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A Study on Interleukin-10 -1082 G/A (rs1800896) Polymorphism in Acute Lymphoblastic Leukemia Patients

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ME, IRM and NM conceptualized the study. ME and GM performed data curation. ME, IRM and NM performed the formal data analysis and investigation, wrote the article. IRM, NM and GM administered and supervised the project, reviewed and edited the manuscript. NM and IRM performed validation of results and data visualization.

Key words
Interleukin-10, Acute lymphoblastic leukemia, Single nucleotide polymorphism, Biochemical risk factors, Clinical risk factors

ABSTRACT

In Pakistan, the prevalence of leukemia is 5.3% among all ages, while in children, the prevalence of acute lymphoblastic leukemia (ALL) is 19.8%. In the present study, 200 patients with acute lymphoblastic leukemia (n=200) and ethnically matched control samples were gathered from nearby tertiary care hospitals to investigate the frequency of the IL-10 gene's polymorphism at -1082 (G/A). After extracting genomic DNA, the polymorphism at a location (-1082 G/A) was identified by an allele-specific polymerase chain reaction in ALL patients and normal healthy controls. These results were also confirmed by Sanger sequencing of the randomly chosen samples. The study revealed that genetic variation of ALL patients at -1082 of the human IL-10 gene was found in 79% with the AA allele, 19% with GG, and 2% with the GA allele. As for healthy subjects, the AA allele was found in only 8%, GG was 49%, and GA was found in 43% of Pakistanis. So, among ALL patients of the Pakistani population, the AA genotype at locus -1082 was revealed as the predominant genotype. Moreover, this study also indicated that family history of cancer, radiation exposure, and age groups are considered the most important risk factors in the progression of ALL among the people of Pakistan.

INTRODUCTION

This kind of leukemia or blood cancer occurs when immature white blood cells (WBCs) in the red portion of the bone marrow proliferate uncontrollably and become blast cells. According to Sung *et al*. (2020), the estimated number of new cases of leukemia in both genders with all ages was in Asia (48.6%), Europe (21.1%), and North America (14.3%). In Pakistan, the prevalence of leukemia among all ages and genders is 5.3% (Tufail and Wu, 2023). In children of both genders, the prevalence of acute lymphoblastic leukemia (ALL) is 19.8% (Tufail and Wu, 2023).

There are so many risk factors for Acute lymphoblastic leukemia. It is found to be the most prevalent type of leukemia among children, especially those children who are below 15 years of age. It contributes to 30% of all cancer diagnoses. Studies also show that ALL occurrences are more than AML, about 5 times more than AML, especially among children (Belson *et al*., 2007). So many epidemiologic studies of ALL among children have observed possible risk factors such as genetic, infectious, and environmental factors. Environmental factors are ionizing radiation and nonionizing radiation; ionizing radiation is the most important factor in the environment, paternal exposure to nuclear radiation before childbirth or in utero radiation exposure, and exposure to ionizing radiation after birth; some environmental factors are weekly associated such as nonionizing radiations (Shu *et al*., 2002).

If the genetic variation can be found more significant than 1% of the population is called Single nucleotide polymorphism (SNP) (Wang *et al*., 1998). SNPs are utilized as genetic markers because they allow for detecting any gene mutation (Taylor *et al*., 2001; Srinivasan *et al*., 2016). Single nucleotide polymorphism can be considered genomic markers due to being linked through several disorders such as high blood pressure, diabetes, Alzheimer's disease, different types of cancer, migraines,

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and schizophrenia (Kaur *et al*., 2019).

The highly varied class of proteins known as interleukins (IL), or cytokines, regulates or cascades the actions of both innate and adaptive immunity. Many white blood cells produce interleukins, but in the body, a particular lymphocyte creates a unique kind of IL that can cascade into various other sorts (Zhang and An, 2007; Arango and Descoteaux, 2014). IL-10 protein is produced by several cells in which monocytes and lymphocytes are primarily involved. This cytokine exhibits the many spectrums of inflammation and immunoregulation (Saxena *et al*., 2015).

Helper T-cells produce many chemical mediators, i.e., IL-1 type B, IL-2, IFN-y, and A type of tumor necrosis factor is critical to cell-mediated immunity. In the host defense mechanism, they are crucial in the fight against viruses and intracellular infections. Major histocompatibility class 2 (MHC2) antigens are found on the surface of various antigen-presenting cells (APC), including endothelial cells, macrophages, and different B cells. They are crucial to the immunological response (Carey *et al*., 2012).

