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Incidence of *PAX6* single nucleotide polymorphisms in congenital iridofundal coloboma and other congenital ocular abnormalities: a tertiary care hospital experience from central India

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Purpose *of this study is to identify selected single nucleotide polymorphisms (SNP) of the PAX6 gene and to assess their correlation with congenital iridofundal colobomas and other congenital ocular anomalies.* ***Material and methods****. It was a case-control study done on 45 patients aged from 75 days to 58 years (mean age 29.36 ± 14.4 years) with irido-fundal coloboma and 45 healthy controls aged 35.23 ± 13.92 years. Ocular examination was done by using slit-lamp microscopy inspection, fundoscopy and intraocular pressure measurement. Genotyping was done by using the polymerase chain reaction — restriction fragment length polymorphism (PCR-RFLP) method.* ***Results****. Two irido — fundal coloboma patients showed CT (+/−) heterozygous genotype of rs667773 SNP and the rest were wild-type CC (−/−) homozygous genotype. All controls showed CC (−/−) wild-type homozygous genotype. PAX6 SNP rs3026354 showed CC (−/−) wild-type homozygous genotype condition in all patients. Neither CG (+/−) heterozygous nor homozygous GG (+/+) genotype was reported in patients and controls. SNP rs662702, genotype pattern was CC (−/−) wild type homozygous in all patients and controls. CC genotype frequency was 95.56 and CT genotype was 4.4% while C allele frequency was 97.78 and T allele frequency was 2.22 % in rs667773 C>T SNP. rs3026354C>G SNP had 100 % CC genotype and C allele frequency in both case and control populations. SNP rs 662702C>T showed 100 % CC genotype and C allele frequency in the case and control respectively.* ***Conclusion.*** *The elevated frequency of the CC genotype with C allele was more common in irido fundal patients. Two heterozygous CT genotype of rs667773C>T SNP were reported in two irido-fundal coloboma patients.*

Keywords: polymorphism; mutation; gene; polymerase chain reaction; restriction fragment length polymorphism; single-nucleotide polymorphism
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Частота выявления однонуклеотидного полиморфизма *PAX6* при врожденной иридофундальной колобоме и других врожденных глазных аномалиях: опыт больницы третичного уровня из центральной Индии

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Цель *работы — выявление отобранных однонуклеотидных полиморфизмов (SNP) гена PAX6 и оценка их корреляции с врожденной иридофундальной колобомой и другими врожденными аномалиями глаз.* ***Материал и методы****. В исследование, проведенное в формате случай-контроль, вошли 45 пациентов с иридофундальной колобомой в возрасте от 75 дней до 58 лет (в среднем 29,36 ± 14,4 года) и 45 здоровых лиц (средний возраст 35,23 ± 13,92 года). Офтальмологическое обследование включало биомикроскопию, фундоскопию и тонометрию. Генотипирование проводилось методом полимеразной цепной реакции с анализом полиморфизма длин рестрикционных фрагментов (ПЦР-ПДРФ).* ***Результаты.*** *У двух пациентов с иридофундальной колобомой был выявлен гетерозиготный генотип CT (+/−) SNP rs667773, а у остальных был гомозиготный генотип CC (−/−) дикого типа. У всех контрольных лиц был выявлен гомозиготный генотип CC (−/−) дикого типа. PAX6 SNP rs3026354 характеризовался CC (−/−) дикого типа гомозиготного генотипа у всех пациентов. Ни гетерозиготный CG (+/−), ни гомозиготный GG (+/+) генотипы не были зарегистрированы у пациентов и лиц контрольной группы. Паттерн генотипа SNP rs662702 был CC (−/−) дикого типа гомозиготным у всех пациентов и лиц контрольной группы. Частота генотипа CC составила 95,56%, а генотипа CT — 4,4%, в то время как частота аллеля C составила 97,78%, а частота аллеля T — 2,22% в rs667773 C>T SNP, частота SNP rs3026354C>G генотипа CC и аллеля C была 100% как в популяции пациентов, так и в контрольной группе. Частота SNP rs 662702C>T генотипа CC и C аллеля также была 100% у пациентов и в контроле.* ***Заключение.*** *Повышенная частота CC генотипа с C аллелем была более распространена у пациентов с иридо-фундальной колобомой. Два гетерозиготных CT генотипа rs667773C>T SNP были зарегистрированы у двух пациентов с иридо-фундальной колобомой.*

