

Association of *FOXE3*-p.Ala170Ala and *PITX3*-p.Ile95Ile Polymorphisms with Congenital Cataract and Microphthalmia

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Abstract

Purpose: To investigate the association of *FOXE3*-p.Ala170Ala (rs34082359) and *PITX3*-p.Ile95Ile (rs2281983) polymorphisms with congenital cataract and microphthalmia in a western Indian population.

Methods: *FOXE3*-p.Ala170Ala (c.510C>T) and *PITX3*-p.Ile95Ile (c.285C>T) polymorphisms were genotyped in 561 subjects consisting of 242 cases with congenital cataract, 52 with microphthalmia, and 267 controls using polymerase chain reaction-restriction fragment length polymorphism. Approximately 10% of samples were randomly sequenced for each single nucleotide polymorphism to confirm the genotypes. The prediction of mRNA secondary structure for polymorphism *FOXE3*-p.Ala170Ala and *PITX3*-p.Ile95Ile was performed.

Results: A significantly high frequency of T allele and a borderline significance in the frequency of TT genotype of *FOXE3*-p.Ala170Ala was observed in microphthalmia cases, as compared to controls [T allele: OR: [CI] = 1.8 [1.15-2.72], $P = 0.0115$; TT: OR [CI] = 2.9 [1.14-7.16], $P = 0.0291$]. The frequency of CC genotype was significantly low in microphthalmia cases when compared to controls (CC: OR [CI] = 0.5 [0.24-0.86], $P = 0.0150$). There was no significant difference in the allele and genotype frequencies of *PITX3*-p.Ile95Ile between cases and controls. A slight free energy change was observed in the secondary structure of mRNA between the *FOXE3*-p.Ala170Ala C-allele (-917.60 kcal/mol) and T-allele (-916.80 kcal/mol) and between *PITX3*-p.Ile95Ile C-allele (-659.80 kcal/mol) and T-allele (-658.40 kcal/mol).

Conclusion: The present findings indicate that *FOXE3*-p.Ala170Ala 'T' allele and 'TT' genotype could be predisposing factors for microphthalmia while 'CC' genotype might play a protective role against it. A reduction in the free energy change associated with *FOXE3*-p.Ala170Ala 'T' allele could further contribute towards disease risk.

Keywords: Congenital Cataract; Forkhead Box E3; Microphthalmia; Paired-like Domain Transcription Factor 3

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INTRODUCTION

Congenital cataract and microphthalmia are vision-threatening eye disorders that account for 10-11%

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of childhood blindness.^[1,2] Congenital cataract represents lens opacity present at birth^[3] and microphthalmia is characterized by the presence of small eye within the orbit.^[1] Both disorders occur either in isolation or as part of a syndrome and are highly heterogeneous.^[1,4] Several chromosomal, monogenic, and environmental factors have been identified as causes for the occurrence of congenital cataract and microphthalmia.^[1,5,6] Nevertheless, genetic causes have been suggested to play a major role in these conditions.^[1,7]

Several transcription factor genes are known to be indispensable for vertebrate eye development. Two such genes: forkhead box E3 (*FOXE3*) and paired-like domain transcription factor 3 (*PITX3*), play an important role in ocular development and map to chromosome 1p32 and 10q25 respectively. In a normal eye, *FoxE3* controls lens epithelial proliferation and closure of lens vesicle,^[8] and *Pitx 3* controls lens epithelial maintenance and lens fiber differentiation.^[9] In humans, mutations in *FOXE3* and *PITX3* are responsible for varying degree of eye phenotypes such as congenital cataract, anterior segment mesenchymal dysgenesis, and microphthalmia.^[10-15] However, genetic association studies associating polymorphisms in *FOXE3* and *PITX3* genes and these eye disorders are sparse.

