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Mutational analysis of *CRYAA* gene of cataract and investigating risk assessment factors responsible for eye diseases in district Buner, KPK, Pakistan

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ABSTRACT

This research has been designed to analyze the risk factors of major eye diseases and the genetic alterations contributing to the manifestation of such diseases. For this purpose, data was collected from 256 patients diagnosed by an ophthalmologist by using a specialized questionnaire. Blood samples were collected from 100 patients to perform a genetic investigation of cataracts. Whole genomic DNA was extracted from blood samples via the phenol-chloroform method. The purified DNA was used as the template for the amplification of about 400 bp fragments amplifying exons 1 and 2 of the CRYAA gene. The statistical analysis showed that 68% of individuals were blind due to cataracts. During molecular analysis, nucleotide sequences obtained have resulted in one silent mutation that occured at 20 positions in exon 2. It was replacing A>G which in turn substitutes the lysine at position 70 for arginine. It was interpreted by statistical analysis that this mutation did not result in a significant change in the CRYAA gene. In addition, protein analysis showed no significant changes in the structure of normal and mutated genes. At last, it is concluded that environmental risk factors play a major role in the studied diseases as compared to genetic factors. It is recommended to extend the study to a larger population to study all exons of the CRYAA gene as well as develop better estimates of the magnitude of the problems of visual loss and eye diseases in the Pakistani population.

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Introduction

Human eyes play a crucial role in our life as they help us to see and understand the world around us. Eye disorders are considered a major anomaly across the globe, cataract is one of the disorders that cause blindness, if left untreated (1). A cataract is caused by the opacity of the lens resulting in the cloudiness of the eye. A clouding of the lenses in the eye leads to the breakdown of tissue which causes protein clumping at that point. Moreover, there are many modifiable and non-modifiable risk factors like age, hypertension, diabetes, steroid use, trauma, smoking, and family history (2). Many people in developing countries suffer from cataract blindness i.e., Africa 60%, India 25% Afghanistan 15% (3). In the United States, cataract is the main cause of low vision in 40 years of age and above. It is expected that 30.1 million Americans will have a cataract by the year 2022 (4). A study carried out in 2009 by the Pakistan National Blindness and Visual Impairment revealed that cataract prevalence is getting higher mostly because of environmental risk factors (5). Every year about fifteen million cataract surgeries are performed worldwide. It raised to 5 million in the last 5 years (6). It is expected to increase five-fold in individuals over 55 years during the next fifty years (7). Cataracts cannot be treated by medication, so surgery is the last option to recover vision loss (8). In childhood or early life occasionally, cataracts can be caused by a particular syndrome. For instance, the following chromosome abnormalities linked with the cataract, trisomy 18 (Edward's syndrome), 1q21.1 deletion syndrome, Down syndrome, Patau's syndrome, cri-du-chat syndrome, and Turner's syndrome (9).

The injury, infection, or inflammation is caused to the cornea when expose to the atmosphere. As the result, the anatomy and physiology become impaired and lost their capacity (10). The occurrence of corneal scarring may depend on the health, hygiene, and nutrition of a population (11). After the cataract corneal scar is one of the most prominent causes of blindness in Africa along with glaucoma and cataract (12).

It has been reported by the world health organization that when the image is not correctly focused on the retina a refractive error can occur (13). This causal disease consists of different types such as myopia (nearsightedness), Hyperopia (farsightedness), and presbyopia (14). A condition named astigmatism is occurred due to an uneven curve in the cornea causing distorted vision (15). The fundamental reason for blindness is a visual impairment that is caused by a refractive error in kids which contributes to 3% of blindness in southern Indian school students and in China

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significantly more (myopia occurrence is 21.6 percent and hyperopia prevalence is 2.7 percent) (16). The three most prevalent types of refractive errors are as follows: Myopia (nearsightedness) - the inability in seeing distant objects clearly (17). Hyperopia, also known as Hypermetropia (farsightedness), is a condition in which it is difficult to see close objects clearly. Astigmatism is a condition characterized by distorted vision caused by an unevenly curved cornea (15). The refractive error also leads to blindness. 3 percent of blindness in southern Indian schoolchildren is caused by myopia, while the incidence in China is substantially higher (myopia prevalence is 21.6 percent, hyperopia prevalence 2.7 percent) (16). Myopia is believed to be responsible for 5.6 percent of all blindness among school-going children in the United States (17).

