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이 논문을 의학 박사 학위신청 논문으로 제출함

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조선대학교 대학원

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# 초록

# 백내장에서 TDRD7 유전자의 역할

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**제목** : 실시간 중합효소 연쇄반응 검사를 통한 백내장 환자의 혈장 TDRD7 mRNA의 비교

**목적** : 백내장은 전 세계적으로 실명을 일으키는 주요한 원인이지만, 지금까지 유전자 관련 분야에 대해서는 거의 알려진 바가 없었다. 최근에, 백내장의 발생에 TDRD7 유전자가 중요한 역할을 함이 밝혀졌다. 이에 저자들은 인간의 말초혈액를 정량적으로 분석하여, 혈장 TDRD7 mRNA의 유용성을 평가하고자 하였다.

**방법** : 백내장으로 진단받은 40명, 정상 대조군 30명의 정맥에서 mRNA를 추출하여 중합효소 연쇄반응 검사를 시행하였다. 대상 환자들의 고혈압 또는 당뇨병의 유무를 문진하였고, 혈중 콜레스테롤과 식전 포도당 농도를 측정하였으며, 안과 검진을 통하여 백내장의 형태와 단계를 조사하였다. 혈장 TDRD7 mRNA를 측정하기 위해 ΔΔCt 방법을 이용하여 분석하였고, 상대적인 정량을 위해 ABL1 mRNA를 사용하였다.

**결과** : ABL1 mRNA를 이용하여 보정한 plasma TDRD7 mRNA의 $2^{-\Delta\Delta Ct}$ 값은 대조군에서 1.52±0.63, 백내장 환자군에서는 1.05±0.34 로, 백내장 환자군에서 대조군에 비해 낮았으며, 이는 통계학적으로 의의가 있었다(p=0.048). 백내장의 형태에 따라 비교해 보면, cortical type 과 mixed type이 nuclear type과 posterior subcapsular type보다 plasma TDRD7 mRNA의 $2^{-\Delta\Delta Ct}$ 값이 낮았다(p<0.05). 연령에 따른 plasma TDRD7 mRNA의 $2^{-\Delta\Delta Ct}$ 값을 비교한 것은 통계학적으로 유의한 차이를 보이지 않았다(p>0.05).

**결론** : 이 연구는 말초혈액을 통해 TDRD7 유전자와 백내장과의 관련성을 정립하는 첫 번째 연구로서 의의가 있다. 저자들은 백내장 환자에서 TDRD7 mRNA가 정상 대조군보다 낮게 표현됨을 통해 인간 백내장과 TDRD7 유전자의 돌연변이는 강한 인과관계가 있다고 생각한다. 혈장 TDRD7 mRNA 수치는 백내장의 잠재적인 표식자로 유용할 것이라 생각된다.

# Introduction

Cataract is globally the first cause for blindness, and is the cause for blindness of about 15 million people which account for more than half of the blind. It shows a prevalence rate of 50 % of adults in developing countries and 50% of adults over the age of 65 in advanced countries like USA.[1] According to a report, cataract occurs with 32.8-43.5% of adults over the age of 40, and it is said that the prevalence rate noticeably increases as the age increases to show initial sign of cataract with almost 100% of elderly at the age of 80s.[2] The factors which are known to have directly influence on occurrence of cataract up to now include ocular trauma, intraocular surgery, endophthalmitis, long-term use of steroid, and congenital cataract.[3] However, the causes for most of cataract have not been found and, through many researches, risk factors of cataract are determined to be systemic disease such as age, gender, smoking, myopia and diabetes as well as ultraviolet irradiation.[4-7]