In our study on mutation screening in candidate genes for anophthalmia/microphthalmia, we observed a higher frequency of two polymorphisms: *FOXE3*-p.Ala170Ala (c.510C>T, rs34082359) and *PITX3*-p.Ile95Ile (c.285C>T, rs2281983), in isolated microphthalmia cases as compared to controls.^[16] This prompted us to investigate the association of these polymorphisms in congenital cataract and isolated microphthalmia cases using a larger number of samples. Both these polymorphisms are synonymous and do not change the amino acid sequence. However, synonymous single nucleotide polymorphism (SNP) contributing towards the risk of several disorders are very well defined.^[17-19] Genetic association studies of this kind have the potential to bring about major advancements in the field of congenital cataract and microphthalmia through diagnostics and risk prediction, as well as through modification in the therapeutic approaches, depending on the genetic profile of the patients. This is the first study to test for the association of polymorphisms in *FOXE3* and *PITX3* with congenital cataract and microphthalmia.

METHODS

Clinical Settings and Recruitment of Subjects

This study adhered to the tenets of the Declaration of Helsinki and was approved by the institutional review committee. All the subjects belonged to western

India (Gujarat, Rajasthan, Maharashtra, and parts of Madhya Pradesh). All study subjects, or their guardians, were informed about the nature of the study and informed consent was obtained from them. Peripheral blood was obtained from 561 subjects consisting of 242 cases with congenital cataract (females, *n* = 138; males, *n* = 104), 52 with microphthalmia (females, *n* = 32; male, *n* = 20), and 267 age- and ethnicity-matched normal healthy controls (females, *n* = 144; males, *n* = 123). Diagnosis of congenital cataract was made using a slit lamp biomicroscope and classification of the type of cataract was based on zone and morphology of lens opacification. Congenital cataract cases consisted of nuclear (*n* = 42), lamellar (*n* = 59), posterior subcapsular (*n* = 46), membranous (*n* = 28), sutural (*n* = 15), and total (*n* = 52) cataracts. A globe with a total axial length at least two standard deviations less than the mean for age was diagnosed as microphthalmia.^[20] The inclusion criteria comprised of isolated microphthalmia and congenital cataract diagnosed between the ages of 0.1-1 year. The controls had no family history of ocular malformations. Subjects with traumatic cataracts, eye disorders except for congenital cataract and microphthalmia, viral infections, chromosomal abnormalities, systemic diseases, neurodevelopmental disorders, and inborn errors of metabolism were excluded.

Single Nucleotide Polymorphism Genotyping

Genomic DNA was isolated from blood using the salting-out method.^[21] The *FOXE3*-p.Ala170Ala and *PITX3*-p.Ile95Ile polymorphisms were selected for genotyping based on observations from our previous study. Genotyping of *FOXE3*-p.Ala170Ala and *PITX3*-p.Ile95Ile was carried out using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Primers for PCR-RFLP were designed using primer3.0 (<http://primer3.ut.ee/>) online program. *FOXE3* was amplified using primers *FOXE3*-FW: 5'-CGCAAGTGGCAGAACAGCAT-3' and *FOXE3*-RV: 3'-TAGCAGGAGTTGAGTCCAG-5' that gives an amplicon of length 769 base pairs (bp) while *PITX3* was amplified using primers *PITX3*-FW: 5'-GGGAGCCAGCGAGTGGCTTAGGAG-3', and *PITX3*-RV: 3'-GGGTGGAACCGCTGGCCTCCG-5' that gives an amplicon of length 374 bp. The polymorphisms were analyzed using RestrictionMapper version 3.0 (www.restrictionmapper.org/) and were found to create a gain of *DdeI* restriction enzyme site for T allele of *FOXE3*-p.Ala170Ala and loss of *MboI* restriction enzyme site for T allele of *PITX3*-p.Ile95Ile. About 10% of samples were randomly selected and the genotypes for each SNP confirmed by Sanger's bi-directional sequencing using BigDye® Terminator V3.1 Cycle sequencing kit and run on the ABI 3130xl Genetic Analyzer.