Ключевые слова: полиморфизм; мутация; гены; ПЦР; RFLP; SNP
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PAX6 is located on human chromosome 11p13, *PAX6* spans 22 kilobases and contains 14 exons encoding a protein with 422 amino acids. The *PAX6* gene codes a transcriptional regulator that controlled development of forebrain, pancreas and ocular tissues, including corneal epithelium, lens and retina in human [1]. *PAX6* gene is also a master control gene in the development of ocular tissues in invertebrates [2]. Mutations in *PAX6* gene lead to a variety of hereditary ocular malformations of the anterior and posterior segment, including aniridia, coloboma of the iris, keratitis, congenital cataracts, Peter's anomaly, and optic nerve defects [3, 4]. A total of 300 mutations in the *PAX6* gene resulted in different disease phenotypes, many of which resulted from gain-of-function or loss-of-function mutations [5]. Mutations or intragenic deletions of *PAX6* were the major causes of aniridia and iris coloboma, however, rare cases could be associated with large chromosomal deletions or rearrangements [6]. Ocular coloboma is a congenital eye disorder characterized by partial absence of the iris and fundus coloboma. The aim of this study was to establish the correlation between *PAX6* gene polymorphism and the development of irido-fundal coloboma.

MATERIAL AND METHODS

The study included 45 patients with congenital iris colobomas or fundal iris colobomas. Among the patients, the age ranged from 75 days to 58 years, with a mean of 29.36 ± 14.4 years. With 18 males and 27 females, the male to female ratio was 2 : 3. A total of 45 healthy controls were also

Table 1. SNPs, Primer sequence, and PCR Conditions
Таблица 1. SNP, последовательность праймера и условия ПЦР

PAX6 Genes SNPs Однонуклеотидные полиморфизмы гена *PAX6*	Primer sequence Последовательность праймера	PCR Conditions Условия ПЦР
rs667773C>T	5'-TGGCACAATATGGAAAATCAA-3'F 5'-CGGAGCAAACAGGTTTAAAGA-3'R	One cycle at 95 °C for 5 min, 35 cycle of 95 °C for 30s, 62 °C for 40 s and 72 °C for 40 s and one final extension cycle at 72 °C for 10 min Один цикл при 95 °C в течение 5 мин, 35 циклов при 95 °C в течение 30 с, 62 °C в течение 40 с и 72 °C в течение 40 с и один заключительный цикл удлинения при 72 °C в течение 10 мин
rs3026354C>G	5'-CTCCCAAGCTCCCTAAGCCA-3' F 5'-CGCCCGAGGCTCTGTACGGC-3'R	One cycle at 95 °C for 5 min, 35 cycle of 95 °C for 30 s, 63 °C for 40 s and 72 °C for 40 s and one final extension cycle at 72 °C for 10 min Один цикл при 95 °C в течение 5 мин, 35 циклов при 95 °C в течение 30 с, 63 °C в течение 40 с и 72 °C в течение 40 с и один заключительный цикл удлинения при 72 °C в течение 10 мин
rs662702C>T	5'-ACCAGACTGTGCTACTTTGC-3' F 5'-ATTGAGATTCATTGCTCCGG-3'R	One cycle at 95 °C for 5 min, 35 cycle of 95 °C for 30 s, 62 °C for 40 s and 72 °C for 40 s and one final extension cycle at 72 °C for 10 min Один цикл при 95 °C в течение 5 мин, 35 циклов при 95 °C в течение 30 с, 62 °C в течение 40 с и 72 °C в течение 40 с и один заключительный цикл удлинения при 72 °C в течение 10 мин

recruited for comparison of allele frequencies. The control group had a mean age of 35.23 ± 13.92 years and included 12 males and 33 females.

The study was conducted in the tertiary care hospital of Madhya Pradesh, India. The study was approved by Institutional Human Ethical Committee of college and all participants gave their written informed consent for the study. A pretested questionnaire was applied to obtain detailed relevant information of ophthalmological examination. Study design was case-control. All patients were informed verbally as well as in written form about the purpose and the method of the research and the voluntary nature of participation in the study. A total 45 cases of congenital ocular coloboma attended in ophthalmology out-patient department (OPD) for routine checkup and 45 age and sex matched normal individuals without any ocular disorder were enrolled as controls. Patient diagnosed with presence of deficient iris tissue and presence of coloboma in retina on clinical examination was included in study. Acquired Coloboma subjects and patients not willing for study were excluded from study. Both the patients and controls underwent ophthalmologic examination including bilateral naked eyes visual acuity, eye ball movement, lid, conjunctiva, cornea, sclera, anterior chamber, iris, pupil, lens, and fundus by using slit-lamp microscopy inspection, fundoscopy, retinoscopy, and intraocular pressure measurement. Visual acuity was examined by Snellen chart and converted in logMAR value. Examination of ocular anterior and posterior segments was performed with a slit-lamp biomicroscope and a binocular indirect ophthalmoscope.

