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Galactokinase gene mutations and age-related cataract. Lack of association in an Italian population

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Purpose: To investigate possible associations between sequence changes in the galactokinase gene (*GALK1*) and age-related cataract in a European population.

Methods: Persons without lens opacities and persons with clinically significant age-related cataract were selected from those participating in the Collaborative Italian-American Clinical Trial of Nutritional Supplements and Age-Related Cataract or from those attending the Section of Ophthalmology of the University of Parma for cataract surgery. Type and severity of the opacities were assessed by slit-lamp and retro-illumination lens photographs. Mutations in *GALK1* were identified by PCR amplification of individual exons and flanking sequences and sequencing using fluorescent terminator technology in an ABI 377 Prism or 3100 automated DNA sequencer.

Results: DNA samples were obtained from 115 individuals with clear lenses and from 185 individuals with cataract (106 with any nuclear, 88 with any cortical, and 25 with any posterior sub capsular cataract). 157 of the 185 patients with cataract (85%) were age-matched with a control within an age range of plus or minus 1 year. SNPs causing amino acid changes in the galactokinase protein were identified in exon 4; I184M, 1/115 control versus 0/185 cataractous individuals, $p=0.38$, exon 6; G274D, 0/115 control versus 1/185 cataractous individuals, $p>0.99$, and exon 7; V338A, 0/115 control versus 1/185 cataractous individuals, $p>0.99$. Thus, there were no significant differences in the distribution of sequence alterations resulting in amino acid changes between control and cataractous individuals. Eighty samples showed a C to T transition 43 bases into intron 7 (46 cataracts and 34 controls). Testing the distribution of the intron 7 findings showed Hardy-Weinberg equilibrium for both cases ($p=0.73$) and controls ($p=0.51$). There was no difference in C/T distribution between cases and controls ($p=0.27$).

Conclusions: In this northern Italian population age-related cataract does not appear to be associated with *GALK1* alleles. Since this is due to a lack of sequence changes in both affected and control individuals, this study cannot rule out the possibility of an association in other populations.

Galactokinase (EC 2.7.1.6) phosphorylates galactose to form galactose-1-phosphate, allowing its conversion to UDP-galactose, from which it can be isomerized to UDP-glucose or incorporated into glycolipids and glycoproteins. Galactokinase deficiency is an autosomal recessive disorder (OMIM 230200) characterized by hypergalactosemia and the primary clinical manifestation of cataract formation at birth or in the first few months of life. In galactosemia, while some galactose is oxidized to galactonate, most of it is reduced by aldose reductase to form galactitol, which accumulates in the lens with subsequent hyperosmotic disruption of lens fiber cells. Heterozygous galactokinase deficiency has been reported to be significantly increased in Caucasian patients developing bilateral idiopathic cataract between 20 and 55 years of age, especially in the presence of a high galactose diet [1]. The incidence of heterozygous galactokinase deficiency has been estimated to be approximately 1 in 309 in Caucasians by enzyme activity, corresponding to an allele frequency of approximately 3×10^{-6} [2]. The human galactokinase gene (*GALK1*), cloned in 1995 and mapped to chromosome 17q24, consists

of 8 exons and 7 introns and encodes a protein of 392 amino acids [3]. All known galactokinases share three highly conserved regions containing a galactokinase signature sequence (exon 1) and two separate ATP binding motifs (exons 3 and 7). A total of 24 mutations in *GALK1* resulting in galactokinase deficiency have been described so far [3-7]. Most of these mutations are confined to individual families, but some have been observed in individuals of Costa Rican/European descent (Q382X) and of eastern European origin (Bosnian, Romani Gypsies, P28T).

