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Report

A Genetic Factor for Age-Related Cataract: Identification and Characterization of a Novel Galactokinase Variant, "Osaka," in Asians

Yoshiyuki Okano,[1] Minoru Asada,[1] Akie Fujimoto,[2] Akira Ohtake,[4] Koichiro Murayama,[5] Kwang-Jen Hsiao,[6] Kyuchul Choeh,[7] Yanling Yang,[8] Qixiang Cao,[9] Juergen K. V. Reichardt,[10] Shizuhiro Niihira,[3] Takuji Imamura,[1] and Tsunekazu Yamano[1]

[1]Department of Pediatrics, Osaka City University Graduate School of Medicine, [2]Osaka City Environment and Public Health Association, and [3]Department of Human Development and Welfare, Faculty of Human Life Science, Osaka City University, Osaka; Departments of [4]Pediatrics and [5]Ophthalmology, Saitama Medical School, Saitama, Japan; [6]Department of Medical Research and Education, Veterans General Hospital–Taipei, and Institute of Genetics, National Yang-Ming University, Taipei; [7]Department of Pediatrics, Eulji Medical College, Taejon, South Korea; [8]Department of Pediatrics, The First Hospital of Peking University, Peking; [9]Neonatal Screening Center, Shanghai Children Hospital, Shanghai; [10]Institute for Genetic Medicine and Department of Biochemistry and Molecular Biology, Kech School of Medicine, University of Southern California, Los Angeles

Galactokinase (GALK) deficiency is an autosomal recessive disorder characterized by hypergalactosemia and cataract formation. Through mass screening of newborn infants, we identified a novel and prevalent GALK variant (designated here as the "Osaka" variant) associated with an A198V mutation in three infants with mild GALK deficiency. GALK activity and the amount of immunoreactive protein in the mutant were both 20% of normal construct in expression analysis. The K_m values for galactose and ATP-Mg^{2+} in erythrocytes with homozygous A198V were similar to those of the healthy adult control subjects. A population study for A198V revealed prevalences of 4.1% in Japanese and 2.8% in Koreans, lower incidence in Taiwanese and Chinese, no incidence in blacks and whites from the United States, and a significantly high frequency (7.8%; $P < .023$) in Japanese individuals with bilateral cataract. This variant probably originated in Japanese and Korean ancestors and is one of the genetic factors that causes cataract in elderly individuals.

Galactokinase (GALK) deficiency (MIM 230200) is an autosomal recessive disorder characterized by hypergalactosemia and cataract formation if the child is not kept on a lactose-free diet. GALK (EC 2.7.1.6) catalyzes the first step of galactose metabolic pathway and is involved in the conversion of galactose to galactose-1-phosphate. The incidence of GALK deficiency is about 1/1,000,000 in whites (Segal and Berry 1995). In Japan, the incidence of GALK deficiency is also rare (1/1,000,000), on the basis of a mass-screening program for newborn infants with galactosemia (Aoki and Wada 1988). The initial studies on GALK deficiency at a molecular level resulted in the construction of a full-length human GALK cDNA, which is 1.35 kb long and encodes a peptide of 392 amino acids (Stambolian et al. 1995). Subsequent studies identified the GALK genome on chromosome 17q24, which consists of eight exons spanning 7.3 kb (Stambolian et al. 1995; Bergsma et al. 1996; Asada et al. 1999). Mutational analysis of patients with complete or near-complete GALK deficiency identified 14 mutations in Europe and the United States (Stambolian et al. 1995; Kolosha et al. 2000), 5 mutations in Japanese (Asada et al. 1999), and 1 prevalent mutation in the Romani population of Bulgaria (Kalaydjieva et al. 1999).

