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MOLECULAR ANALYSIS OF RETINITIS PIGMENTOSA AND OPTIC NEUROPATHY IN PAKISTANI FAMILIES

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Abstract

Abnormal functioning or impairment of eye leads to visual disturbance. Optic neuropathy and retinitis pigmentosa are heterogeneous group of genetic disorders characterized by progressive loss of vision and degeneration of neurons respectively. The present research was conducted to identify the epidemiology and molecular analysis of autosomal recessive retinitis pigmentosa and optic neuropathy among the population of Punjab, Pakistan. Blood samples were collected from the families having at least 2 affected individuals of optic neuropathy and RP. DNA was extracted using Qiagen minikit. Touchdown PCR and Polyacrylamide gel electrophoresis were used to detect the amplification and linkage analysis of reported loci respectively. Two families of optic neuropathy (ONO01 and ONO02) and two families of RP (RP01 and RP02) were included in this study. Families ONO01 and ONO02 consisted of two affected individuals with optic neuropathy. All the normal and affected individuals were homozygous for markers D11S1979 and D11S4197 thus no linkage was seen in these families. Family RP01 consisted of one deceased and three affected individuals with autosomal recessive RP. Family RP02 consisted of one deceased and one affected individual with autosomal recessive RP. Affected and normal individuals of both RP families were homozygous for markers ZNF513, TULP1, RP1 and MERTK, thus no linkage was also seen in these families. It is suggested that an elaborate genomic analysis should be conducted for the identification of causative genes and environmental factors responsible for retinitis pigmentosa and optic neuropathy phenotype in these families.

Keywords
Retinitis Pigmentosa, Optic Neuropathy, Genetic Disease

1. Introduction

Autosomal recessive retinitis pigmentosa (RP) and optic neuropathy are the heterogeneous group of eye disorders. RP is one of the commonest forms of the blindness affecting 1 in 3,000 to 1 in 5,000 individuals worldwide

(Chizzolini *et al.*, 2011). The genes affected may remain unknown while there is strong association of cousin marriages (Hartong *et al.*, 2006). Autosomal recessive retinitis pigmentosa is the most prevalent in Pakistan (Heckenlively *et al.*, 1998). It consists for 50-60% of all cases worldwide (Zafar *et al.*, 2017). RP is characterized by progressive loss of vision. Patients exhibit night blindness in the early stages followed by a progressive reduction in the visual field (Ferrari *et al.*, 2011). ERG (Electroretinography) show completely extinguished or severely diminished rod response while the cone response is normal in early stages but is undetectable with the progression of the disease (Scholl & Zrenner, 2000). The fundus changes include attenuation of the retinal vessels, bone spicule-like pigmentation in the mid-peripheral retina and a pale optic nerve (Hamel, 2006). Ocular findings show atrophic changes in the photoreceptors followed by the presence of melanin-containing structures in the vascular layer of retina (R Sparrrow *et al.*, 2010).

The genetic mode of inheritance has significant role in the prognosis of the disease. But knowing the exact mode of inheritance is not possible (Kalloniatis & Fletcher, 2004). The inheritance can be X-linked (10–15%), autosomal recessive (15–20%) or autosomal dominant (Mansergh *et al.*, 1999; Kajiwara *et al.*, 1994). Mutations in ZNF513, TULP1, RP1 and MERTK genes were analyzed in the current study. These genes were selected as they were found to be more common in causing retinitis pigmentosa (Li *et al.*, 2010; Paloma *et al.*, 2000; Riazuddin *et al.*, 2005, Shahzadi *et al.*, 2020).

Optic neuropathy is characterized by degeneration of few or most of the optic nerve (Newman *et al.*, 2005). It can be bilateral or symmetric based on nutritional (folate or vitamin B12) deficiency, toxic or genetic defects (González-Quevedo *et al.*, 2018; Brandariz-Núñez *et al.*, 2019). Optic nerve fiber layer and retinal ganglion cells are damaged in genetically inherited optic atrophies (Lotti *et al.*, 2015). The retinal ganglion cells form 1.2 million nerve fibers. Death of these ganglion cells is the key feature of optic neuropathy (Yu-Wai-Man *et al.*, 2011).

