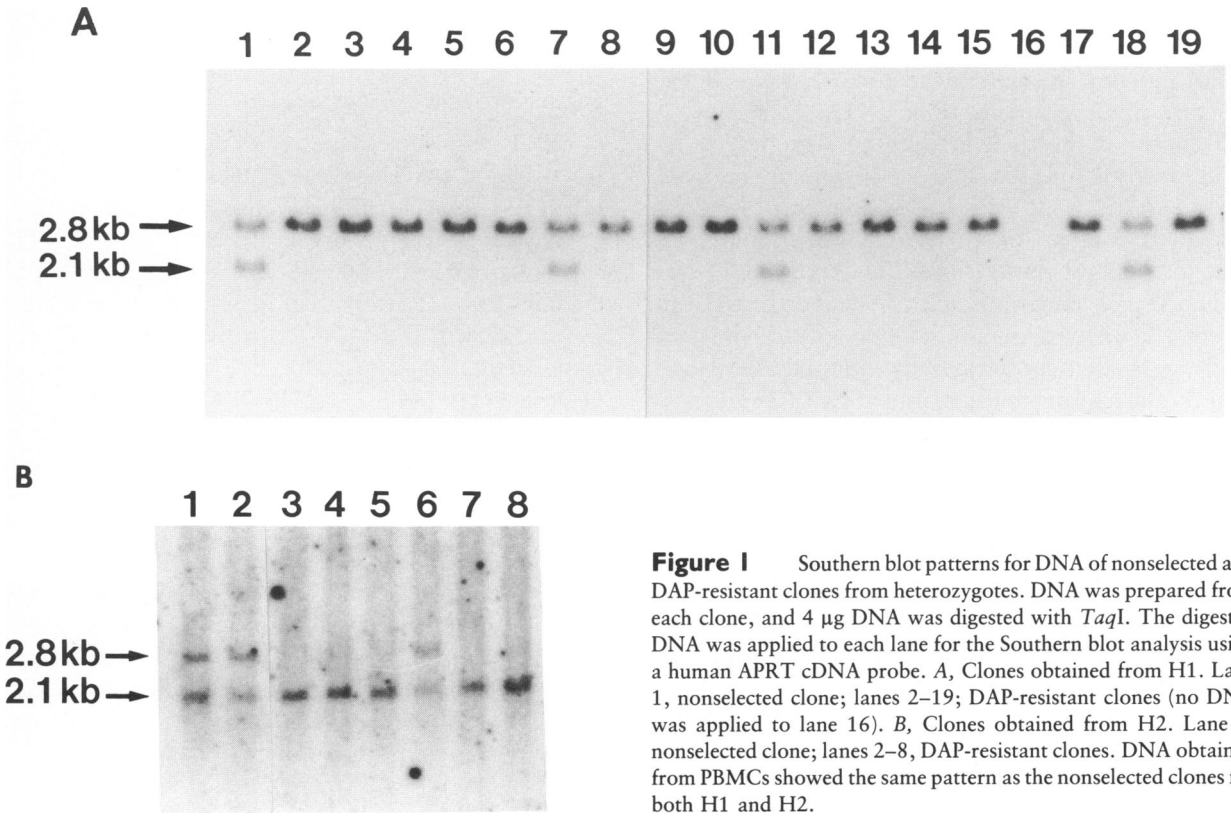

Figure 1 Southern blot patterns for DNA of nonselected and DAP-resistant clones from heterozygotes. DNA was prepared from each clone, and 4 μg DNA was digested with *Taq*I. The digested DNA was applied to each lane for the Southern blot analysis using a human APRT cDNA probe. *A*, Clones obtained from H1. Lane 1, nonselected clone; lanes 2–19; DAP-resistant clones (no DNA was applied to lane 16). *B*, Clones obtained from H2. Lane 1, nonselected clone; lanes 2–8, DAP-resistant clones. DNA obtained from PBMCs showed the same pattern as the nonselected clones for both H1 and H2.

that, in H1, the germinal mutation and the 2.8-kb *Taq*I RFLP were on the same chromosome and the normal allele was associated with the 2.1-kb *Taq*I RFLP. On the other hand, in H2, the germinally normal and mutated alleles were associated with 2.8-kb and 2.1-kb *Taq*I RFLP, respectively. In order to analyze mutational events at the DNA level, it was important that the germinally mutated and normal alleles show different RFLP bands as in H1 and H2.

