# Exploration of Molecular Factors Impairing Superoxide Dismutase Isoforms Activity in Human Senile Cataractous Lenses

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**Purpose.** To explore different molecular factors impairing the activities of superoxide dismutase (SOD) isoforms in senile cataractous lenses.

**Methods.** Enzyme activity of SOD isoforms, levels of their corresponding cofactors copper (Cu), manganese (Mn), zinc (Zn), and expression of mRNA transcripts and proteins were determined in the lenses of human subjects with and without cataract. DNA from lens epithelium (LE) and peripheral blood was isolated. Polymerase chain reaction–single strand conformation polymorphism (PCR-SSCP) followed by sequencing was carried out to screen somatic mutations. The impact of intronic insertion/deletion (INDEL) variations on the splicing process and on the resultant transcript was evaluated. Genotyping of IVS4+42delG polymorphism of *SOD1* gene was done by PCR–restriction fragment length polymorphism (RFLP).

**Results.** A significant decrease in Cu/Zn- and Mn-SOD activity ($P < 0.001$) and in Cu/Zn-SOD transcript ($P < 0.001$) and its protein ($P < 0.05$) were found in cataractous lenses. No significant change in the level of copper ($P = 0.36$) and an increase in the level of manganese ($P = 0.01$) and zinc ($P = 0.02$) were observed in cataractous lenses. A significant positive correlation between the level of Cu/Zn-SOD activity and the levels of Cu ($P = 0.003$) and Zn ($P = 0.005$) was found in the cataractous lenses. DNA sequencing revealed three intronic INDEL variations in exon4 of *SOD1* gene. Splice-junction analysis showed the potential of IVS4+42delG in creating a new cryptic acceptor site. If it is involved in alternate splicing, it could result in generation of SOD1 mRNA transcripts lacking exon4 region. Transcript analysis revealed the presence of complete SOD1 mRNA transcripts. Genotyping revealed the presence of IVS4+42delG polymorphism in all subjects.

**Conclusions.** The decrease in the activity of SOD1 isoform in cataractous lenses was associated with the decreased level of mRNA transcripts and their protein expression and was not associated with either modulation in the level of enzyme cofactors or with INDEL variations.

Keywords: superoxide dismutase, cofactors, somatic mutation, splice-transcript variant, senile cataract

Despite having a powerful antioxidant system, the aging human lens often falls prey to oxidative stress (OS) owing to constant exposure to factors producing reactive oxygen species (ROS).[1] Superoxide dismutase (SOD) is most important in this milieu as it provides the first line of defense against deleterious ROS. Three isoforms of SOD, cytosolic copper/zinc–dependent Cu/Zn-SOD (or SOD1), mitochondrial manganese-dependent Mn-SOD (or SOD2), and an extracellular (EC) Cu/Zn-dependent EC-SOD (or SOD3), have been identified. All SOD isoforms catalyze the conversion of highly reactive, more dangerous superoxide anion ($O_2^{\cdot-}$) into less reactive hydrogen peroxide ($H_2O_2$) and molecular oxygen. Hydrogen peroxide is then converted into water by either catalase (CAT) or glutathione peroxidase (GPX).[2] However, failure in any of the conversion processes or imbalance between the production of ROS and the antioxidant enzymes results in tissue damage, which leads to OS-induced cataract.[3] Cataract is defined as a gradual, painless loss of lens transparency that impedes the passage of light to the retina and impairs vision. It is one of the leading causes of blindness in elderly individuals over the age of 50 years. It is estimated that there are 16 million cases of cataract worldwide, with approximately 50% of the cases originating from Africa and Asia.[4] Surgical intervention is the only available remedy at present.

Activity levels of antioxidant enzymes SOD, CAT, and GPX are impaired in human senile cataractous lenses.[5–11] A few studies have suggested that impaired SOD isoforms activity in cataractous lenses could be associated with physiological and molecular factors such as abnormal levels of cofactors,[12] diabetes-induced glycation,[13,14] and genetic polymorphisms of *SOD* genes.[15] Other molecular factors such as production of aberrant SOD transcripts as a result of alternate splicing,[16–18] mutations in coding and noncoding regions,[19,20] and epigenetic control through promoter hypermethylation[21] have also been reported in other diseases. However, the precise molecular mechanisms and/or factors impairing SOD isoforms activity in senile cataractous lenses have not been well established to date.

Thus, the present study was designed to explore the molecular factors impairing SOD isoform activity in senile cataracts. The level of SOD isoforms activity and their cofactors such as copper, zinc, and manganese, and their mRNA transcripts and proteins were assessed. Screening of mutations and/or single nucleotide polymorphisms in the coding and noncoding regions of *SOD1* was carried out. Further, an attempt was also made to find the association of IVS4+42delG polymorphism of *SOD1* with senile cataracts.

## Methods

### Subjects and Samples

The present study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Ethical Committee. All patients of age $\geq$ 50 years were clinically examined and diagnosed by the chief ophthalmologist (ARV). Using slit-lamp observation, the type of cataract was categorized by the zone of opacification and the grade was categorized by the degree and color of opalescence using the lens opacification classification system (LOCS) III.[22] Patients with primary cataract were included. Written informed consent was obtained from all the participants.

Anterior lens epithelium (LE) and peripheral cortical fibers along with the lens nucleus (LN) were obtained from the cataract patients recruited for extracapsular cataract extraction surgery at Raghudeep Eye Clinic, Ahmedabad ($n = 134$: nuclear [47], cortical [46], and posterior subcapsular [41]; mean age, 67.1 $\pm$ 11.8 years). Human donor eyes were obtained from C.S. Samaria Red Cross Eye Bank, Ahmedabad. From these donor eyes, whole lenses were extracted within 6 to 8 hours of death and examined for the presence of opacity. Clear lenses thus obtained ($n = 37$; mean age, 61.4 $\pm$ 10.1 years) were included in the study. Lens epithelium and LN were separated from the clear lenses under an operating microscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany). Peripheral venous blood (2–5 mL) was collected from the cataract ($n = 100$; mean age, 65.4 $\pm$ 9.2 years) and noncataract control ($n = 100$; mean age, 66.4 $\pm$ 8.7 years) subjects.