Four mutations have been identified only in Japanese individuals [4] and the A198V mutation, known as the Osaka variant, has been reported to show an allele frequency of 4.1% in a sample of 291 Japanese controls, and to be associated in homozygotes with an 80% reduction of Galk activity [8]. The Osaka variant was found in the same study to have an allele frequency of 2.8% in Koreans but to be rare in other Asian populations and absent in Caucasians. In a group of 148 Japanese patients over 55 years of age and undergoing surgery for age-related cataract, the frequency of the mutation was found to be significantly increased (7.8%, $P<0.023$) compared to that in the controls and 2 homozygous and 19 heterozygous individuals were identified [8]. Taken together with the results of

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Stambolian, et al. [1] associating heterozygous *GALK1* deficiency with presenile cataract, these data suggested that as yet uncharacterized *GALK1* alleles resulting in mild deficiency might contribute to age-related cataract in European populations. In this study we investigated the occurrence of mutations in *GALK1* from 185 individuals with age-related cataract and 115 age-matched controls with clear lenses in a Northern Italian population.

METHODS

Subjects: We included in the study a total of 300 individuals, 185 with age-related cataract and 115 with clear lenses. Most subjects (135 of those with cataract, 73%; and 115 of the controls, 100%) were participants in the Collaborative Italian-American Clinical Trial of Nutritional Supplements and Age-related Cataract (CTNS). Fifty subjects (27%) of the cataract group were patients admitted to the Institute of Ophthalmology of the University of Parma for age-related cataract surgery. CTNS is an ongoing, National Eye Institute supported, thirteen year study whose primary objective is to evaluate the safety and efficacy of a vitamin-mineral supplement containing recommended daily allowance (RDA) dosages in preventing age-related cataract or delaying its progression.

In CTNS lens status is assessed twice at baseline and then at each annual visit by grading slit-lamp and retro-illumination lens photographs according to a modification of the Age-Related Eye Disease Study (AREDS) lens opacities grading system [9]. A revised scale for nuclear opalescence was adopted after densitometric evaluation of the slit-lamp standard photographs suggested that the introduction of an additional standard between original standards 5 and 6 would improve scoring. Nuclear severity grade is therefore assigned on a scale defined by 8 standards with decimal grades ranging from 0.9 to 8.1. Both cortical and posterior sub-capsular opacities are graded on retro-illumination photographs by dividing the pupillary area into 17 sub-fields by superimposing the photograph to be graded on a grid of concentric circles with eight radiating spokes. Estimates of each sub-field weighted by area are combined to calculate the percent involvement of the central 5 mm circle and the entire visible area of the lens. The same photographic technique and grading system were utilized for the non-CTNS patients with age-related cataract included in this study.

In this study a person was classified as having clear lenses if both eyes had a grade <3.0 for nuclear opalescence (N), <5% within the 5 mm circle for cortex (C), and <0.5% within the 5 mm circle for posterior subcapsular opacities (PSC). A participant was designated as having a clinically significant cataract if at least one eye had a severity grade >5.0 for N, and/or >25% involvement in the central 5 mm circle for C, and/or >25% involvement outside the 5 mm circle, and/or >12.5% involvement in the central 5 mm circle for PSC. Early cataracts were therefore not included in this study, leaving a free interval between completely clear lenses and clinically significant types of cataract. A cataract was identified as pure if only one type of opacity had a severity grade sufficient to qualify as a clinically significant opacity, and the severity of

the other types of opacity, if present, was compatible with a definition of no opacity. All other types were classified as mixed cataracts.

Eligible persons were systematically selected from the CTNS cohort or from those attending the Institute of Ophthalmology for cataract surgery on the basis of the last available lens severity photographic grading score which, in most cases, was performed not more than 6 months before blood drawing for the study. All participants signed a written informed consent form agreeing to participate in the ancillary genetics study. The ancillary study was approved by the CTNS DSMC and by the University of Parma and National Eye Institute IRBs and adhered to the tenets of the Declaration of Helsinki.

Genomic DNA extraction and sequencing: Human blood samples (18 ml) were collected in citrate anticoagulated tubes and frozen at -80 °C. Genomic DNA was isolated using a standardized protocol that included cell lysis with anionic detergent, high salt precipitation of proteins, ethanol precipitation to concentrate DNA followed by further purification of DNA with a buffered phenol/chloroform mixture [10]. After a final precipitation with alcohol the DNA pellet was dissolved in TE (Tris-EDTA 10 mM, pH 8.0).

