Protective association of A-T-T haplotype of DMT1 gene against risk of human age-related nuclear cataract

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ABSTRACT

Background: Age-related cataract (ARC) is profoundly associated with oxidative stress. Iron plays a pivotal role in generating oxidative stress and promoting deleterious irreversible damage to the macromolecules. Divalent metal transporter 1 (DMT1) mediates the uptake of iron into the cell. Aberrant transcript expression of DMT1 gene in lenses of human ARC was reported. The present investigated the genetic association between DMT1 gene polymorphisms and risk of ARC.

Methods: DNA from peripheral blood of ARC subjects (n = 764) and age-matched controls (n = 794) was isolated. Genotyping of three single-nucleotide polymorphisms (SNPs) – rs224589 (C/A), rs1048230 (T/C), and rs2285230 (T/C) – of DMT1 gene was performed by polymerase chain reaction (PCR)-restriction fragment length polymorphism technique. Level of DMT1 transcript expression was determined by quantitative real-time PCR analysis using RNA from lens epithelial and fiber cells.

Results: Nuclear cataract showed a higher frequency of CC genotypes (OR = 1.40; 95% CI = 1.01–1.95; p = 0.04) of SNP rs224589 and a significantly lower frequency of A-T-T haplotype (OR = 0.63; 95% CI = 0.42–0.92; p = 0.02) than that of controls. The A-T-T haplotype demonstrated a dominant protective effect against disease risk when compared to the more common haplotype (C-T-T) (p = 0.01). The haplotype pairs C-T/T/C-T-T and A-C-C/A-C-C showed a higher level of transcript expression of DMT1 than C-T-T/A-T-T haplotype pair (p < 0.05). Further, a novel genetic variation (c.1328A>G; p.N443S) in exon 3 of DMT1 gene was observed in a subject with nuclear cataract.

Conclusions: The results highlighted a protective association of A-T-T haplotype against the risk of ARC.

Introduction

Cataract, an opacification of eye lens, obscures the visual path and impedes the sense of vision. This painless loss of vision occurs principally – but not exclusively – in the elderly. Age-related cataract (ARC) is the leading cause of visual disability and is an increasingly important problem worldwide. It is the cause of blindness in 51% of the world’s 39 million blind people (1). Implantation of an artificial intraocular lens is the most commonly preferred treatment for it (2,3).

Development of ARC is influenced by multiple factors and is found to be associated with oxidative stress (4–6), diabetes (7), renal failure (8), hypertension (9), sunlight (10), myopia (11), infrared radiation (12), ultraviolet radiation (13,14), cigarette smoking (15,16), and obesity (17). A few family-based linkage studies in the past reported a potential association of diverse genetic loci (1q31, 2p24, 2q11, 4q32, 6p12–q15, and 15q13) and candidate genes (HSF4, GJA8, EPHA2, and GST) with ARC (18). Candidate gene association studies revealed exciting results on the association of a few single-nucleotide polymorphisms (SNPs) of SOD1, SOD2, SOD3, CAT, GPX, GST, XPD, XCC1, and WRN genes (19–24) with ARC. Since these genes account only partially for the genetic predisposition to ARC, a genetic association study of this kind is needed to explore the involvement of other genes in the development of ARC.

The genes responsible for regulation of transition metals’ homeostasis could be the ideal candidates, because beyond the normal threshold level the transition metals are found to augment redox reactions culminating in oxidative stress via Haber-Weiss and Fenton reactions. A plethora of reports ascertain the presence of transition metals such as aluminum, chromium, copper, manganese, nickel, iron, and zinc in human lenses, and a few of these transition metals play a pivotal role in generating oxidative stress (6,25–32). Among these metals, iron is considered to be one of the most important elements as it acts as an essential cofactor for many important proteins such as hemoglobin, cytochromes, oxygenases, flavoproteins, and redoxins. However, unlike other metal ions, iron not only causes considerable oxidative stress but also promotes deleterious irreversible damage to the macromolecules such as DNA, proteins, and lipids by causing the release of hydroxyl radical (OH·) (33). An increased level of lenticular iron in nuclear cataract and a role...
of iron-mediated OH\(^{-}\) production in the etiology of age-related nuclear cataract have also been reported (28).