Recently, gene-related researches have been widely conducted over the whole field of medicine and biology thanks to advancement of genetic engineering, and, in ophthalmology as well, gene related researches are conducted for retinal vascular diseases, age-related macular degeneration, glaucoma and cataract.[8,9,10] While cataract represents a common end stage of mutations in a potentially large number of genes acting through varied mechanisms in practice, most inherited cataracts have been associated with a subgroup of genes encoding proteins of particular importance for the maintenance of lens transparency and homeostasis. The increasing availability of more

detailed information about these proteins and their functions is making it possible to understand the pathophysiology of cataracts and the biology of the lens in general. Recently, Lachke et al has found that RNA granules which play a key role in messenger RNA (mRNA) processing can affect eye development, leading to juvenile cataracts in humans and mice. They also verified a malfunctioning gene, TDRD7, in a mouse strain that develops cataract.[11] TDRD7 is a scaffold protein of which the specific function is unknown. It has been identified in complexes with proteins which regulate dynamics of cytoskeleton, movement of cytocentrum, transportation of mRNA and organs which translate proteins. TDRD7 encodes a component of cytoplasmic RNA granules which are involved in determining the fate of mRNAs. Component of specific cytoplasmic RNA granules involved in post-transcriptional regulation of specific genes probably acts by binding to specific mRNAs and regulating their translation. If TDRD7 fails to build an essential protein, the lens cannot be normally developed. Lachke et al discovered the protein missing in the children and a type of structure known as RNA granules in a lens of a mouse.[11] RNA granules serve to regulate mRNAs in the cell. The TDRD7 mutation affects the regulation of mRNA, and as a result this misregulation was implicated in causing the cataracts. This work is the first study which has suggested that RNA granules are important in TDRD7 for lens transparency.

For such a reason, the authors intended to look into the usefulness of plasma TDRD7 mRNA in differentiating cataract from normal condition by quantitatively analyzing human peripheral blood.

# Subjects and Methods

## Patient Population

The objects were the 70 patients who visited the Department of Ophthalmology of Chosun University Hospital from May till September, 2011. The contents of the survey and examination were explained to all the objects, whose consents were received orally or in writing. The questionnaire was made of gender, age and life habit variables such as smoking status, drinking status, and daily life, and survey items about personal history. Medical examination was comprised of measurement of height, weight, blood pressure, fasting plasma glucose, and total cholesterol and genetic test for ABL1 and TDRD7. As to the genetic examination, we obtained approval from Institutional Review Board (IRB) of Chosun University Hospital. The blood for test was taken before noon the next day after letting the objects to keep their stomachs empty for 12 hours or longer after eating dinner. For serum cholesterol, plasma glucose and gene analysis, peripheral blood (4~5mL) was taken into vacuum tubes which are EDTA (Ethylene Diamine Tetraacetic Acid) treated, and transported being stored in ice from the time it was taken till centrifugation. The blood which arrived at the test room was centrifuged for 10 minutes at 3,000 rpm at 4° C before being tested. Total cholesterol and plasma glucose were measured by a commercial analytical system (Architect ci16200 analyser, Abbott Laboratories, IL, USA).

## ABL1 and TDRD7 Gene Expression Measurement

Total RNA was extracted from each venous blood sample with the

EasyRed RNA extraction kit (Intronbio, Seoul, Korea) according to manufacturer's protocol. The cDNA synthesis was performed by Superscript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. For quantification of TDRD7 transcript relative to the ABL1 control gene, we used the primers that were specific for TDRD7 (Forward: 5'-ACGAGTAGAGATCACAAATG-3'; Reverse: 5'-TCTGCTAAACAGCACTTAAT-3') and ABL1 (Forward 5'-CTGCTAGAGAAGGACTACC-3'; Reverse: 5'-TCGTCTGAGATACTGGATTC-3'). Real-time PCR reactions with the above primers were performed in a total volume of 20 μL using Phusion flash (Thermo Scientific, Wilmington, Delaware) with EvaGreen (Biotium, Hayward, CA) according to the manufacturer's recommendations. Thermocycling was performed using a CFX96 Real-time PCR Detection System (Biorad, Hercules, CA) with the following conditions: 1 cycle of 98ºC for 10 seconds, 40 cycles of 98ºC for 1 seconds and 55ºC for 15 seconds. The amplication curves of 2 genes, TDRD7 and ABL1 mRNA, are illustrated in Figure 1.