Statistical Analysis

Allele and genotype frequencies were determined using (<http://analysis.bio-x.cn/myanalysis.php>).^[22] Fisher's exact test was used to check the strength of association of SNPs between cases and controls. Odds ratio at 95% confidence interval (CI) was calculated using <http://www.hutchon.net/confidor.htm>. Correction for multiple testing was done by dividing 0.05 by the total number of SNPs evaluated. *P* values <0.05 were considered as statistically significant.

In silico Analysis

The functional effect of the two SNPs, *FOXE3*-p. Ala170Ala and *PITX3*-p. Ile95Ile, were checked using tools such as PROVEAN,^[23] SIFT,^[24] and mutation taster^[25] according to the standards and guidelines set by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology.^[26]

Analysis of RNA Secondary Structure

The *in silico* modeling was done by RNAfold web server <http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi> to predict the secondary structure of mRNAs for *FOXE3*-p. Ala170Ala and *PITX3*-p. Ile95Ile.

RESULTS

The study consisted of 242 congenital cataract cases (females: 57%, males: 43%; mean age: 6.11 ± 2.14 years), 52 microphthalmia cases (females: 61.5%, males: 38.5%; mean age: 5.19 ± 2.43 years), and 267 age and ethnicity matched controls (females: 53.9%, males: 46.1%; mean age: 5.83 ± 2.10 years). Congenital cataracts consisted of lamellar (24.4%), total (21.4%), posterior subcapsular (19.0%), nuclear (17.4%), membranous (11.6%), and sutural (6.2%) cataracts.

Genotyping by PCR-RFLP of *FOXE3*-p. Ala170Ala yielded four fragments (512, 189, 47, and 21 bp) for C allele and five fragments (339, 189, 173, 47, and 21 bp) for T allele when digested with *DdeI* (C↓TNAG) restriction enzyme, and *PITX3*-p. Ile95Ile yielded one fragment (374 bp) for T allele and two fragments (253 and 121 bp) for C allele when digested with *MboI* (↓GATC) restriction enzyme. The three possible genotypes of both polymorphisms were scored using agarose gel electrophoresis [Figure 1a and c]. The corresponding electropherograms are shown in Figure 1b and d.

The allele and genotype frequencies of both polymorphisms were in Hardy-Weinberg equilibrium in both cases and controls. The frequency of *FOXE3*-p. Ala170Ala T allele was found to be significantly increased in microphthalmia cases when compared to controls (OR [CI] = 1.8 [1.15-2.72], *P* = 0.0115). The frequency of CT and TT genotype

of *FOXE3*-p. Ala170Ala was found to be significantly high in microphthalmia cases when compared to controls (CT: OR [CI] = 2.0 [1.04-3.98], *P* = 0.0459; TT: OR [CI] = 2.9 [1.14-7.16], *P* = 0.0291) [Table 1]. The genotyping of *PITX3*-p. Ile95Ile did not show any significant difference in the allele and genotype frequencies between cases and controls [Table 2]. A significant difference in the frequency of CC genotype of *FOXE3*-p. Ala170Ala was observed between microphthalmia cases and controls using dominant model (OR [CI] = 0.5 [0.24-0.86, *P* = 0.0150) [Table 3]. After correcting the *P* value for multiple testing (*P* = 0.025), the frequency of *FOXE3*-p. Ala170Ala T allele was found to be significantly associated with microphthalmia and there was a borderline significance in the association of TT genotype with microphthalmia. The *FOXE3*-p. Ala170Ala CC genotype was found to confer protective effect towards the development of microphthalmia. There was no significant difference in the allele and genotype frequencies of *FOXE3*-p. Ala170Ala and *PITX3*-p. Ile95Ile among the different cataract types and the controls (data not shown). *In silico* analysis by Provean, SIFT, and mutation taster classified both *FOXE3*-p. Ala170Ala and *PITX3*-p. Ile95Ile as 'neutral', 'tolerated', and 'polymorphisms' respectively. A slight change in free energy was observed between C allele (-917.60 kcal/mol) and T allele (-916.80 kcal/mol) of *FOXE3*-p. Ala170Ala [Figure 2a and b] and C allele (-659.80 kcal/mol) and T allele (-658.40 kcal/mol) of *PITX3*-p. Ile95Ile [Figure 2c and d].