Divalent metal transporter 1 (DMT1), an isoform of natural resistance-associated macrophage protein 2 (NRAMP2), mediates transport of ferrous iron from the lumen of the intestine into the enterocyte and export of iron from endocytic vesicles. It has an affinity not only for iron but also for other divalent cations including cadmium, cobalt, copper, lead, manganese, nickel, and zinc. DMT1 gene is located on chromosome 12q13 in humans and expresses four major isoforms; two with iron-responsive elements (1A/+IRE and 2/+IRE) and two without iron-responsive elements (1A/-IRE, and 2/-IRE) (34). Mutations or polymorphisms of DMT1 gene may have an impact on human health by disturbing metal trafficking (34,35) and augmenting systemic and tissue overload of divalent metal ions (36). Association of different genetic polymorphisms of DMT1 with age-related macular degeneration (37), Alzheimer’s disease (38), hereditary hemochromatosis (39), microcytic anemia (40,41), Parkinson’s disease (42), and Wilson’s disease (43) have been reported.

Previous study from our laboratory showed a higher level of divalent metal ions such as copper, iron, manganese, and zinc and a differential expression of DMT1 gene transcripts in human cataract lenses (6,32). Therefore, we hypothesize that DMT1 genotypic variations might contribute to the aberrant expression of DMT1 followed by a significant increase in the endogenous iron level and consequent lens opacities through oxidative damage. Further, paucity of information on the relationship between genetic polymorphisms of DMT1 gene and its influence in transcript expression in the development of human cataracts prompted us to investigate the potential association of SNPs of DMT1 gene with human ARC.

Materials and methods

Subjects and samples

The present study adheres to the tenets of the Declaration of Helsinki and was approved by the Institutional Ethical Committee. Patients, after pupil dilatation, were categorized using a slit lamp biomicroscope and graded using the Lens Opacities Classification System (LOCS III) (44). LOCS III consists of grading of nuclear opalescence and color (N; 0.1–6.9), cortical opacity (C; 0.1–5.9), and subcapsular opacity (P; 0.1–5.9). Patients were divided into five groups according to the type of the cataract: group 1, nuclear cataract (NC); group 2, cortical cataract (CR); group 3, subcapsular cataract (SC); group 4, mixed cataract (MC), and group 5, hypermature cataract (HMC). Patients were selected for the pure cataract group, if only one parameter of the LOCS grading system was higher than LOCS grading-2 and the patient was categorized under MC, if an eye presented more than one parameter with LOCS grading-2. Patients having a history of smoking, diabetes, hypertension, previous ocular surgery, ocular inflammation, myopia, uveitis, glaucoma, and under steroid therapy were excluded from the study. A written informed consent was obtained from the participants before the standard preoperative examinations were carried out. About 2 ml of peripheral venous blood and postsurgical lens aspirate containing lens epithelial cells and fiber cells were obtained postoperatively from the patients recruited for cataract surgery. Two milliliters of peripheral venous blood were also collected from non-cataract control subjects who attended the clinic for general eye checkup.

Selection of SNPs

Eighteen SNPs spanning 40.37 Kb of genomic region on chromosome 12 coding for DMT1 (SLC11A2) gene were selected for the preliminary screening, and only three SNPs (rs2285230, rs1048230, and rs2245859) spanning 17.3 Kb were selected for genotyping based on the availability of polymerease chain reaction – restriction fragment length polymorphism (PCR-RFLP) genotyping method.

