

PAX6 mRNA transcript analysis in various ocular/ non-ocular tissues

Abstract

PAX6 is a critical regulatory gene located on human chromosome 11p13, is expressed in many ocular tissues, and non-ocular tissues like brain, and pancreas. The expression of mRNA transcript has been investigated in this study since *PAX6* is likely to play a vital role in the development of eye. RNA was extracted from various ocular tissues including lens epithelium, retina, iris, non-ocular tissues Trabecular Meshwork cells (TM cell line), HeLa cells. Besides the mutant and wild type blood cells were also included and total RNAs were subjected to convert in to Complementary DNA (cDNA). The high sensitive reverse transcriptase polymerase chain reaction (RT-PCR) was performed to monitor the mRNA transcript level to detect the expression profiles of the ocular/non-ocular tissues taken for this study. The competitive RT-PCR analysis was carried out using the house keeping gene beta actin as a control in both the ocular and non-ocular tissues. The level of beta actin expression in ocular/non-ocular tissues was equal and showed 540bp amplification. Subsequently the *PAX6* mRNA transcript was analysed in these tissues. The expression level of *PAX6* was observed in the ocular tissues such as retina, iris and lens epithelium which expressed approximately 600, 855, 1013 and 1055bp amplification. In the non-ocular HeLa cells the *PAX6* expression was abnormally high and showed approximately 1.2kb amplification. No *PAX6* expression was observed in the wild type WBC cells; however the mutant WBC amplified an irrelevant transcript. Sequencing the irrelevant transcript revealed the homology hint to various chromosomes particularly of hypothetical LOC441763 mRNA. RT-PCR amplification confirms the presence of various *PAX6* mRNA expressions in ocular and non-ocular tissues. Lens epithelium, retina and iris tissues have significant *PAX6* expression while the retina contains an overwhelming majority of mRNAs encoding crystallins and other genes but still express *PAX6* isoforms at high levels. HeLa is a fibroblast based cell line and it would not expect a fibroblast to express a lot of *PAX6* However *PAX6* gene expression might be high in these cells for some reason. The *PAX6* gene partial expression was found in mutant WBC and not in wild type WBC cells, the real fact for this reason is unknown. *PAX6* mutations should be analyzed with effective functional assays, which will be useful for future study.

Keywords: *PAX6* mRNA transcript, TM cell lines, cDNA, RT-PCR, WBC lymphocytes

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Abbreviations: *PAX6*, paired box gene 6; mRNA, messenger ribonucleic acid; cDNA, complementary deoxyribonucleic acid; RT-PCR, reverse transcriptase polymerase chain reaction; WBC, white blood cells; TM Cells, trabecular meshwork cells; HeLa Cells, cancer cell lines

Introduction

PAX6 is a critical regulatory gene located on human chromosome 11p13, is expressed in many ocular tissues, and non-ocular tissues brain, and pancreas.¹ *PAX6* encodes a specific paired and homeo DNA binding transcription factor, which is essential for eye development.² *PAX6* extends over 22kb and contains 14 exons and intron sequences, in addition, a CCAGCATGC translation start site at exon 4, a TAA stop codon in exon 13. The transcription start site and promoter region with TATA, CAAT, and GC regulatory elements, and three possible polyadenylation signals have all been characterized.³ *PAX6* heterozygous mutation causes aniridia (eye without iris) and other related phenotypes¹ whereas the homozygous *PAX6* cause anophthalmia (absence of one or both eyes) and neonatal lethality.⁴ Human *PAX6* is transcribed as a 2.7kb mRNA and encodes a 422-amino-acid protein that includes the paired box, the homeo box, and a third possible DNA-binding motif, the PST domain (Proline,

Serine, and Threonine-rich sequence).¹ Interestingly, *PAX6* contains an alternative mRNA splice-site in the paired domain. This resulted in a 42-nucleotide insertion; the insertion allows the carboxy terminal sub-region of the paired domain to recognize a novel DNA sequence. *PAX6* contains an alternative mRNA splice-site which allows to regulate the restricted set of genes depends on the mRNA splicing. These two major forms of the protein, *PAX6* and *PAX6* (5a) are highly conserved among metazoans (Multicellular animals).⁵ However the over expression of *PAX6* (5a) was detected in human congenital cataract.⁴ In addition *PAX6* (5a) up regulation was observed in transgenic mouse have resulted in lens abnormalities.⁶ Therefore, *PAX6* transcription factor, which is required for proper eye development, is expressed by normal lens epithelial cells but not in mature fiber cells.⁶⁻⁸ The expression of *PAX6* mRNA transcript have been investigated since *PAX6* is likely to play a vital role in the development of eye therefore the high sensitive RT-PCR was performed to monitor the *PAX6* mRNA transcript level in various ocular tissues like lens epithelium, retina, iris, and non-ocular tissues TM cell line, HeLa RNA and WBC cells.

