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Correlation of Rheumatoid Arthritis with Interleukin-38 and its Polymorphism (rs6743376)

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Abstract

Among autoimmune disorders, rheumatoid arthritis is one of the most prevalent. The IL-1 family and the IL36 subfamily both include interleukin-38. IL-38 is anti-inflammatory cytokine. IL-38 is essential for inflammation control and host defense. In the Pakistani population, this research intends to evaluate the relationship flanked by IL-38 levels and its polymorphism (rs 6743376) C>A and rheumatoid arthritis. Eighty samples in total were included and divided into sick and control groups. IL-38 levels were measured through ELISA. For the SNP of IL-38, an allele-specific polymerase chain reaction assay was applied. All subjects' serum levels of CRP, RF, and ACCP were assessed. 70% of patients had negative CRP tests, while 30% had positive CRP tests. All of the patients' RF and ACCP tests came back positive. As the p-value was smaller than (0.001), a significant difference was observed in the mean ESR test values between patients and controls. IL-38 concentration was higher in the RA having a mean value of 26.8621. In comparison to

homozygous allele (CC) and heterozygous allele (AC), patients with homozygous allele (AA) are more common. Contrarily, the control group revealed that the homozygous allele (CC) was more prevalent than the homozygous allele (AA) and heterozygous allele (AC). When compared to homozygous allele (CC) and heterozygous allele (AC), IL-38 rs6743376 (A/A) is related with a higher risk of developing RA illness. In contrast to heterozygous (A/A) allele, homozygous (C/C) allele and heterozygous (A/C) allele are protective in RA. According to this study, IL-38 homozygous alleles (CC) and heterozygous alleles (AC) are associated with a lower probability of developing RA than heterozygous alleles (AA).

Keywords: Anti-inflammatory cytokines, Autoimmune diseases, Interleukin-38**,** IL-38 rs6743376, Rheumatoid arthritis, IL polymorphism

Introduction

The immune system's unchecked response to the body's autoantigen is what causes autoimmunity. Screening external antigens, such as pathogenic microorganisms, poisons, and other dangerous bodily parts, is a critical function of the immune system. (Sármay, 2021). The most prevalent autoimmune illness is rheumatoid arthritis (RA). It is a persistent inflammatory condition (Dakhil, 2017).

In extreme situations, this results in persistent discomfort that lasts for a long time, instability, and other serious problems include joint deformity and deterioration of the bones (Ebel & O'Dell, 2021). Immune cell infiltration of the synovium, which results in the loss of bone, cartilage, and joint function, is a hallmark of the RA pathophysiology. Invasion of the synovial membrane by T lymphocytes. They stimulate synovial fibroblasts and macrophages. Rheumatoid factors (RFs), cytokines, and anti-citrullinate protein antibodies (ACPA) are all produced by B-cells (Gierut, Perlman, & Pope, 2010). Macrophages start releasing amounts of cytokines and harmful enzymes in inflamed joints. Mast cells, killer cells, and DCs are immune system cells that have a major contribution in the pathogenicity of RA (Lopes et al., 2017). The stimulation and development of immune cells like T cells and B cells depends heavily on interleukins (ILs). The range of interleukins includes interleukin-1 (IL-1) and interleukin-38 (IL-38) (Dinarello, 2018). Other cytokines, chemokines, and other substances are started by interleukins of the IL-1 class. Certain disorders are treated with immunotherapy using interleukin-2 (IL-2). The cytokine

interleukin-10 (IL-10) has anti-inflammatory properties. Autoimmune disorders can arise as a result of interleukin-18 (Furman et al., 2019). Tumor necrosis factor (TNF) is a key cytokine in RA that starts the destruction of joints and inflammation (Liu et al., 2021). Both the interleukin-1 family and the interleukin-36 subfamily contain interleukin-38. This interleukin is essential for inflammation control and host defense. IL-1 is essential for starting innate and adaptive immunity (Xu & Huang, 2018). The anti-inflammatory reaction of IL-38. T cell production is reduced by IL-38 (Akdis et al., 2016). Only a few IL-38 polymorphisms have been investigated thus far. They include rs17042888 and rs 6743376. On chromosome 2, rs6743376 is a missense single nucleotide polymorphism (SNP) of the IL-38 gene that is close to the IL1RN-IL1F10 gene. In some disorders, this polymorphism has a role in triggering inflammatory reactions. The variation rs6743376 and IL-1F10 genes are associated with a lowering ratio of forced vital capacity (FVC) ratios (Kim et al., 2020). Inflammatory autoimmune illnesses are becoming more prevalent (Xie et al., 2020). IL-38 SNPs rs 6743376 were linked to a lower risk of illnesses like RA and T2DM (Walker et al., 2017). Effect of IL-1 and its variations on RA and cardiovascular patients (rs (6743376) and rs (1542176) (Consortium, 2015). The polymorphisms of several cytokines (TNF- and IL-6) and their roles in the pathophysiology of rheumatoid arthritis are crucial.