In Pakistan, to the best of the author's knowledge, there is no published data that cover all the risk factors and molecular aspects investigated in the present work. In the medical profession, past research on eye conditions has only estimated the fraction of risk factors; however, the current research has shed light on descriptive and analytical features of the disease, as well as molecular aspects of the disease. This work aims to discover and define the biochemical risk factors for cataracts in individuals over 40 years of age in connection to numerous clinical background factors such as age, sex, trauma, smoking, ultraviolet radiation, diabetes, hypertension, and prescription medication.

Materials and Methods

Sample size and sampling technique

This study is carried out on the patients of District Buner Khyber Pakhtunkhwa, located in Northwest Pakistan. Data and blood samples were collected from two tertiary care hospitals, i.e. Shifa eye center and District Head Quarter Hospital, Buner. Patients with selected eye diseases of all ages visiting these hospitals for treatment were recruited for this study. Data were collected from five different levels/Phases that is the preparatory phase, data collection phase, data analyzing phase, coding data phase, and blood sampling phase.

A random sampling technique was used to collect the data via a specially designed questionnaire. Data was taken from 256 patients for the assessment of risk factors while blood samples were taken from 100 patients for molecular study.

Preparatory phase

In the preparatory phase, a questionnaire was constructed containing 6 sections as shown in Table 1.

The initial communication was established with the administration of the hospitals (where the data was collected), specifically with the authorities in charge of the diabetes patients and with the hospital's medical officers. During this phase, the investigator delivered a letter to the authorities of the participating hospitals in which the objectives and significance of the study were described, as well as a questionnaire and a letter of request from the supervisor, to obtain permission to collect data.

Data collection phase

In the eye center, the patient's blood sample was taken to check the presence or absence of another disease which are involved in the loss of vision. The data was collected by the researchers themselves and with the assistance of a trained enumerator by interviewing and filling out the prescribed questionnaires.

Coding of data

Since eye disease is a dichotomous variable because it has categories Yes and No, and is a dependent variable

Table 1. The number of questions and information obtained from each section of the questionnaire.

Section	Disease name and information	Number of questions
1	Demographic information	9
2	Cataract	17
3	Glaucoma	11
4	Corneal opacity	11
5	Refractive error	4
6	Other diseases	5

Table 2. Gender, age, types, and number of the risk factor for each disease.

Variable	Question types	Code
Gender	Male, female	0, 1
Age	1-20 , 21-30	0 ,1, 2
	31-60 , 61-80,81-100,>100	3 , 4
Disease	Cataract, glaucoma, corneal opacity, refractive error, other disease	1,2,3,4,5,
Cataract	All questions about cataracts denoted from Yes or No	0,9
Glaucoma	All questions of glaucoma denoted from Yes or No	0, 9
Corneal opacity	All question of corneal opacity denoted from Yes or No	0,9
Refractive error	Types of refractive error	1 , 2
		3 , 4
Other disease	Types of other diseases, causes and surgery	1,2,0 and 9

so coded as 0 or 1 and its independent factors Age, Gender, blindness, Surgery and for the section containing two options (Yes and No) 1 and 9 coded respectively as indicated in Table 2. All questions about cataracts are denoted by Yes or No.

Blood sampling and obtaining informed consent

Blood samples were collected from the cataract patients through informed consent. 5 ml of blood was collected through (Shifa) disposable syringes into EDTA tubes. The blood was then transferred to the Human Genetics lab at Hazara University, Mansehra for further analysis. The blood was then centrifuged for 15 minutes at 2300rpm to separate the serum. The separated serum was then transferred to Eppendorf tubes and was stored at -40°C.