Materials and methods

All studies were conducted in accordance with the tenets of the Declaration of Helsinki and the Institutional Review Board of Aravind

Eye Care System is approved by Human Research Protection, United States of America. Whole normal human cadaver globe (60 years old) was borrowed from International Aravind Eye Bank after 4 hours of enucleation and microdissected into retina, iris and lens epithelium. Non-ocular tissues like White blood cells with aniridia (obtained after informed consent), Trabecular Meshwork cells (TM Cells cultured in the lab), HeLa RNA (Supplied by Bangalore Genei RT-PCR kit, Bangalore, India) and the Wild type WBC also obtained. 10mg of each tissue and 1.5ml of wild type blood was collected in the presence of an anticoagulant, preferably EDTA or citrate or heparin or acid citrate dextrose and processed to isolate RNA by QIAamp RNA blood mini kit (QIAGEN, GmbH, Germany). QIAamp spin columns are silica-gel based membrane with microspin technology. During the procedure for purification of RNA from ocular and non-ocular tissues were selectively lysed using highly denaturing conditions to inactivate the *RNases*. After homogenization, the lysate briefly spun through QIAshredder spin column, where the RNA binds to the

silica based matrix. The contaminants were washed with appropriate buffers then the intact cellular RNA was collected in fresh tube using RNase-free water. For the optimal results the ocular/non-ocular tissues were processed immediately after the collection since the mRNA have different stabilities. The isolated total RNA was stored at -200C in DEPC water for approximately 3-6 months. Ribonucleases (*RNases*) are highly stable and difficult to inactivate hence all the plastic wares and glasswares were treated thoroughly with a solution containing 0.1M NaOH and 1mM EDTA followed by RNase-free water.

Primers

Sequence specific primers were used for RT-PCR analysis. Primers used were synthesised from Microsynth, Switzerland. Besides the published primer⁹ another set of primers designed by ABI prism software to amplify the paired domain, PST domain and to amplify the whole *PAX6* gene. Details of the primers are sorted out in (Table 1).

Table 1 Primers used in RT-PCR analysis to amplify *PAX6* gene transcript

S. No.	Primer name	Nucleotide sequence	Reference
1	C139	5'AGAGTGGACAGACATCCGAGA3'	Hanson et al. ⁹
2	C 402	5'AATCTTGGCCAGTATTGAGAC3'	Hanson et al. ⁹
3	C127	5'GCCAGAGCCAGCATGCAGAAC3'	Hanson et al. ⁹
4	C401	5'ATCAGGTTCACTTCCGGGAAC3'	Hanson et al. ⁹
5	RTF	5'ACAACCAGAAAGGATGCCTCA3'	ABI prism software
6	RTR	5'CCCATCTGTTGCTTTTCGCTA3'	ABI prism software
7	RTPST	5'CAGATGTGAAGGAGGAAACCG3'	ABI prism software
8	b-actin F	5' CGTCTTCCCCTCCATCG 3'	ABI prism software
9	b-actin R	5' CTCGTTAATGTCACGCAC 3'	ABI prism software

RTF, reverse transcript forward primer; RTR, reverse transcript reverse primer; RTPST, reverse transcript proline serine threonine region; b-actin, beta actin

RNA quantity/quality analysis

The concentration and purity of RNA determined in to two methods. Gel estimation analysis by visualization and measuring its absorbance at 260 nm and 280 nm in spectrophotometer. The absorbance reading should be greater than 0.15. One unit at 260 nm corresponds to 40µg of RNA per ml. The ratio between the 260/280 nm provides the purity of RNA. The importance of using full-length RNA for reverse transcription depends on the intactness.

DNase I digestion

The RNA samples (2µg) were treated with *RNase free* one µl of *DNase I* with one µl of 10X buffer and stored at 370C for one hour then the reaction stopped by adding one µl of EDTA (25mM) followed by heating to 650C for 10min to remove possible genomic DNA contamination prior to cDNA conversion. *DNase* buffer contains cations hence the RNA was heated to degrade the cations in the buffer solution.