Amplication of an endogenous control may be performed to standardize the amount of TDRD7. For the quantification of gene expression, we used ABL1 mRNA as the endogenous control. The relative expression levels of TDRD7 mRNA, normalized to ABL1 mRNA, were calculated using the $2^{-\Delta\Delta Ct}$ value. In order to apply the $2^{-\Delta\Delta Ct}$ method, the results of real-time RT-PCRs were represented as cycle threshold (Ct) values. The Ct value was defined as the cycle at which a sample crosses a threshold which is significantly above the background fluorescence and within the exponential phase of the amplification. The average from three Ct measurements was calculated for both the TDRD7 and ABL1 mRNA. ΔCt was determined as the mean of the triplicate Ct values for the TDRD7 genes minus the mean of the triplicate Ct values for the ABL1

gene. The ΔΔCt represented the difference between the two groups for a given target gene, more precisely ΔΔCt = ΔCt (cataract group) - ΔCt (the mean of normal group). The x-fold higher expression of a given target gene in cataract group compared to normal group was calculated as $2^{-\Delta\Delta Ct}$. All assays were executed in triplicate.

### Ophthalmologic Examination

Past history of ophthalmologic diseases such as ocular trauma and cataract surgery was checked, and whether the objects have systemic diseases such as hypertension, diabetes or cardiovascular diseases and the family history were investigated. In addition to uncorrected visual acuity, corrected visual acuity was examined additionally in the case of spectacled objects, and the best corrected visual acuity was measured after checking refractive state through a manifest refraction test. The state of the lens was classified by LOCS III through an examination using a slit lamp microscope after mydriasis.[12] If, in one eye or two eyes, there was nuclear opacity which belongs to the second stage or higher in LOCS III classification, or cortical opacity of second stage or higher, or posterior subcapsular opacity of the first stage or higher, or if there is an pseudophakic eye after cataract surgery, those objects were classified as cataract patients and otherwise, as control group members. Cataract patients were classified into nuclear, cortical, posterior subcapsular or mixed types. In the case of unilateral pseudophakic eye, classification was done depending on the condition of the other eye and the cases of bilateral pseudophakic eye were excluded from the classification.

### Statistical Analysis

Data analysis was done using SPSS for Windows, version 17.0

(Chicago, IL, USA) and the actual numbers, percentage, average and standard deviation were obtained as descriptive statistics in order to study general characteristics of the patient group and the control group. Comparison of general characteristics of the patient group and the control group was conducted using t test and $\chi 2$ test, and, as to comparison of genetic values of the patient group and the control group, the case where the p value is found to be below 0.05 by independent sample t test was determined to be statistically significant. To compare TDRD7 gene values of different types of cataract, one-way ANOVA was used while, to compare TDRD7 gene values of normal eyes of different age objects, Correlation Analysis was used. The case where the p value is below 0.05 was determined to be statistically significant.

# Results

Among total 70 objects, 40 (57.1%) were cataract patients while 30 (42.9%) belonged to the control group, and 33 (47.1%) were males and 37 (52.9%) were females. The average age of the whole object was 52.67± 12.96 years and the age range of the object was between 0 and 89 years. The age of the cataract patient was significantly higher showing a value of 62.68±13.40 while that of the control group was 38.60±23.99($p<0.001$). The frequencies of medical history of the cataract patients related to hypertension and diabetes were higher than those of the control group showing values of 37.5% and 25.0% respectively, which were statistically significant ($p<0.05$). Though the total cholesterol of the cataract patients was higher than that the control group showing a value of 187.38±47.87 mg/dl while that of the control group was 174.67±44.96 mg/dl, it was not statistically significant ($p>0.05$). Though the serum glucose level of the cataract patients was higher than that of the control group showing a value of 109.63±32.72 mg/dl, while that of the control group was 95.31±15.45 mg/dl, it was not statistically significant ($p>0.05$) (Table 1). As a result of classifying cataracts of 39 patients excluding one patient with bilateral pseudophakic eyes, in accordance with the positions of the opacity, the cataracts were found to be 19 nuclear types, 10 cortical, 6 posterior subcapsular and 4 mixed types.