As a co-stimulatory molecule on macrophages, IL-10 down-regulates the production of Th1 cytokines and MHCII antigens. Additionally, IL-10 promotes B cell survival, proliferation, and antibody production (Couper *et al*., 2008). The cytokine IL-10 can inhibit the synthesis of NF-kappa B. It also regulates the JAK-STAT signaling pathway (Carey *et al*., 2012).

Numerous investigations exhibited that variation of the IL-10 gene at locus -1082 significantly affects the expression of genes and then involved cellular and molecular pathways of antitumor activity (Crawley *et al*., 1999; Hoffmann *et al*., 2001; Kingo *et al*., 2005). Several studies (de Deus *et al*., 2012; Zhou *et al*., 2014; Namazi *et al*., 2018) show the association of leukemia with this promoter region. The association of IL10 at locus -1082 G/A was also shown in the prevalence of breast cancer in the Pakistani population (Aijaz *et al*., 2023). Another recent study shows that IL-10 gene polymorphism at 1082 locus, AA genotype was found at 6.3%, AG genotype was 57.8% and GG genotype was found to be 35.9% in the children with ALL (El-Baiomy *et al*., 2023). Therefore, the human IL-10 gene at this locus -1082 (G/A) was chosen as a biomarker in ALL patients of the Pakistani population with acute lymphoblastic leukemia. Further investigation is necessary on a larger group of individuals who have acute lymphoblastic leukemia.

MATERIALS AND METHODS

There were two groups of subjects: 200 patients and 100 healthy controls. To find out the genetic variation of the human IL-10 gene at -1082, we have searched literature (Settin *et al*., 2011), which was then assessed using a variety of bioinformatics tools, including the Primer 3 program manufactured by Korean company Macrogen.

The present study's sample of participants was chosen using inclusion-exclusion criteria. Newly diagnosed ALL patients who have not yet begun chemotherapy met the inclusion criteria.

Collection of blood samples

Two hundred leukemia patients from several nearby tertiary care hospitals had their blood samples taken, and 100 healthy people served as the control group. Data on laboratory results and clinical histories of cancer patients were gathered. Following informed consent, 5 ml disposable syringes were used to draw the required volume of venous blood (1-3 ml), which was then placed into ethylene diamine tetra acetic acid (EDTA) vials and kept at -20°C until needed.

Extraction of DNA

DNA was extracted using the kit method (Vivantis, Cat. No. GF-BD-100). The DNA concentration was analyzed at 260 nanometers (nm) using a spectrophotometer; then it was stored for further use at -20°C.

Molecular analysis

SNP analysis was done by allele-specific PCR. Using this technique, SNP at the IL-10 gene at locus -1082 (G/A) was found using two allele-specific reverse primers and one forward primer. The reverse Primer sequence was 5′-AGCAACACTCCTCGTCGCAAC-3′ and allele-specific primer sequences were 5′-CCTATCCCTACTTCCCCC-3′ and 5′-CCTATCCCTACTTCCCCT-3′ for detection of G and A allele for the IL-10 SNP analysis (Settin *et al*., 2011). The total volume of each PCR reaction was 25 µL and the following ingredients were used: forward primer 0.5 µL (stock 10 pmol/µL, each reverse primer 0.5 µL (stock 10 pmol/µL), Taq polymerase 0.5 µL (stock 5U/µL), dNTPs 2 µL (stock 2mM), genomic DNA 2 µL (3 µg), $MgCl_2$ 2.5 µL (stock 25 mM).

PCR conditions included initial denaturation at 5°C for 4 min, which was followed by 30 cycles, with each cycle consisting of denaturation at 95°C for 40 sec, annealing at 61°C for 60 sec, extension at 72°C for 60 sec, and final extension at 72°C for 10 min. The PCR reaction was then terminated at 4°C. On a 2% agarose gel, the 139 bp amplified PCR products for the IL-10 gene, which corresponds to alleles G and A, were examined. Additionally, five randomly chosen patient samples with

breast cancer underwent sequencing analysis.