Methodology

In the University Institute of Medical Lab Technology (UIMLT) division of The University of Lahore, a case-control study was carried out. It was determined that there would be an 80-person sample size, and subjects were divided into two groups: 40 people with rheumatoid arthritis and 40 healthy people. The method utilized was a straightforward random sampling one. Patients from Lahore's Bajwa and Latif hospitals who had rheumatoid arthritis symptoms and indicators were largely chosen. Written informed consent was obtained. Following that, demographic information was entered on a standardized questionnaire, including age, gender, symptoms, family history, other chronic conditions, and history of medical treatment. Interviews were conducted with healthy persons who had no prior history of RA or any other autoimmune disease, and responses were recorded on the same pre-designed proforma.

Sample collection/processing and selection

Each person's blood was drawn in the quantity of five ml. and administered into two vacutainers: one ordinary tube and the other an EDTA tube. 2ml of blood was placed in a gel vacutainer (Tube-2) for serum separation, and 3ml of blood was collected in EDTA vial (Tube-1) for DNA extraction. Tube-1 was kept at -20° C until the Iranpur method of DNA extraction (Iranpur-Mubarakeh & Esmailizadeh, 2012). After promptly centrifuging plain tube-2 at 2000–3000 rpm for 10 minutes, serum was divided into two aliquots and kept at –80°C until an ELISA method was used to determine the presence of IL-38. The patient group for the study included both male and female patients with ages ranging from 18 to 60 who were clinically diagnosed according to ACR criteria. As healthy controls, males and females between the ages of 18 and 60 who had no infections, RA, or other autoimmune diseases were included.

C-reactive protein (CRP) Qualitative Test

On the glass slide, different circles were made with one drop (50 microliter) each of the patient serum, the +ve and -ve control solutions. One drop (45 microliter) of the CRP latex reagent was added to each of the sample and control circles after being gently stirred. Using the disposable mixer and spreading the mixture over the entire test area, the sample and reagent were properly mixed on the glass slide. The slide was manually shaken for roughly two minutes before being examined for agglutination.

Rheumatoid Factor (RF) Qualitative Test

On separate circles on the latex agglutination card, 50 µl of each positive and negative control were put, along with 50 µl of sample-on-sample on the same reaction card. After mixing, one drop (45 micro-l) of RF latex reagent was added to each circle. Using a disposable mixer, the glass slide was correctly blended. The blended mixture was then spread over the entire test area, and the card was tilted while the combination was combined with disposable mixing sticks. The slide was shaken with a rotator for roughly two minutes, and then the presence or absence of clumps was checked.

Anti-Cyclic Citrullinated Peptide Antibody (ACCP) Test

The 96-well coated strip plate was employed. Before adding the samples to the wells, sample diluent was multiplied by 100 to dilute each sample. Following that, 20ml of wash buffer concentration was 25x diluted with ultrapure water. Controls and samples were run twice. In the control wells, 100 µl of both positive and negative controls were introduced. Plate was kept on incubation at 37°C for 30 minutes. A total of 250 µl of 1x wash buffer were used to perform five separate washings on each well. Each wash had a 30-second window before being completely aspirated. The plate was blotted on absorbent paper after the last wash. Each well (other than the Blank well) received 100 µl of HRP-conjugate antibody before incubation for 30 min at 37°C. Each well was scrubbed five more times. Each well received 50 µl each of substrate solutions A and B, which were then incubated at 37 °C in the dark for 10 minutes. Each well received 50 µliters of stop solution, which immediately turned the blue tint to yellow. Elisa reader was set at 450 nm for determining the optical densities of the solutions.