Hypergalactosemia results in accumulation of galactitol in the lens, which is synthesized by the reduction of galactose by aldose reductase. The osmotic phenomenon of galactitol causes swelling within lens fiber cells and eventually leads to cataract formation. Galactose metabolism is suspected to be related to cataractogenesis in the presence of complete (as well as partial) enzyme deficiency in the galactose metabolic pathway, high adult jejunal lactase activity, and/or large consumption of lactose (Couet et al. 1991). Age-related cataract is a mul-

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Address for correspondence and reprints: Dr. Yoshiyuki Okano, Department of Pediatrics, Osaka City University Graduate School of Medicine, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan. E-mail: okano@med.osaka-cu.ac.jp

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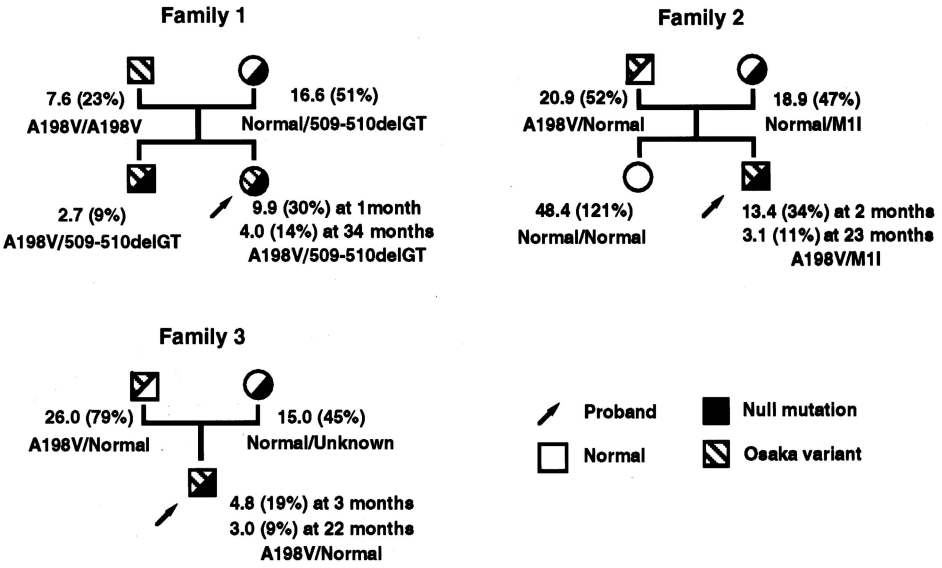

Figure 1 Pedigrees of families 1–3. Genotype and GALK activity (in nmol/min/g hemoglobin) measured in erythrocytes of male (*squares*) and female (*circles*) members of the families. Data in parentheses represent the percentage of GALK activity relative to that in healthy adult control subjects (25.2–40.0 nmol/min/g hemoglobin). The age at which GALK activity was measured is indicated for probands.

tifactorial disease involving host, genetic, and environmental factors. Research in age-related cataract has focused mainly on environmental factors, which seem to be important risk factors. However, Hammond et al. (2000) demonstrated, on the basis of research involving female twins, that genetic factors are most important among several factors in age-related cataract.

In the present study, we report a new variant of GALK, "Osaka," which was detected during mass screening of newborn infants in Japan. We characterized the Osaka variant, genetically and biochemically, and demonstrated its prevalence in Japanese and Koreans. We suspect that the Osaka variant is one of the genetic factors in cataract formation.

Three nonconsanguineous Japanese patients were referred to our hospitals for further investigation of high galactose levels (0.19–0.52 mmol/L) detected during mass screening of newborn infants. All three patients had normal fluorescence in the Beutler spot test for galactose-1-phosphate uridyltransferase (GALT) activity and showed undetectable galactose-1-phosphate and UDP-galactose levels in thin-layer chromatography for galactose metabolites. GALK activity in all three patients at 1–3 months of age ranged from 19 to 34% of adult control subjects, and diminished, in the same patients, to 9%–14% at age 22–34 mo (fig. 1). These results demonstrate that the mutant GALK was under the same age-dependent regulation as the control subjects; the activity in normal infants is two- to three-fold higher than in normal children and adults (Ng et al. 1965). None of the patients had cataract or other clinical manifestations during the neonatal period. Follow-up studies showed a gradual return of blood galactose concentration to the normal range, commencing at age 4 mo, without strict lactose-free diet. In vivo metabolic rate of galactose was determined in our study by the oral galactose–tolerance test (fig. 2). The mean maximum concentration of blood galactose in three patients was

