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Original Article

Effect of EPHA2 gene mutation on congenital cataract and its underlying mechanism

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Abstract: Congenital cataract is a major cause of blindness in children. Ephrin type-A 2 (EPHA2) gene encodes a transmembrane tyrosine kinase receptor. The previous studies showed that EPHA2 gene mutation may be associated with the occurrence of congenital cataract. Therefore, this study aims to investigate the role of EPHA2 gene mutations in congenital cataract and the associated mechanism. The DNA in Blood DNA of 164 patients with congenital cataracts was sequenced to analyze gene mutation of EPHA2. Three kinds EPHA2 mutants were constructed by site-directed mutagenesis and expressed in HLEC-SRA01 epithelial cells. Western-blot assay was used to examine the expression of the mutant EPHA2 protein. Immunofluorescent microscopy was used to observe the localization mutant EPHA2. The effect of EPHA2 mutants on cell survival and antioxidant capacity was measured. Blood DNA sequence results showed that the mutation ratio of EPHA2 is 6.1% and there are three types of mutations, including c.1751C>T, c.2819C>T and c.2875G>A. The whole cell immunofluorescence results indicated that the cellular localization of mutant EPHA2 is abnormal. Cell survival assay and antioxidant capacity assay results showed that EPHA2 mutations decreased the antioxidant capacity of cells, which leads to the cell damage and decreases the cell viability. In conclusion, the EPHA2 gene mutation may lead to congenital cataracts by altering the cellular localization and affecting the function of EPHA2, therefore, affect the cellular antioxidant capacity and cell survival, and eventually lead to the occurrence of cataracts.

Keywords: Eephrin type-A 2, congenital cataract, cellular localization, cell survival, oxidative stress

Introduction

Cataract is a type of eye disease that leads to the highest rate of blindness, which forms a cloudy in lens of eyes caused by the congenital or acquired reasons. Actually, the congenital cataract is the leading cause for blindness in children [1]. About 30% to 50% of congenital cataracts is related to the changes of the genes [2]. Study of congenital cataract-related gene expression and the roles they played in the pathogenesis of congenital cataract is significant in prevention and treatment of blindness in children caused by congenital cataract [3].

Currently, a total of 27 genes have been identified to be associated with congenital cataracts, of which EPHA2 is a recently identified gene that is closely related to congenital cataract [4-7]. EPHA2 belongs to the family of RTKs, and encodes a transmembrane tyrosine kinase receptor. The autosomal visible or invisible mutation of EPHA2 may cause cataract in clinial [8]. Approximately 5% of congenital cataract is caused by the EPHA2 mutation, and the previous study has found that the EPHA2 mutation in mice also led to the cloudy of lens [9]. It is reasonable to speculate that EPHA2 plays a pivotal role in the formation of the lens in mammals. Therefore, this study aims to investigate the role of EPHA2 mutation in the pathogenesis of congenital cataract.

Material and methods

Subjects

A total of 164 patients with congenital cataract (46 males and 36 females) from Hangzhou Red Cross hospital between May 2014 and November 2015 were involved in this study. The patients with an average age of 2.1 ± 0.9 years (ranges from 3 months to 8 years). All of the patients were diagnosed as congenital cataract

Table 1. Primer sequences used for mutagenesis

Mutation site	Primer	Sequence (5'-3')
WT	Eph-wtF	CTAGCTAGCATGGAGCTCCAGGCAGCCCGC
	Eph-wtR	ACGCGTCGACTCAGATGGGGATCCCCACAGT
c.1751C>T	Eph1751-F	CTGAAGCTCCTGAAGACATACGTGGATCCCCAC
	Eph1751-R	GTGGGGATCCACGTATGTCTTCAGGAGCTTCAG
c.2819C>T	Eph2819-F	GGTGGTGCAGATGATCAACGACGATATCAAGAG
	Eph2819-R	CTTGATATCGTCGTTGATCATCTGCACCACCTTC
c.2875G>A	Eph2875-F	CGCATCACCTACAGCCTGCTGG
	Eph2875-R	CAGCAGGCTGTAGGTGATGCGC

through visual examination, B-ultrasound scan, pupil examination, and slit lamp eye examination in clinical. The other causes for the blurred vision, including corneal disease, diabetic retinopathy, retinal detachment, were also excluded from this study. All of the patients have gave their consents and approved this study.