The genetically mode of inheritance can be Mendelian (X-linked, autosomal recessive and autosomal dominant) or non-Mendelian (mitochondrial) (Villegas-Perez *et al.*, 1998; Novotny *et al.*, 1996). LHON (Leber's hereditary optic neuropathy) is maternally inherited disease because it occurs due to the mutation in 18 mitochondrial genes. Three most important mitochondrial mutations related to this disorder are G3460A, G11778A, and T14484C. These three genes transcribed into subunit I of complex I, subunit IV of complex I and subunit VI of complex I respectively. Family history is observed in 43-65% cases of LHON, while singleton cases are more common (Johnson, 1990). Mutation in mt-DNA is either homoplasmic, if all the cells having copy of a mutated DNA, or hetroplasmic, if few cells

contain mutated copy of mt-DNA (Puomila *et al.*, 2000).

Retinitis pigmentosa can be detected with the help of fundus examination, OCT (optical coherence tomography), FAF (auto fluorescence imaging) and functional assessment of vision. Genetic subtyping may also be a definitive test for diagnosing the disorder (Naviaux, 2000). The current treatments available for retinitis pigmentosa and optic neuropathy are not highly effective. Vitamin A and E may protect the photoreceptors as Vitamin A is essential for the formation of light sensitive rhodopsin. Pharmacologically retinitis pigmentosa can be treated by regulating ion channels through different drugs which may enhance or inhibit the activity of rods and cones (Kumar *et al.,* 2018). We conducted present study to identify the epidemiology and molecular analysis of autosomal recessive retinitis pigmentosa and optic neuropathy among the population of Punjab.

2. Experimental Work

Identification of families was done by visiting Mayo Hospital, Layton Rehmat Ulla Benevolent Trust and various schools of special education in Lahore. Performa were filled to gather information about disease history, number of affected individuals and their residential addresses for sample collection and further information. Affected individuals of the families were clinically assessed through fundoscopy. Electroretinography testing system and visual evoked potential was done by medical specialist to confirm and differentiate the disorders of retinitis pigmentosa and optic neuropathy.

2.1 Collection of Biological Samples

Affected families were personally visited and interviewed about affected individuals, consanguineous marriages and sib-ship. Each family was provided with the information about the research and its possible benefits according to their level of satisfaction. Their current age, age of onset, consanguinity, ethnic background and other relevant information were recorded and familial transmission pattern of the disease was assessed. Affected families were given a specific number and name. Blood sample of the whole family was drawn and stored at 4◦C until further analysis.

2.2 Pedigree Analysis

Detailed and complete pedigrees of families were drawn to understand the mode of inheritance of the disorder (Figure 1-Figure 4). In each pedigree drawing circles and squares represent females and males respectively**.** The generations in the family represent by Roman numerals while each member of a particular generation represent by an Arabic numeral. Black shaded symbols represent the affected individuals and white symbols denote normal individuals. Deceased members of the family are represented by a symbol with a line crossing it.

2.3 Extraction and Analysis of Genomic DNA

DNA was extracted using DNA extraction Qiagen minikit. DNA was quantified using a Nano drop Spectrophotometer. The DNA was diluted to 10X and stored at 4◦ C until further

use. Housekeeping gene (119-bp of β-globin gene) was amplified to detect the quality of extracted DNA. Positive samples for the amplification of β-globin gene were furthered used for the screening of selected markers.