### Determination of SOD Activity

Each of the LE and LN samples was homogenized separately in a homogenization buffer (100 mM sodium pyrophosphate buffer, pH 7.4). The enzyme source was obtained by centrifugation at 10,000*g* for 15 minutes at 4°C. The total protein level was determined using the Micro BCA protein estimation kit (Pierce Biotechnology, Rockford, IL). The levels of total Cu/Zn-SOD and Mn-SOD activities were determined using SOD Activity Assay Kit (BioVision Inc., Milpitas, CA) according to the manufacturer's instruction, with slight modifications. The activity of Mn-dependent SOD was discriminated from Cu/Zn-dependent SOD by incubating the enzyme source with 5 mM sodium cyanide because Cu/Zn-dependent SODs are sensitive to cyanide.[11] SOD activity was expressed as a unit activity per milligram of protein.

### Estimation of Cofactors

Lens epithelium and LN of the same lens samples were pooled separately in acid-washed amber glass vials and dried under reduced pressure at 37°C in a hot air oven for the first 1 hour, and then dried overnight at 60°C (for approximately 16 hours). The dry weight of the samples was obtained, and digested in a 5-mL mix of nitric acid and perchloric acid (3:1) in a closed chamber overnight or until the solid particle was no longer visible. The digested contents were preheated at 100°C in a microwave oven for the first hour and then at 200°C until the contents became colorless and the acid content was reduced to 2 to 3 mL. The digested contents were cooled, mixed with the required volume of distilled water, filtered through a Whatman No. 42 filter paper (GE Healthcare Life Sciences, Piscataway, NJ), and made up to 50 mL with HPLC-grade distilled water. The quantities of Cu, Zn, and Mn were estimated using Spectra AA220 Zeeman flame atomic absorption spectrophotometer (Varian Australia Pty Ltd., Mulgrave, Victoria) and expressed as micrograms per gram of dry tissue weight.

### Quantitative Real-Time PCR Analysis

Lens epithelium and LN of the same lenses were pooled and washed with sterile PBS, pH 7.4. The lenses were homogenized in 2 mL TriZol reagent (Invitrogen Inc., Carlsbad, CA) and total RNA was extracted. cDNA was synthesized using First Strand cDNA Synthesis Kit (Invitrogen Inc.) according to the manufacturer's instructions in a 20-µL reaction volume containing 1.0 µg total RNA). *SOD* gene isoforms were amplified in a 20-µL reaction volume containing 1X of SYBR green master mix (Roche Diagnostics GmbH, Mannheim, Germany), 100 ng cDNA, and 10 pmol each of forward and reverse primers (Supplementary Table S1) using standard operating program, which consists of preincubation at 95°C for 10 minutes, amplification at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 10 seconds in a LightCycler 480II real-time PCR system (Roche Diagnostics GmbH). Level of β actin (*ACTB*) gene expression was kept as a control to normalize the expression status. The difference in the level of relative expression between the clear and the cataract samples was calculated based on the $2^{-\Delta\Delta C_P}$ function using the Relative Expression Software Tool (REST) 2009.[23]

### Western Blot Analysis

Lens epithelium and LN of the same lenses were pooled and lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer (10 mmol/L tris-HCl, 150 mmol/L NaCl, 1.0% NP40, 0.25% deoxycholate, 0.1 mmol/L phenylmethanesulfonyl fluoride (PMSF), 0.1 mmol/L iodoacetamide, 10 µg/mL leupeptin, and 10 µg/mL aprotinin; pH 7.4) at 4°C for 30 minutes. The supernatant was obtained by centrifugation at 12,000*g* for 10 minutes. Approximately 50 µg total protein was separated by 12% SDS-PAGE, and subsequently blotted onto a nitrocellulose (NC) membrane (100 V for 1 hour). The membrane was blocked in an incubation buffer (10 mmol/L tris-HCl, 50 mmol/L NaCl, 0.05% Tween 20, pH 7.5, containing 5% BSA) at room temperature (RT) for 1 hour followed by incubation with anti-SOD polyclonal antibodies (rabbit anti-SOD1; GenScript, Piscataway, NJ; and rabbit anti-

SOD2 and anti-SOD3; Abcam Inc., Cambridge, MA) overnight at 4°C. Nitrocellulose membrane was washed thrice, incubated with goat anti-rabbit IgG-horse-radish peroxidase conjugate for 2 hours at RT, and then visualized using diaminobenzidine (DAB)/$H_2O_2$ detection method. The band intensity of SOD1 protein levels was normalized using β-actin protein levels. The relative band intensity between SOD1 and β-actin was calculated using ImageJ (provided in the public domain by http://rsbweb.nih.gov/ij/) program.

### Screening of Mutations and Single Nucleotide Polymorphisms (SNP)

DNA was extracted from LE and peripheral blood using the NucleoSpin Blood Genomic DNA Extraction Kit (Macherey-Nagel GmbH & Co., Düren, Germany) according to the manufacturer's instruction. Primers for all exons of *SOD1* gene were designed[19] (Supplementary Table S2) to amplify 5′ and 3′ splice sites along with coding sequences. Polymerase chain reaction was performed using 1X SappireAmp Fast PCR Master Mix (TaKaRa Bio Inc., Shiga, Japan) in a 50-μL reaction mix consisting of 100 ng genomic DNA and 50 pmol each of forward and reverse primers. The thermal reaction comprised 1 cycle of initial denaturation at 94°C/1 min, and 40 cycles of second denaturation at 98°C/5 s; annealing at 55°C to 58°C for 5 seconds; extension at 72°C/10 s, and a final extension at 72°C/3 min. All amplicons were resolved using 2% agarose gel and visualized by UV-transilluminator upon ethidium bromide staining. Single strand conformation polymorphism (SSCP) analysis[24] using 10% polyacrylamide gel followed by silver staining[25] was done for all amplicons. The gels were photographed, and the bands that showed a mobility shift were sequenced (First BASE Laboratories, Selangor Darul Ehsan, Malaysia) using the BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), in an Applied Biosystems Prism 3130xl Genetic Analyzer. Sequence homology and/or nucleotide polymorphisms in the coding and noncoding regions were assessed using the Basic Alignment Sequence Tool (BLAST) (provided in the public domain by www.ncbi.nlm.nih.gov/blast) program.