DNA extraction and amplification of DNA loci

Genomic DNA was extracted from the blood samples using the modified phenol-chloroform method developed by (Goode *et. al.,* 2011). Commercially available Thermo scientific PCR kit catalog EP0402 was used for DNA amplification. The genomic DNA was added to each tube of the final reaction mixture, which was made in a 25µl volume. The reaction tubes were then inserted in the applied bio-system Thermal Cycler 2720.

Data analysis

All of the variables were pre-coded to facilitate computer analysis to perform certain necessary calculations about the questionnaires. Age was treated as a continuous variable in the epidemiological investigation, while it was treated as a discrete variable in the analytical analysis. The records were reviewed to ensure that the information had been entered correctly. The data analysis was carried out with the help of SPSS version 20.0 for Windows. The data was collected in the order of the Buner district in the overall sample of 256 people. Contingency tables were created, and relevant tests, such as figures, tables, and binomial tests, were used to evaluate the results of the study. CRYAA gene PCR amplified product was delivered to Beijing Genome Institute China with primer for further sequencing of the fragmented gene. By using Bio Edit software, we were able to further evaluate the sequence data of the required fragment for mutations.

Ethical approval

This study was reviewed and approved by the ethical research committee (Advanced Studies and Research Board) of Hazara University Mansehra, Khyber Pakhtunkhwa Pakistan [No. HU/R&P/ASRB/2015/1995]. The study was conducted following approval guidelines and prior permission was granted by the higher authority of DHQ Hospital Buner, KP, Pakistan.

Results

Demographic information on eye diseases

From collected data from 256 samples, cataract was the major disease responsible for blindness among the population of District Buner. Out of which 57.8% were cataracts followed by glaucoma at 9.7%, corneal opacity was 7.8% while the refractive error was 20.3% and other diseases were only 4.6%. In a rapid-assessment study in southern Punjab, the prevalence has been shown two times more than among men (18). The diseased conditions can be visualized in Fig 1.

Gender-wise prevalence

Out of 256 samples, a total number of 148 individuals were affected by cataracts including 52.7% of men and 47.3% of women as shown in Fig 2.

Age-wise prevalence

The samples were divided into different age groups as shown in Table 2. A higher prevalence of cataracts was seen among those individuals whose age was above 60 years indicating that age plays a crucial role in cataracts as indicated in Fig 3.

Major eye diseases that affect the population of the study area

A cataract is the major cause of blindness in district Buner. Further cataract is divided into four subtypes namely subcapsular cataract (30.4%), cortical cataract (25.7%), nuclear cataract (35.8%) and congenital cataract (8.1%). Among these types, the nuclear cataract is most abundant as elucidated in Fig 4.

Demographic information

Based on the questionnaire cataract was the disease

Figure 1. Typical images of different eye disorders. (A) normal eye, (B) cataract eye, (C) glaucoma eye, (D) corneal opacity eye and (E) myopic eye.

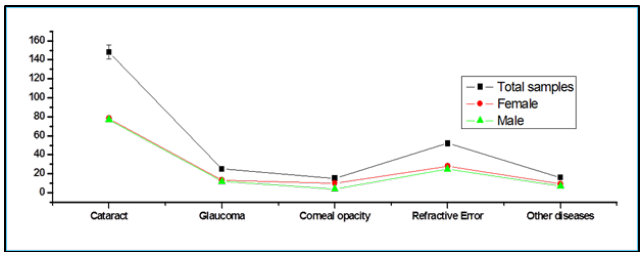

Figure 2. Affected patient ratio from different eye diseases between males and females.

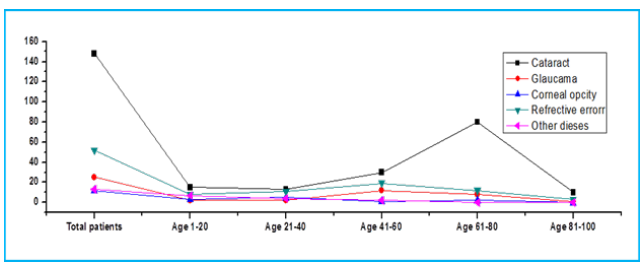

Figure 3. Age-wise percentage of the affected patient with different eye diseases.