2.4 Linkage Analysis

Linkage of selected genes was analyzed by touchdown PCR using microsatellite markers for each locus (Table 1). Forward primer was labelled with FAM dye. PCR reaction was made in a total volume of 25µl, containing 1.5ul MgCl2, 2.5ul buffer, 0.5ul dNTPs, 18.5ul PCR water and 0.2ul Taq polymerase, 1ul of template DNA, and 0.3ul of forward and reverse primers. The PCR was carried out through 30 cycles. At initial stage, denaturation was done at 96˚C for 6min. During cycle; denaturation was done at 95˚C for 30 sec then annealing at 63˚C for 30sec. However, the final stage was carried out at 72˚C for 30 sec. The PCR products were examined on 2.5% agarose gel. The amplified product was then run on a vertical 10% polyacrylamide gel for Linkage analysis.

2.5 Amplification of Mitochondrial gene through PCR

Extracted DNA was amplified by PCR using 25ng of DNA from each individual in a final volume of 20µl, followed by agarose gel electrophoresis. Primers were designed from primer 3 (table 2). PCR reaction was made in a total volume of 20 microliter containing 2 microliter of template DNA, 10 microliter of red dye master mix, 1 microliter of reverse and forward primer and 6 microliter of PCR water.

The PCR was carried out through 30 cycles. Initially denaturation was done at 96^{o}C for 6min, denaturation during the cycle was done at 95^{o}C for 30sec, then annealing at 63^{o}C for 30sec finally extension was carried out for 30sec at 72^{o}C.30 cycles were used.

3. Results

After the study of 2285 cases, 71 cases of optic neuropathy were identified from different areas of Punjab, Pakistan. The major diagnosis was severe loss of visual impairment followed by complete blindness. The clinical details for optic neuropathy patient samples are shown in table 3 which include three variables age group, gender and visual acuity.

The prevalence of low to severe vision impairment was 59.15% and that of blindness was 32.39% (Figure 1). Data also showed male biasness; 46 out of 71 patients were males and 25 were females.

Figure 1: Symptoms of visual acuity among patients.

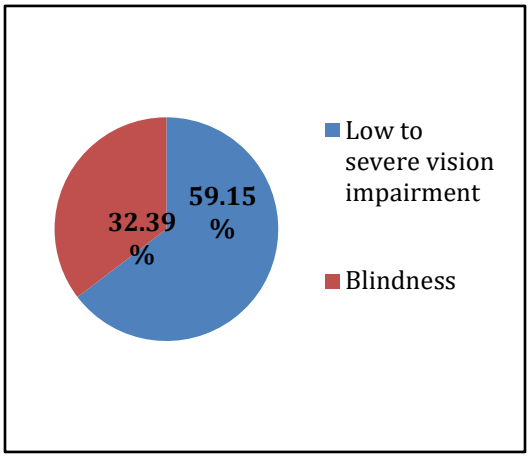

Table 1: Microsatellite Markers Used For Linkage Analysis

Disorder	Locus	Cytogenetic location	Markers	Distance cM*	Tm	Mg++ (mM)
RP	ZNF513	2p23.3	D2S174	48.64	53	1.5
			D2S2247	49.19	53	1.5
			D2S223	40.56	53	1.5
			D2S165	49.69	53	1.5
	TULP1	6p21.31	D6S1611	57.12	55	3
			D6S1645	57.12	55	3
			D6S439	57.12	55	3
			D6S963	57.12	55	3
	RP1	8q12.1	D8S1737	48.77	53	3
			D8S2332	69.65	53	3
			D8S2332	68.63	53	3
			D8S2326	45.67	53	3
	MERTK	2q14.1	D2S2278	27.05	54	1.5
			D2S168	27.7	54	1.5
Optic Neuropathy	OPA7		D11S2015	87.89	53	1.5
			D11S4197	87.89	53	1.5
				87.89		
			D11S1979		53	1.5

Table 2: Markers Used For Analysis Of Mitochondrial Genes

Primer	Melting Temperature	Length (bp)	Product Size	Sequence
mt-ND4 F	55.45	18	196	CAGCCATTCTCATCCAAA
mt-ND4 R	55.28	20	196	AGGCTTGCTAGAAGTCATCA