### Splice-Junction and Alternate-Splice Transcript Analysis

Splice-junction analysis was done using algorithms, SCAN and RI of SEQUENCE WALKER[26,27] program to determine the potential effect of insertion/deletion (INDEL) variations on native donor and acceptor sites. Further, the information contribution at each newly formed cryptic acceptor and donor site was determined. Reverse Transcription PCR was performed using three specific primer pairs (Supplementary Table S3)[28] to check the presence of alternate splice-transcripts. Amplicons were resolved using 2% agarose gel.

### Genotyping by PCR-Restriction Fragment Length Polymorphism (RFLP)

The potential loss or gain of restriction sites owing to INDEL variations on exon4 was determined using the restriction mapper online tool (provided in the public domain by www.restrictionmapper.org). DNA was isolated from LE of cataract and cadaveric donor eyes and from peripheral blood of cataract and control subjects. Exon4 was amplified, digested with 0.1 to 1 U of *TatI* enzyme at 65°C overnight. Digested products were resolved using 4% agarose gel.

### Statistical Analysis

Statistical analyses were performed with SPSS. Differences between the means of the two variables were evaluated by the Student's *t*-test and the Mann-Whitney *U* test. The Pearson correlation analysis was done to check the influence of modulated levels of cofactors on corresponding enzymatic activity in the cataractous lenses. A $P$ value of $<0.05$ was considered statistically significant.

## Results

### SOD Isoforms Activity

The LE showed a higher level of SOD activity, almost 15- to 20-fold, as compared with the LN of both clear ($n = 10$) and cataractous lenses ($n = 29$). However, when compared with clear lenses, cataract lenses showed a decreased level of total Cu/Zn- and Mn-SOD ($P < 0.05$; Figs. 1A, 1C, Supplementary Table S4) in both LE and LN. There was a significant decrease in the level of total Cu/Zn- and Mn-SOD activity in LE and LN of nuclear, cortical, and posterior subcapsular cataracts ($P < 0.05$; Figs. 1B, 1D) except Mn-SOD in LE of cortical cataract ($P = 0.45$). Further, the level of total and Cu/Zn-SOD activity in LE and LN of cataractous lenses was approximately 50% less than in the clear lenses. The level of Mn-SOD activity was negligible when compared with total activity in both clear and cataract lenses.

### Cofactors

There was an increase in the level of manganese and zinc ($P < 0.05$) and no change in the level of copper ($P = 0.36$) in cataractous lenses (Fig. 1E, Supplementary Table S5). Further, the increase in Zn and Mn was solely contributed by cortical cataract ($P < 0.05$; Fig. 1F). Pearson correlation analysis showed a positive correlation between the level of Cu/Zn-SOD activity and the levels of Cu ($P = 0.003$) and Zn ($P = 0.005$) and no correlation between the levels of Mn-SOD activity and Mn ($P > 0.05$) in the cataractous lenses.

### Transcript Expression and Protein Level

The level of *SOD1* transcript expression ($P < 0.001$) was significantly decreased in cataractous lenses. However, the levels of *SOD2* ($P = 0.73$) and *SOD3* ($P = 0.33$) expression were not significantly altered (Fig. 1G, Supplementary Table S6). Downregulation of *SOD1* and no change in *SOD2* was observed in nuclear, cortical, and posterior subcapsular cataract (PSC) cataracts ($P < 0.05$); however, an upregulation of *SOD3* was observed in nuclear cataract ($P < 0.05$). Further, Western blot analysis revealed a significant decrease ($P < 0.05$) in the level of SOD1 protein in cataractous lenses (Fig. 1H). However, immunoreactivity against specific antibodies for SOD2 and SOD3 proteins in clear and cataractous lenses could not be detected even after developing the blot with Enhanced Chemi-Luminescence Detection System (Thermo Fisher Scientific Inc., Rockford, IL).

### SNPs and Mutation Screening

SSCP analysis was carried out for all 5 exons of *SOD1* gene in both control LE (C) ($n = 7$) and cataract LE (M) ($n = 20$). Mobility shifts were found in exon2 (cases, $n = 12$ [60%]) and exon4 (control, $n = 4$ [57%]; cases, $n = 12$ [60%]) amplicons (Fig. 2A). DNA sequencing and subsequent blast analysis revealed three INDEL variations (IVS4+13_14insA,