From DAP-resistant clones obtained from the two heterozygotes, 82 mutant clones were analyzed. The extracted DNA from each clone was digested with *Taq*I and subjected to Southern blot analysis, using the cDNA probe for the human APRT gene. Of 42 clones from the heterozygote H1, 33 (79%) showed the loss of the 2.1-kb bands (fig 1*A*). On the other hand, 31 out of 40 clones (78%) obtained from the heterozygote H2 had lost the 2.8-kb bands (fig. 1*B*). Thus, 79% (H1) or 78% (H2) of the somatic mutant cells in vivo had lost the germinally normal APRT alleles.

In the remaining somatic mutant clones (nine, or 21%, for H1 and nine or 22%, for H2), Southern blot analysis showed no change (fig. 1). Thus, all of these clones had a 2.1-kb/2.8-kb *Taq*I RFLP pattern. These results indicate that a smaller fraction (approximately 22%) of all the somatic mutant cells at the *aprt* locus probably had changes in the genome leading to the loss of function but without major alterations in the structure of the genomic APRT DNA.

For many of the allele-loss mutants, the intensities of the bands associated with the germinally mutated alleles (*APRT*J* and *APRT*Q0*) seemed to have increased in comparison to normal clones and non-allele-loss mutant clones (fig. 1). The amount of DNA applied to each lane was nearly equal, as deduced from the ethidium bromide staining of the gels. These data may suggest that the germinally mutated alleles had been duplicated in many of the allele-loss mutants. Comparison of the intensities of the bands, however, is probably not sufficient to prove the doubling of the amounts of the gene, and further analysis is necessary. Nevertheless, it was confirmed that, in 78% of the DAP-resistant clones we obtained, loss of the germinally intact allele was the mechanism of the mutation, thereby excluding the possibility that some epiphenomenon not affecting genetic materials was the cause of the APRT deficiency.

In the allele-loss mutants derived from H1 with the genotype of *APRT*1/APRT*J*, retention of the germinally mutated allele and the loss of the normal allele were further confirmed by the in vitro amplification of a part of the genomic APRT sequence and subsequent probing with oligonucleotides. This procedure clearly reveals whether the genomic DNA has the *APRT*J* or non-*APRT*J* allele (or both). As shown in figure 2, DNA from all the allele-loss mutants had retained the *APRT*J* allele but had lost the *APRT*1* allele, as shown by the positive hybridization to the *APRT*J*-specific probe but not to the non-*APRT*J*-specific probe. On the other hand, non-allele-loss mutant clones from the same individual showed positive hybridization to both probes.

Figure 2 Synthetic oligonucleotide hybridization after in vitro amplification of part of genomic APRT sequence. Amplified DNA was dot blotted onto nylon membranes which were subsequently hybridized to a labeled oligonucleotide probe specific for either the *APRT*J* or the non-*APRT*J* sequence. DNA was from PBMCs from a control subject (lane 1), a homozygote with a genotype of *APRT*J/APRT*J* (lane 2), H1 (lane 3), and DAP-resistant T cell clones from H1 (lanes 4–9). DNA in lanes 4, 5, 6, 7, 8, and 9 was the same as that in lanes 11, 18, 12, 13, 14, and 15 in fig. 1*A*, respectively.

Enzyme Activities in T Cell Colonies

APRT enzyme activities were measured in cell extracts of the DAP-resistant T colonies. As shown in table 3, 12 T cell colonies from three different heterozygotes with a genotype of *APRT*1/APRT*Q0* showed virtually no enzyme activities. In contrast, 13 colonies from three different heterozygotes with a genotype of *APRT*1/APRT*J* showed significant enzyme activities. Thus, the two different types of heterozygotes (*APRT*1/APRT*Q0* and *APRT*1/APRT*J*) could be clearly differentiated by measuring the APRT enzyme activities in the DAP-resistant T cell colonies.