Detection of EPHA2 gene mutation in patients with congenital cataract

Total amounts of 5 ml peripheral blood were collected from each person and treated with EDTA as anticoagulants. TRI pure LS Reagent (BioTech, USA) was used to extract the total RNA. After measurement of concentration, the RNA was used as a template to reverse transcripted into the cDNA at 37°C for 2 h. Primers were designed according to the mRNA sequence of EPHA2 gene (Genebank: NM_004431) (EPHA2-Forward: ATGGAGCTCCAGGCAGCCC, EPHA2-Reverse: TCAGATGGGATCCCCACAGTG). The primers were synthesized by the Sangon Inc. Co. (Shanghai, China). PCR products were analyzed by using the electrophoresis, and then sequenced by Shanghai Shenggong (Shanghai, China). The examined gene sequences were aligned and blasted with the cDNA sequence of wild-type EPHA2 to analyze the gene EPHA2 mutation.

Construction of different EPHA2 mutants

Three EPHA2 mutants, including c.1751C>T, c.2819C>T, and c.2875G A, were constructed by using wild type EPHA2 in pQCXIP vector (pQCXIP-EPH-wt) as templates and were named as pQCXIP-EPH-1751, pQCXIP-EPH-2819, or pQCXIP-EPH-2875, respectively. Primer sequences used for mutagenesis are listed in **Table 1**. Q5® Site-Directed Mutagenesis Kit (NEB) is used to construct different EPHA2 mutants. Briefly, wild type EPHA2 was used as a template to perform PCR reaction by using primers designed with mutations. PCR products were treated with KLD enzymes (DpnI restriction endonuclease and alkaline phosphatase) to remove the methylated template DNA and phosphorylate DNA terminus. After ligation, DNA was transformed into E. coli competent cells to get single colonies for sequencing.

Cell culture and transfection

Human lens epithelial cell line, SRA04, was obtained from American Type Culture Collection (ATCC, USA). The cells were cultured in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum and 5000 U/ml penicillin and 5000 μg/ml streptavidin (Life Technologies Inc. Co. USA). Three EPHA2 mutants, pQCXIP-EPH-1751, pQCXIP-EPH-2819, and pQCXIP-EPH-2875 and wild-type pQCXIP-EPH-wt were transfected into the HLEC-SRA01 cells by using LipofectamineTM 2000 transfection kit (Invitrogen, USA). Briefly, HLEC-SRA01 cells were seeded into 96-well plates for 24 h before transfection. Serum-free medium was used to dilute vector DNA and Lipofectamine 2000, and the two diluted solutions were mixed and incubated at room temperature for 20 min and added directly to the cells. Four hours after culture, the medium was replaced with the fresh DMEM. Meanwhile, the pQCXIP-EPH-wt was assigned as a control [10].

Western-blot measurement of EPHA2 expression

Forty-eight hours post transfection, the HLEC-SRA01 cells were collected and employed to detect the expression of EPHA2 by using the western-blot assay. The proteins were extracted by using the lysis buffer, and dissolved in the DMEM. Then, the extracted proteins were examined by using the electrophoresis, and transferred to the PVDF membranes. Subsequently, the membranes were blocked with 5% free-fat milk in DMEM solution. The membranes were incubated with the mouse anti-human Myc monoclonal antibody (1:3000, Santa Cruz, USA) in DMEM solution. The membranes were washed with 1 × PBST (PBS+ 0.5% Tween 20) (5 min for 3 times), and incubated with the HRP-labeled goat anti-mouse IgG polyclonal