Sequence analysis

Using an ABI sequencer, primers IL-10 (F) 5′-ATCCAAGACAACACTACTAA-3′ and IL-10(R) 5′-TAAATATCCTCAAAGTTCC-3′ were used to sequence five randomly selected samples of ALL (Turner *et al*., 1997). Using sequencing primers, the product size was amplified to 588 bp. The sequencing findings were subsequently analyzed for SNP identification after peak corrections.

RESULTS

Our analysis of the ALL type in patients, whether B-ALL or T-ALL, displays that B-ALL with 176 (88%) was more common than T-ALL with 24 (12%) in the Pakistani population. Patients with ALL were categorized into three subgroups: group 1(0 to 6 years), group 2 (7 to 12 years), and group 3 (13 to 18 years). Data showed that a maximum of ALL patients were present in the first group (124), which means that ALL occurs at an early age in this population as compared with the second (64) and the third group (12), as shown in Table I.

To identify the variations in the types and age categories of ALL patients, the chi-squared test ($\chi 2$) was employed. The significance was determined using a p-value at the 0.05 significant threshold. The chi-square ($\chi 2$) value is 188 for ALL patient categories and p values .000*** indicate significant differences between ALL types. The chi-square ($\chi 2$) value is 400 for various age groups of ALL patients, displaying a 0.000*** p-value suggesting a substantial difference between age groups associated with ALL disorders (Table I).

Table I. Allocation of ALL patients (group and age-wise) (n=200).

Category	Frequency (No of patients)	%	χ^2	P value
Distribution of ALL types among patients				
B-ALL	176	88	188	0.000***
T-ALL	24	12		
Distribution of ALL patients age-wise				
Group 1 (0-6 Y)	124	62	400	0.000***
Group 2 (7-12 Y)	64	32		
Group 3 (13-18 Y)	12	6		

Note: *** represent the statistical significance at 0.001

Clinical history parameters analysis

Consanguinity, use of low-quality water filters for drinking, being a male, and being in a rural area are the most important factors for ALL (Fig. 1). The statistical analysis of various risk factors such as consanguinity, gender, family history of cancer, locality, use of filtered water, radiation exposure, chemical exposure, and age groups with ALL. There exists a strong association between a family history of cancer ($p = 0.0000$), filtered water ($p < 0.0001$), locality ($p < 0.0000$), radiation exposure ($p = 0.001$), and age group ($p < 0.00001$). The presence of consanguinity ($p = 0.0023$), gender ($p = 0.0002$), and chemical exposure ($p = 0.0123$) plays a protective role (Table II).

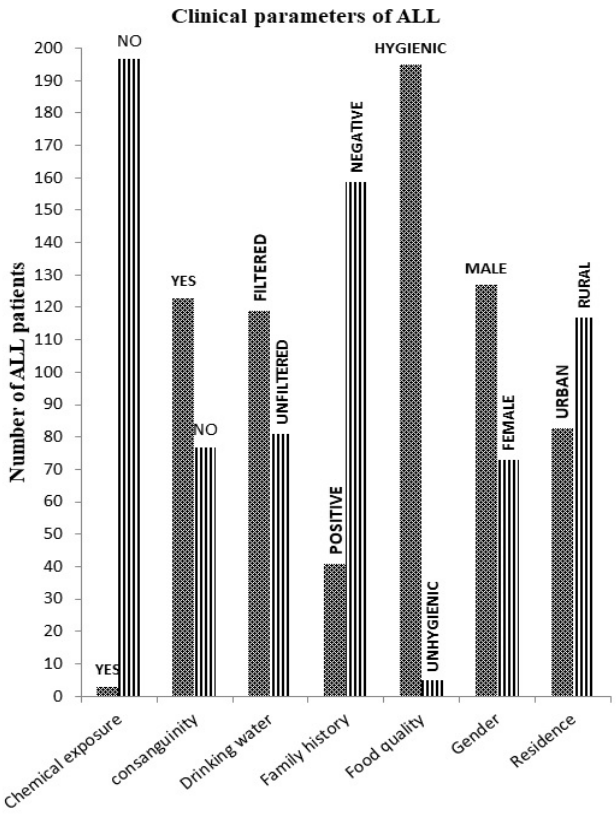

Fig. 1. The different clinical parameters determined in ALL patients (n=2010).

Figure 2 showed that there were significant changes in levels of lactate dehydrogenase (LDH), number of white blood cells (WBCs), levels of hemoglobin (Hb), platelets, and red blood cells in ALL patients.