Quantification of IL-38

Used were purified monoclonal IL-38 antibody-coated microwells of Elisa plates. Prior to use, all samples and reagents from the kit were stored at 18 to 25°C for 30 minutes. To create a 120ng/L standard stock solution, 120 µl of the standard and 120 µl of standard diluent were added. The standard was kept on moderate agitation for setting down within 15 minutes, before making standard dilutions. By serially diluting the standard stock solution (120ng/L), 4 solutions in ng/L were generated. The zero standard (0.0 ng/L) is standard diluent. Following that, 20ml of wash buffer concentration was 25x diluted with distilled water until 500 ml of 1x wash buffer remained. A standard solution of 50 µl was added to the standard well, followed by the addition of 40 micro-l of a sample and 10 µl of an IL-38 antibody. Streptavidin-HRP in a volume of 50 microliter was added to both the standard and sample wells. The dish was thoroughly mixed, sealed, and incubated for 1 h at 37°C. The sealer was taken off when the incubation was finished, and the working wash buffer was used to wash the plate five times. Wells should be immersed in a minimum of 0.35 ml of wash buffer for 30 to 1 minutes for each wash. then rinse. 50 µl of substrate(S) solution A was added into each well before receiving 50 µl of “S” solution B. The plate was sealed with a fresh coating and let to sit at 37°C in the dark for ten minutes. 50 µl of stop solution was added to each well, instantly turning the blue tint to yellow. Ten minutes after adding the stop solution, the plates

were read at 450 nm. Using a microplate reader, the optical density (OD value) of each well was calculated.

DNA Extraction

For the DNA extraction, red blood lysis buffer, nucleic cell lysis buffer, saturated sodium chloride, 50X TAE buffer, and 0.5X running buffer were created. Using the Gel Doc system (InGenuis3, UK), gel was examined under ultra violet (U.V.) light following the determination of DNA using PCR. With the help of SPSS version 23, data input and analysis were completed.

Results and discussion

There were 82.5% female participants and 17.5% male participants. In the case group, 7 males (18%) and 33 females (83%) were present, compared to 24 men (60%) and 16 women (40%) in the control group. The average age of the 80 participants was 33.59 13.513 years, as shown in Figure 5. Patients' ages ranged from 45.55 to 11.30, on average. The control group's average age was 25.62 6.20 years. None of the female participants were expecting a child.

Table-1: Demographic data of all subjects.

Parameter		Patients n(40)	Controls n(40)
Gender n(%)	Male	7(17.5%)	24(60%)
	Female	33(82.5%)	16(40%)
Age Mean ± SD		45.55±11.30	25.62±6.20
Marital Status married	Married	25(62.5%)	8(20%)
	Never	15(37.5%)	32(80%)
Pregnancy women	Yes	0(0%)	0(0%)
	No	33(82.5%)	16(40%)