Diagnosis of Heterozygotes among Family Members

The present method was also used to test family members other than putative heterozygotes to assess the practicality of the method (table 4). Thirteen members from six different families were examined. DAP-resistant T cells were detected in seven of nine siblings, one of one grandmother, none of one aunt, and none

Table 3

APRT Activities in Nonselected and DAP-resistant T Cell Colonies from Probable Heterozygotes

Heterozygote (Genotype) and T Cell Colony	APRT Activity (nmol/min/mg protein)
H.T. (*APRT*/APRT*J*):	
Nonselected:	
2	1.93
3	1.80
5	1.31
7	2.23
DAP-resistant:	
1^r	1.31
2^r	1.47
3^r	1.69
5^r	1.39
8^r	1.50
12^r	1.50
13^r	1.97
Y.S. (*APRT*1/APRT*J*):	
DAP-resistant:	
1^r	3.05
2^r	2.64
K.S. (*APRT*1/APRT*J*):	
DAP-resistant:	
1^r	2.71
2^r	1.98
3^r	0.79
4^r	2.36
H.H. (*APRT*1/APRT*Q0*):	
Nonselected:	
1	2.27
2	2.95
4	2.80
8	1.00
DAP-resistant:	
1^r	0.04
3^r	0.01
4^r	0.00
5^r	0.02
6^r	0.00
7^r	0.00
T.S. (*APRT*1/APRT*Q0*):	
DAP-resistant:	
1^r	0.02
2^r	0.00
3^r	0.00
5^r	0.00
7^r	0.07
K.K. (*APRT*1/APRT*Q0*):	
DAP-resistant:	
1^r	0.01

of two nephews. In total, eight of 13 family members were diagnosed as heterozygotes, while the other five were diagnosed as normal.

Screening for Heterozygotes in the General Population

We then used this method to detect heterozygotes in two separate populations in Japan. Five milliliters of blood was obtained from each individual. In total, 433 individuals from two areas were examined. When more than 5×10^6 PBMCs were recovered from an individual, culture in 48 wells (10^5 cells/well) was set up. When the total number of PBMCs obtained from an individual was less than 5×10^6, the culture was set up in as many wells as possible. In order to avoid false negative results, the data on individuals for whom less than 20 wells were set up for culture were not included unless DAP-resistant T cells were detected in the cultures. This cutoff point was determined by data from the putative heterozygotes, as mentioned above. Only eight individuals were excluded for this reason, and the results from 425 individuals were assessed. The growth of DAP-resistant T cells was detected in cultures from two individuals. Thus, there were 35 positive wells of 48 for one individual (I. T. from Tokyo), and 33 of 42 wells had DAP-resistant T cells for the other one (S.F. from Fukuoka). Thus, these two individuals were considered to be heterozygotes. From these results, the frequency of heterozygotes was calculated to be 0.47%. If the mutant alleles are assumed to distribute evenly, and the probability of finding a certain number of heterozygotes in a randomly selected sample is assumed to follow a Poisson distribution, then 95% confidence limits for this value are calculated to be 0.05%–1.7%.

Confirmation of Heterozygosity by Enzyme Assay and Molecular Biological Techniques

APRT enzyme activities comparable to nonselected normal T cells were detected in cell extracts of six DAP-resistant T cell colonies from individual I.T., while virtually no enzyme activities were detected in three DAP-resistant T cell colonies from individual S.F. (table 5). Thus, we can conclude that I.T. has a genotype of *APRT*1/APRT*J*, while S. F. has a genotype of *APRT*1/APRT*Q0*. DNA from I.T. and S.F. was submitted to the PCR allele-specific oligonucleotide hybridization procedure in order to distinguish *APRT*J* and non-*APRT*J* alleles. Figure 3 indicates that DNA from PBMCs from I.T. had both

Table 4

Diagnosis of Heterozygotes among Family Members

Family	Genotype of Propositus	Family Member	Total Wells	Positive Wells	% Positive Wells
2	*APRT*J/APRT*J*	M.S. (grandmother)	24	20	83
		A.M. (aunt)	24	0	0
4	*APRT*J/APRT*J*	K.I. (brother)	48	0	0
5	*APRT*J/APRT*J*	K.E. (brother)	48	0	0
8	*APRT*J/APRT*Q0*	T.S. (brother)	48	32	67
		Y.S. (sister)	48	35	73
		K.S. (sister)	48	36	75
10.....	*APRT*J/APRT*J*	S.N. (sister)	48	25	52
		M.S. (sister)	48	24	50
11.....	*APRT*Q0/APRT*Q0*	K.K. (brother)	48	17	35
		Y.K. (sister)	48	20	42
		H.K. (nephew)	48	0	0
		I.K. (nephew)	48	0	0

*APRT*J* and non-*APRT*J* alleles, while DNA from S.F. had only non-*APRT*J* alleles.