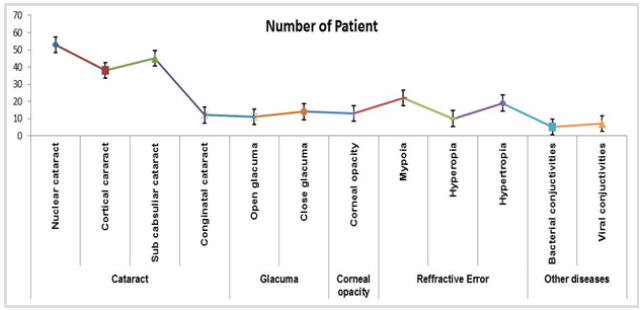

Figure 4. Different eye disorders and their incidence rate.

of focus. In the collected data, total samples were 256 of which 57.8% of people are affected by cataracts including both males and females. The collected result shows that cataract is a major disease that is responsible to cause 68% of blindness among the population of district Buner.

Risk factors of cataract

Results of the current study interpreted that 68% patients were suffering from cataracts in the study area. There are certain potential risk factors responsible for the high prevalence of cataracts. These risk factors include trauma (20%p=0.000), UV light (03% p=0.000), diabetes (46% p=0.324), hypertension (53% p=0.511), obesity (32% p=0.000), smoke (22% p=0.000), Corticosteroid medication (10% p=.000), statin medicine for cholesterol reduction (08% p=.000), previous eye injury or inflammation (40% p=0.014), previous eye surgery (21% p=.000), hormone replacement therapy (05% p=.000), clinical data available (08% p=.000), family background of cataract disease (11% p=.000), cousin marriages (43% p=0.100) and any Syndrome (08% p=.000). A major risk factor is hypertension followed by diabetes. Other studies are inaccordance with the current study. Individuals with uncontrolled hypertension have more risk of developing cataracts than those with mild hypertension as elucidated in Table 3.

Genetic investigation of cataract

The sequenced samples were then analyzed by aligning them with the control *i.e. HBB* gene by using U-gene software and the online bioinformatics tool BLAST. The results obtained after analysis can be visualized in Fig 5-7.

Proteomic analysis for *CRYAA gene*

The mutation occurs at 20 positions in exon-2 which result in replacing A>G; as a result, substitutes the lysine at position 70 for arginine. The functional effect of the mutation was verified by using Sift software (19). SIFT: Predicting amino acid changes that affect protein function. These results are supported by PROVEAN (20) and mutation Taster (21). This shows that the mutation is tolerated and has no significant effect on protein-coding as the function of both amino acids is almost similar. The Swiss

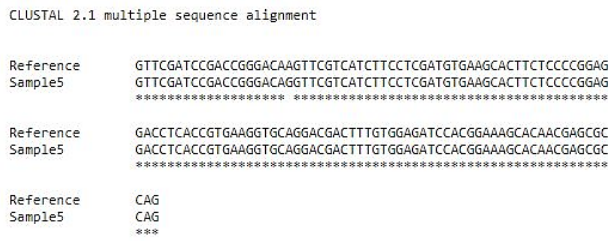

Figure 5. Sequence alignment of *CRYAA* gene Exon 2 with the normal sequence.

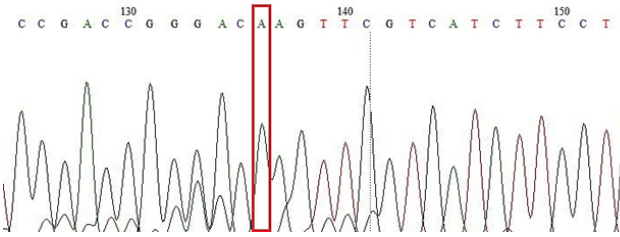

Figure 6. Normal chromatogram sequence of *CRYAA* gene.