RT-PCR and cDNA Conversion

RT-PCR and cDNA Conversion

Generally reverse transcription reactions are performed with one of the primers of random hexamers or oligonucleotides (Oligo dTs) or an oligonucleotide that can hybridize with the specific RNA under

the study. However, in this study two types of first strand cDNA (Complementary DNA) conversion were performed in all the RNA samples.

- RNA with the mixture of oligo-dT and random hexamers
- RNA with the mixture of oligo-dT and gene specific primer

One µg of total RNA was heated just before reverse transcription to denature any secondary structure, which could impede the reverse transcriptase from copying the RNA, at 750C for 3 min. The equal amount of oligo-dT and random hexamer (50 µM) added to the final concentration of 5µM,¹⁰ with 10.5µl of DEPC water again kept in PCR at 700C again for 5 min. The samples were placed immediately in ice. To the samples the following components were added 1X buffer (500mM Tris-HCl, 750mM KCl, 30mM MgCl₂, 50mM DTT), 10mM dNTPs mix, 20U of RNase Inhibitor, 200 U of Moloney Murine Leukemia Virus-Reverse Transcriptase enzymes (Ambion, USA) and the nuclease free DEPC water to the final concentration of 30µl set up. The reaction mixture was incubated at 420C for 1 hour and 950C for 10 min to inactivate the reverse transcriptase enzyme followed by chill on ice. In both types of cDNA conversion gave the consistent results hence either one type can be followed for further study.

PCR analysis

All the resulting cDNA was then amplified with primers specific to

both *PAX6* and β -actin in a total reaction volume of 20 μ l. The reaction mix containing 1X polymerase chain reaction (PCR) buffer (10mM Tris-HCl, 50mM KCl) 2mM of MgCl₂, 0.25 μ M of forward and reverse primer and 1 unit of Taq DNA polymerase enzyme (Fermentas life science). The *PAX6* gene specific and the house keeping gene β -actin primers were used. PCR parameters consist of 5 min denaturing step at 950C followed by 35 cycles (940C for 1 min, 600C for 2 min and 720C for 2 min) and the final extension at 720C for 10 min. For β -actin gene the reaction mix contains 1X buffer, 1.5mM MgCl₂, 0.2 μ M primer and 1 unit of Taq DNA polymerase enzyme (Fermentas). The PCR conditions for β -actin were 940C for 3 min and then cycles for 30 times at 940C for 1.30 min, 520C for 1 min and 720C for 2 min and then 720C for 5 min final extension.

Post PCR electrophoresis

RT-PCR amplified products were resolved and visualized on 2% agarose gel for both *PAX6* and β -actin. Electrophoresis was carried out at 100V for 60 min in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). Gels were stained with ethidium bromide (5 μ g/ml) nucleic acid stain. Longer products resolve better on a gel with slightly less agarose.

Minus-RT controls

The importance of minus-reverse transcriptase as control in the RT-PCR experiments to avoid contamination (genomic). This could help that the template for the PCR product was from cDNA and not from the genomic DNA. In the negative control sample all the necessary components were added except the RT enzyme.

Sequencing

The aberrant band expressed in mutant WBC was re-amplified and purified by QIAGEN columns. The PCR products were sequenced directly on an Applied Biosystems (ABI) model 3730 automated sequencer.

Results and discussion

The expression patterns of *PAX6* were analyzed by high sensitive RT-PCR analysis from various ocular tissues of normal cadaver globe obtained from International Aravind Eye Bank and micro dissected. To detect the *PAX6* transcript since the eyes formed through serious of interactions between the cells and the embryonic origins that result in lineages of ocular tissues. The total RNA was isolated was resolved on 1% agarose gel to check the integrity of RNA. The extracted RNA subjected for two types of cDNA conversion. These two types of cDNA conversion performed to amplify the *PAX6* transcript from the ocular/non-ocular tissues and no significant changes were observed. The competitive RT-PCR analysis was carried out using the house keeping gene beta actin as a control in both ocular and non-ocular tissues. All the three ocular and four non-ocular tissues such as TM cell HeLa RNA, wild type WBC, mutant WBC RNA was used to amplify the house keeping gene β -actin. Both the ocular/non-ocular tissues expressed the β -actin gene equal levels and showed 540bp amplification. Majority of the genes commonly considered to have a housekeeping function (e.g., β -actin and GAPDH) exhibit considerably variable expression levels from one tissue type to another. However, expression profiles for the maintenance/housekeeping genes exhibit similar patterns for each specific tissue type (Figure 1A). As a primary attempt three samples of ocular tissues such as lens epithelium, normal WBC lymphocytes, and the HeLa RNA were subjected to