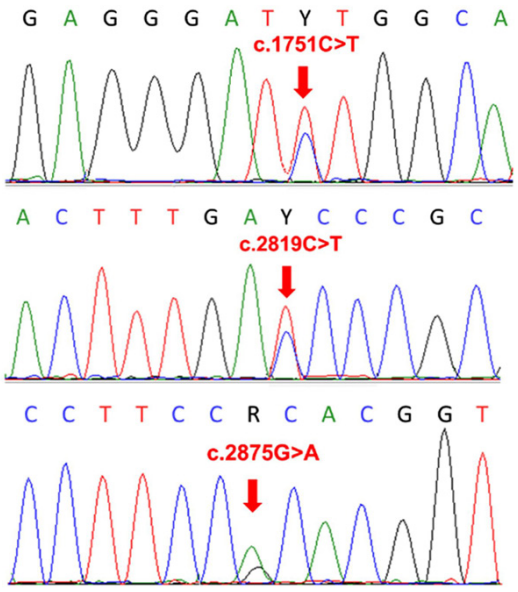

Figure 1. Sequencing results of EPHA2 gene.

Table 2. Distribution of EPHA2 mutation in patients with congenital cataract

Mutation	Cases (n=164)	Percentage
Wild type	154	93.9%
c.1751C>T	4	2.4%
c.2819C>T	4	2.4%
c.2875G>A	2	1.3%
Total	10	6.1%

antibody (1:2000, Santa Cruz, USA). The incubated membranes were then washed with 1 × PBST (5 min for 3 times). The reactive signals were visualized by using the ECL kit (PE Applied Biosystems, USA). The EPHA2 expression images of the immunoblot were scanned by using the Gel imaging analysis system, and digitalized, saved as the TIF format.

Immunofluorescence

Localization of EPHA2 in HLEC-SRA01 was detected by using the immunofluorescence assay. Briefly, 48 h after transfection, the HLEC-SRA01 cells were collected, digested by using trypsin (final concentration of 0.25%), re-suspended with 1 × PBS (washing 5 min for 3 times). Then, the cells were fixed by using the 4% paraformadehyde, and blocked with the 10% FBS in DMEM. The cells were then incubated with the mouse anti-human Myc monoclonal antibody (1:2000, Santa Cruz, USA) for 2 h at 37°C. The cells were then centrifuged (500 × g for 5 min), washed with 1 × PBS (5 min for 3 times). Subsequently, the cells were incubated with rhodamine-labeled goat anti-mouse polyclonal antibody (1:1000, Santa Cruz, USA) for 1 h at 37°C. Total of 10 μl cell suspensions were transferred to the slides, mixed with anti-quench mounting solutions and observed under fluorescence microscope [10].

Analysis of cell survival and antioxidant capability

To measure the effect of EPHA2 mutation on HLEC-SRA01 cell viability, cell counting Kit-8 (Enzo Life Sciences, USA) was used to detect the relative cell death. HLEC-SRA01 cells were digested by using trypsin (0.25% concentration), and re-suspended by using × PBS, and then seeded into 96-well plates. 200 μM of H_2O_2 was added to each well and incubated for 4 h. CCK8 was added to each well and incubated for another 4 h. Finally, the absorbance was measured at the wavelength of 450 nm.

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Stat View 5.0 software was used to analyze the data. One-way ANOVA was used to compare the differences between groups. The value of $P<0.05$ represents the statistical significance.

Results

Analysis of EPHA2 gene mutation in patients with congenital cataract

The EPHA2 gene mutation analysis results for the patients with congenital cataract were shown in **Figure 1** and **Table 2**. Approximately 93.9% of the patients with congenital cataract illustrated the wild type EPHA2, and 6.1% of patients with congenital cataract illustrated the mutant EPHA2. The percentage of c.1751C>T, c.2819C>T and c.2875G>A mutation was 2.4%, 2.4% and 1.3%, respectively.