Individual exons of *GALK1* were amplified by the PCR as described by Stambolian, et al. [3]. PCR products were analyzed on 1% agarose gels and purified using a Quick Step 2, 96 well PCR purification kit (Edge Biosystems, Gaithersburg, MD) and concentrated by ethanol precipitation. Sequencing in the forward and reverse directions using the same exon specific primers as were used for PCR amplification was performed using an Amplitaq FS cycle sequencing kit (ABI, Foster City, CA) with dye-labeled terminators according to the manufacturer's instructions. Sequencing reactions were purified using Performa DTR 96 well short plat kit (Edge Biosystems, Gaithersburg, MD) and sequences were analyzed on an ABI 3100 DNA analysis system (ABI).

Statistical analysis: The frequencies of sequence changes in the case and control groups were compared using Fisher's exact test. Hardy-Weinberg equilibrium of the sequence change in intron 7 was assessed by comparing the observed and expected C-T pair frequencies by Fisher's exact test. The Breslow test of homogeneity was applied to test whether the C-T pair distributions of the case and control groups were equal. All statistical analysis was performed with Statistical Analysis System (SAS) software.

RESULTS & DISCUSSION

The distribution of participants by lens status, age, and other factors are shown in Table 1. Of the 185 patients with cataract who participated in the study, 106 had a nuclear cataract (79 pure form, 74.5%), 88 had a cortical cataract (66 pure form, 75%), and 26 had a posterior sub-capsular cataract (7 pure form, 26.9%). 27 patients with N cataract had a mixed type of opacity (11PN, 14 CN, 2 CPN), 22 patients with C cataract had a mixed type of opacity (14 CN, 6 CP, 2 CPN), and 19 patients with P cataract had a mixed type of opacity (11 PN, 6 CP, 2 CPN). The mean age of controls was 68 years (range 59 to 78 yrs) and the mean age of cataracts was 71 years (range

50 to 80 yrs). 157 of the 185 patients with cataract (85%) were age-matched with a control within an age range of plus or minus 1 year (the same participant with clear lenses could act as control for patients with different types of opacities).

Eight single nucleotide polymorphisms (SNPs) were identified in the exons of *GALK1* and their flanking intronic sequences (Table 2). Three coding SNPs resulted in amino acid changes in 3 individuals; 615C->G (I184M) in 1 individual without cataracts, 884G->A (G274D) in 1 individual with cortical cataracts, and 1076T->C (V338A) in one individual with nuclear cataracts. In addition, there were three coding SNPs not resulting in amino acid changes; 252G->A (L63L) in 1 patient with nuclear cataracts, 315G->A (E84E) in 1 individual with cortical cataracts, and 1119G->A (T352T) in 1 individual with mixed nuclear and posterior subcapsular cataracts. Finally, two intronic SNPs were detected. The first was a G to A change 34 bases into intron 4 in two individuals, one with a nuclear cataract and one control individual. The second was a frequent polymorphic change, C to T 43 bases into intron 7, which was seen in 80 individuals, 76 heterozygous (44 cataractous and 32 control individuals) and 4 homozygous (2 cataractous and 2 control individuals). As can be seen from Table 2, none of these changes showed a statistically significant association with cataracts, nor did they show significant association in their aggregate. The results were not different if analyzed only for the 157 age-matched cases. The intron 7 SNP was in Hardy-Weinberg equilibrium in both cataract and control groups, and was distributed evenly between them.