amplify the *PAX6* gene using 5' paired domain primer. When the RT-PCR products were run on 2.5% agarose gel the lens epithelium showed two *PAX6* mRNA transcripts (*PAX6* and *PAX6* 5a isoforms) of approximately 900bp and 1.2kb amplification (Figure 1B, lane 3). The *PAX6* expression might be abnormally high in HeLa RNA that showed approximately 1.2kb (Figure 1B, lane 1). However, neither *PAX6* nor *PAX6* (5a) isoforms was observed in the wild type WBC (Figure 1B, lane 2). The *PAX6* mRNA transcript was analyzed in the ocular tissues such as retina, iris, and lens epithelium were showing approximately 600, 800, 1013 and 1055bp (Figure 2A, lane 2-4). The various *PAX6* mRNA transcripts are consistent with previous published literature.¹¹ Similar pattern of *PAX6* expression profile was also obtained in the non-ocular tissue RNA taken in this study.

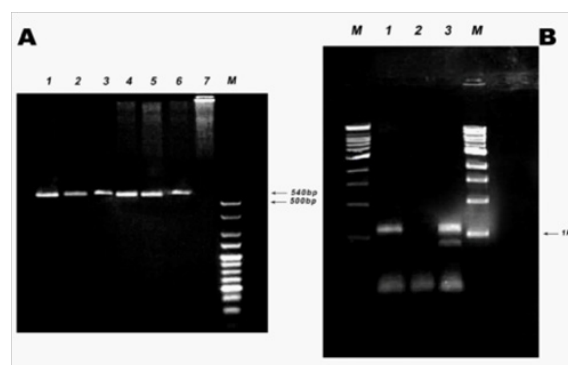


Figure 1 (A) Expression of housekeeping gene (β -actin) amplification of various ocular/non-ocular tissues. Lane 1-Retina, lane 2-iris, lane 3-lens epithelium, lane 4-normal WBC without DNase I, lane 5-TM cell line, lane 6-WBC with DNase I digestion, lane 7-WBC RNA without RT enzyme as negative control. M- 50bp marker. (B) *PAX6* expression profiles in HeLa RNA and lens epithelium. M-1 kb marker, lane 1-HeLa RNA showing the unusual *PAX6* expression and no isoform of 5a. lane 2-wild type WBC RNA, lane 3-Lens epithelium showing both isoforms of *PAX6* and *PAX6* (5a).

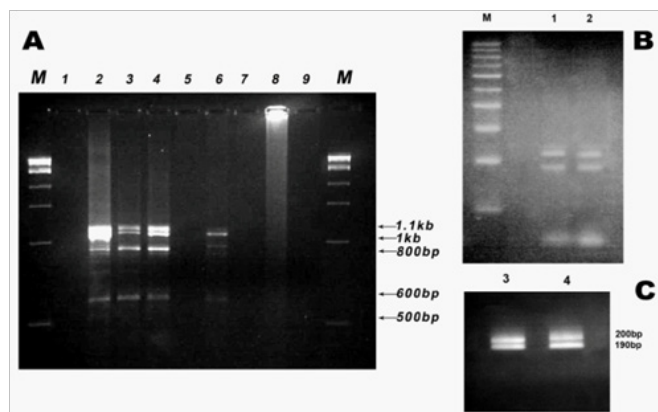


Figure 2 (A) *PAX6* mRNA transcripts of various ocular tissues by RT-PCR analysis. M-1kb marker, 1-Empty, 2-Retina cDNA with oligo dTs and gene specific primers, 3-Iris, 4-lens epithelium, 5- Wild type WBC cDNA without DNase I treatment, 6-TM cell line with oligo dTs and gene specific, 7-empty, 8- Wild type WBC cDNA with DNase I treatment, 9-Retina cDNA without RT enzyme as negative control. (B) *PAX6* expression pattern in affected individual & amplicon purification. M-100bp Marker, lane 1-Mutant mother showing the amplification, lane 2-Affected proband shows the same amplification. (C) Lane 3, 4-Showing approximately 190bp, 200bp of purified RT-PCR product for sequencing.