### TDRD7 Gene Measurement

The ABL1 and TDRD7 values in the control group were 26.95±0.91 and 29.68±1.07 respectively, and those in the cataract group were

26.17±1.25 and 29.11±1.34 respectively. ΔCt was determined as the mean of the triplicate Ct values for the TDRD7 genes minus the mean of the triplicated Ct values for the ABL1 gene. ΔΔCt represented the difference between the two groups for a TDRD7 gene, more precisely ΔΔCt = ΔCt (cataract group) - ΔCt (normal group). The mean and standard deviations for all ΔCt and ΔΔCt values (cataract versus normal group) are given within Table 2. The actual expression level of a TDRD7 gene was analyzed using the $2^{-\Delta\Delta Ct}$ value. The results, including standard deviations, are listed in Table 2. In the result of adjusting TDRD7 value in reference to ABL1 mRNA, the TDRD7 expression level of the cataract patients was significantly lower than that of the control group statistically showing a value of 1.05±0.34 while that of the control group was 1.52±0.63. (p=0.048). In other words, we can say that cataract increases as TDRD7 gene decreases (Table 2). To allow a better overview and to show the wide spectrum of expression rates, all $2^{-\Delta\Delta Ct}$ values (with standard deviations) are illustrated within a single figure (Figure 2).

The result of comparing the genetic values of different types of cataract showed that the TDRD7 expression levels of cortical type and mixed type were lower than those of nuclear type and posterior subcapsular opacity type, which was statistically significant ($p<0.0001$) (Table 3).

The result of measuring TDRD7 gene expression levels of the normal eyes of different age objects showed that there was no statistically significant correlation between two variables (p=0.785) (Figure 3).

# Discussion

In this study, we can say that expression level of plasma TDRD7 mRNA in patients with cataract is significantly lower than that in normal controls statistically. Cataract accounts for about 50% of the cause for blindness and the only way to treat cataract is to remove the lens through surgery. Cataract surgery globally became the most frequently executed surgery among the aged.[13] Understanding about the causes for cataract and risk factors can be helpful in groping for non-operative method to delay occurrence of or prevent cataract. Most known gene mutations which lead to the congenital cataract come from the genes which encode structural protein of the lens. The gene mutations are involved in generation of transcription factors which regulate the expression of structural genes account for the subset of mutations which cause cataract.

### Correlation between TDRD7 Gene and Ophthalmologic Disease

Though precise transcriptional regulation of gene expression is essential for development of vertebrate, the role of post-transcriptional regulatory mechanism is less clear. While cytoplasmic RNA granules act in the post-transcriptional control of gene expression, but it is not well known to what the extent it is involved in organogenesis. Lachke et al suggested that RNA-containing granules can regulate the subcellular localization of mRNA as well as the processing which plays an important role in transparency of the lens.[11] Cytoplasmic RNA granules play the role of determining whether mRNAs undergo degradation, stabilization, or intracellular

localization. The structure of the lens of a vertebrate requires a very high concentration of specialized cytoplasmic proteins, the crystalline; a specialized intermediate filament cytoskeleton to stabilize the structure of lens[14]; and cellular membranes with high concentrations of both the lens-specific water channel, aquaporin 0, and lens-preferred connexins, which allow the lens to transport nutrients and waste products despite its lack of blood vessels[15]. A number of the genes encoding these proteins are highly expressed in the lens, leading to a highly biased transcriptome[16]. Lachke et al. have reported the identification of Tudor domain-containing 7 protein, or TDRD7, as an RNA granule component with a highly enriched and conserved pattern of developmental expression in the ocular lens. They suggested that human TDRD7 mutations result in cataract formation via the misregulation of specific, developmentally critical lens transcripts.[11]