GALK1 was a logical candidate gene for age-related cataract as suggested by a number of observations; (a) complete *GALK1* deficiency results in galactosemia with congenital cataracts [11], (b) some *GALK1* alleles resulting in partial deficiency are associated with presenile cataracts in the presence of a diet rich in galactose [1], and (c) the Osaka (A198V) variant, with about 20% of residual activity due to a shorter

half life, appears to contribute to a significant fraction of age related cataracts in Japanese [8]. The Osaka variant was also found in Koreans as well as some Chinese, although not, so far, in European or African populations. The Osaka variant was not present in the Northern Italians studied here, either among cataractous or control individuals. Neither were any of the alleles that were found associated with age-related cataract, although the *GALK1* activity of these allelic variants was not determined. The upper 95% confidence limit on the occurrence of any of these sequence changes among these cataract patients doesn't exceed 3.0%. This rarity excludes *GALK1* sequence changes as a significant cause of age-related cataract in this population.

The original design was a case-control study with case-control pairs matched on age. Following Schlesselman's sample size formula for the McNemar test analysis, it was determined that for a minimum power of 80% with two-sided 0.05 level testing, 100 pairs would be adequate to detect relative risks of from 4 (assuming a 5% mutation prevalence in controls) to 10 (assuming a 1% prevalence in controls). In the course of the study the difficulty in recruiting older controls led to the abandonment of pair matching in favor of an age-adjustment in the analysis stage. At study's end, the observed prevalences were much lower than assumed at the outset. Consequently the post hoc power for detecting odds ratios even as high as 10.0, by Fisher's exact test, was negligible (less than 1%) for all except the Intron 7 comparison, which, although not significant, was capable of detecting a two-fold increase in odds with 80% power.

It should be noted that the lack of association seen in this population was likely due to the paucity of allelic variants detected, and thus does not in any way speak to the ability of the Osaka or other allelic variants of *GALK1* to contribute to cataracts when present. The study population is a fairly homogeneous Northern Italian population and these results may be extrapolated to some degree to other European populations, but certainly not to African or Asian population groups. However, in this Northern Italian population, specific *GALK1* alleles do not appear to be associated with age-related cataract.

TABLE 1. DISTRIBUTION OF PARTICIPANTS BY LENS STATUS, AGE, AND OTHER FACTORS

	Nuclear opacities	Cortical opacities	PSC opacities	Controls (clear lenses)
Total number of lenses	106	88	26	115
Pure cataracts	79	66	7	
Mixed cataracts	27	22	19	
Mean severity score (pure type SD)	5.6 (0.6)	43.3 (20.3)	32.0 (19.9)	
Median age (range)	72 (50-80)	71 (59-79)	68 (61-77)	69 (59-78)
Female	46.2%	52.3%	44.0%	40.0%
Hypertension	22 (20.7%)	22 (25.0%)	11 (42.3%)	17 (14.8%)
Diabetes	7 (6.6%)	10 (11.4%)	7 (26.9%)	6 (5.2%)
Smoking (ever)	58 (54.7%)	32 (36.4%)	9 (34.6%)	60 (52.2%)
Myopia >10 D	1 (0.9%)	0 (0%)	1 (3.8%)	0 (0%)
IOP >21	1 (0.9%)	2 (2.2%)	2 (7.6%)	0 (0%)

Some individuals with mixed cataract types are counted in more than one category. The condition titled "Hypertension" indicates anti-hypertensive therapy or systolic >170 and/or diastolic >95.

TABLE 2. DISTRIBUTION OF *GALK1* GENE SEQUENCE CHANGES AND ASSOCIATED AMINO ACID CHANGES AMONG CATARACTOUS AND CONTROL INDIVIDUALS.

Exon/ intron	SNP	Codon change	Cataracts	Controls	p value
Exon 2 (56-118)	c. 252G->A	L63L	1(N)	0	>0.99
	c. 315G->A	E84E	1(C)	0	>0.99
Exon 4 (159-203)	c. 615C->G	I184M	0	1	0.38
Intron 4	IVS4+34G->A		1(N)	1	>0.99
Exon 6 (264-314)	c. 884G->A	G274D	1(C)	0	>0.99
Exon 7 (315-368)	c. 1076T->C	V338A	1(N)	0	>0.99
	c. 1119G->A	T352T	1(PN)	0	>0.99
Intron 7	IVS7+43C->T SNP rs743554		48	36	0.27

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