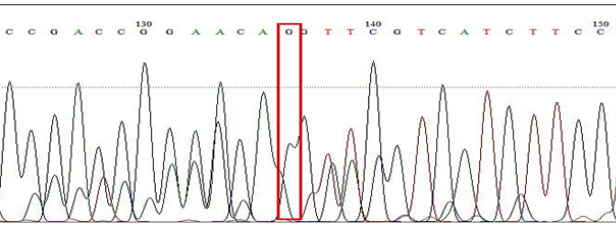

Figure 7. Mutated chromatogram sequence of *CRYAA* gene.

Table 3. Risk factor %age responsible for Cataract in Buner.

Risk Factors	Overall %age for YES	P-value
Blindness	68%	0.000
Trauma	20%	0.000
Ultraviolet radiations	03%	0.000
Diabetes	46%	0.324
Hypertension	53%	0.511
Obesity	32%	0.000
Smoke	22%	0.000
Cortico steroid medication	10%	0.000
Statin medicine for cholesterol	08%	0.000
Previous eye injury or inflammation	40%	0.014
Previous eye surgery	21%	0.000
Hormone replacement therapy	05%	0.000
Clinical data available	08%	0.000
Do you have family background of cataract disease	11%	0.000
Cousin marriages	43%	0.100
Any syndrome	08%	0.000

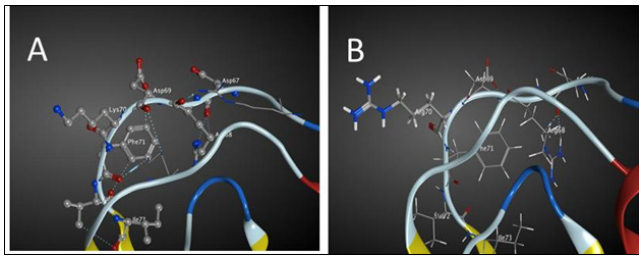

Figure 8. Protein structures of *CRYAA*, (**A)** represents normal protein and (**B**) is a mutated protein structure.

Model was used for the prediction of protein structure. The structures were analyzed with chimera software. The mutation was found in the strand and there is no significant change in the protein structure.

Fig 8 shows the structure of a protein in the normal gene and the mutated one. It can be seen that there is no significant difference in both the structures as the amino acid formed by the *CRYAA* gene in normal conditions is lysine which is replaced by arginine when this mutation occurs in the gene. Hence the functions of both amino acids are similar at large, so it is not going to result in a big change in coding respective proteins. As a result, although there is a mutation in the gene it would not result in the disturbance of body function that may affect the function of the eye.

Discussion

Eye diseases are serious health problems globally that may lead to blindness in certain cases. A cataract is the major cause of blindness followed by glaucoma and corneal opacity. Studies carried out in different provinces of Pakistan show that women are more likely to get blindness as compared to men (22,23). It has been assessed that there are 1,140,000 (962,000– 1,330,000) blind grown-ups in Pakistan (24,25). In the present study, patients recruited were mostly affected by conjunctivitis 41.7% are male and 58.3% were female; therefore, females are more highly affected by conjunctivitis than males. The previous study shows that males are more exposed to environmental pollutants as they spend most of their time outdoors. By contrast, females remain indoors due to cultural, social as well as religious causes (26). Probably due to this, females are more affected than males.

Among the types of cataracts, nuclear cataract is the most predominant cataract type in South Asia. This kind of cataract is as often increasing in women (27). In case of glaucoma, angle-closure glaucoma was most prevalent. These results are supported by other studies which show that angle-closure glaucoma is more common among East Asian populations (28). Other ocular defects were also prevalent in the studied population. This study shows that adults over 40 have high prevalence rates of nearsightedness, emmetropia, and Hypermetropia. An extensive report that included grown-ups more than 40 in the US, Western Europe, Australia, and Norway (25,29) were found to have predominance rates of Hypermetropia and nearsightedness (myopia).