DAP-resistant T Cells in Normal Individuals

We identified only two DAP-resistant clones in samples from 432 normal individuals (3.6×10^9 cells in total). We assayed APRT activities in the cell extracts from the mutant clones and confirmed that they were completely deficient in the enzyme activity. The calculation done according to our method (Hakoda et al. 1990) indicates that the frequency of $aprt^{-/-}$ mutant T cells in $aprt^{+/+}$ individuals is 8.4×10^{-9}. We analyzed DNA from both of the individuals (N1 and N2) as well as the $aprt^{-/-}$ mutant clones. N1 showed a homozygous pattern for the common *Taq*I RFLP (fig. 4*A*). N1 also showed a homozygous pattern for another common RFLP detected by *Sph*I (Arrand et al. 1987) (fig. 4*B*). Although the mutant clone from N1 had the same RFLP patterns as the genomic DNA from N1 (fig. 4*A, B*), such data are not so informative for determining the mechanisms involved in generating these mutant T cells. However, N2 showed a heterozygous pattern for the common *Sph*I RFLP (fig. 4*B*). The mutant $aprt^{-/-}$ clone obtained from N2 showed the same heterozygous RFLP pattern as the genomic DNA from N2, thereby indicating that some minor changes not causing major structural alterations in the genome had occurred in both of the two germinally normal *aprt* alleles of N2 (fig. 4).

Table 5

APRT Activities in DAP-resistant T Cell Colonies from Heterozygotes Detected by Screening

Subject and DAP-resistant T Cell Colony	APRT Activity (nmol/min/mg protein)
I.T.:	
2^r	2.28
3^r	1.97
4^r	3.07
6^r	1.93
7^r	2.15
8^r	1.01
S.F.:	
12^r.....	.00
27^r.....	.04
29^r.....	.00

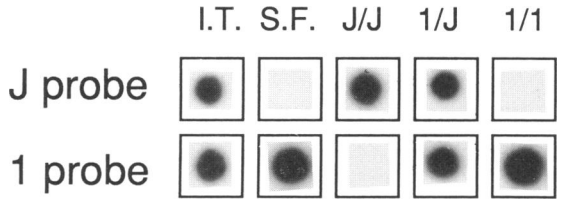

Figure 3 Detection of *APRT*J*-specific sequence in genomic DNA of I.T. but not in that of S.F. DNA from I.T., S.F., and individuals with genotypes of *APRT*J/APRT*J* (*J/J*), *APRT*1/APRT*J* (*1/J*), or *APRT*1/APRT*1* (1/1) was used for the PCR followed by allele-specific oligonucleotide hybridization on dot-blotted samples, as described in Material and Methods. Either PN-3 with the *APRT*J*-specific sequence (J probe) or PN-4 with the non-*APRT*J* sequence (1 probe) was used as the radiolabeled probe.

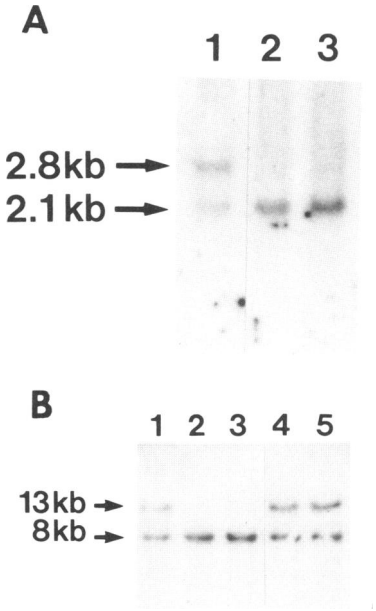

Figure 4 Southern blot patterns for DNA obtained from PBMCs and DAP-resistant mutant clones from two normal individuals. DNA was digested with *TaqI* (*A*) or *SphI* (*B*) and subjected to electrophoresis. DNA was from PBMCs of a control individual heterozygous for both RFLPs (lane 1), PBMCs from N1 (lane 2), a DAP-resistant T cell clone from N1 (lane 3), PBMCs from N2 (lane 4), and a DAP-resistant T cell clone from N2 (lane 5).