DISCUSSION

Normal eye development requires sequential activation and silencing of thousands of genes in a spatial and temporal manner. Several tissue-specific transcription factors control these developmental processes. An alteration in any of these transcription factors can have serious consequences for normal ocular development and could lead to partial or total blindness. In humans, mutations in several transcription factor genes have been reported to cause disruption of vertebrate eye development or maintenance.^[10-15,27-32] This led to the hypothesis that polymorphisms in these genes may also predispose towards the risk of congenital eye disorders. In our previous studies on mutation screening in candidate genes for congenital cataract and microphthalmia, we observed that the frequency of two polymorphisms *FOXE3*-p. Ala170Ala and *PITX3*-p. Ile95Ile was higher in congenital cataract and microphthalmia cases as compared to controls. Hence in the present study, we evaluated the association of polymorphisms in *FOXE3* and *PITX3* transcription factors with the risk of developing congenital cataract and microphthalmia.

The polymorphisms *FOXE3*-p. Ala170Ala and *PITX3*-p. Ile95Ile tested in the present study are

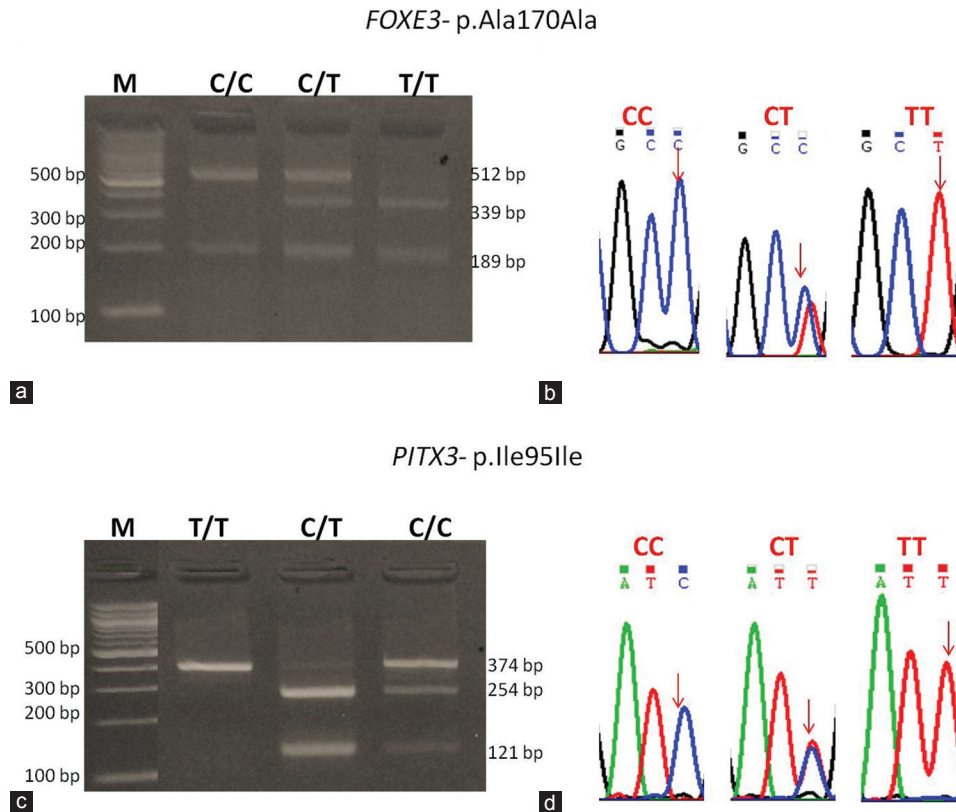


Figure 1. (a and c) Agarose gel electrophoresis showing PCR-RFLP patterns for *FOXE3*-p.Ala170Ala, and *PITX3*-p.Ile95Ile after digesting with *DdeI* and *MboI* restriction enzyme respectively. (b and d) Electropherograms confirming all the three possible genotypes of *FOXE3*-p.Ala170Ala and *PITX3*-p.Ile95Ile respectively.

Table 1. Distribution of *FOXE3*-p.Ala170Ala in the study groups

Study groups	<i>FOXE3</i> -p.Ala170*Ala				