Cell transfection

In order to investigate the possible roles of the three EPHA2 mutations in the pathogenesis of congenital cataract, the PCR and cloning techniques were used to construct wild-type EPHA2,

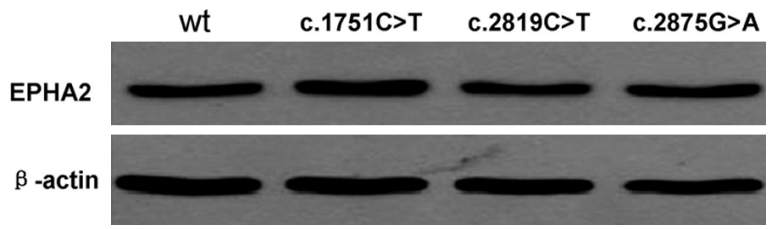

Figure 2. Western-Blot measurement of EPHA2 expression in transfected cells.

c.1751C>T, c.2819C>T and c.2875G>A mutation expression vectors. Three mutants and wild-type EPHA2 were transfected into HLEC-SRA01 cells. Western-blot assay was used to measure the expression EPHA2 gene and its mutants (**Figure 2**). Data showed that all HLEC-SRA01 cells are able to translate EPHA2 gene, which suggests that the mutants were successfully constructed and transfected.

Immunofluorescence detection of different EPHA2 mutants expression

Expression of different EPHA2 mutants was measured by the immunofluorescence assay (**Figure 3**). Since the antibody can't cross the cell membrane, it can only react with the EPHA2 localized on cell surface. We found that cells expressing the wild-type EPHA2 showed obvious fluorescence, whereas cells expressing the mutant EPHA2 showed no apparent fluorescence, which suggests that the EPHA2 mutation might result in abnormal localization.

Effect of EPHA2 gene mutation on cell proliferation

The proliferation capability of HLEC-SRA01 cells expressing different EPHA2 mutants were shown in **Figure 4**. Data showed that that the transfection of wild-type EPHA2 significantly decreased the mortality rate of HLEC-SRA01 cells compared to HLEC-SRA01 cells transfected with empty vector ($P<0.05$). Transfection of mutant EPHA2 significantly increased the mortality rate of HLEC-SRA01 cells compared to HLEC-SRA01 cells transfected with wild-type EPHA2 ($P<0.05$).

Measurement of antioxidant capacity

H_2O_2 was added to the cells and the cell death was measured to evaluate the antioxidant capacity (**Figure 5**). Viability of HLEC-SRA01 cells transfected with wild-type EPHA2 had significant higher antioxidant capacity compared with that of HLEC-SRA01 cells transfected with empty vector ($P<0.05$). Viability of HLEC-SRA01 cells transfected with mutant EPHA2 had significant lower antioxidant capacity compared with that of HLEC-SRA01 cells transfected with wild type EPHA2 ($P<0.05$).

Discussion

EPHA2 gene belongs to the EPH subfamily of transmembrane tyrosine kinase receptor family. Molecular biology research of congenital cataracts and other diseases proved that EPHA2 gene mutation may play an important role in the incidence and progression of congenital cataracts [8, 11]. This study aims to to investigate the association between EPHA2 mutations and congenital cataract. Three type mutations of EPHA2 gene were found in patients with congenital cataract by employing the gene sequencing. The three mutations of EPHA2 were introduced into HLEC-SRA01 for expression. Whole-cell immunofluorescence re-sults showed that EPHA2 mutation resulted in abnormal cellular localization. Cell survival and antioxidant capacity measurements results showed that HLEC-SRA01 cells transfected with wild-type EPHA2 had higher cell viability and antioxidant capacity, compared with that of HLEC-SRA01 cells transfected with mutant EPHA2.