About 2 ml venous blood was collected in EDTA vacutainers from patients and controls for DNA extraction. DNA was extracted by kit method (Geneaid Biotech Ltd, Taiwan). Single nucleotide polymorphisms (SNP) of the *PAX6* gene were selected from the published literature [7–9]. Primers for two of the three SNPs were manually designed for polymerase chain reaction — restriction fragment length polymorphism (PCR-RFLP). Restriction enzymes were selected with the help of NEB cutter software. Oligonucleotide (BR Biochem, India) concentration was 25 pico moles and 2.5 U Taq pol (BR Biochem) while 0.2 mM DNTPs (BR Biochem, India) each and 1.5 mM $MgCl_2$ concentration was used for all SNPs to 50 μL reactions. Amplification was performed using thermo cycler (IGENE LABSERVE) machine. Appropriate restriction enzymes were used according to the manuals of the manufacturer. Molecular investigation was done as per previous published literature [10]. Details of the SNPs, primer sequence, and PCR conditions are given in table 1. Amplified product was analysed by horizontal electrophoresis in Ethidium Bromide containing 2% Agarose gel while restriction enzyme digested sample were analyzed on a 3% Agarose gel. Frequency distribution used to compare the various parameters and t-test (Graphpad software, Version 4) was applied to compare the means of group. The odd ratio (OR), its standard error &95% confidence interval are calculated according to [11]. P value <0.05 was considered statistically significance.

RESULTS

Congenital ocular anomalies had been documented in three cases. For the case group, the mean visual acuity (VA) was 1.65 ± 0.83 logMAR in the right eye (RE) and 1.26 ± 0.55 logMAR in the left eye (LE), while for the control group it was 0.30 ± 0.40 and 0.32 ± 0.46 respectively. A statistically significant difference was found between RE and LE when the values were compared between the cases and controls. The data on demographics and visual acuity are provided in table 2.

The 45 patients studied had 40 bilateral iris colobomas, while 22 had unilateral iris colobomas. Nine unilateral cases involved RE and 13 involved LE. A total of 35 patients had choroidal colobomas, of which five had isolated choroidal colobomas, while the remaining 30 patients had both iris and choroidal colobomas.

Table 2. Demographic details & visual acuity data (Snellen measurement of visual acuity converted to *logMAR* visual acuity measurements)
Таблица 2. Демографические данные и данные об остроте зрения (значения остроты зрения по Снеллену, преобразованные в значения остроты зрения logMAR)

Characters Показатели	Case Пациенты	Control Контроль	p-value Значение p
Age, years Возраст, лет (Mean ± SD)	29.36 ± 14.4	35.23 ± 13.92	0.0524
Visual acuity Острота зрения (mean log MAR) OD OS	 1.65 ± 0.83 1.26 ± 0.55	 0.30 ± 0.40 0.32 ± 0.46	 < 0.0001 < 0.0001

In the remaining 25 patients with unilateral choroidal coloboma, 10 patients had RE and 15 patients had LE involvement. Among the 29 patients, microcornea was the most common congenital ocular anomaly. There were 14 patients with nystagmus and 8 with microphthalmos. Nine patients were found to have congenital cataracts. In addition to bilateral congenital anomalies, there were 2 cases of disc colobomas, 2 cases of lens colobomas, 1 case of optic disc pits, 1 case of morning glory syndrome, 1 case of Bergmeister's papilla, 1 case of persistent hyperplastic primary vitreous (PHPV) and 1 case of persistent hyaloid arteries among the study patients.

Three SNPs of interest were amplified by simple PCR. A 2% agarose gel was used to visualize rs667773, rs3026354 and rs662702 PCR products at 581, 511 and 551 bp respectively. Based on the manufacturer's instructions, the amplified PCR product was digested with BccI, BsrI, and Hpy188I enzymes. A 3% agarose gel was used to check digested products. Based on restriction digestion of the PCR product, the rs667773 SNP fragmented product size was 581bp, 391bp, and 190bp.