The official name of the TDRD7 gene is "tudor domain containing 7" and is also called by other names such as CATC4, KIAA1529, PCTAIRE2BP, RP11-508D10.1, and TRAP. It is located on the long(q) arm of chromosome 9 at position 22.33. More accurately, it is located from base pair 100,174,301 to base pair 100,258,406 on chromosome 9. TDRD7 is a member of a large family of Tudor domain containing proteins that mutually interact with methylated arginine residues on other proteins. Many members of the TDRD family, including TDRD7, are found in RNA granules cytoplasmic complexes of protein and RNA that regulate gene expression by influencing RNA degradation, stabilization, and the intracellular localization of RNA.[17] Though TDRD7 transcripts and proteins are found in a variety of cells, especially of the male germ line, but TDRD7 expression is very high in lens fiber cells and may control the production of extremely high amounts of structural

proteins of lens. TDRD7 is a Tudor domain RNA binding protein which is expressed in lens fiber cells, is required for the posttranscriptional control of mRNAs which are essential for normal lens development and RNA granule function. Lachke et al. show that TDRD7 is found in a granular distribution in lens fiber cells and colocalizes with concentrations of RNA found in the cytoplasm of these cells.[11] They demonstrate that human organogenesis defects can result from perturbation of a distinct, tissue-specific RNA granule component that regulates the posttranscriptional steps of developmentally critical mRNAs. Simple RNA distribution within lens fibers would presumably be inefficient because of the relatively long distance between the lens fiber cell nucleus and the fiber cell tips as well as the high concentration of proteins in the fiber cell cytoplasm.[18] Accordingly, when we look into the matter retrospectively, a requirement for a lens-preferred RNA transport mechanism may not be surprising. Notably, the lens epithelial cells express mRNAs for lens structural proteins that are not translated into proteins until the onset of fiber cell differentiation , which is also when the onset of TDRD7 expression occurs.[19] In addition, another possible role for TDRD7-containing RNA granules would be to regulate the translation of fiber cell structural proteins. They found that reduced TDRD7 expression in a lens epithelial cell line caused statistically significant changes in expression level for 6% of the genes expressed in those cells, including some crystallins.[11] Overall, they demonstrated that the vertebrate lens contains several classes of RNA granules and show that TDRD7-containing granules are crucial for normal function of lens. These findings can be opened up a new field of investigation that will need to elucidate both the physiological and biochemical functions that regulate RNA granules in the lens.

## Risk factors for cataract

When we look into different risk factors of cataract, there are risk factors which affect specific types of cataract. For example, smoking increases prevalence of nuclear cataract, and steroid is related to occurrence of posterior subcapsular cataract.[5] The result of comparing gene values of different types of cataract in this study showed that the TDRD7 expression ratios of cortical type and mixed type were lower than those of nuclear type and posterior subcapsular type, and this can be inferred that, while many senile cataracts are of nuclear type, many of cataracts caused by genetic mutation can be of cortical type.

Though blood cholesterol is not known as a risk factor of cataract, a case wherein posterior subcapsular cataract has occurred in two eyes of a lathosterolosis patient showing a defect of cholesterol biosynthesis was recently reported[20] and Klein et al suggested that ingestion of statin which is widely used for the purpose of preventing cardiovascular disease by lowing the blood cholesterol value lowers incidence rate of nuclear cataract.[21] In this study, though the total cholesterol value of the cataract patients was higher than that of the control group, it was not statistically significant. Furthermore, we can think of a hypothesis that TDRD7 gene is involved in lowering the incidence of cataract by influencing cholesterol.

The result of comparing age and the expression ratio of TDRD7 gene of the patients with normal eyes showed that there is no statistically significant correlation between two variables. Even though we know that the ages of the cataract patients are higher than those of the control group members, we think that the effect of age can be excluded for such a reason.

There can be several differences between the previous studies and

our study. There are few studies on the relation between TDRD7 gene and cataract. Though Lachke et al in their study derived a result by dyeing fiber cells of lens, we inferred the result statistically using the gene analysis taken from peripheral blood. Of course, the number of objects of this study, which was 70, may be insufficient to verify statistical significance and, as the objects were residents of local region, regional difference may have been overlooked. As TDRD7 gene can be related to specific diseases separately from TDRD7 gene, if additional studies are conducted on the relation between TDRD7 gene and cataract, the effect of TDRD7 gene can be more directly established.