By analyzing the gene sequence and mutations of EPHA2 (**Figure 6**), we found that two of the three mutations, EPHA2 c.2819C>T and c.2875G>A, are located in the SAM domain. SAM domain is a conserved domain of regulatory proteins and transcription factors, which can promote protein-protein interactions [12]. Mutations occurred in the SAM domain may cause a variety of diseases [13, 14]. Both EPHA2 c.2819C>T and c.2875G>A mutations can result in changes of amino acid's polarity, therefore, lead to protein mis-folding or abnormal protein function. Park *et al.* found that mutations occur in the SAM domain of EPHA2, which are likely to lead to decreased expres-

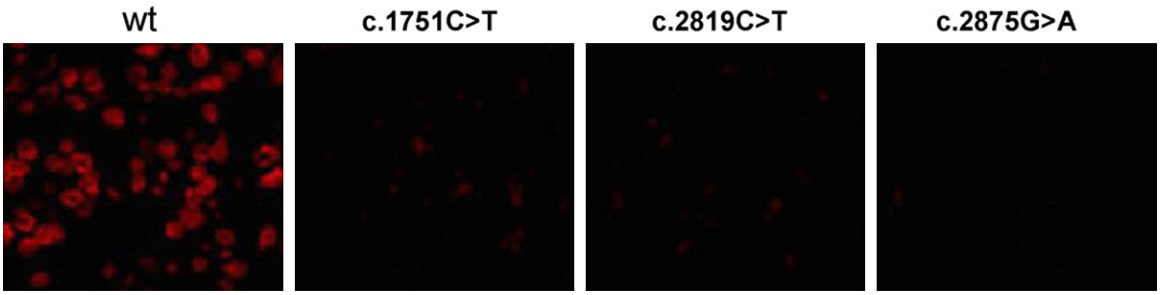

Figure 3. Immunofluorescence measurement of localization of EPHA2 its mutants.

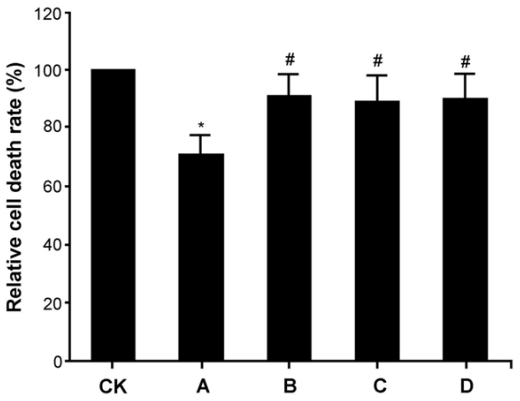

Figure 4. Effect of EPHA2 gene mutation on cell proliferation. CK: HLEC-SRA01 transfected with empty vector; A: HLEC-SRA01 transfected with wild type EPHA2; B: HLEC-SRA01 transfected with c.1751C>T mutant; C: HLEC-SRA01 transfected with c.2819C>T mutant; D: HLEC-SRA01 transfected with c.2875G>A mutant. *P<0.05, compared with control group; #P<0.05, compared with A group.

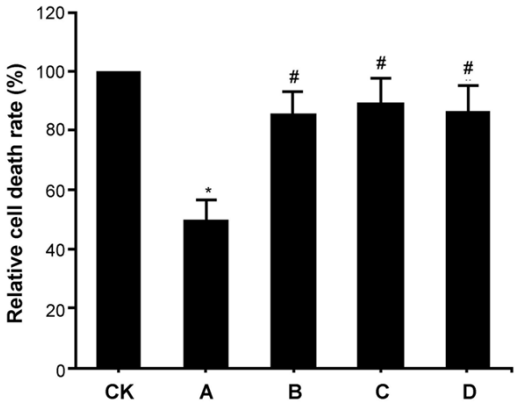

Figure 5. Effect of EPHA2 mutation on cells' antioxidant capacity. CK: HLEC-SRA01 transfected with empty vector; A: HLEC-SRA01 transfected with wild type EPHA2; B: HLEC-SRA01 transfected with c.1751C>T mutant; C: HLEC-SRA01 transfected with c.2819C>T mutant; D: HLEC-SRA01 transfected with c.2875G>A mutant. *P<0.05, compared with control group; #P<0.05, compared with A group.