Figure 3 shows the frequency distribution of different clinical parameters in ALL patients, including hepatomegaly (112), splenomegaly (134), lymphadenopathy (146), fever (138), bleeding (24), pallor (84), bruises (30), and pain in bone in (26) patients.

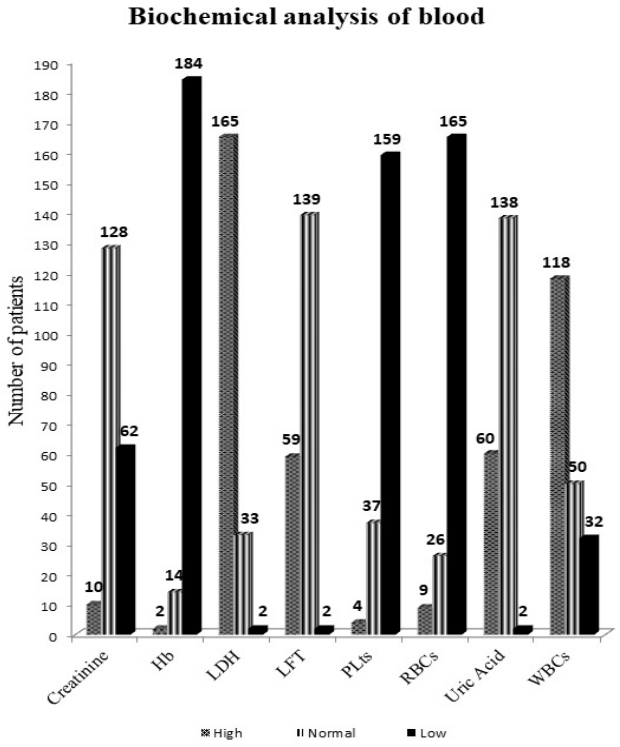

Fig. 2. Analysis of blood parameters in ALL patients (n=200).

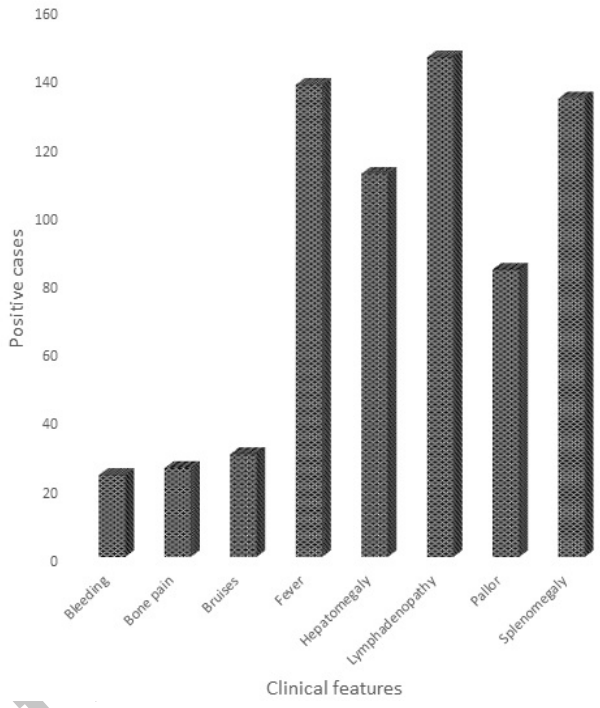

Fig. 3. Frequency of clinical parameters among ALL patients (n = 200).

Table II. Association of various parameters with ALL.

Category	Status	ALL (n=200)	Control (n=100)	Odds ratio	95% CI	Chi-square (P-value)
Consanguinity	Yes	123	79	0.4246	0.2427 to 0.7428	9.28** (0.002314)
	No	77	21			
Gender	Male	127	84	0.3314	0.1806 to 0.6082	13.43*** (0.000248)
	Female	73	16			
Family history of cancer	Yes	41	2	12.6352	2.9892 to 53.4083	18.58*** (0.000016)
	No	159	98			
Filtered Water	Yes	119	4	35.2593	12.4714 to 99.6854	84.89*** (< 0.00001)
	No	81	96			
Locality	Rural	117	24	4.4639	2.6058 to 7.6468	31.85*** (< 0.00001)
	Urban	83	76			
Radiations exposure	Yes	28	2	7.9767	1.8602 to 34.2057	10.67** (0.001091)
	No	172	98			
Chemical exposure	Yes	3	7	0.2023	0.0512 to 0.8001	6.26* (0.012359)
	No	197	93			
Age group	1-7 years	134	21	7.6378	4.3441 to 13.4289	56.49*** (< 0.00001)
	8-14 years	66	79			

Note: *** represent the statistical significance at 0.001, ** represent the statistical significance at 0.01, * represent the statistical significance at 0.05 levels.