Table 3: Clinical data of OA patients

Clinical Characteristics of Optic neuropathy Patients		No. Of Patients(%)
Age (years)	1-15	52.11
	16-30	26.76
	31-45	4.22
	46-60	8.45
	61-75	7.04
	>75	1.4
Gender	Male	46

	Female	25
	>0.3	8.45
	0.3-0.1	12.67
	<0.1-0.05	22.53
	<0.05-0.02	23.94
Visual Acuity	<0.02	14.08
Total Blindness		18.03

3.1 Genetic Characterization

The families affected with optic neuropathy were named as ONO01 and ONO02 and the families affected with RP were named as RP01 and RP02. All the families belonged to Punjabi ethnic group. The genes of optic neuropathy were D11S4197 and D11S1979 and the genes of RP family were ZNF513, TULP1, RP1 and MERTK respectively.

3.2 Linkage Analysis of Optic neuropathy

3.2.1 Family ONO01

The pedigree was drawn up to five generations. In this family two affected (V: 1 and V: 3) members were found (Figure 2). Blood samples were collected from all the alive family members. Linkage analysis was performed with already reported genes. During screening of this family, all the affected and normal individuals were homozygous for markers D11S4197 and D11S1979, thus no linkage was seen in the family.

3.2.2 Family ONO02

The pedigree was drawn up to four generations. In this family two affected (IV: 1 and IV: 2) members were found (Figure 3). Blood samples were collected from all the family members. Linkage analysis was performed with already reported genes. During screening of this family, all the affected and normal individuals were homozygous for markers D11S4197 and D11S1979, thus no linkage was seen in the family.

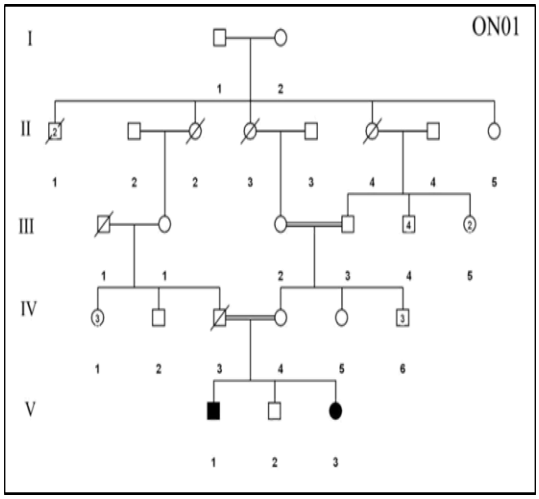

Figure 2: Pedigree of ON01.Squares represent males, circles shows females; filled symbols show affected individuals. Double line shows cousin marriage and diagonal line represents deceased family members.

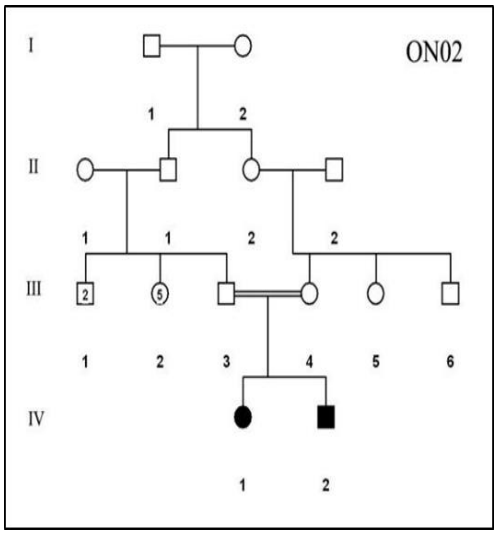

Figure 3: Pedigree of ON02. Squares represent males, circles show female. Double line indicates cousin marriage and filled symbols show affected individuals.