Discussion

The importance of an accurate diagnosis of healthy carriers of disease-related genes is twofold. First, it is important for genetic counseling, and second, it can be used to determine the frequency of such disease-causing genes in human populations. In the case of determination of the frequency in human populations, normal variations are likely to blur the distinction between normal and heterozygous individuals unless the difference is qualitative rather than quantitative. In case of X-linked disorders, the distinction between the heterozygotes and normal females can be qualitative because of Lyonization (Lyon 1961). For example, each heterozygote for hypoxanthine-quanine phosphoribosyltransferase (HPRT) deficiency contains both normal and deficient somatic cells, and such a mosaic state can be detected by culturing the fibroblasts with 6-thioguanine, an HPRT-dependent cytotoxic drug (Rosenbloom et al. 1967). Our data showed that a mosaicism also exists in heterozygotes for an autosomal recessive disorder, APRT deficiency, although the ratio of deficient to normal somatic cells is much lower. Due to the presence of in vivo somatic mutant T cells in the heterozygotes, we designed a method for the diagnosis of the heterozygotes for APRT deficiency which distinguishes heterozygotes from normal subjects qualitatively (all-or-nothing fashion) rather than quantitatively.

Data on deficient APRT alleles suggested that the fraction of newly generated mutant genes among deficient genes in the population is very low (Kamatani et al., in press). Therefore, it is feasible to assume that all 15 putative heterozygotes examined (not only all children but all parents as well) are indeed heterozygotes. DAP-resistant T cell colonies were readily detected in all the 15 putative heterozygotes for two different types of APRT deficiencies; thus, a false negative diagnosis using our method is unlikely. The detection of a heterozygous state in eight of 13 other family members (62%) is consistent with calculated probabilities of being heterozygotes. In contrast, no DAP-resistant T cells were obtained from a large number of mononuclear cells (in total, 1.2×10^9 cells) obtained from seven control individuals. These initial experiments suggested that the DAP-resistant T cells are not detected in normal individuals using our method.

With the same method, two different Japanese populations were screened for heterozygotes. Among a total of 425 individuals randomly selected from two areas, two heterozygotes were detected. The authenticity of the diagnosis was validated by two different methods for the two subjects. APRT enzyme activities in all DAP-resistant colonies from S.F. were virtually zero, thereby confirming that all the resistant clones were real mutants and that S.F. has a genotype of *APRT*1/APRT*Q0*. For I.T., however, the enzyme activities in the resistant clones did not confirm the heterozygosity; rather, it was confirmed by identification of a mutant sequence specific to the *APRT*J* alleles in DNA from the PBMCs, using a molecular biological technique. These data confirm that the two individuals are indeed heterozygotes and that a false positive diagnosis with our method is unlikely.

Although the number of heterozygotes detected is small, the frequency (0.47%; 95% confidence limit, 0.05%–1.7%) is compatible with the value (1.2%) estimated from the number of patients with homozygous APRT deficiency (Kamatani et al. 1987*b*). Since the individuals examined were selected from only two areas (800 km apart), the frequency of heterozygotes obtained here may deviate from the actual value, be-

cause the distribution of heterozygotes may not be random. Further study to examine individuals from different areas is needed to obtain a precise value.

Our results also contribute to the knowledge of somatic cell genetics. Thus, in addition to four heterozygotes we examined in our previous paper (Hakoda et al. 1990), 21 heterozygotes were found to possess DAP-resistant T cells, at rather high frequencies, although accurate frequencies were not determined for the latter subjects. The high-frequency mutational events at the *aprt* locus in somatic cells is a common in vivo phenomenon in heterozygotes in humans.