Genotyping of DMT1 polymorphisms by PCR-RFLP

DNA was extracted from the blood using the NucleoSpin Blood genomic DNA extraction kit (Macherey-Nagel GmbH & Co. KG, Germany) as per the manufacturer’s instruction. Primers for the genotyping of selected SNPs (rs224589, rs1048230, rs2285230) were obtained from previous studies (40,41). PCR was performed using 1X SappireAmp Fast PCR Master mix (TaKaRa Bio Inc., Japan) in a 20 μl reaction mix consisting of 20 ng genomic DNA and 10 pmoles each of forward and reverse primers (Supplementary Table 1). The thermal reaction comprised of one cycle of initial denaturation at 94°C/3 min and 40 cycles of second denaturation at 94°C/5 s, annealing at 60°C/5 s, extension at 72°C/10 s, and a final extension at 72°C/3 min. The amplicons were digested using specific restriction endonucleases (MnlI (rs224589); MboI (rs1048230); RsaI (rs2285230)) in a 20 μl reaction mix consisting of 10 μl of ampiclon, 1 unit of restriction endonucleases, and 1X buffer (New England Biolabs, Hitchin, UK). The reaction mixture was incubated at 37°C for 4 h. The digested amplicons were separated by electrophoresis using a 2% agarose gel. The digested pattern was visualized by UV-transilluminator upon ethidium bromide staining. Allele and genotypes were interpreted and scored based on the specific digestion pattern.

Sanger’s bidirectional sequencing

Sanger’s bidirectional sequencing was performed to confirm the genotypes of all three SNPs for randomly selected samples. The PCR amplicons were purified using the nucleo-spin gel extraction kit (Macherey-Nagel, Germany) adhering to the manufacturer’s instruction. Sanger’s bidirectional sequencing was carried out commercially using the BigDye®Terminator V3.1 Cycle Sequencing kit and run on the ABI 3130xl Genetic Analyzer. Nucleotide variations were examined using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The possible impact of amino acid substitution on protein function was analyzed using web-based tools including Polymorphism Phenotyping version 2 (PolyPhen 2.0) (http://genetics.bwh.harvard.edu/pph2/) (45), Sorting Intolerant From Tolerant (SIFT) (http://sift.jcvi.org/) (46), Protein Variation Effect Analyzer (PROVEAN) (http://provean.jcvi.org/index.php) (47), and Mutation Taster (www.mutationtaster.org/)
A Polyphen score of >0.85 is interpreted as probably damaging and a score ranging from 0.15 to 0.85 is interpreted as possibly damaging. A SIFT score below 0.05 indicates that the amino acid change is not tolerated. A PROVEAN score of ≤−2.5 is deleterious whereas a score of >−2.5 is neutral. The prediction of the secondary structure was carried out using the Chou and Fasman Secondary Structure Prediction server (http://cho-fas.sourceforge.net/index.php) (49). The Human Splicing Finder (http://www.umd.be/HSF3/index.html) was used to assess the possible impact of nucleotide variations on mRNA splicing (50). Analysis of amino acid conservation across species was done using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (51).

Quantification of DMT1 mRNA expression

Lens aspirate containing lens epithelial and fiber cells was transferred to 50 ml sterile conical screw-capped tubes and centrifuged at 3000 × g for 10 min at 4°C. The pellet was washed with sterile phosphate buffered saline, pH 7.4, homogenized in 1 ml of TriZol reagent, and total RNA was extracted. Total RNA concentration was determined by Qubit RNA Assay Kit in a Qubit Fluorometer as per the manufacturer’s instruction. (Thermo-Fisher Scientific, USA). Complementary DNA (cDNA) was synthesized using First strand cDNA synthesis Kit (Invitrogen Inc., USA) as per the manufacturer’s instruction in a 20 μl reaction volume containing 250 ng of total RNA. Iron-responsive elements (IRE) and non-IRE forms of DMT1 were amplified in a 20 μl reaction volume containing 1X SYBR green master mix, 10 ng cDNA, and 10 pmole each of forward and reverse primers (Supplementary Table 1) using standard operating program. This program consists of pre-incubation at 95°C for 1 min, amplification at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 10 s in a LightCycler 480II instrument. Expression level of the target gene was normalized by the expression of level of house-keeping gene (GAPDH). The difference in the level of expression between the house-keeping and target genes was calculated based on the $2^{-ΔCt}$ function.