3.3 Linkage Analysis of Autosomal Recessive Retinitis Pigmentosa

3.3.1 Family RP01

In this family one deceased (I: 2) and three affected (II: 2, II: 4 and III: 1) members were found (Figure 4). Blood samples were collected from all the alive family members. Affected individuals had poor night vision. The disease was severe and progressive in this family, showing clear signs of RP. No clinical record of the family was found. Linkage analysis was performed with already reported regions of autosomal recessive RP by using 4 polymorphic markers. During screening of this family, all the affected and normal individuals were homozygous for markers ZNF513, TULP1, RP1 and MERTK, thus no linkage was seen in the family.

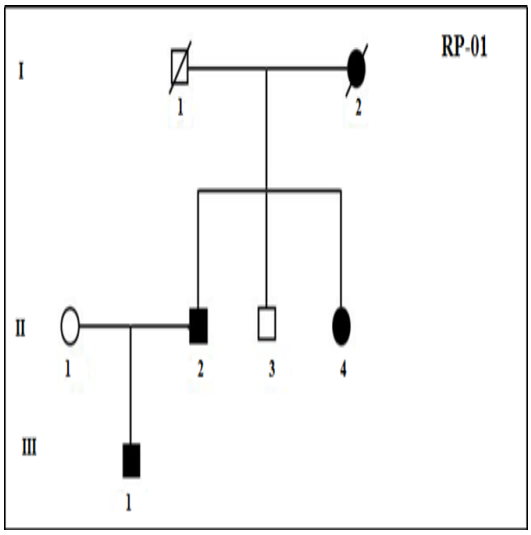

Figure 4: Pedigree of the family RP-01 affected with autosomal recessive pigmentosa. Squares and circles represent males and females respectively**.** The generations in the family were denoted by Roman numerals while each member of a particular generation was denoted by an Arabic numeral. Black shaded symbols represent the affected individuals and white symbols denote unaffected individuals. Deceased members of the family are represented by a symbol with a line crossing it.

3.3.2 Family RP02

In this family one deceased (I: 1) and one affected (II: 2) members were found (Figure 5). Blood samples were collected from all the alive family members. Affected individuals had poor night vision. No clinical record of the family was found. All the affected and normal individuals were homozygous for markers ZNF513, TULP1, RP1 and MERTK, thus no linkage was seen in the family.

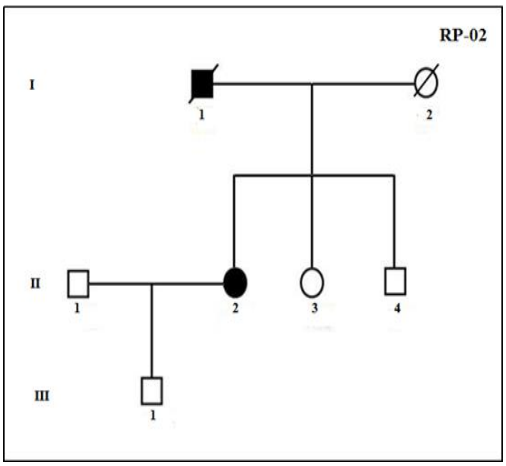

Figure 5: Pedigree of the family RP-02 affected with autosomal recessive pigmentosa. Squares and circles represent males and females respectively**.** The generations in the family were denoted by Roman numerals while each member of a particular generation was denoted by an Arabic numeral. Black shaded symbols represent the affected individuals and white symbols denote unaffected individuals. Deceased members of the family are represented by a symbol with a line crossing it.