A total of 2 irido-fundal colobomas were CT(+/−) genotypes, whereas all the others were CC(−/−) genotypes. Agarose gel pictures are given in Fig. 1 & 2. TT homomozygous condition was not reported in the patient. CC−/− wild type genotypes were found in all controls. The ancestral allele was C since the reference SNP allele was C/T. As a result of PCR analysis of the rs3026354 SNP, all patients showed CC(−/−) wild type homozygous genotype conditions. The heterozygous CG (+/−) genotype or the homozygous GG (+/+) genotype was not observed in either patients or controls. SNP reference alleles were C/G and ancestral allele was C. The restriction digestion of rs662702 SNP produced a 551bp PCR product, and the genotype pattern was CC−/− wild type homozygous in both patients and controls, while reference SNP alleles were C/T, and ancestral allele was C. In the rs667773 C>T SNP, the CC genotype frequency was 95.56, the CT genotype frequency was 4.44%, the C allele frequency was 97.78, and the T allele frequency was 2.22%. As a result of the rs3026354C>G SNP, 100% of the cases and controls had C allele frequencies and CC genotypes. SNP rs662702C>T displayed CC genotypes and C allele frequencies in cases and controls, respectively. The genotype and allele frequency distribution of the studied SNPs are shown in table 3. CT heterozygous genotypes of the rs667773 SNP were found in only 2 patients in this study. It appeared that one of them had choroidal and iris colobomas in both eyes, as well as microcorneas in both eyes. On the other hand, the choroidal coloboma was limited to the right eye in the other case.

Fig. 1. PCR Amplicon of *PAX6* rs667773 SNP
Рис. 1. ПЦР-ампликон SNP *PAX6* rs667773

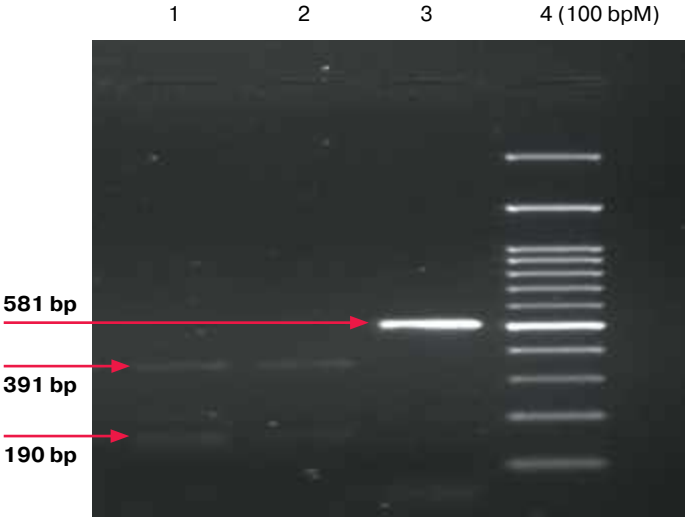

Fig. 2. PCR product restriction enzyme analysis of *PAX6* rs667773 single nucleotide polymorphism using BccI restriction enzyme. Digested samples were analysed by horizontal electrophoresis on a 3% agarose gel. Lane 1 and 2 resulted in heterozygote condition with CT genotype. Lane 3 resulted in an undigested 581bp fragment of genotype CC. Lane 4 is a 100bp DNA ladder
Рис. 2. Анализ рестриктазой продукта ПЦР однонуклеотидного полиморфизма *PAX6* rs667773 с использованием рестриктазы BccI. Расщепленные образцы анализировали с помощью горизонтального электрофореза в 3% агарозном геле. Дорожки 1 и 2 показали гетерозиготное состояние с генотипом CT. Дорожка 3 показала нерасщепленный фрагмент генотипа CC длиной 581 пн. Дорожка 4 представляет собой лестницу ДНК длиной 100 пн

Table 3. Genotype and allele frequency of *PAX6* SNPs in case versus control. The odd ratio (OR), its standard error and 95% confidence interval are calculated according to [11]
Таблица 3. Генотип и частота аллелей *PAX6* SNP у пациентов и в группе контроля. Коэффициент вероятности (OR), его стандартная ошибка и 95%-ный доверительный интервал (ДИ) рассчитаны по [11]