Though this study has a significance in that it is the first study on the relation between TDRD7 gene and cataract using Koreans as the objects, if proactive studies are conducted for a bigger number of objects selected from a bigger number of regions in the future, the relation between TDRD7 gene and cataract can be more clearly established.

In conclusion, as the TDRD7 gene expression levels of the cataract patients found in the test conducted for 70 objects living in local region in this study were significantly lower than those of the control group statistically, we can say that there is a strong causal relation between occurrence of human cataract and mutation of TDRD7 gene.

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Table 1. Clinical characteristics of the cataract group and normal control group

| Variables | Cataract (n=40) | Control (n=30) |
|---|---|---|
| Mean age (mean±SD, yr)* | 62.68±13.40 | 38.60±23.99 |
| Male/Female (n) | 18/22 | 15/15 |
| Ocular trauma history (n) | 2 | 1 |
| Hypertension (%)* | 37.5 | 16.7 |
| Diabetes mellitus (%)* | 25.0 | 6.7 |
| Serum total cholesterol (mean±SD, mg/dl) | 187.38±47.87 | 174.67±44.96 |
| Serum glucose (mean±SD, mg/dl) | 109.63±32.72 | 95.31±15.45 |

* Statistically significant ($p<0.05$)

**Table 2.** Relative quantitation using the comparative Ct method in cataract group and normal group.

| | Cataract | Normal | *p* value |
|---|---|---|---|
| *ABL1* | 26.17±1.25 | 26.95±0.91 | 0.542 |
| *TDRD7* | 29.11±1.34 | 29.68±1.07 | 0.063 |
| ΔCt | 2.61±0.95 | 3.03±0.75 | 0.051 |
| ΔΔCt | −0.10±0.95 | 0.32±0.75 | 0.051 |
| $2^{-\Delta\Delta Ct}$ | 1.05±0.34 | 1.52±0.63 | 0.048 |

The results were represented as cycle threshold (Ct) values.

There is an inverse correlation between Ct values and the amount of target mRNA: higher amounts of target mRNA have lower Ct values, and lower amounts of target mRNA correspond to a higher Ct values.

ΔCt was determined as the mean of the triplicate Ct values for the TDRD7 genes minus the mean of the triplicated Ct values for the ABL1 gene.

ΔΔCt represented the difference between the two groups for a TDRD7 gene, more precisely ΔΔCt = ΔCt (cataract group) - ΔCt (normal group).

The mean and standard deviations for all ΔCt and ΔΔCt values (cataract vs normal group) are given within Table 2.

The actual expression level of a TDRD7 gene was analyzed using the $2^{-\Delta\Delta Ct}$ value.

The results, including standard deviations, are listed in Table 2.