sion of EPHA2 protein. Further studies have found that the mutations in SAM domain would accelerate EPHA2's degradation via ubiquitin-mediated protein degradation pathway and ultimately affect EPHA2 function [15]. The c.1751C>T mutation can lead to substituting a proline by a leucine at position 584 in the protein. This mutation is located on EPHA2's transmembrane region, which may also affect the cellular localization.

EPHA2 belongs to Eph-ephrin signaling pathway, which plays an important role in a variety of physiological and developmental processes, including the nervous system, skeletal system, and vascular system, *and etc.* [12-15] Park *et al.* found that EPHA2 gene mutations including c.1751C>T, c.2819C>T, c.2875G>A, c.2826-9G>A, were closely related with the incidence of congenital cataracts [15]. These results are consistent with the present study. Only three mutations, c.1751C>T, c.2819C>T and c.2875G>A were found in this study, which may be because of the genetic differences between eastern and western population [20]. Studies on EPHA2 and congenital cataract were focused on the effect on EPHA2 on the maintenance of lens transparency [21, 22]. The results of this study also indicated that EPHA2 gene mutation may reduce the cellular antioxidant capacity, and result in decreased cell viability. Since the lens cells under normal physiological conditions are under a higher oxidative stress, long-term accumulation of oxidative stress can lead to necrosis or dysfunction of lens cells.

Congenital cataract, associated with genetics, is the leading cause of blindness in children. Expression changes of genes involved in the development and maintenance of lens are likely to lead to congenital cataracts. The sensitive gene mutation screening and the investigation

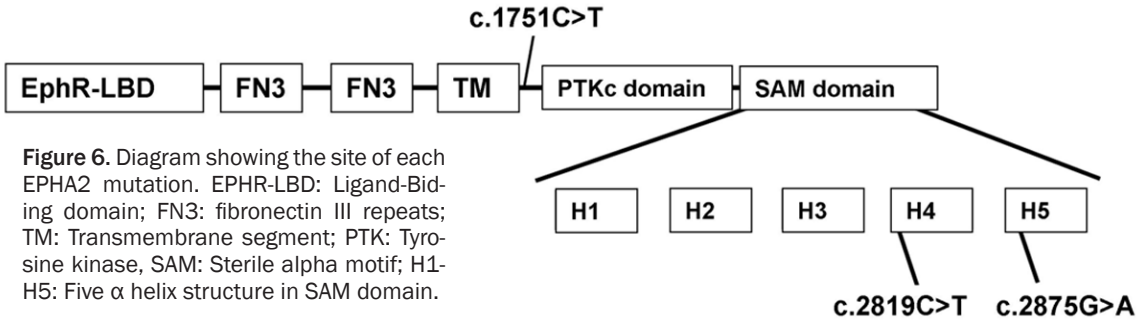

Figure 6. Diagram showing the site of each EPHA2 mutation. EPHR-LBD: Ligand-Biding domain; FN3: fibronectin III repeats; TM: Transmembrane segment; PTK: Tyrosine kinase, SAM: Sterile alpha motif; H1-H5: Five α helix structure in SAM domain.

for the mechanism of relevant gene mutations are significant and critical in preventing and treating the congenital cataract.

In summary, this study explored and investigated the EPHA2 gene mutations in congenital cataract patients as well as its role in the expression of EPHA2, which provides a theoretical basis for prevention of congenital cataract and treatment of congenital cataract by gene therapy.

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Disclosure of conflict of interest

None.

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