IL-10 gene polymorphism

The target is amplified by attaching reverse primer (G) to the template found that contains the IL-10 gene that has been altered -1082 (G). The -1082 (A) mutation, on the other hand, for every one of the two mutant-specific opposite primers when allele-specific primer (A) is attached to the SNP site gives the 179 base pairs product size (Fig. 4). A total of 200 ALL individuals were identified; 38 (19%) had homozygous allele GG, 158 (79%) had homozygous mutant allele AA, and 4 (2%) had heterogeneous equally allele-specific allele G and allele-specific A (Table III).

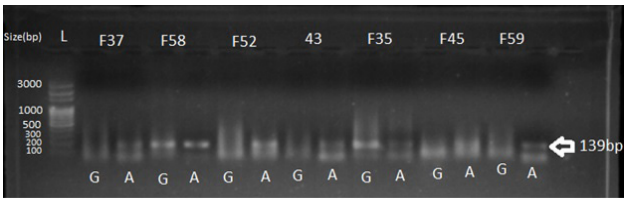

Fig. 4. Polymorphism of the IL10 gene in a gel (2%): L represent 100 bp ladder, seven samples were analyzed in which F37, F52, F43, F45, and F59 show the AA genotype. Sample F58 was heterozygous (GA) and sample F35 was having GA genotype. The product size was 139 bp.

Table III. Frequency distribution of genotypes and alleles among ALL patients and control groups.

Geno-type	ALL (n=200) (%)	Control (n=100) (%)	(χ2)	P-value
Distribution of genotype frequencies				
AA	158 (79%)	8 (8%)	152.9565	<0.000***
AG	4 (2%)	43 (43%)		
GG	38 (19%)	49 (49%)		
Frequencies distribution of alleles				
A	320 (80%)	59 (29.5%)	113.5129	0.000***
G	80 (20%)	141 (70.5%)		

Note: *** represent the statistical significance at 0.001.

The spreading of genotypes with AA, AG, and homozygous GG genotypes concerning their frequency and alleles A and G are shown in Table III. A chi-square test was used to find the difference among AA, Ag, and GG genotypes and alleles A and G. Table III shows that a 152.9565 value of the chi-square test with a 0.000*** p-value was obtained, which shows the significant difference among ALL patients and healthy subjects (Table III).

The gene in ALL cases, the prevalence is 320 (80%), and the allele (G) is 80 (20%). The alleles A and G for the control group are 59 (29.5%) and 141 (70.5%), respectively. The chi-square (χ2) value is 113.5129, and the p-value is .000*** indicating significant variations across the allele groups (Table III).

SNP validity was confirmed through sequencing. The Sanger sequencing method was used to obtain the results following the sequencing. More analysis was carried out to identify the SNP causing the IL-10 gene at locus -1082 polymorphism. The heterozygous has both GA genotypes; the mutant has the AA genotype, and the wild type has the GG genotype.

At the -1082 locus, SNP(A) was detected in every leukemia sample (Fig. 5). The IL10 gene's A allele at locus -1082 polymorphism is indicated by the red color in the molecular analysis, according to Clustal W analysis (Fig. 6).

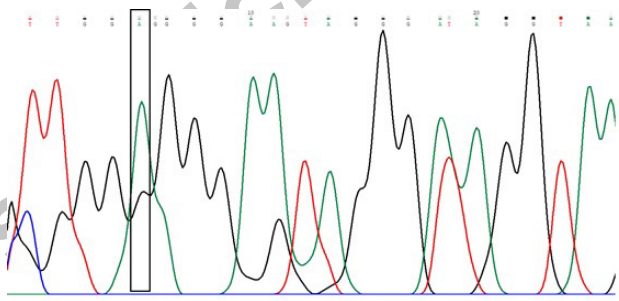

Fig. 5. The detection of SNP (A) nucleotide at IL-10 gene location -1082 in the ALL-patients.