	Cytosine	Thymine	CC	CT	TT
Controls, n=267	373 (0.699)	161 (0.301)	132 (0.494)	109 (0.408)	26 (0.097)
[†] CCA, n=242	326 (0.674)	158 (0.326)	109 (0.450)	108 (0.446)	25 (0.103)
[‡] MT, n=52	59 (0.567)	45 (0.433)	16 (0.308)	27 (0.519)	14 (0.269)
Control versus CCA; [§] OR (95% [¶] CI)	Reference	1.1 (0.86-1.46)	Reference	1.2 (0.83-1.73)	1.2 (0.64-2.13)
Fisher exact value	-	0.4170	-	0.3495	0.6452
Control versus MT; OR (95% CI)	Reference	1.8 (1.15-2.72)	Reference	2.1 (1.05-4.0)	2.9 (1.14-7.16)
Fisher exact value	-	0.0115	-	0.0459	0.0291

*Ala, alanine; [†]CCA, congenital cataract; [‡]MT, microphthalmia; [§]OR, odds ratio, [¶]CI, confidence interval. *P*<0.05 was considered as statistically significant. Values are expressed as count (frequency)

Table 2. Distribution of *PITX3*-p.Ile95Ile in the study groups

Study groups	<i>PITX3</i> -p.Ile95*Ile				
	Cytosine	Thymine	CC	CT	TT
Controls, n=267	267 (0.500)	267 (0.500)	67 (0.251)	133 (0.498)	67 (0.251)
[†] CCA, n=242	230 (0.475)	254 (0.525)	61 (0.252)	108 (0.446)	73 (0.302)
[‡] MT, n=52	49 (0.471)	55 (0.529)	11 (0.212)	27 (0.519)	14 (0.269)
Control versus CCA; [§] OR (95% [¶] CI)	Reference	1.1 (0.86-1.41)	Reference	8.9 (0.58-1.37)	1.2 (0.74-1.93)
Fisher exact value	-	0.4514	-	0.6608	0.5409
Control versus MT; OR (95% CI)	Reference	1.1 (0.74-1.71)	Reference	1.2 (0.58-2.64)	1.2 (0.54-3.01)
Fisher exact value	-	0.5942	-	0.7069	0.6654

*Ile, isoleucine; [†]CCA, congenital cataract; [‡]MT, microphthalmia; [§]OR, odds ratio, [¶]CI, confidence interval. *P*<0.05 was considered as statistically significant. Values are expressed as count (frequency)

Table 3. Dominant and recessive models for FOXE3-p.Ala170Ala and PITX3-p.Ile95Ile