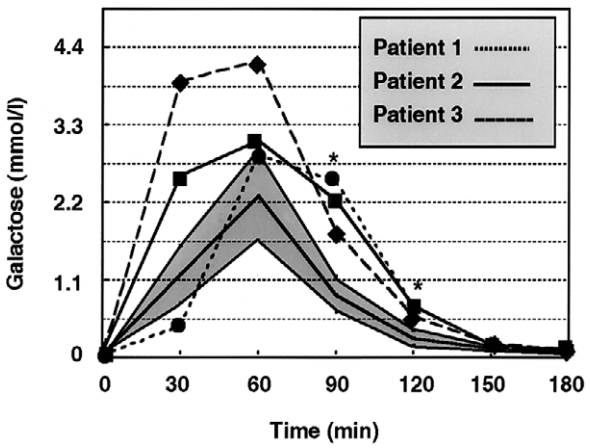

Figure 2 Oral galactose–tolerance test in three patients and six healthy control subjects. The oral galactose–tolerance test was performed using a dose of 1 g of galactose/kg of body weight (maximum 50 g). Patient 1, age 1 year; patient 2, age 1 year; patient 3, age 9 mo. *Shaded area,* mean ± SD data of the healthy control subjects, aged 30–43 years, with normal GALK, GALT, and UDP-galactose 4′-epimerase in erythrocytes. An asterisk (*) denotes a P value <.05, relative to the control subjects.

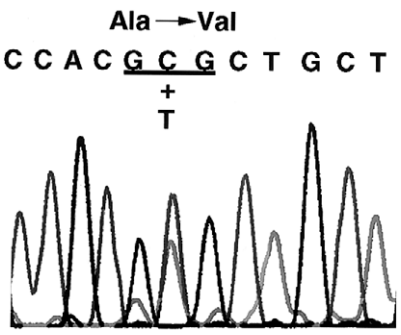

Figure 3 Identification of missense mutation in patients with the Osaka variant. Each exon and its flanking intronic region was amplified with a pair of human GALK-specific oligonucleotide primers (one primer was biotinylated) using PCR. The amplified products were purified to single-strand DNA, using magnetic beads coated with streptavidin M280 (Dynal), and were sequenced with dye terminator methods using an ABI autosequencer (310 Genetic Analyzer). Primers used for the PCR amplification in exon 4 of the human GALK gene are as follows: biotin-sense primer of 5′-GAATCTCCCTGGAGTGTCATT-3′ and antisense primer of 5′-CAGGCAGTGGGCACACTCCA-3′. Sequencing primer is sense primer of 5′-TCATTGAAGCCACTGCT-GCT-3′. The C and T bands appear at the same position in the patients' sequences, resulting in a C→T transition and replacement of alanine by valine at codon 198.

3.42 ± 0.07 mmol/l at 60 min (mean ± SD in healthy adult control subjects 2.45 ± 0.86 mmol/l). Furthermore, blood galactose levels at 90 and 120 min (2.2 ± 0.36 and 0.69 ± 0.082 mmol/l, respectively) were significantly higher than those of the control subjects (0.90 ± 0.26 and 0.21 ± 0.078 mmol/l, respectively; $P < .001$ each, Student's *t*-test). Galactose metabolic rate in these patients was lower than in the control patients.