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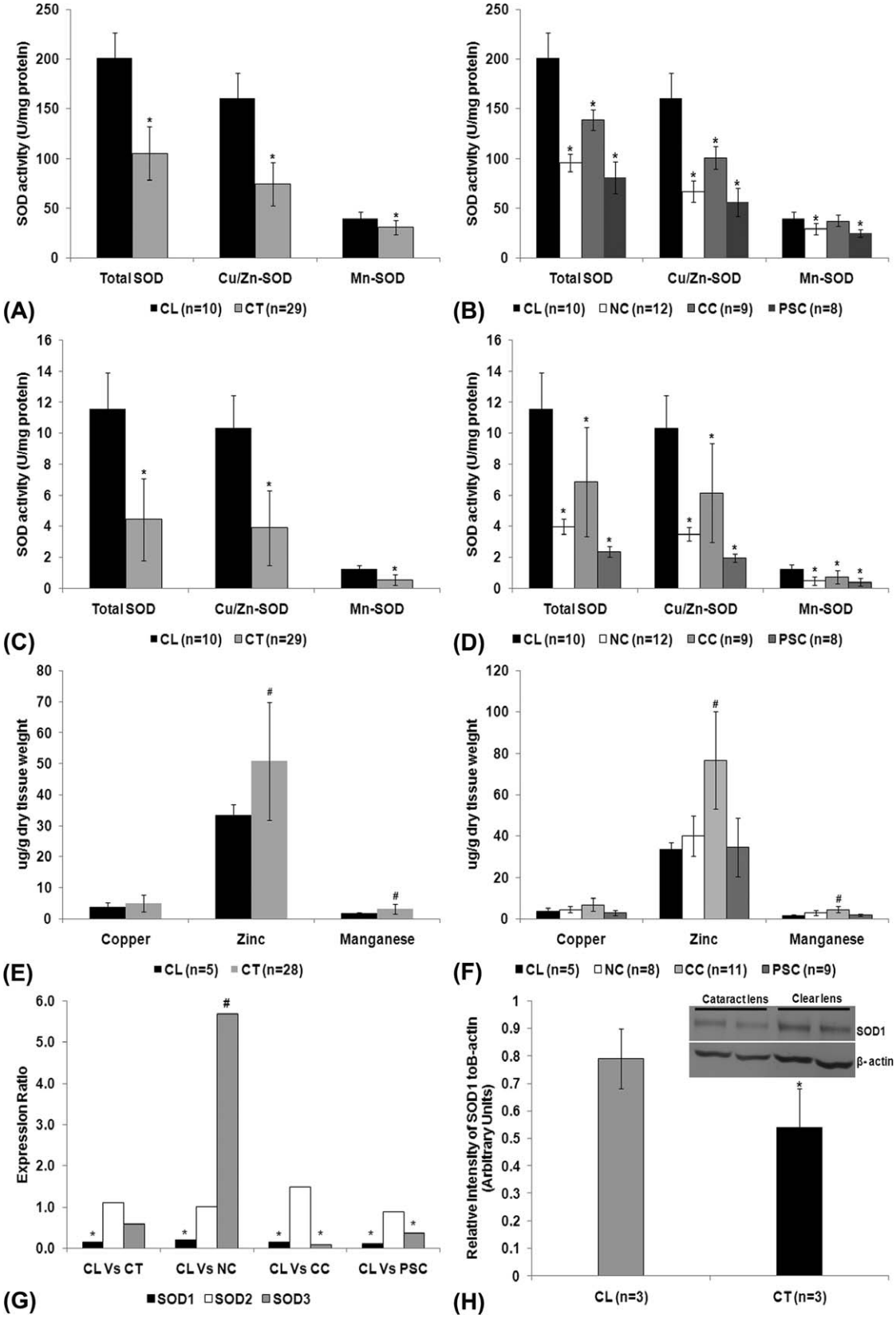


**Figure 1.** SOD activity in lens epithelium (**A**), lens nucleus (**C**), and lens epithelium and lens nucleus of different types of cataract (**B, D**). Values are expressed as mean ± SD; * indicates significant decrease in the level of activity ($P < 0.05$). Estimation of cofactors in lenses (**E**) and in different types of cataracts (**F**); values were expressed as mean ± SD; # indicates a significant increase in the level of cofactors ($P < 0.05$). (**G**) Real-time quantitative PCR analysis of SOD isoform transcripts in clear ($n = 12$) and cataractous ($n = 54$) lenses and also in different types of cataracts. Values were expressed as means expression ratio. * indicates significant downregulation ($P < 0.05$) and # indicates significant upregulation ($P < 0.05$). (**H**)

Western blot (*inlet*) and densitometric analysis of SOD1 protein levels in lenses. Relative band intensity analysis revealed a significant decrease in the level of SOD1 protein (*$P < 0.05$) in the cataractous lenses. CL, clear lens; CT, cataract lens; NC, nuclear cataract; CC, cortical cataract; PSC, posterior subcapsular cataract; Vs, versus.

IVS4+42delG, and IVS4+57_58insT) in the 3′ region of the exon-intron4 boundary (Figs. 2B, 2C).

### Alternate-Splice Transcript Analysis

Splice-site nucleotide substitutions can be analyzed by comparing the individual information contents (Ri, bits) of the normal and variant splice-junction sequences.[26,27,29,30] Theoretically, splice sites need to have Ri values of at least 0.0 bits to exist either as acceptors or as donors, but, empirically, it has been found that sites below 2.4 bits are not generally functional, and sites above 2.4 bits are strong enough to act as either donors or acceptors.[26,27,29,30] We presumed that the observed INDEL variations in exon4 of cataractous samples could have influenced the strength of native splice sites and abolished their function. Splice-junction analysis of the normal (GenBank accession number L44138.1) and variant exon4 sequence showed no influence of INDELs on the strength of native-acceptor and native-donor splice sites, each having strength of 8.9 bits, which is strong enough to be involved in normal splicing events (Fig. 3A). Though we did not find any changes in the strength of native splice sites because of all three observed INDEL variations, the IVS4+42delG variation was found to have some effect on two other weak cryptic acceptor splice sites (CASS1 and CASS2). This particular SNP wiped off the CASS1 and enhanced the strength of the CASS2 from 2.2 to 4.0 bits (Fig. 3B). We believed that the increase in strength of CASS2 could make it able to be involved in alternate splicing events, whereby the resulting transcript might lack the complete exon4 coding region and retain part of proximal intron4 upstream to exon5 region. Next, we performed RT-PCR using primers that could amplify the entire coding region of *SOD1* mRNA transcript (exon1 through exon5) to check the presence of alternatively spliced transcripts in cataractous condition. But, RT-PCR showed amplicons with 395-bp size in both clear ($n = 3$) and cataractous samples ($n = 36$), which indicates the absence of alternatively spliced *SOD1* mRNA transcripts (Fig. 4A).