Statistical analysis

Allele, genotype, haplotype frequencies, linkage disequilibrium, and Hardy–Weinberg equilibrium (HWE) were calculated using SHEsis online tool (http://analysis.bio-x.cn/myanalysis.php). Chi-squared or Fisher’s exact test (two-sided) was applied to test the association between the alleles, to assess the genotypes in relation to the cases and controls, and to test deviation of the genotype distribution from HWE. The OR and 95% CIs were calculated to estimate the strength of the association of genetic polymorphisms between cases and controls (http://www.hutchon.net/confidort.htm). Bonferroni corrected $p$ value of less than 0.025 ($p$ value/no.of SNPs tested) was set to test the significance in allele, genotype, and haplotype frequencies between controls and cases. The effect of haplotype or haplotype features on disease risk was calculated by expectation–conditional-maximization (ECM) algorithm using CHAPLIN software (52,53). Different genetic models such as dominant (MM vs. Mm+ mm), recessive (mm vs. Mm+MM), over-dominant (MM+mm vs. Mm), co-dominant (MM vs. Mm vs. mm), and additive and multiplicative models were checked, and the most significant models were applied. For additive model the genotypes MM, Mm, and mm were inferred, respectively, as lowest, intermediate, and higher risk for disease, and for multiplicative model the genotypes MM, Mm, and mm were inferred, respectively, as highest, intermediate and lower risk for disease. The significance of difference between two independent proportions was calculated by Z-test with 2-tailed probability. Continuous variables are presented as mean ± SE and $p$ values of <0.05 were considered statistically significant. Power of the study design/association was calculated using CaTS Genetic Power Calculator (54) (http://csg.sph.umich.edu/abecasis/CaTS).

Results

Demographic status

No significant age ($p = 0.90$) and gender ($p = 0.09$) difference was observed between cases and controls (Table 1). Among cataracts, the prevalence of mixed cataract (26.6%, 203/764) was found to be the highest followed by nuclear (22.5%, 172/764), cortical (19.0%, 145/764), subcapsular (17.5%, 134/764), and hypermature (14.4%, 110/764) cataract.

SNP genotyping and Sanger’s bidirectional sequencing

Genotyping of rs224589 (c.309 + 44A>C) yielded three fragments (216, 100, and 36 bp) for allele A and four fragments (183, 100, 36, and 33 bp) for allele C when digested with MnlI (Figure 1a (i)). While rs1048230 (c.1254T>C) yielded two fragments (196 and 31 bp) for C allele, and a single fragment (229 bp) for T allele when digested with MboI (Figure 1a (ii)). The SNP rs2285230 (c.*583T>C) produced a single fragment (231 bp) for T allele and two fragments (147 and 84 bp) for C allele when the amplicons (231 bp) were digested with RsaI.

Table 1. Demographic status of subjects.

|                | Controls | Cases     | Types of cataract |
|----------------|----------|-----------|-------------------|
|                | n = 794  | n = 764   | CR    | NC   | SC   | MC   | HMC  |
| Female/male    | 366/428  | 320/444   | 0.09* |      |      |      |      |
| Mean age (years) ± SD | 60.1 ± 12.1 | 59.0 ± 8.6 | 59.5 ± 8.7 | 59.7 ± 7.9 | 51.9 ± 6.1 | 61.1 ± 8.5 | 55.4 ± 5.7 |
| $p$ Value      |          | 0.11*     |       |      |      |      |      |

CR: cortical cataract, NC: nuclear cataract, SC: subcapsular cataract, MC: mixed cataract, HMC: hyper mature cataract; SD: standard deviation *indicates $p$ value for female/male ratio between cases and controls; # indicates $p$ value for age between cases and controls.
Sanger’s bidirectional sequencing analysis confirmed all the genotypes of each SNP (Figure 1b (i-ix)).

**Association of allele and genotype frequencies of SNPs with disease risk**

All the three tested SNPs were in complete linkage disequilibrium ($D' = 1.00$, $r^2 = 0.54$ between rs224589 and rs1048230; $D' = 1.00$, $r^2 = 1.00$ between rs1048230 and rs2285230) and all the genotypes of the three SNPs were in HWE for both cases and controls ($p > 0.05$).