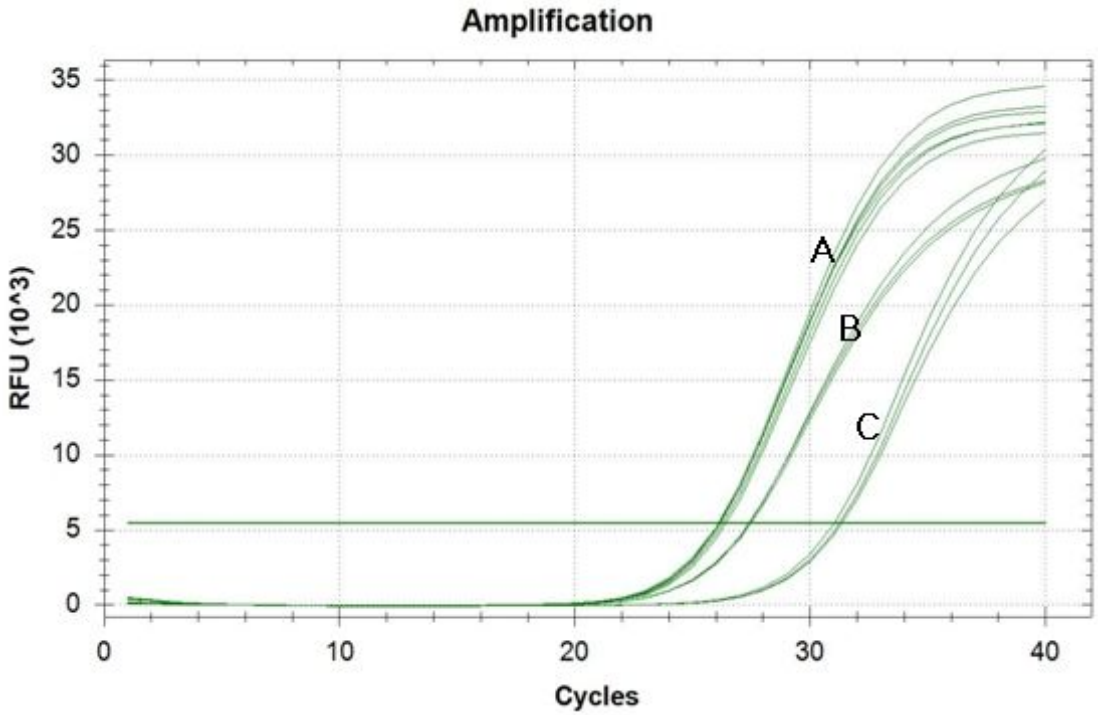
Table 3. Relative quantitation using the comparative Ct method in the type of cataract

| | Nuclear | Cortical | PSC | Mixed | *p* value |
|---|---|---|---|---|---|
| *ABL1* | 26.49±1.60 | 25.97±0.70 | 25.63±1.02 | 25.86±0.30 | 0.022 |
| *TDRD7* | 29.68±1.71 | 29.59±0.82 | 29.41±1.05 | 30.41±0.96 | 0.725 |
| $2^{-\Delta\Delta Ct}$ | 0.79±0.30 | 0.59±0.28 | 0.67±0.51 | 0.34±0.20 | <0.0001 |

PSC : posterior subcapsular opacity

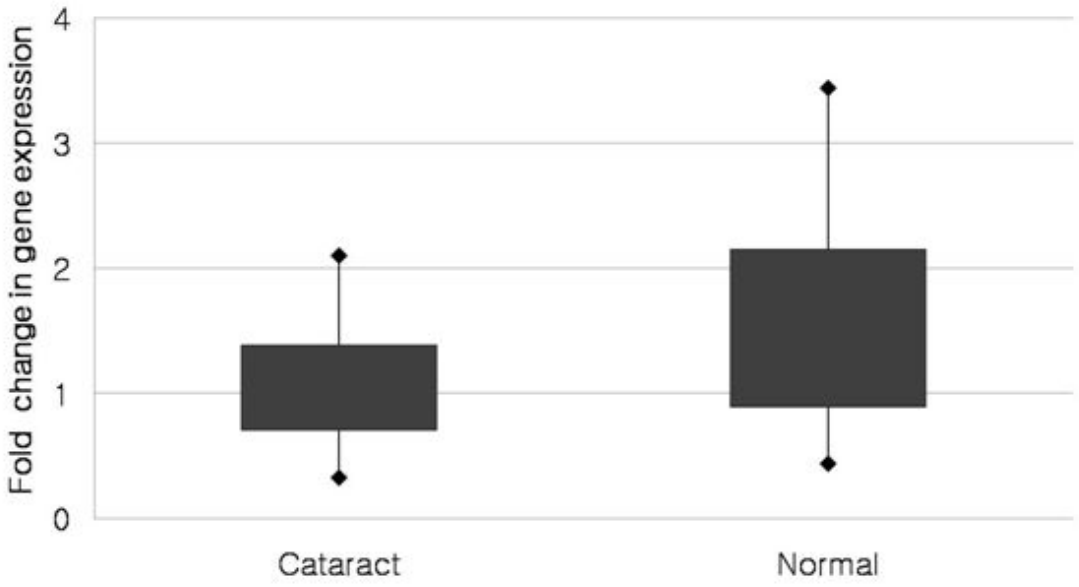
The results were represented as cycle threshold (Ct) values.
The actual expression level of a TDRD7 gene was analyzed using the $2^{-\Delta\Delta Ct}$ value including standard deviations.