The wild type WBC cDNA with or without *DNase I* treatment does not show *PAX6* expression profile and this concludes that the *PAX6* is not expressed in wild type WBC cells, However, the housekeeping gene (β -actin) was expressed at equal levels. TM cell line RNA also showed various *PAX6* mRNA transcripts approximately of 1000, 800 and 600bp amplification (Figure 2A, lane 6). No amplification was found in the negative controls of ocular retina RNA (Figure 2A, lane 8) and non-ocular wild type WBC (Figure 2A, lane 9). The banding pattern of *PAX6* isoforms in ocular and non-ocular tissues is shown in (Table 2). After optimization of RT-PCR in wild type WBC, the further analysis was performed in familial aniridic patients whom the mutation was confirmed by genomic PCR (c.710delC) in mother and child.¹² The both mutant mother and child WBC RNA was subjected for the *PAX6* expression after RT-PCR the products were resolved

on 2% agarose gel showed unusually two bands of unexpected size amplification approximately 190bp, 200bp (Figure 2B). The unusual PCR products were then reamplified and purified for sequencing purpose (Figure 2C). The sequencing chromatogram is shown in Figure 3A and the blast hit alignments (Figure 3B) identified 85% of homology refers to various chromosomes (Figure 3C), but the prime hit to Homo sapiens chromosome 16 genomic contig, reference assembly (Accession Number NT_010393). The evidence based on nucleotide supports the gene description is similar to Homo sapiens hypothetical LOC441763, mRNA (ACCESSION XM_930284) however, 15% resembling as *PAX6* (Homo sapiens paired box gene 6 (Aniridia, keratitis), *PAX6* transcript variant 2, mRNA) homology in the blast search (Figure 3D).

Table 2 Description of the *PAX6* mRNA transcript isoforms products in ocular and non-ocular tissues

S. No	Ocular tissues	Non-ocular tissues	Beta actin	Isoforms of <i>PAX6</i> and <i>PAX6</i> (5a)
1	Retina		540bp	600bp, 800bp, 1kb, 1.1kb
2	Iris		540bp	600bp, 800bp, 1kb, 1.1kb
3	Lens Epithelium		540bp	600bp, 800bp, 1kb, 1.1kb
4		Wild WBC without DNase I digest	540bp	-
5		TM Cell Line	540bp	600bp, 800bp, 1kb
6		Wild WBC with DNase I digest	540bp	-
7		HeLa Cell line	540bp	1.2kb
8		Mutant WBC	-	190bp, 200bp
9	Retina cDNA (without RT enzyme)		Negative control	Negative control
10		WBC RNA without RT enzyme	Negative control	Negative control

Bp, base pair; cDNA, complementary DNA; RT, reverse transcriptase; TM cell line, trabecular meshwork cells; WBC, white blood cells

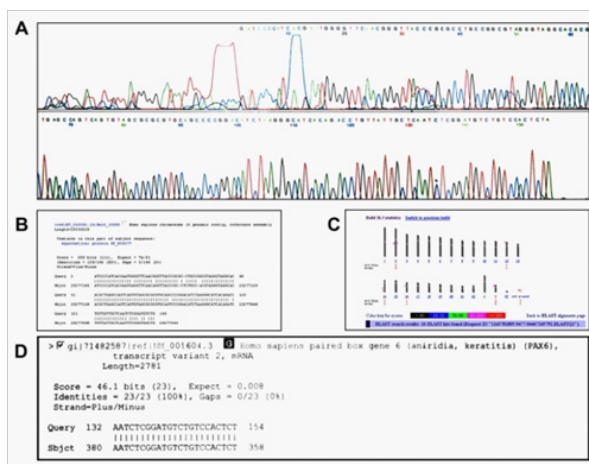


Figure 3 (A-3) sequencing result of aberrant banding pattern in mutant WBC lymphocytes. (B) Homology result indicates the blast hit identified 85% of homology refers to Homo sapiens chromosome 16 genomic contig, reference assembly, However, the evidence based on nucleotide supports the description of gene, is similar to Homo sapiens hypothetical LOC441763 (LOC441763), mRNA. Figure (C) Homology of nucleotide sequences that correlates to other chromosomes (Red mark shows the location). Figure 3D-15% homology of mutant WBC lymphocytes shows *PAX6*.

Partial *PAX6* is illegitimately expressed in mutant WBC lymphocytes and not in wild type WBC lymphocytes hence it needs to explore the function of *PAX6* mRNA in mutant. These results suggest that the *PAX6* gene expression patterns of is highly variable in each ocular/non-ocular tissue types and most likely related to differences in metabolic activity hence the human tissues of an individual may

contribute to gene-specific expression pattern. In this study the normal cadaver eye (60 years old) had been taken because the tissue ocular tissues specific genes are expressed more, which is an important model to understand the molecular basis in patterning the expressing gene of *PAX6*. The expression level of *PAX6* is moderately high in ocular tissues like cornea, lens epithelium, lens etc., hence cadaver globe

was borrowed and the analysis done as a control. Approximately 451 genes have been expressed in normal human tissues and designated as housekeeping genes. These genes display significant variation in expression pattern in tissues,¹³ but no attempt have been made to amplify these genes except β -actin.