No significant difference in allele frequency between controls and cases for SNP rs224589 was observed. However, an insignificant difference in allele frequencies between cases and controls for SNPs rs1048230 (OR = 1.16, 95%CI = 0.98–1.36, $\chi^2 = 3.02$, $p = 0.08$) and rs2285230 (OR = 1.16, 95%CI = 0.98–1.36, $\chi^2 = 3.02$, $p = 0.08$) was seen. Further, a borderline difference in the frequency of ‘C’ alleles of rs1048230 (OR = 1.33, 95%CI = 1.04–1.69, $\chi^2 = 5.12$, $p = 0.02$) and
Haplotype distribution and related OR values.

| Subjects | A-C-C | A-T-T | C-T-T |
|----------|-------|-------|-------|
| Cases (n = 764) | 395 (0.26) | 171 (0.11) | 962 (0.63) |
| OR (95% CI), \( \chi^2 \) (p) | 1.16 (0.98–1.36), 3.02 (0.08) | 0.77 (0.62–0.95), 5.98 (0.02) | 1.01 (0.87–1.17), 0.02 (0.89) |
| CR (n = 145) | 36 (0.12) | 178 (0.62) |
| OR (95% CI), \( \chi^2 \) (p) | 0.86 (0.59–1.26), 0.59 (0.44) | 0.95 (0.73–1.22), 0.19 (0.66) |
| NC (n = 172) | 32 (0.09) | 230 (0.67) |
| OR (95% CI), \( \chi^2 \) (p) | 1.04 (0.79–1.36), 4.62 (0.02) | 1.20 (0.94–1.53), 2.09 (0.15) |
| SC (n = 134) | 42 (0.16) | 395 (0.26) |
| OR (95% CI), \( \chi^2 \) (p) | 1.13 (0.79–1.62), 0.46 (0.50) | 0.95 (0.73–1.24), 0.13 (0.72) |
| HMC (n = 110) | 31 (0.11) | 116 (0.29) |
| OR (95% CI), \( \chi^2 \) (p) | 1.24 (0.95–1.71), 1.81 (0.18) | 0.98 (0.73–1.31), 0.02 (0.90) |
| MC (n = 203) | 252 (0.62) |
| OR (95% CI), \( \chi^2 \) (p) | 1.24 (0.95–1.71), 1.81 (0.18) |

*Frequency <0.03 in both control and case has been dropped.
cataract, the SNPs rs1048230 (ORR = 1.92, 95% CI = 1.12–3.30, χ² = 5.72, p = 0.02) and rs2285230 cataract (ORR = 1.92, 95% CI = 1.12–3.30, χ² = 5.72, p = 0.02) were found to have borderline difference compared to control groups (Table 3).

**Association of haplotype frequencies of SNPs with disease risk**

In general, no significant difference in the distribution of haplotypes A-C-C and C-T-T was found between cases and controls. In contrast, the distribution of A-C-C haplotype was significantly higher in mixed cataract (OR = 1.33, 95% CI = 1.04–1.69, χ² = 5.13, p = 0.02) when compared to control group. In contrast to this condition, a significant reduction in the distribution of A-T-T haplotype was noted in cases (OR = 0.77, 95% CI = 0.62–0.95, χ² = 5.98, p = 0.02), nuclear (OR = 0.63, 95% CI = 0.42–0.92, χ² = 5.68, p = 0.02), and mixed (OR = 0.63, 95% CI = 0.44–0.90, χ² = 6.38, p = 0.01) groups as compared to control groups (Table 4). Further, the distribution of haplotype pairs C-T-T/A-T-T was found to be significantly decreased in cataract cases compared to controls (p = 0.01), albeit the absence of difference in distribution of other haplotype pairs between cases and controls (Table 5). The ECM algorithm (CHAPLIN) revealed a dominant effect of A-T-T haplotype (Wald = −2.58, AIC = 4473.14, LR = 6.71, p = 0.01) on disease risk (Table 6), i.e. the subjects with one copy of the haplotype face the same risk as those with two copies. The distribution of A-T-T haplotype is decreased in cataract cases implying a protective role, and in fact, the results of ECM analysis indicate that even a single copy of A-T-T haplotype can exhibit a protective influence against disease risk and can act as negative modifier of disease phenotype.