We suspected a novel allelic variant in the human GALK gene in our patients, on the basis of the following features: (1) slightly elevated blood galactose levels identified through mass screening in newborn infants, (2) GALK activity only 10% of that in control subjects, and (3) low metabolic rate of galactose by galactose-tolerance test. The above results cannot be explained by only two concepts of normal and null or near-null enzyme deficient-type of GALK activity, but can be clearly, theoretically explained by the existence of a novel variant. Therefore, we propose the name "Osaka variant."

To characterize the GALK gene of the Osaka variant and to confirm Mendelian segregation, we performed sequence analysis in three families. Among the three patients, we found a common missense mutation of C→T transition at nucleotide position 593 of the GALK genomic gene (GenBank accession numbers L76927 and AF084935) in exon 4, resulting in the replacement of alanine (GCG) by valine (GTG) at codon 198 (A198V) (fig. 3). GALK activities and transmission of the A198V mutation in three GALK deficient families are shown in figure 1. All three patients showed a compound heterozygote of the A198V mutation and severe phenotype mutation of GALK activities and genotypes. In patient 1, we identified another mutation: two nucleotide deletions of 509-510 del GT (Asada et al. 1999). Patient 2 had another mutation; a missense mutation of M1I with GALK activity of 3% of normal on expression analysis (Kolosha et al. 2000). In family 1, the father (GALK activity was 23% of that in adult healthy control subjects) was homozygous for the A198V mutation. The above results indicate that the A198V mutation is transmitted from parents (fathers) to patients, and is associated with a reduction of GALK activity to 20% of that in the control subjects.

To determine whether the A198V mutation was associated with a low enzyme activity and to assess the potential GALK activity of the A198V mutation, we reconstructed A198V mutant in the expression vector pCDNA3 by site-directed mutagenesis. Mutant and normal GALK cDNAs were introduced into COS cells by electroporation with a Gene Pulser (Bio-Rad) as described previously (Ashino et al. 1995). The normal GALK cDNA inserted into COS cells in a transient expression assay led to 8–10-fold stimulation of GALK activity (10.7 U/mg protein) compared to the endogenous background (1.35 U/mg protein). GALK activity and immunoreactive amount of the A198V construct was established by correcting the transfection efficiency into COS cells (fig. 4). GALK activity of A198V construct was reduced to 18.5% of normal construct, which corresponded to 20% GALK activity in erythrocytes of the father with A198V/A198V in family 1. Immunoreactive protein of the A198V construct was also reduced to 20% of normal construct by the densitometric analysis. The A198V substitution was a causal gene for partial GALK deficiency in the probands, and the low GALK activity correlated with the reduced amount of

Table 1

Distribution of A198V Variant among Various Races and the Frequency of A198V Variant in Patients with Cataract

Population	Total Alleles	A198V Alleles	Frequency (%)
Japanese	582	24	4.1
Japanese with cataract	296	23	7.8
Koreans	288	8	2.8
Taiwanese	264	1	.38
Chinese:			
Peking	140	1	.71
Shanghai	138	0	0
United States:			
Whites	188	0	0
Blacks	10	0	0