### Genotyping of IVS4+42delG Polymorphism

IVS4+42delG polymorphism results in loss of the restriction site for *Tat1* enzyme, which would otherwise yield two fragments of sizes 197 and 52 bp upon digestion in the native condition. Polymerase chain reaction–RFLP analysis of genomic DNA from LE ($n = 27$) and peripheral blood revealed the absence of restriction fragments in both cataract ($n = 100$) and noncataract control ($n = 100$) samples (Fig. 4B).

## Discussion

The aging human lens resides continuously in an OS atmosphere, which is believed to be one of the key factors in the gradual loss of lens transparency and subsequent cataract development.[1,5–11] In our present study, we observed a much higher level of SOD activity in the LE than in the LN irrespective of the lens type. This indicates that a majority of defense enzymes against oxidative stress, especially SOD in lens, are derived from the lens epithelium.[6,10] The lens epithelium also expresses all three isoforms of SOD.[11] In our present study, we found lower levels of SOD1 and SOD2 activity in cataractous lenses irrespective of the type of cataracts. However, we could not estimate SOD3 activity as it was not possible to distinguish their activity from that of SOD1; these two enzymes are functionally similar in terms of their cofactor requirements and sensitivity to cyanide. Further, we observed that in cataractous lenses, SOD1 contributed only 60% to 70% of the total SOD activity as opposed to 90% in clear lenses.[31] Although, in the present study, we found decreased or no change (in LE of cortical cataract) in the level of SOD2 activity in cataractous lenses, the level of activity was negligible in both clear and cataract lenses. Though we observed a higher level of SOD1 and no significant difference in SOD2 activity in cortical cataract activity than in nuclear and PSC, overall there was a significant decline in the levels of both SOD1 and SOD2 activity in different types of cataract. This suggests that a modulation in the level of SOD2 activity may not have any direct impact on the formation of different types of age-related cataract.

All SODs require trace elements as cofactors for their catalytic activity.[32] Contradictory results have been published in relation to the level of trace elements in the cataract condition. A few studies have reported decreased levels of Zn[12,33] and Mn,[34] and a few have reported increased levels of Cu[35–37] and Zn[36,38] in senile cataractous lenses. In the present study, we found a higher level of Mn and Zn and no change in the level of Cu cofactors of SOD isoforms in cataractous lenses, especially in cortical cataract. Despite the presence of equimolar concentration of cofactors, the lens system failed to restore the optimal level of SOD activity in cataractous lenses. Therefore, we presumed factors other than the level of cofactors might be responsible for impairing SOD isoform activity. So, we performed mRNA expression analysis and found a significant downregulation of *SOD1* and *SOD3*, but not of *SOD2* in all types of cataract. Western blot analysis further confirmed the downregulation of SOD1 protein levels in cataractous lenses. But, we do not have any clue for the loss of immunoreactivity in Western blot for SOD2 and SOD3 in both clear and cataractous lenses. However, slow proteolysis, low new-protein synthesis, and extensive denaturation of proteins have been suggested as some of the reasons for loss of immunoreactivity.[39] The present study suggests that the modulation of SODs, especially SOD1, has a very devastating effect on the antioxidative potential of the lenses. As a result, the system cannot counteract the increasing generation of $O_2^{\cdot-}$, which could have resulted in lens damage and the subsequent loss of lens transparency.[1,11] Recent studies have demonstrated that SOD1 is selectively expressed in lens tissue,[40] and overexpression of the same was reported to prevent cataract formation in an in vitro whole lens model.[41] As mRNA expression analysis, followed by Western blotting and enzyme activity experiments unequivocally revealed a significant decrease in the level of *SOD1* mRNA and protein expression, and a subsequent decline in their activity, we believe that the impairment of SOD1 activity could be one of the risk factors in the cataractogenesis.

*SOD1* is ubiquitously expressed and is composed of two equal subunits each containing a catalytic Cu ion and a stabilizing Zn ion.[32] *SOD1* encodes a 645-bp transcript that codes for a 154–amino acid protein product. *SOD1* has always been a subject of research in several age-related and systemic diseases including cataract,[10] amyotrophic lateral sclerosis (ALS),[20] cancer,[42] diabetes,[43] and cardiovascular diseases.[44] Several mutations[20] and splice variants[16,17] in the *SOD1* gene have been reported to abolish its expression and enzymatic