**Association of haplotypes with expression of IRE and non-IRE form of DMT1**

An analysis based on qPCR revealed a significant lower level of expression of IRE (DMT1-IRE) and non-IRE (DMT1-nonIRE) forms of DMT1 in haplotype pairs C-T-T/C-T-T (p < 0.05) than in the common or major haplotype pair C-T-T/C-T-T (Figure. 2(a,b)). Although a higher level of expression of DMT1-IRE was observed in A-C-C/A-C-C than wild-type C-T-T/C-T-T haplotype pair (Figure 2b), it was not statistically significant (Figure 2b). The haplotype pairs A-T-T/A-C-C and A-T-T/A-T-T showed reduced level of expression of both DMT1-IRE and DMT1-nonIRE.

**Mutation screening and in silico analysis of mutant DMT1**

PCR followed by Sanger’s bidirectional sequencing revealed a nucleotide variation c.1328A>G in exon 3 of DMT1 gene (Figure. 3(a,b)) resulting in substitution of an amino acid asparagine (N) by serine (S) at position 443 (p.N443S) in a patient with nuclear cataract, whereas this replacement was found to be absent in control subjects (Figure 3a). Protein alignment of DMT1 showed that the amino acid asparagine is evolutionarily conserved among different species (Figure 3c). Secondary structure prediction of wild-type (Figure 3d) and mutant protein (Figure 3e) of DMT1 showed an addition of helix at around amino acid position 450 owing to substitution of amino acid asparagine by serine. The observed amino acid variation is found to occur at 10th transmembrane domain of DMT1 protein (Figure 3f) closer to the iron-binding region. All the four in silico analyses (i.e. Polyphen, SIFT, Provean, and Mutation Taster) revealed a damaging effect of N443S substitution on the protein function. Human Splice Finder analysis revealed that the mutation c.1328A>G occurring in the late exonic positions created a new exonic ESS (Exonic Splice Silencer) site that has a potentiality to cause alteration of splicing.

**Discussion**

Human eye lens harbors the oldest cells of the body that were formed at the beginning of early embryological development. Instead of substituting older ones with newer cells, the later one covers and compresses the former cells and forms concentric rings of secondary fiber cells. Therefore, any physiopathological change that had occurred at the course of lens development is likely to persist throughout the life and possibly increase in severity with the advancing age. Our previous studies (6,32) along with few others (25–31) reported the

| Table 5. Distribution of different haplotype pairs. |
|-----------------------------------------------|
| **Haplotype pair** | **Controls**, n (%) | **Cases**, n (%) | **p Value** |
|-------------------|------------------|----------------|-----------|
| Common haplotype pair |
| C-T-T/C-T-T | 304 (0.40) | 310 (0.41) | 0.36 |
| C-T-T/A-C-C | 242 (0.32) | 240 (0.31) | 0.69 |
| Rare haplotype pair |
| A-C-C/A-C-C | 45 (0.06) | 58 (0.08) | 0.13 |
| A-T-T/A-C-C | 36 (0.05) | 39 (0.05) | 0.60 |
| A-T-T/A-T-T | 21 (0.03) | 15 (0.02) | 0.37 |

*Z-test (2-tailed probability) for proportion was performed.

| Table 6. Effect of haplotype pair (C-T-T/A-T-T) on disease risk. |
|-----------------------------------------------|
| **Haplotype pair** | **Effect** | **Wald** (p-value) | **AIC** | **Robust score** (p-value) | **LR** (p-value) |
|-------------------|---------|----------------|-----|-----------------|-------------|
| C-T-T/A-T-T | Recessive | −0.12 (0.91) | 4479.84 | 0.01 (0.91) | 0.01 (0.91) |
| C-T-T/A-T-T | Dominant** | −2.58 (0.01) | 4473.14 | 6.71 (0.01) | 6.71 (0.01) |
| C-T-T/A-T-T | Multiplicative | −2.34 (0.02) | 4474.32 | 5.38 (0.02) | 5.54 (0.02) |
| C-T-T/A-T-T | General | −2.59 (0.01) | 4475.07 | 6.84 (0.03) | 6.78 (0.03) |