SNPs Однонуклеотидные полиморфизмы	Case Пациенты n = 45	Control Контроль n = 45	Odd ratio 95% CI Коэффициент вероятности 95% ДИ	p
	genotype allele frequency генотип частота аллелей	genotype allele frequency генотип частота аллелей		
rs667773C>T	CC{43(95.56)} CT{2(4.44)} TT(0) C{88 (97.78)} T{2(2.22)}	CC{45(100)} CT(0) TT(0) C{90(100)} T(0)	5.22 0.244 to 112.06	0.29
rs3026354C>G	CC{45(100)} CG (0) GG (0) C{90 (100)} G{(0)	CC {45(100)} CG(0) GG(0) C {90(100)} G(0)	1.0 0.0194 to 51.49	1.0
rs662702C>T	CC{45(100)} CT (0) TT (0) C{90 (100)} T(0)	CC{45(100)} CT(0) TT(0) C{90(100)} T(0)	1.0 0.0194 to 51.49	1.0

DISCUSSION

One of the most extensively studied genes, *PAX6* performs a variety of roles in oculogenesis and contributes to many human congenital ocular malformations. Coloboma patients and those with other ocular congenital anomalies can have the *PAX6* gene sequenced to determine novel sequence changes and clinical phenotypes that may shed light on disease pathogenesis. There was no evidence of an association between *PAX6* and coloboma in a study in the north Indian population, but it was a pilot study and suggested expanding the study to large populations [12]. The *PAX6* gene SNPs rs662702 and rs667773 were associated with extreme myopia in Japanese patients [13]. As a result of our study, two patients with irridofundal coloboma carried a heterozygous CT genotype of rs667773C>T SNP. In this study, we focused on the population of Vindhyan region and conducted a hospital-based study. In three of the studied *PAX6* SNPs, the CC wild type genotype was observed in both patients and controls. A recent study by Y.Tsai et al. found rs667773 to be associated with high myopia among Chinese Taiwanese [10]. In an independent study conducted in China, *PAX6* mutations were associated with microcornea [14]. Mutations in *PAX6* coding regions are associated with thinning of the outer and inner retinal layers of the macula, consistent with fewer neurons in the macula and abnormal foveal formation [15]. As a result of this study, we demonstrated the use of PCR-RFLP for the detection of SNPs in the *PAX6* gene in iridofundal colobomas. The frequency of *PAX6* SNPs in iridofundal colobomas was determined using a rapid and inexpensive method. As well, this study confirms that CC wild type homozygous genotypes and C alleles are most common in case and control groups. In Tunisia, a nonsense mutation (p.Q89X) was found in a family with aniridia and congenital cataracts [16]. As well as the coloboma mutations, N. Azuma et al. also identified new mutations in pedigrees with optic-nerve malformations, including hypoplasia/aplasia of the optic-nerve, persistent hyperplastic primary vitreous, and morning glory disc anomaly [17]. There may be a correlation between vitamin A levels and coloboma, since vitamin A may be an important ocular marker. Deficiency of vitamin A occur in the majority of Indians, which affects their vision. Vitamin A is essential for the expression of many genes involved in ocular morphogenesis and eye development. It is unable to close the optic fissure in embryos deficient in vitamin A.

Limitation of the study. The correlation between the polymorphism of the *PAX6* gene and the iridofundal coloboma was not established because of the small sample size. A larger study of the population must be conducted in order to determine the genotype associated with iridofundal colobomas.

CONCLUSION

Very few studies were evaluated the association of *PAX 6* gene with choroidal fundal coloboma or other congenital ocular anomalies. A large part of human ocular tissue development is controlled by the *PAX6* gene. *PAX6* encodes a transcriptional regulator involved in regulating development of the forebrain, pancreas, cornea, lens, and retina of the human eye. Using *PAX6* gene polymorphism as a marker for iridofundal colobomas, we sought to establish a correlation between the two. We identified only 2 patients with irridofundal coloboma who had a heterozygous CT genotype for rs667773C>T. Due to the small number of heterozygous genotypes and/or alleles present in the 2 cases with different clinical phenotype patterns, correlation studies could not be performed. Wild type CC homozygosity has been reported for three SNPs in the *PAX6* gene in both patient and control groups. It was found that iridofundal colobomas had an ancestral allele of C for rs667773, rs3026354, and rs662702 polymorphisms of the *PAX6* gene.

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