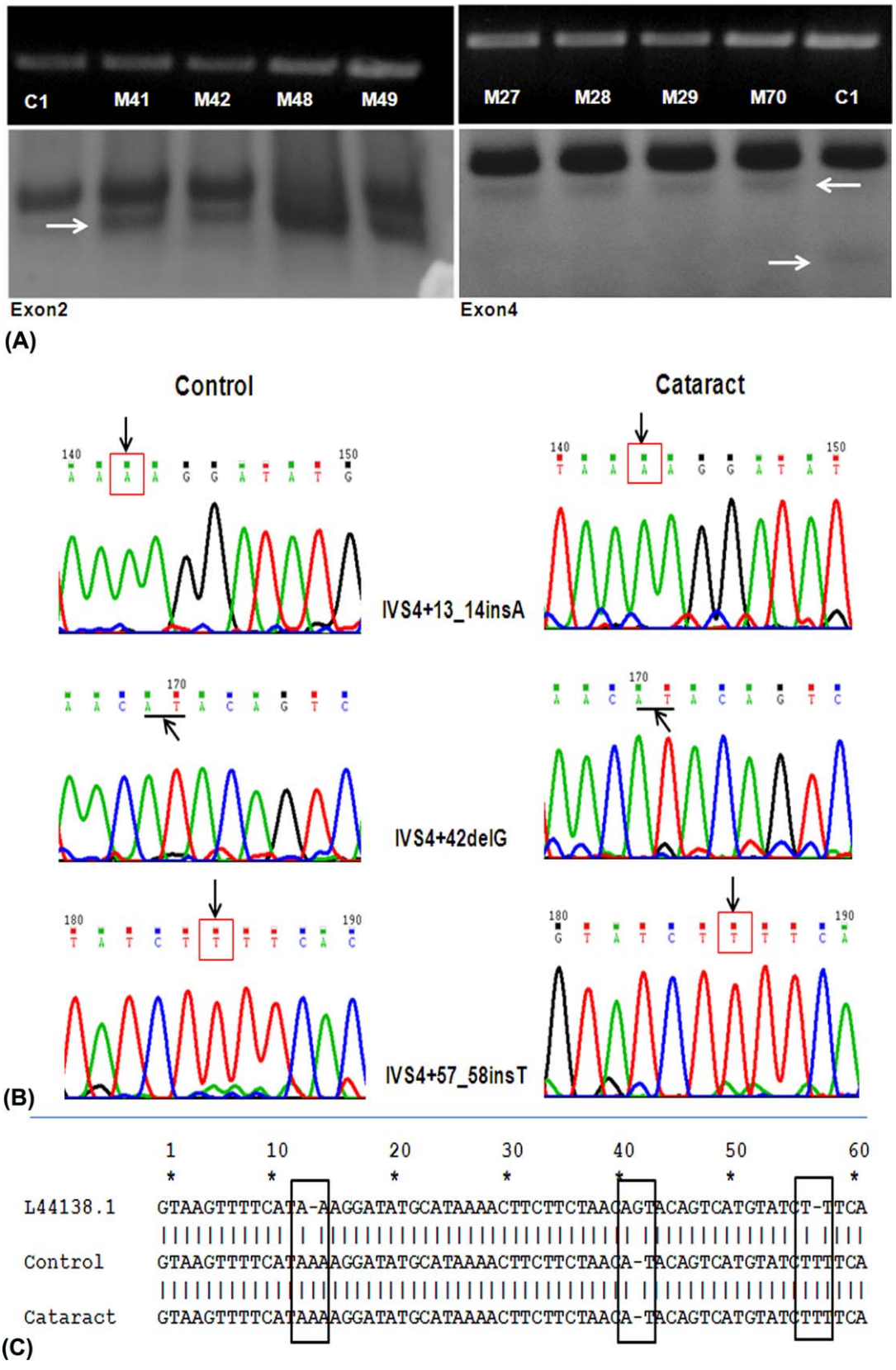


**Figure 2.** (**A**) Polymerase chain reaction amplicons of exon2 and exon4 of *SOD1* gene resolved in a 2% agarose gel (*upper panel*) and SSCP analysis (*lower panel*). C, control samples; M, cataract samples; *arrows* indicate mobility shifts. (**B**) Sequence chromatogram of exon4 of *SOD1* gene from control and cataractous LE DNA samples. *Arrows* point to the site of nucleotide variation (insertion variations are indicated by *boxes* and deletion variations are indicated by *underline*). (**C**) BLAST analysis of exon4 of controls and cataractous samples against the GenBank sequence (L44318). *Numbers* denote the intronic nucleotide position; *boxes* represent the site of the nucleotide polymorphisms.