Study groups	FOXE3-p.Ala170*Ala		PITX3-p.Ile95*Ile	
	Dominant	Recessive	Dominant	Recessive
	CC versus (CT+TT)	TT versus (CC+CT)	CC versus (CT+TT)	TT versus (CC+CT)
Controls, n=267	132/135 (0.506)	26/241 (0.903)	67/200 (0.749)	67/200 (0.749)
*CCA, n=242	109/133 (0.550)	25/217 (0.897)	61/181 (0.748)	73/169 (0.698)
[†] MT, n=52	16/36 (0.692)	9/43 (0.827)	11/41 (0.788)	14/38 (0.731)
Control versus CCA; [†] OR (95% ^{**} CI)	0.8 (0.59-1.19)	1.1 (0.60-1.91)	1.0 (0.67-1.50)	1.3 (0.87-1.90)
Fischer exact value	0.3296	0.8829	1.0	0.2329
Control versus MT; OR (95% CI)	0.5 (0.24-0.86)	1.9 (0.85-4.43)	0.8 (0.39-1.65)	1.2 (0.56-2.15)
Fischer exact value	0.0150	0.1420	0.6012	0.8618

*Ala, alanine; [†]Ile, isoleucine; [‡]CCA, congenital cataract; [†]MT, microphthalmia; [†]OR, odds ratio; ^{**}CI, confidence interval. $P < 0.05$ was considered as statistically significant. Values are expressed as count (frequency)

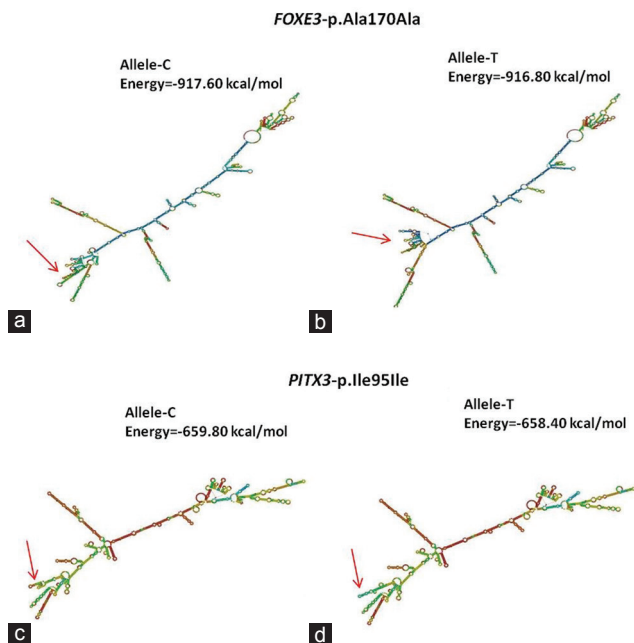


Figure 2. Predicted RNA secondary structure and energies for FOXE3-p.Ala170Ala (a) C allele and (b) T allele and PITX3-p.Ile95Ile (c) C allele and (d) T allele measured using RNA fold. The alteration in secondary structure has been shown by an arrow. The stability of the RNA structure is determined using the free energy.

synonymous variations that do not change the protein sequence. However, synonymous SNPs are gaining much attention for their ability to cause changes in protein expression, conformation, and function, and also for their contribution towards human disease risk and other complex traits.^[17-19] In our study, the FOXE3-p.Ala170Ala T allele and TT genotype (borderline) was found to be associated with the risk of microphthalmia while the CC genotype was found to have a protective effect towards its development.

During the translation process, the stability of mRNA has a profound impact on the expression level of protein. Any alteration in the free energy change

of the secondary structure of mRNA can reduce the translation efficiency up to four-fold.^[33] In the RNA secondary structure, a decrease in predicted free energy change (ΔG) has been suggested to reduce the relative expression of the protein.^[33] In our study, we observed a minor free energy change in the secondary structure of FOXE3 mRNA between the C allele (-917.60 kcal/mol) and T allele (-916.80 kcal/mol) and also of PITX3 mRNA between the C allele (-659.80 kcal/mol) and T allele (-658.40 kcal/mol). Hence, we believe that the T allele of FOXE3-p.Ala170Ala might contribute to decreased FOXE3 mRNA expression when compared to C allele carriers which might further increase disease susceptibility. However, this idea needs to be functionally validated by *in vitro* studies.