**Figure 1.** Comparison of gene expression of ABL1 and TDRD7 genes in cataract group versus normal group by quantitative real-time RT-PCR.



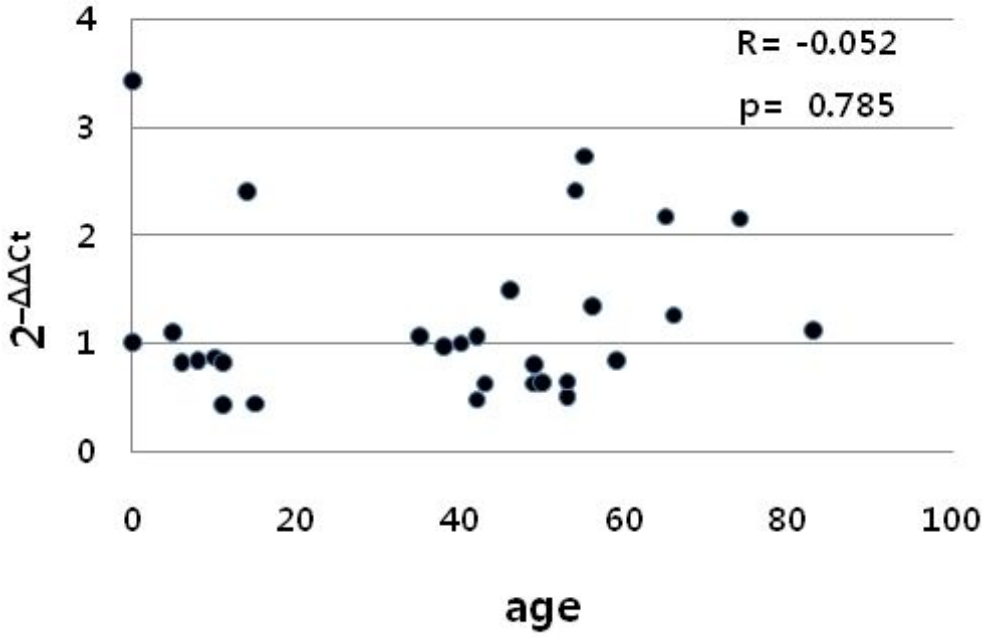

Amplication curves for A) ABL1, B) TDRD7 (normal group), C) TDRD7 (cataract group). The target-specific fluorescence signal of SYBR Green fluorescence emission (detection range 515-545 nm) is plotted against the number of PCR cycles. Curves of ABL1 are given in 6 left, TDRD7 (normal group) in 3 middle, and TDRD7 (cataract group) in 3 right. All real-time RT-PCR experiments were carried out in triplicate. The threshold level is given by a horizontal line. The cycle at which the mean amplication curve of ABL1 or TDRD7 real-time RT-PCRs crosses the threshold (Ct value) is indicated by a vertical line.

**Figure 2.** Normalized plasma TDRD7 mRNA in the patient group amount relative to that in normal control group




Fold change was expressed as $2^{-\Delta\Delta Ct}$ ($\Delta$Ct [TDRD7-ABL1] of each case - average $\Delta$Ct [average TDRD7 Ct-average ABL1 Ct] of normal control group).

**Figure 3.** Correlation analysis between age and TDRD7 gene expression levels in the blood of normal group by PCR




Expression of TDRD7 was confirmed by PCR assay.

No significant correlation (Pearson correlation test, r=-0.052, p=0.785) was observed between age and TDRD7 gene expression levels of the normal eyes.

To compare these data, we converted TDRD7 gene expression (expressed as $2^{-\Delta\Delta Ct}$) results between the interval value 0-4. Linear regressions, as well as the correlation coefficient R and p values are indicated in figure.