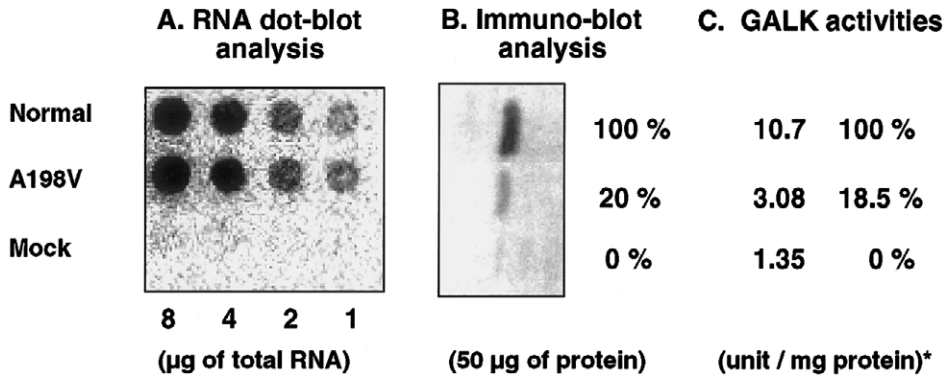

Figure 4 Analysis of GALK mRNA, immunoreactive protein, and GALK activity in COS cells transfected with normal or A198V human GALK cDNAs constructs. *A,* GALK mRNA levels in cell extracts were determined by dot-blot hybridization for serially diluted samples containing 1, 2, 4, or 8 μg of total RNA with a GALK cDNA probe labeled with [α-^{32}P] dCTP. The levels of GALK activity and immunoreactive protein were corrected with transfection efficiency by these levels of GALK mRNA. *B,* Immunoreactive GALK was identified by Western blotting, using a rabbit anti-human GALK antibody, goat anti-rabbit IgG-peroxidase, and ECL system (Amersham Pharmacia Biotech). The density of each band was quantified by scanning with a Bio-Rad Imaging Densitometer (GS-700). *C,* GALK activity was determined twice to ensure reproducibility using ^{14}C-galactose and a diethylaminoethyl cellulose column-DE52, as described elsewhere (Shin Buehring et al. 1977). GALK activities in normal, A198V, or mock constructs were expressed as nmol phosphorylated galactose/min/mg protein. The level of GALK activity in the A198V mutant construct was expressed as a percentage between those of normal constructs (100%) and endogenous background (0%).

GALK protein. These results define A198V as novel and as the first mutation detected from a variant with a mild clinical phenotype. So far, two variants have been identified: the Philadelphia and Urbino variants (Tedesco et al. 1972, 1977; Magnani et al. 1982). The Osaka variant appears to differ from the Philadelphia and Urbino variants with respect to the following features: (1) Philadelphia and Urbino variants have been detected in blacks and in Italians, respectively, whereas the Osaka variant was detected in Japanese and Koreans, and (2) GALK activity of Philadelphia and Urbino variants is 70% and 50%, respectively, of that in the control subjects.

To determine whether the cause of low GALK activity in the A198V mutation is a reduced catalytic rate or a low number of GALK molecules, we determined the kinetic parameters of GALK activity (K_m and V_{max}) in erythrocytes with A198V/A198V (the father of patient 1) or normal/normal for galactose and ATP-Mg^{2+}. K_m and V_{max} values were calculated using linear regression analysis and extrapolated intercepts from Michaelis-Menten and Lineweaver-Burk plots. The K_m values for galactose in normal and mutant were 1.06×10^{-4} and 1.17×10^{-4} moles, respectively. The V_{max} values for galactose in normal and mutant were 27.0 and 5.89 nmol/min/g hemoglobin, respectively. The K_m values for ATP-Mg^{2+} in normal and mutant were 3.09×10^{-4} moles and 3.30×10^{-4} moles, respectively. The V_{max} values for ATP-Mg^{2+} in normal and mutant were 26.9 and 9.17 nmol/min/g hemoglobin, respectively. The K_m values for galactose and ATP-Mg^{2+} in erythrocytes of patients homozygous for A198V mutation were similar those in normal. The V_{max} in erythrocytes of A198V/A198V genotype was one-third to one-fifth of that in normal/normal. Furthermore, the amount of immunoreactive protein of A198V was 18.5% of normal. The low GALK activity in A198V mutation is caused by instability of mutant GALK proteins. Functional abnormality of the A198V mutation is also suspected, because of the position of this mutation in the alignment of the predicted galactokinase protein. The A198V mutation in exon 4 was not in the conserved sequence of the homologous enzymes from *Streptomyces lividans* (Adams et al. 1988) and was not in the signature sequence and the ATP-binding domain (Debouck et al. 1985; Tsay and Robinson 1991). It is speculated that the A198V mutation does not have strong effects on the structure of GALK protein.