Our study did not find any significant association of FOXE3-p.Ala170Ala with congenital cataract and PITX3-p.Ile95Ile with both congenital cataract and microphthalmia. This suggests that in addition to mutations in candidate genes and an individual's genotype, there are several other modifying factors such as environmental factors, gene-gene interactions etc. that could pose a serious risk towards the onset and maturation of the disease. This study has the limitation of not including the normal, unaffected blood relatives of the study population and also the moderately small sample size (especially microphthalmia) and hence to substantiate the present findings, similar studies using the blood relatives and large cohorts in different populations is essential.

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Conflicts of Interest

There are no conflicts of interest.

REFERENCES

- Verma AS, Fitzpatrick DR. Anophthalmia and microphthalmia. *Orphanet J Rare Dis* 2007;2:47.
- Yi J, Yun J, Li ZK, Xu CT, Pan BR. Epidemiology and molecular genetics of congenital cataracts. *Int J Ophthalmol* 2011;4:422-432.
- Churchill A, Graw J. Clinical and experimental advances in congenital and paediatric cataracts. *Philos Trans R Soc Lond B Biol Sci* 2011;366:1234-1249.
- Cui XJ, Lv FY, Li FH, Zeng K. Correlations of single nucleotide polymorphisms of CRYAA and CRYAB genes with the risk and clinicopathological features of children suffering from congenital cataract. *Medicine (Baltimore)* 2017;96:e7158.
- Shen C, Wang J, Wu X, Wang F, Liu Y, Guo X, et al. Next-generation sequencing for D47N mutation in cx50 analysis associated with autosomal dominant congenital cataract in a six-generation Chinese family. *BMC Ophthalmol* 2017;17:73.
- Santana A, Waiswo M. The genetic and molecular basis of congenital cataract. *Arq Bras Oftalmol* 2011;74:136-142.
- Pichi F, Lembo A, Serafino M, Nucci P. Genetics of congenital cataract. *Dev Ophthalmol* 2016;57:1-14.
- Blixt A, Mahlapuu M, Aitola M, Pelto-Huikko M, Enerbäck S, Carlsson P, et al. A forkhead gene, FOXE3, is essential for lens epithelial proliferation and closure of the lens vesicle. *Genes Dev* 2000;14:245-254.
- Ho HY, Chang KH, Nichols J, Li M. Homeodomain protein pitx3 maintains the mitotic activity of lens epithelial cells. *Mech Dev* 2009;126:18-29.
- Garcia-Montalvo IA, Pelcastre-Luna E, Nelson-Mora J, Buentello-Volante B, Miranda-Duarte A, Zenteno JC, et al. Mutational screening of FOXE3, GDF3, ATOH7, and ALDH1A3 in congenital ocular malformations. Possible contribution of the FOXE3 p.VAL201MET variant to the risk of severe eye malformations. *Ophthalmic Genet* 2014;35:190-192.
- Liu H, Liu H, Tang J, Lin Q, Sun Y, Wang C, et al. Whole Exome sequencing identifies a novel mutation in the PITX3 gene, causing autosomal dominant congenital cataracts in a Chinese family. *Ann Clin Lab Sci* 2017;47:92-95.
- Verdin H, Sorokina EA, Meire F, Casteels I, de Ravel T, Semina EV, et al. Novel and recurrent PITX3 mutations in Belgian families with autosomal dominant congenital cataract and anterior segment dysgenesis have similar phenotypic and functional characteristics. *Orphanet J Rare Dis* 2014;9:26.
- Saboo US, Penke D, Mahindrakar A, Uddaraju M, Sankurathri C, Gong X, et al. Exome sequencing reveals novel homozygous FOXE3 mutation in microphthalmos with staphylocomatous malformation. *Ophthalmic Genet* 2017;38:295-297.