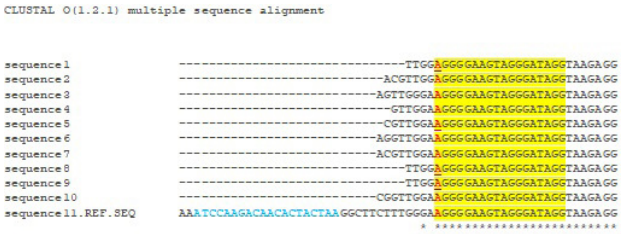

Fig. 6. Results of the ClustalW analysis are displayed. The A allele in the IL10 gene at locus -1082 polymorphism is indicated by the red color in the molecular analysis. Yellow highlights denote allele-specific primers.

DISCUSSION

This study's main goal was to investigate the molecular analysis of the G/A polymorphism in the human *IL-10* gene at locus -1082 in Pakistani patients with acute leukemia. Under ideal circumstances, this SNP was examined using the allele-specific PCR method. For this study, we have selected 200 ALL patients and 100 healthy volunteers based on the inclusion criteria. The clinical

histories of these patients were also maintained.

The frequency of this important illness varies greatly; in the US, blood cancer is identified in one person every three min, and in 2020, 178,520 cases of leukemia, lymphoma, or myeloma will be reported (Cancer Factors and Figures, 2020). Childhood ALL, or acute lymphoblastic leukemia, is the most frequent type of malignancy. Less than seven participants out of a million had either leukemia (4.6) or lymphoma (2.6) for those under the age of twenty. This includes children, adults, and young people. Between 2012 and 2016, the percentage of participants under 20 years old, including children, young adults, and young people, who had leukemia or lymphoma was 4.8% (Sarah, 2021). Sung *et al*. (2020) reports that there are 6261 (5.3%) reported deaths and 8305 (4.7%) new cases of leukemia in Pakistan (Sung *et al*., 2021).

Information revealed that, of 200 ALL patients, 197 (98.5%) had never been exposed to chemicals, except 3 instances (1.5%), which did not appear to be a meaningful risk factor for leukemia. The remaining patients had positive family histories, with 79.5% of ALL negative cases having a negative family history. Compared to females (36.5%), males (63.5%) are more likely to develop ALL. According to data on residence or locale, 41.5% of people resided in urban regions and 58.5% lived in rural ones. According to earlier research, 24% of leukemia cases had a familial history, and 21% of cases were caused by chemical exposure. Compared to women (39%), men had a higher incidence of ALL (61%). The location region is comparable to the current research as well, with 46% of patients residing in urban areas and 54% in rural areas (Karakosta *et al*., 2016).

IL-10 is produced by thymocytes, B cells, and T cells. It is believed to be a significant cytokine with immunoregulatory and moderating effects on immune response activation and suppression (Lyer and Cheng, 2012). According to Gonzales *et al*. (2018), in addition to having both tumor-inhibiting and tumor-promoting properties, IL-10 activation encourages the growth and development of many immune cell types. Consequently, it plays a role in the development and metastasis of malignancies within the body.

The SNP of the human IL-10 gene and its genetic variation in the pathophysiology of leukemia patients were investigated in the current study. The data indicates a connection between the AA genotype and the development of ALL. This is the first study in Pakistan to look at the connection between ALL and the IL-10 genotype. A large sample size is required in childhood cancer studies to ensure the validity and accuracy of the current findings. From a biological perspective, we may state that mutant alleles at this locus may cause aberrant proteins by changing the transcription and translation of this gene. In neonates, the upstream signaling cascade's cellular activity will cause ALL development.

CONCLUSION

The main risk factors for ALL include consanguinity, drinking water filtered with low quality, being male, and living in a rural region. The molecular investigation discovered IL-10 at a locus (-1082 G/A) variation to predict risk for ALL patients in the Pakistani community. Future developments in gene therapy and medicine design may benefit from this research if more intricate molecular analyses and mechanisms are found.

DECLARATIONS

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IRB approval

The University of Sargodha's Board of Advanced Studies and Research, Pakistan, approved the study protocol, and all procedures complied with the Helsinki Declaration.

Ethical statement

This study received ethics approval, with informed consent obtained from participants. Confidentiality was maintained, and all ethical guidelines were followed to ensure participant safety and voluntary participation.

Statement of conflict of interest

The authors have declared no conflict of interest.

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