The non-ocular tissues like normal WBC cells, mutant WBC, TM cell lines and HeLa cells were also analyzed for the expression pattern of *PAX6*. Since *PAX6* was first identified in 1991,¹⁴ more information has been accumulated during the past decade. *PAX6* is essential for normal vision however, the genes directly regulated by *PAX6* are unknown.¹⁵ In this present data lens epithelium, retina and iris tissues have significant *PAX6* expression while the retina contains an overwhelming majority of mRNAs encoding crystallins and other genes but they still express *PAX6* isoforms at high levels. Surprisingly in HeLa RNA showed *PAX6* expression since the HeLa cells are a fibroblast based cell line and it would not expect a fibroblast to express, a lot of *PAX6*. However *PAX6* expression might be high in these cells for some reason. To gain into the transcriptional competence the corresponding level of β -actin was examined. β -actin has been used as standard determination of absolute levels of genes expressed in a population of eukaryotic cells. The housekeeping gene b-actin was taken as a control in analyzing the ocular and non-ocular tissues because b-actin is essential for the cell proliferation, activation, and differentiation. In this report the all the ocular/non-ocular tissues showing equal level of beta actin expression. B-actin commonly assumed to have constant expression level which could provide new standards for quantitative controls of expression gene. The TM cell line selected because the cells cultured *in-vitro* has high transcriptional modification during culture and the number of cells is relatively high. The transcriptional activity has strongly modulated by cell density. Indeed the expression of both isoforms was dramatically increased in proliferative cells.¹⁶

Various studies in the development of eye and gene regulation provide substantial evidences that the single gene *PAX6* play crucial role not only in eye but also in non-ocular tissues like brain, olfactory, pituitary, pancreas, and spinal cord expression. However, it has been documented in HeLa RNA, which expresses *PAX6* aberrantly in this study.¹⁷ Evidences showed that *PAX6* plays autonomous role in lens^{18,19} and non-autonomous role in other ocular cells.^{20,21} Even though equal amount (50ng) of RNA was taken as a standard for the analysis, hence the *PAX6* mRNA in adult retina showing more intensity when compared with non-ocular tissues taken in this study. This was confirmed by several repeated RT-PCR analysis and the intensity was observed so high in retinal cells. This concludes that the *PAX6* is highly expressed in retina, which is consistent with previous publication.²² In this report, the abnormal bands detected in aniridic patients and the sequencing result revealed that the 15% homology similar to *PAX6* nucleotide with C-terminal ends. Hence, the expressed bands were not an artifact of RT-PCR method, but a true biological phenomenon. The real function of alternatively spliced isoform is still controversial and the importance of the isoform might play role in mutants. The *PAX6* partial expression in mutant WBC cell was found and not in normal WBC cells, the real fact for this reason is unknown. However, in lymphocytes *PAX6* is definitely expressed at extremely low levels, therefore lymphocytes RNA was done PCR with several methods but failed to get amplification. The study gives further evidence that the mutant transcription was analyzed and is consistent with previous literature.²³ The absence of *PAX6* expression was observed in wild type WBC cells. In contrary, the molecular studies on *PAX6* missense

mutations produce putative abnormal amino acid substitution resulted in aniridia and related phenotypes likewise the *PAX6* frame shift mutations needs further exploration to detect the exact mechanism with the natural *PAX6*.^{5,12,24}

Conclusion

The opposing debate is why the mutations are analyzed in the genomic level in blood cells but not in the mRNA level, that mean the mRNAs are not produced in WBC cells or in truncated form or it is degraded hence it needs to be explored further for the tissue specific expression. To conclude the various types of mutations should be analyzed at least to the mRNA level instead of genomic/protein level because very less transcript information is available. *PAX6* mRNA transcript are present in ocular/non-ocular tissues hence the *PAX6* mutations should be analyzed with effective functional assays, which will be useful to find the transcript level. Further studies will focus on the expression levels that are altered in *PAX6* and other ocular tissues evaluating their expression pattern during eye development and its functional role.

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Conflicts of interest

Author declares that there is no conflict of interest.

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