In the current study, conjunctivitis was divided into two types bacterial conjunctivitis and viral conjunctivitis. Most people have conjunctivitis due to allergies ales and trauma. Individuals with serious hypertension have a higher danger of cataracts than those with mild hypertension. A few examinations demonstrated a linear positive relationship between blood pressure and cataract (30). According to a study, the hazard components of cataracts include aging, smoking, ultraviolet radiation exposure, and hereditary variables while the epidemiological proof is as yet disputable for antioxidants, alcohol utilization, and supplement use (31). Hypertension and diabetes have been perceived as hazard factors for cataracts in a few investigations (32). Nearsightedness and in rare cases eye injury are also one of the main causes of glaucoma. These results are supported by other studies which show that higher elevated ocular pressure leads to a greater risk of glaucoma (33). Other studies on corneal opacity show that corneal visual deficiency stays most noteworthy in developing countries. Infectious keratitis, visual injury, and corneal opacities cause an expected 1.5– 2.0 million new instances of unilateral visual deficiency every year (34).

The structure of a protein in the normal gene and the mutated one can be seen in Fig 8. It can be seen that there is no significant difference in both the structures as the amino acid formed by the *CRYAA* gene in normal conditions is lysine which is replaced by arginine when this mutation occurs in the gene. Hence the functions of both amino acids are similar at large, so it is not going to result in a big change in coding respective proteins. As a result, although there is a mutation in the gene, it would not result in the disturbance of body function that may affect the function of the eye. Utilizing sequencing tools, we recognized a novel missense mutation replacing A>G in the *CRYAA* gene. To date, there is a sum of 26 different mutations that have been recognized in *CRYAA* (35), including 21 missense mutations, 1 regulatory mutation, and 4 small deletions.

Conclusion

The present study concludes that the prevalence of cataracts is high in the study area. Prolonged cataract leads to blindness. A potential risk factor responsible for cataracts is hypertension followed by diabetes and cousin marriages. Mostly elder people are more affected by angle closure glaucoma than an adult due to aging. In the case of glaucoma nearsightedness and eye, injury are playing the main role. Blindness caused by corneal opacity was rarely found in the population of district Buner. Foreign bodies striking with eyes and eye injury are the main risk factor responsible for corneal opacity. Myopia and presbyopia affected more individuals after cataracts in district Buner and were mostly found in adults. Genetic analysis of cataracts shows that one silent mutation occurs at 20 positions in exon 2 which resulted in replacing A>G which in turn substituted the Lysine at position 70 to Arginine. Bioinformatics analysis shows that this mutation did not result in a significant change in the *CRYAA* gene. Protein analysis showed no significant changes in the structure of the normal and mutated genes. It can be concluded that environmental risk factors play a major role in the studied diseases as compared to genetic factors. It is recommended to extend the study to a larger population with the genetic analysis of all the exons of the *CRYAA* gene as well as developing better estimates of the magnitude of the problems of visual loss and eye diseases in the population of Pakistan.

Further studies are needed to evaluate the risk factors associated with studied eye diseases. Besides risk factors, it may be significant to study the complete gene that is res-

ponsible for the cataract in this region. This may help to decrease the risk of eye diseases and possible prevention, as well as develop better estimates of the magnitude of the problems of visual loss and eye diseases in the population of Pakistan. Genetic Counseling and prenatal diagnosis are suggested for the rest of the family members of the cataract patients. This may reduce the frequency of cataracts. A large-scale study needs to be carried out to find out the risk factors and the results should be communicated and shared with health and policy-making departments to implement the findings of research studies.

Acknowledgements
None.

Conflict of Interest
Authors declare no conflict of interest.

Ethical approval
This study was reviewed and approved by the ethical research committee (Advanced Studies and Research Board) of Hazara University Mansehra, Khyber Pakhtunkhwa Pakistan [No. HU/R&P/ASRB/2015/1995]. The study was conducted following approval guidelines and prior permission was granted by the higher authority of DHQ Hospital Buner, KP, Pakistan.

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