Recessive effect: subjects with one copy of the haplotype have the same risk as those subjects with no copies, dominant effect: subjects with one copy of the haplotype have the same risk as those subjects with two copies, multiplicative effect: subjects with one copy of the haplotype are at an intermediate risk (on log scale), with respect to those subjects with zero or two copies. General effect: subjects with one copy of the haplotype have a general change in risk compared to those with zero or two copies.
presence of elevated levels of divalent metals in human lenses. It is believed that the elevated iron concentration could be as a result of gradual accumulation in the growing lens owing to individual’s genetic predisposition.

The involvement of DMT1 in the first line of non-transferrin mediated iron uptake in epithelial cells and a differential expression of it in human cataract lenses (32) make DMT1 as a suitable candidate for understanding the transition metals mediated cataractogenesis. Therefore, we analyzed the distribution of alleles, genotypes, and haplotypes of three informative SNPs such as rs224589, rs1048230, and rs2285230 of DMT1 gene in age-related cataracts and also in age-matched controls to predict a potential genetic association of SNPs with the risk of cataract.

In the present study, the nuclear cataract exhibited a higher distribution of CC genotype of rs224589 with a dominant association while hypermature and mixed cataracts showed a recessive association of CC genotypes of SNPs rs1048230 and rs2285230 with the risk of cataract development. It was reported that the ‘C’ variant of rs224589 increases the risk of age-related macular degeneration and Parkinson’s disease, while the ‘A’ variant plays a protective role (40,41). It was also shown that individuals with CC genotypes of rs224589 tend to have a higher concentration of iron in blood than those with AA and CA genotypes (42).

He et al. (40) reported the presence of haplotype C-C of SNPs rs224589 and rs1048230 in Hans Chinese population and Kelleher et al. (36) have shown that C-T, A-C, and A-T haplotypes of rs224589 and rs1048230 as commonest in Ireland population. However, we noticed haplotypes comprising of C-T-T, A-T-T, and A-C-C, but not of C-C-C alleles of SNPs rs224589, rs1048230, and rs2285230 in our study population. Therefore, the current findings are in good agreement with Kelleher et al. (36) and not with He et al. (40).

The effect of haplotype or haplotype features on disease risk calculated by ECM algorithm allows for dominant, recessive, multiplicative, and general modeling of specific haplotype

Figure 2. Haplotype-based DMT1 gene expression: (a) expression of non-IRE DMT1, (b) IRE form of DMT1 in lens epithelial cells of human cataract lenses. C-T-T: haplotypes formed by combination of all major alleles, A-T-T: haplotypes formed by combination of minor and major alleles; A-C-C: haplotypes formed by combination of all minor alleles. Sample size for each haplotype pair was as C-T-T/C-T-T (n = 39); A-T-T/A-T-T (n = 3); A-C-C/A-C-C (n = 7); C-T-T/A-T-T (n = 15); C-T-T/ A-C-C (n = 28); A-T-T/A-C-C (n = 7). *Indicates p value less than 0.05 and ** indicates p value less than 0.01.
Figure 3. Mutation analysis of DMT1 gene: chromatogram showing (a) wild-type nucleotide sequence of exon 13, (b) mutant sequence of exon 13 having a nucleotide variation (c.1328A>G) leading to change in codon (AAT>AGT), and substitution of amino acid asparagine (N) by serine (S) at position 443 (p.N443S), (c) protein alignment of DMT1 showing conservation of amino acid asparagine (N) among different species and mutant amino acid serine (S) at position 443 in Homo sapiens. Amino acid sequences that are underlined highlight the highly conserved region among species. Secondary structure prediction of (d) wild-type DMT1 protein, (e) mutant DMT1 protein showing an addition of helix at around 450 amino acid position owing to substitution of amino acid asparagines (N) by serine (S) at position 443. (f) A model illustration of DMT1 protein showing 12 transmembrane domains and intracellular and extracellular loops. The observed amino acid change is located at 10th transmembrane domain, and the actual iron binding region lies in the 4th extracellular loop between 7th and 8th transmembrane domains. Open boxes indicates the region of changes and arrow indicates the exact change.
features on disease risk. The decreased frequencies of A-T-T haplotype and C-T-T/A-T-T haplotype pairs in cataract cases, especially nuclear and mixed cataracts, may be interpreted to indicate a dominant protective role of A-T-T against disease risk as compared to the more common C-T-T haplotype. Since the A-T-T haplotype acts as a protective haplotype, the C-T-T haplotype could be considered as a potential risk for the onset of age-related cataract. The results further indicated that even a single copy of A-T-T haplotype can have a protective effect against disease risk and can act as a negative modifier of the disease phenotype. At the same time, the increased frequency of A-C-C haplotype observed in mixed cataracts though lesser in incidence cannot be overlooked.