The population frequency of the A198V was analyzed in samples of anonymized control children without a diagnosis and family history of GALK deficiency, selected from hospitals. Genomic DNA was purified from dried blood spots obtained from subjects using the ReadyAmp Genomic DNA Purification System. We examined the frequency of A198V mutation in Japanese, Koreans, Taiwanese, Chinese, and whites and blacks from the United States, using the mutant allele–specific amplification. The A198V mutant gene was easily identified, as shown in figure 5. The frequency of the A198V mutation varied widely according to racial background (table 1). The highest frequency of the A198V mutant gene was among Japanese, amounting to 4.1% in 291 Japanese (24/582 alleles), followed by Koreans (2.8%). Only one of each allele was detected in Taiwanese and Chinese, and no mutant alleles were found in whites and

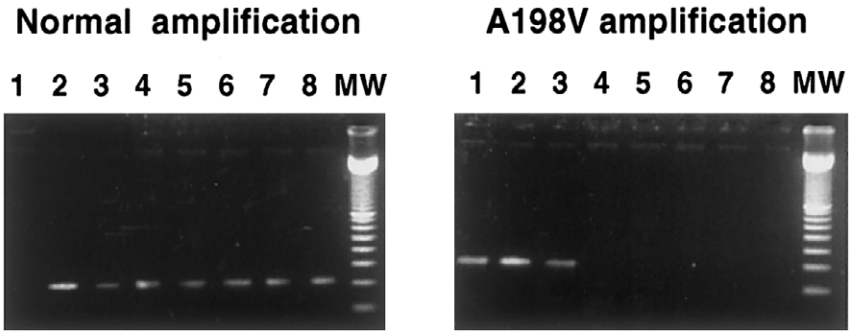

Figure 5 Allele-specific amplification for A198V mutation. Primers A198-AS for normal and 198V-AS for mutant type had the mismatch at the 3′ end and a deliberate G/A mismatch at three bases from the 3′ ends due to improvement of specificity between normal and A198V mutation. The following primer sets were used for PCR: antisense primer of A198-AS: 5′-CCTGCAGTCAATGAGCAACG-3′ for normal, or of 198V-AS: 5′-CCTGCAGTCAATGAGCAACA-3′ for A198V mutation, and sense primer of 198-S: 5′-GAATCTCCCTGGAGTGTCATT-3′ for normal and A198V mutation. *Lane 1,* PCR product (213 bp) shows only A198V amplification, indicating that the subject from whom this sample was taken is homozygous for A198V mutation. *Lanes 2 and 3,* PCR products show both normal and A198V mutant amplifications. The subjects of lanes 2 and 3 are heterozygous carriers of A198V mutation. *Lanes 4 to 8,* PCR products show only normal amplification. These subjects had the normal genotype. MW = molecular weight marker (100-bp ladder).

blacks from the United States. Unfortunately, the sample size of blacks was not sufficiently large to allow a firm conclusion regarding the exact frequency of the A198V mutant gene. Because our data showed that the carrier frequency of the Osaka variant is 1/12 (7.9%), the estimated frequencies of homozygote with the Osaka variant (20% GALK activity) and compound heterozygote with the Osaka variant and severe phenotype (10% GALK activity) is 1/600 and 1/24,000, respectively. We detected 3 patients in 20,000 per year in the annual mass screening, over a period of 4 years. The genetic frequency of the Osaka variant is similar to that of the Duarte variant of GALT deficiency (MIM 230400) in the United States (5.9%) (Elsas et al. 1994; Lin et al. 1994) and Japan (2%) (Ichiba 1989), which is a common variant among various races worldwide and is suspected to have arisen before racial divergence (Lin and Reichardt 1995; Hirokawa et al. 1999). On the other hand, the Osaka variant is common among Koreans and Japanese, with a high frequency of 2.8%–4.1%. However, this variant is rare among other Asians (i.e., Taiwanese and Chinese) and is absent among Caucasians. We suspect that the Osaka variant (A198V mutation) originated in the common ancestors of Koreans and Japanese and that the origin of this variant is relatively new, compared with the Duarte variant.