4. Discussion

The molecular basis of optical diseases is currently under extensive investigation, making use of techniques such as gene mapping and linkage analysis. Basic and clinical eye research, including the prospects of its diagnosis, prognosis and treatment, has witnessed breakthrough progress (Kumar *et al.,* 2018). Our current understanding of ophthalmology, as well as the classification of eye diseases, can substantially improve with these new discoveries in molecular biology. Consanguinity is the key factors in the increased ratio of eye disorders. Consanguineous marriages account for 59% of total marriages in Pakistan (Guadagni *et al.,* 2015). Therefore, the affected Pakistani families might have significant role in determining genetic defects through molecular analysis. The major objective of this research was to explore the epidemiology and molecular analysis of autosomal recessive retinitis pigmentosa and optic neuropathy in Pakistani population.

The survey in 2017 reported that the estimated vision loss burden from the year 1990 to 2025 would be 1.12 million out of 207.7 million people in Pakistan. A major number of these cases are under 20 years of age (Hamamy *et al.,* 2011). If the frequency rate remains the same, the burden of blind persons in Pakistan in the year 2025 will increase tremendously (Hamamy *et al.,* 2011; Hassan *et al.,* 2017). Inherited retinal dystrophies are commonly caused by RP and arRP accounts for 25% of total retinitis cases worldwide (Tam *et al.,* 2005). A study revealed that in Pakistan 20% of childhood blindness is linked with RP while optic neuropathy is a kind of rare disease as 1 in 25,000 individuals suffer from optic neuropathy (Man *et al.,* 2002). Another survey conducted in Pakistan to determine the risk factors of visual impairment and blindness in adults (30 years old) reported that prevalence of optic neuropathy in blind population is 3%. That survey was based on socio-demographic factors and 17314 subjects were examined during that study (Jadoon *et al.,* 2006).

To the best of our knowledge no single study has been conducted to determine solely the prevalence of arOA in Pakistan. So, in present study we examined 3110 subjects from Lahore, Punjab, Pakistan and the prevalence of arOA was found to be 1.57%.This study did not include socio-demographic factors. Our results support the fact that autosomal recessive optic neuropathy is a rare disorder (Man *et al.,* 2002). Out of 49 families, four clinically confirmed optic neuropathy families were interviewed about their history but we were unable to reach the other families. Samples were collected from two enrolled families. Their family history determined the recessive mode of inheritance. Another study in Peshawar, Pakistan reported 2.2% prevalence of visual impairment resulting from optic neuropathy in 270 children. Prevalence calculated during that survey is in accordance with our present study (Hassan *et al.,* 2019).The fact that recessive genetic disorders are major cause of blindness in children is supported by a survey conducted on visual impairment in Edinburgh that includes 107 children. This study showed that 26% of visual impairment was due to autosomal recessive hereditary or genetic disorders (Alagaratnam *et al.,* 2002).

Gender biasness was not observed in our data because of narrow sample size. Although arOA is rare as compared to other eye disorders specially arRP, we conducted molecular analysis on both of these eye disorders in Pakistan. We did not find linkage in families RP1 and RP2 for the genes ZNF513, TULP1, RP1 and MERTK genes. However, few other studies show linkage of these genes in RP families (Li *et al.,* 2010; Paloma *et al.,* 2000; Riazuddin *et al.,* 2005, Shahzadi *et al.,* 2020). Moreover, we did not find linkage of arOA in families OA01 and OA02 to the known loci OPA7. Genetic defects in other loci, environmental and other risk factors might have significant contribution in causing disease in these families.

5. Conclusion

Families affected with autosomal recessive retinitis pigmentosa and optic neuropathies were recruited from Punjab, Pakistan to find linkage analysis. Clinical symptoms of RP and Optic neuropathy were present in all the affected members of selected families. But we did not find linkage for the genes ZNF513, TULP1, RP1 and MERTK genes in RP families. Similarly, we did not find linkage in families affected with optic neuropathy. However, linkage of RP in other families has been reported in literature. An extensive genomic research should be done to find other causative factors responsible for disease in these families. Genetic counselling and other educational programs based on these kinds of genetic disorders should significantly improve our knowledge and identification of mutation of novel genes that may lead to better understanding of vision.

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