Although SNPs prove to be attractive biomarkers owing to their characteristic features such as stable inheritance, high abundance, and diversity within and among populations, the application of individual SNPs has been limited because they are of low penetrance, and their expressivity is relatively difficult to identify (55). Therefore, the haplotype information is valuable in linking DNA sequence variation with the disease. In order to assess the influence of haplotype on the expression of DMT1 in lenses, we performed qPCR analysis for both the IRE and non-IRE forms of this gene. The IRE in the 3' untranslated region of mRNA of DMT1 has the ability to maintain iron homeostasis through regulation of its expression level in response to dietary iron status. Since the non-IRE form of DMT1 is devoid of IRE domain, it lacks this function (39). DMT1-IRE and DMT1-nonIRE are predominantly expressed, respectively, in the epithelial cell line and erythroid precursor cells. DMT1-IRE and DMT1-nonIRE have been reported to play roles as apical iron transporter and endosomal iron transporter, respectively (56). The results obtained in the present study show a higher level of expression of both the forms of DMT1 in subjects with C-T-T/C-T-T and A-C-C/A-C-C haplotype pairs than subjects with C-T-T/A-T-T haplotype pairs. It highlights that C-T-T and A-C-C haplotypes are correlated to higher level of DMT1 expression. These observations suggest that the A-T-T haplotype of DMT1 gene is associated with decreased expression of DMT1 gene through an undetermined mechanism. The increased level of lenticular iron store in human cortical cataracts identified in our previous study (32) could be attributed to the influence of haplotype pairs C-T-T/C-T-T, C-T-T/A-C-C, and A-C-C/A-C-C on transcript expression of DMT1.

Although the attempt to associate DMT1 gene polymorphisms with the risk of cataract development appears to be a pioneer investigation, making a significant correlation of alleles/genotypes of all the three tested SNPs with the risk of cataract development is restricted by the size of the sample used for the present investigation. Detecting a significant association between 'CC' genotype of rs224589 and threat of nuclear cataract with a power of 80% at a level of 0.05 with a genetic relative risk of 1.4 needs a minimum of 600 nuclear cataract cases. Therefore, future studies aiming to determine the role of DMT1 polymorphisms in ARC must focus on large cohorts and different ethnic populations.

The nucleotide variation c.1328A>G in exon 3 of DMT1 gene observed in the present study is believed to alter either the splicing process or the amino acid sequence of DMT1 protein. Further, in silico analyses demonstrated a deleterious effect of this mutation on protein function. It indicates that the substitution of the mutant amino acid asparagine by serine at position 443 (N443S) could result in loss of function of protein.

In conclusion, the genetic polymorphisms in the DMT1 gene contribute to the abnormal expression of DMT1, and the A-T-T haplotype plays a protective role safeguarding the individual from cataract development. Since DMT1 has been implicated in numerous other diseases, it is essential that future research work in this field selects suitable cohorts for evaluating possible association between the DMT1 polymorphisms and relevant diseases.

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Declaration of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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