- Islam L, Kelberman D, Williamson L, Lewis N, Glindzicz MB, Nischal KK, et al. Functional analysis of FOXE3 mutations causing dominant and recessive ocular anterior segment disease. *Hum Mutat* 2015;36:296-300.
- Ullah E, Nadeem Saqib MA, Sajid S, Shah N, Zubair M, Khan MA, et al. Genetic analysis of consanguineous families presenting with congenital ocular defects. *Exp Eye Res* 2016;146:163-171.
- Vidya NG, Rajkumar S, Vasavada AR. Genetic investigation of ocular developmental genes in 52 patients with anophthalmia/microphthalmia. *Ophthalmic Genet* 2018;39:344-352.
- Sauna ZE, Kimchi-Sarfaty C. Understanding the contribution of synonymous mutations to human disease. *Nat Rev Genet* 2011;12:683-691.
- Gotea V, Gartner JJ, Qutob N, Elnitski L, Samuels Y. The functional relevance of somatic synonymous mutations in melanoma and other cancers. *Pigment Cell Melanoma Res* 2015;28:673-684.
- Li X, Chen Y, Qi H, Liu L, Shuai J. Synonymous mutations in oncogenesis and apoptosis versus survival unveiled by network modeling. *Oncotarget* 2016;7:34599-34616.
- Bardakjian T, Weiss A, Schneider A. Microphthalmia/Anophthalmia/Coloboma Spectrum. 2004 Jan 29 [Updated 2015 Jul 9]. In: Adam MP, Ardinger HH, Pagon RA, et al. editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2018.
- Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988;16:1215.
- Shi YY, He L. SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. *Cell Res* 2005;15:97-98.
- Choi Y, Chan AP. PROVEAN web server: A tool to predict the functional effect of amino acid substitutions and indels. *Bioinformatics* 2015;31:2745-2747.
- Ng PC, Henikoff S. SIFT: Predicting amino acid changes that affect protein function. *Nucleic Acids Res* 2003;31:3812-3814.
- Schwarz JM, Rödelberger C, Schuelke M, Seelow D. MutationTaster evaluates disease-causing potential of sequence alterations. *Nat Methods* 2010;7:575-576.
- Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* 2015;17:405-424.
- Macchiaroli A, Kelberman D, Auriemma RS, Drury S, Islam L, Giangiobbe S, et al. A novel heterozygous SOX2 mutation causing congenital bilateral anophthalmia, hypogonadotropic hypogonadism and growth hormone deficiency. *Gene* 2014;534:282-285.
- Huang XF, Huang ZQ, Lin D, Dai ML, Wang QF, Chen ZJ, et al. Unraveling the genetic cause of a consanguineous family with unilateral coloboma and retinoschisis: Expanding the phenotypic variability of RAX mutations. *Sci Rep* 2017;7:9064.
- Somashekar PH, Shukla A, Girisha KM. Intrafamilial variability in syndromic microphthalmia type 5 caused by a novel variation in OTX2. *Ophthalmic Genet* 2017;38:533-536.
- Miao Q, Ping X, Tang X, Zhang L, Zhang X, Cheng Y, et al. Experimental assessment of novel PAX6 splicing mutations in two Chinese families with aniridia. *Gene* 2017;630:44-48.
- Shah MH, Tabanera N, Krishnadas SR, Pillai MR, Bovolenta P, Sundaresan P, et al. Identification and characterization of variants and a novel 4 bp deletion in the regulatory region of SIX6, a risk factor for primary open-angle glaucoma. *Mol Genet Genomic Med* 2017;5:323-335.
- Ammar TH, Ismail S, Mansour OA, El-Shafey MM, Doghish AS, Kamal AM, et al. Genetic analysis of SOX2 and VSX2 genes in 27 Egyptian families with anophthalmia and microphthalmia. *Ophthalmic Genet* 2017;38:498-500.
- Seo SW, Yang J, Jung GY. Quantitative correlation between mRNA secondary structure around the region downstream of the initiation codon and translational efficiency in *Escherichia coli*. *Biotechnol Bioeng* 2009;104:611-616.