In the present study, three patients had 10% of GALK activity and delayed galactose metabolism, on the basis of the results of the galactose-tolerance test. The long-term effects of intermittent episodes of hypergalactosemia caused by partial GALK deficiency on the lens structure and function are not clear. Accordingly, we examined the relationship between A198V mutation and cataract formation to estimate the long-term effects of reduced GALK activity by the Osaka variant and the requirement of strict lactose-free diet in patients with the Osaka variant. We examined the relationship between A198V and cataract by screening for the A198V allele in patients with bilateral idiopathic cataract sufficiently advanced to require cataract surgery in at least one eye. All patients were >55 years of age. Genomic DNA was isolated from lymphocytes, and the detection of A198V mutation was performed as described above. The frequency of A198V mutation in such patients was 7.8%, which was significantly higher than in the general Japanese population (4.1%; $P = .023$; χ^2 test). Among 148 patients with cataract, 2 homozygous and 19 heterozygous patients with the Osaka variant (A198V) were identified. Our results showed a significant correlation between the A198V mutation and cataract. Our three probands and one brother in family 1 still remain on a very mild restriction of lactose intake.

Age-related cataract is a major public health problem because of its high prevalence, which amounts to 15%–20% among sexagenarian subjects (Kahn et al. 1977; Sasaki et al. 1987; Sommer et al. 1991). The risk factors of age-related cataract are multifactorial and include host factors (age, sex, race, and genetic factors) and environmental factors (smoking, food, daylight, medicines, and education). Galactose has also been suspected to influence cataractogenesis for the following reasons: (1) aging is directly associated with lower metabolic capacity and lower erythrocyte GALK and/or GALT activities (Birlouez-Aragon et al. 1993); (2) a chronic impairment of galactose metabolism (partial deficiency of GALT and GALK, including in heterozygotes) is involved in the pathogenesis of presenile and senile cataract (Monteleone et al 1971; Skalka and Prchal

1980; Stambolian et al. 1986; Simonelli et al. 1992); and (3) the consumption of milk and lactase activity, reflecting the amount of galactose absorption, is associated with a high incidence of cataract (Simoons 1982; Rinaldi et al. 1984; Bhatnagar et al. 1989; Simonelli et al. 1989). However, the incidence of typical GALK deficiency is generally low (1/1,000,000) worldwide, and the frequency of carriers is also too low (1/500) to explain the high incidence of the age-related cataract formation. On the other hand, our data showed that the Osaka variant is prevalent, with a carrier frequency of 1/12 (7.9%) in the general population. Furthermore, the Osaka variant tended to concentrate in patients with cataract (the carrier frequency is 1/7 [14.4%]). Another characteristic of the Osaka variant in relation to cataract formation is an 80% reduction of GALK activity. The GALK activity (60% of that in healthy subjects) in carriers of the Osaka variant is similar to that (50%) in carrier for complete GALK deficiency. In this regard, Hammond et al. (2000) reported that genetic factors are most important among the various factors causing cataract in the elderly. The Osaka variant is the first genetic variant that could directly explain the role of genetic factors in the development of cataract in elderly Japanese and Koreans. Further research may allow the identification of similar variants in other races.

The protocols of the studies described in this report were approved by the institutional review boards of Osaka City Environment and Public Heath Association and Osaka City University Graduate School of Medicine. Informed consent for genetic analysis was obtained from all patients or their parents.

Electronic-Database Information

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

GenBank, http://www.ncbi.nlm.nih.gov/Web/Genbank/index.html (for the sequence of the human GALK gene [accession numbers L76927 and AF084935])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for GALK deficiency [MIM 230200] and galactosemia caused by GALT deficiency [MIM 230400])

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