In 78% of the mutant clones, loss of the germinally normal alleles was detectable by the Southern blot analysis. Since the percentage of the allele-loss mutants differed little between clones obtained from the two heterozygotes with the two different germinally mutated alleles (*APRT*J* and *APRT*Q0*), other unexamined heterozygotes are likely to have similar percentages of the allele-loss mutants among the somatic mutant cell populations in vivo. Other researchers and our group investigated molecular changes in the DNA of in vivo mutants deficient at the X-linked *hprt,* and found that major DNA alterations detectable by the Southern blot analysis were not common (around 10%) (Bradley et al. 1987; Nicklas et al. 1987; Hakoda et al. 1989*a*). Thus, the molecular mechanisms leading to the deficiencies seem to differ between these two loci. This difference is likely to be related to the fact that *hprt* is located on the X chromosome while *aprt* is on an autosome. Major gene deletions or chromosomal events (somatic recombination or nondisjunction) in the X chromosome might lead to defects in important cellular functions necessary for cell survival and/or proliferation. If a similar deletion or chromosomal changes do occur in an autosome, the gene on the other homologous chromosome is likely to compensate for the defect. This also explains, at least in part, why the frequency of $aprt^{-/-}$ cells in a heterozygote is much higher than the frequency of $hprt^{-}$ cells. In retinoblastoma cells, loss of one of the two *RB* alleles (loss of heterozygosity) has been frequently observed. Cavenee et al. examined 33 cases and found the loss of heterozygosity involving the *RB* locus in 24 cases (75%) (Cavenee et al. 1986). In many of these cases, alleles on the other homologous chromosome which carries the mutated *RB* alleles were duplicated, thereby indicating that chromosomal events such as mitotic recombination and nondisjunction are mechanisms leading to the homozygous deficiencies at the *RB* locus (Cavenee et al. 1986). In the present investigation, loss of intact *aprt* alleles was observed at a similar high frequency (79%) in $aprt^{-/-}$ mutant T cells in $aprt^{+/-}$ individuals. In many of these allele-loss mutants, the mutated *aprt* alleles seemed to have duplicated. Thus, the molecular mechanisms leading to the homozygous deficiency at the *aprt* locus in somatic cells are probably homologous to those at the *RB* locus in retinoblastoma cells. Our data suggest that the loss of heterozygosity at autosomal loci in somatic cells is considerable in humans.

We identified two $aprt^{-/-}$ clones in normal individuals and calculated the frequency of such mutants to be 8.4×10^{-9}. In one of the two $aprt^{-/-}$ clones from normal individuals, we could show that none of the two *aprt* alleles was lost. To generate this clone in N2, two serial mutational events at two different chromosomes probably occurred. Although the observation that two $aprt^{-/-}$ clones were obtained from normal individuals is important, the general mechanisms involved in such an event await further study on a larger number of mutants.

Recent molecular biological techniques facilitate identification of heterozygotes, just as the method we used in the present report identified *APRT*J*-specific mutation. However, we would like to point out that it is feasible only when the mutational alleles have already been identified at the molecular level. Molecular biological, methods are not sufficient to identify carriers in general populations if the mutational alleles have not been completely identified beforehand. Therefore, use of the methods described here for identifying heterozygotes is needed even when extensively advanced molecular biological techniques and knowledge become available. In our experience of diagnosing more than 50 families with APRT deficiency, the present method is by far more reliable than any other method.

The present novel genetic approach for the diagnosis of heterozygotes by identifying somatic mutant T cells may be applied to other disorders or genetic conditions. For example, thymidine kinase and adenosine kinase are enzymes whose deficiencies can be selected for by purine and pyrimidine analogs. Although genetic deficiencies of these enzymes have not been described, heterozygotes for the deficiencies may exist in the population even if the homozygous deficiencies are lethal. Our method for identifying heterozygotes by detecting somatic mutant cells is now being tested to search for these two enzyme deficiencies. Furthermore, some genes code for proteins expressed on the surface of T cells. The heterozygotes for the defects of

these genes may be detected using antibody-mediated killing of PBMCs and subsequent culturing of the resistant T cells. Killing of T cells with a special HLA antigen with an antibody followed by the culturing of mutant T cells has been successful (Janatipour et al. 1988; Turner et al. 1988).

Acknowledgments

This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture, Japan, and by a research grant, for the study of intractable diseases from the Ministry of Health and Welfare, Japan. We thank Ms. Kuroshima and Ms. Otsuka for excellent technical assistance, Dr. S. Kashiwazaki for support, and M. Ohara for helpful comments.

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