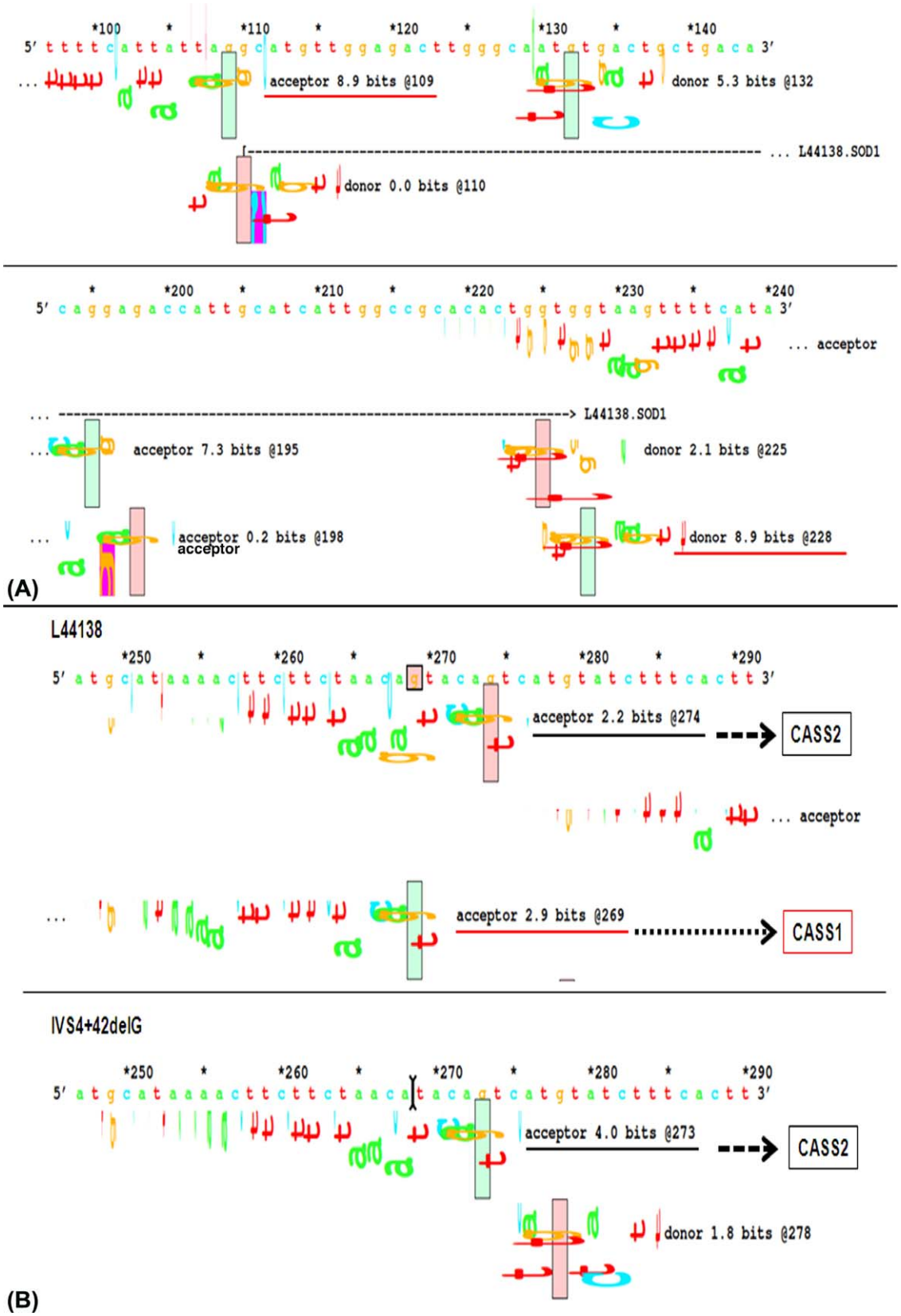


FIGURE 3. (A) Splice-junction analysis of native donor and acceptor splice sites; the strength of both acceptor and donor splice sites are indicated by 8.9 bits at the nucleotide positions 109 and 228, respectively; marked by *underline*. The strength of other possible cryptic splice sites was also shown. (B) Splice-junction analysis of IVS4+42delG polymorphism. This polymorphism wipes out the CASS1 at nucleotide position 269 (shown by *dotted arrow*) and enhances the strength of the CASS2 position 274 from 2.2 bits (*upper panel*) to 4.0 bits (shown by *dashed arrows*) at nucleotide position 273 (*lower panel*).

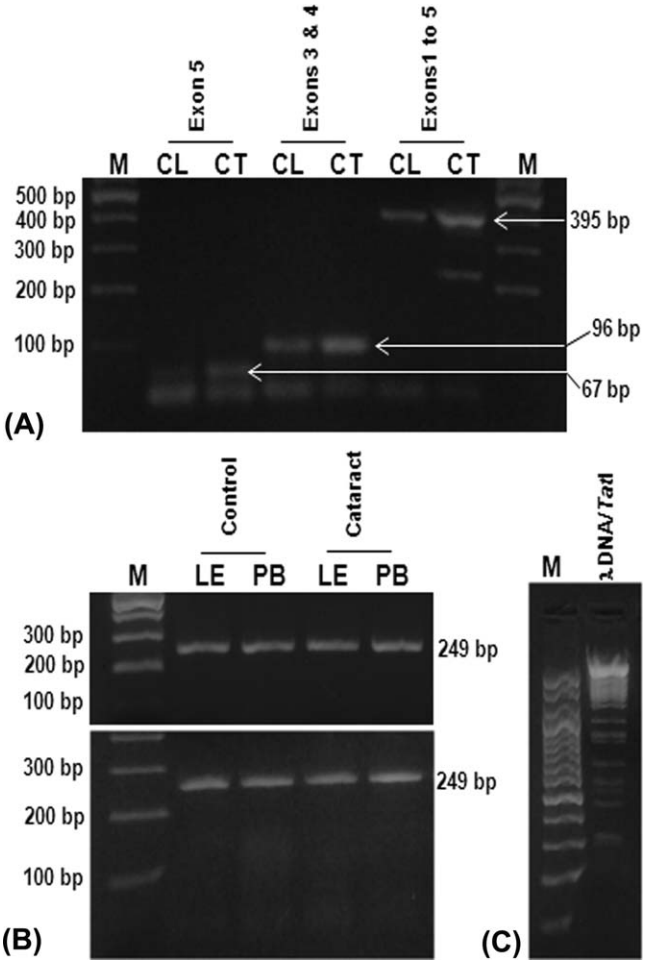


FIGURE 4. (**A**) Real-time PCR analysis of different exonic regions of SOD1 transcripts in clear (CL) and cataract (CT) samples. M, 100 bp DNA ladder; *lanes 2* and *3*, amplicons corresponding to exon 5 (67 bp) region; *lanes 4* and *5*, amplicons corresponding to exons 3 and 4 (96 bp) region; *lanes 5* and *6*, amplicons corresponding to exons 1 to 5 (395 bp) region. (**B**) Polymerase chain reaction–RFLP analysis of IVS4+42delG polymorphism in DNA isolated from LE and peripheral blood (PB) samples. Neither DNA sample showed expected digestion fragments of 192 and 52 bp upon *TatI* restriction enzyme (*lower panel*), which showed intact exon4 fragment of 249 bp similar to undigested exon4 amplicons (*upper panel*), exon4/*TatI* restriction enzyme digestion (*lower panel*). (**C**) Assessment of functional activity of *TatI* restriction enzyme. M, 100 bp DNA ladder; *lane 2*, λ DNA upon digestion with *TatI* restriction enzyme at 65°C for 2 hours showed respective digestion pattern of the enzyme.

activity in amyotrophic lateral sclerosis,[17,19,20] ocular diseases such as keratoconus,[18,45] corneal diseases,[46] and diabetic retinopathy.[47,48]

As several age-related diseases result from the accumulation of somatic mutations, we presumed that similar genetic alteration would also have occurred in LE, which would have impaired the tissue function. Since LE is important for the maintenance of lens homeostasis, we performed mutation screening in *SOD1* from DNA isolated from the LE samples to understand the molecular mechanisms responsible for the impairment of *SOD1* activity. A few samples, upon sequencing, revealed three INDEL variations (IVS4+13_14insA, IVS4+42delG, and IVS4+57_58insT) at 3′ exon-intron4 boundary rather than in the coding region. A similar observation was reported in ALS, in which mutation screening in coding regions of all five exons of *SOD1* revealed no aberrations but revealed an intronic variation and up to 50% decreased activity.[28] So far, three splice-site mutations have been identified in the fourth exon of the *SOD1* gene in ALS patients[48–51] and 7-bp deletion mutations in the second intron (IVS2+50del7 and c.16950del-TAAACAG) in keratoconus patients.[18,45] The INDEL variations that we observed in the present study, however, did not match with the previous studies. Intronic polymorphisms of any gene can cause splice-site aberrations and result in the deletion or insertion of amino acids as well as truncations. Such perturbations will significantly disrupt the structure and stability of the protein, and the result will be a major loss of function.[28] As the observed INDEL variations in our study existed at the splice region, we believed they might have some effect on the alternate splicing mechanism. Therefore, we performed in silico splice-junction analysis using the Sequence Walker Program, which calculates the strength of each of the native and cryptic splice sites.[29,30] Splice-junction analysis of IVS4+13_14insA and IVS4+57_58insT polymorphisms revealed that they do not have much impact on the splicing mechanism as they do not affect the strength of either the native or cryptic splice sites. However, IVS4+42delG polymorphism was found to enhance the strength of a cryptic acceptor splice site 2 (CASS2) from 2.2 to 4.0 bits, which is strong enough to generate a *SOD1* transcript without exon4 during alternate splicing. We performed transcript profiling to check the presence of *SOD1* transcripts lacking exon4 as a result of IVS4+42delG polymorphism. Of interest, our results did not find such transcripts in both clear and cataractous lenses. This finding indicates that the newly created CASS2, owing to IVS4+42delG polymorphism, does not have any direct influence on the *SOD1* transcript splicing process. Further, we evaluated the prevalence of IVS4+42delG polymorphism in the cataractous and control populations and found a loss of restriction sites for *TatI* restriction enzyme in all cataractous and control samples. This indicates that the variation IVS4+42delG might be common in the Western Indian population. Hence, the mechanisms other than genetic mutations and/or polymorphisms might play a major role in the impairment of *SOD1* expression and/or subsequent activity.

To summarize, in the present study, first, we attempted to check the activity level of SOD isoforms and found a decreased level of total SOD, SOD1, and SOD2 activity in cataractous condition. As the enzyme activity is attributed to the availability of cofactors, we decided to assess the level of Cu, Zn, and Mn and found higher levels of Zn and Mn and no change in the level of Cu. Since there is sufficient (as in Cu) or excess (as in Zn and Mn) quantity of cofactors in cataractous lenses, we concluded that the decrease in enzyme activity was not due to cofactors, and we thought that the decrease in activity could be a result of the decreased level of enzyme. So, we performed Western blotting for each enzyme isoform and found a decreased level of SOD1 protein in cataractous lenses, and no immunoreactivity for SOD2 and SOD3 in both clear and cataractous lenses. Further, we wanted to check whether this decreased level of protein expression was due to a decreased level of transcripts. Therefore, we performed quantitative real time (QRT)-PCR analysis and found downregulation of *SOD1* but not *SOD2* and *SOD3* transcripts in cataractous lenses. Since *SOD1* transcript alone was found to be decreased for all three parameters (activity, transcript, and protein levels), we thought that there could be some somatic or localized genetic modulation at the gene level that could have contributed to the decreased expression of *SOD1* transcripts in lens epithelium. Hence, we screened for mutation in all exons of *SOD1* gene from DNA isolated from lens epithelium and found three insertion/deletion (INDEL) variations in the splice sites. Since these variations were found at the splice sites, we

thought to carry out in silico splice-junction analysis to examine whether these variations cause any changes in the native splice sites. One particular variation, IVS4+42delG, was found to increase the strength of CASS2 and to have the potential to be involved in alternate splicing, which could result in exon leaking. Hence, we amplified and sequenced (data not shown) the entire coding region of *SOD1* and found a full-length transcript from exons 1 to 5 (395 bp) in both normal and cataract samples. We concluded that the decrease in transcript and protein levels was not due to alternate splicing of the *SOD1* gene transcript. Parallel to this, we wanted to genotype this particular INDEL variation in both the normal and the cataractous population using PCR-RFLP. We analyzed the potential loss or gain of the restriction site resulting from this particular polymorphism using Restriction Mapper tool and found loss of site for *TatI* restriction enzyme in the variant gene. Results of genotyping showed the presence of the IVS4+42delG polymorphism in all tested samples of both normal and control populations. So, we concluded that this particular polymorphism might be common in the Western Indian population and may not have a major effect on modulating enzyme activity. Since it is purely a correlative study, solely observing changes in different factors that could potentially influence the enzyme activity, it was not possible to rule out that the decrease in SOD activity could be solely attributed to the decrease/loss of enzyme availability. Further, as correlation definitely does not demonstrate causation, we could not justify whether the decrease in SOD activity was a primary or secondary phenomenon to cataractogenesis.

In conclusion, the present study reveals a concomitant decrease in the levels of *SOD1* transcript and protein expression, and enzyme activity in cataractous lenses. Although the decrease in the level of activity was suggested to be associated with suboptimal levels of cofactors, it could not be justified in the present study, as cataractous lenses showed optimal levels of Cu, Mn, and Zn. One of the intronic INDEL variations, IVS4+42delG, was found to have a strong impact on the splicing mechanism and could result in the generation of *SOD1* transcript without exon4. However, the existence of such transcripts was not observed in the present study. PCR-RFLP analysis revealed the presence of this polymorphism in almost all subjects. Future research should focus on the factors that could influence the availability of cofactors for the SOD1 enzyme complex, and epigenetic regulation of *SOD1* gene expression in age-related cataracts.

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