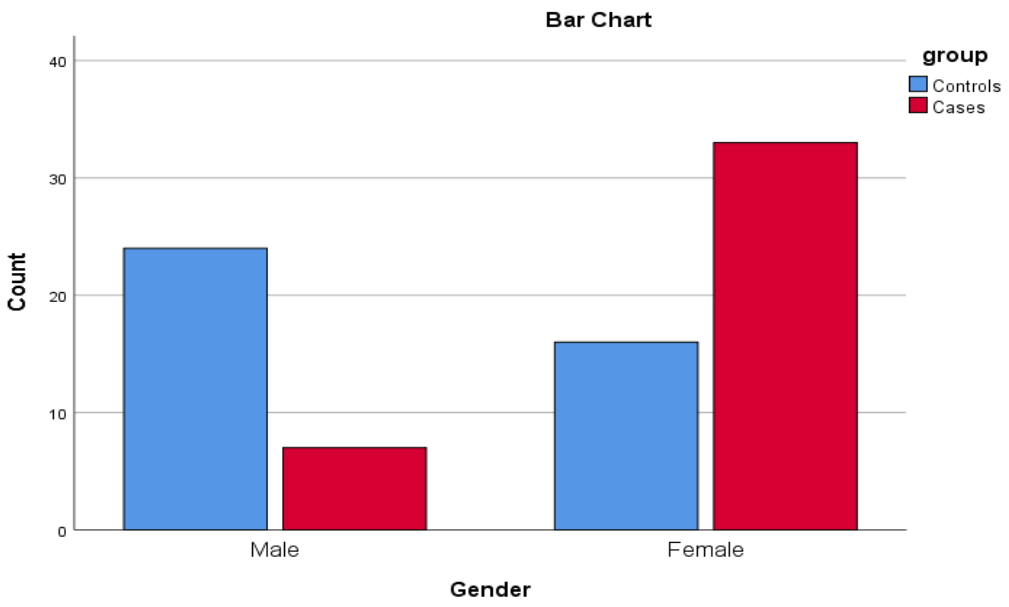

Figure 1: Gender distribution

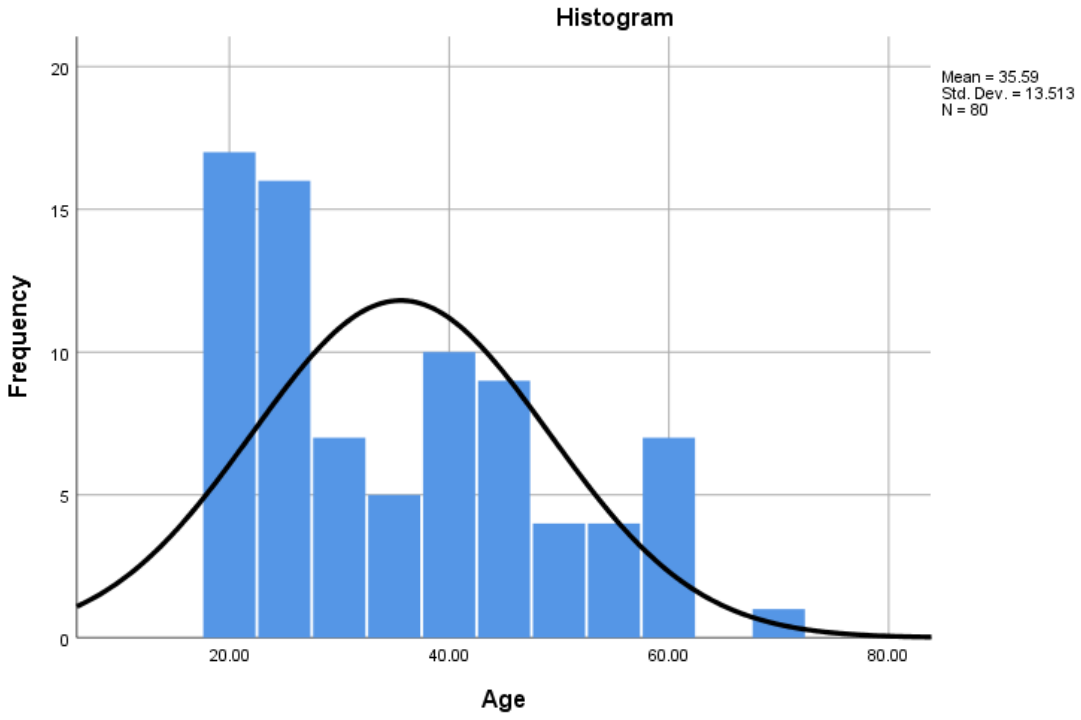

Figure 2: Mean age of studied population.

Laboratory test results for the enrolled participants were displayed in tables 1, 2, and figures 1, 2. Twelve (30%) of the case group's patients had positive CRP test results, while 28 (70%) of them had negative results. All of the patients had positive results for the RF and ACCP testing. All of the healthy controls had negative CRP, RF, and ACCP results.

Table-2: Laboratory parameters in patients group.

Laboratory parameters		Patients
CRP n (%)	Positive	12(30%)
	Negative	28(70%)
RF n (%)	Positive	40 (100%)
	Negative	0 (0%)
ACCP n (%)	Positive	40 (100%)
	Negative	0 (100%)

The patients' mean ESR value was 64.3755. The average ESR in the control group was 8.45 2.85. With a p-value of 0.001, there was a significant difference in ESR levels between the patients and the control group.

Table-3: **Comparison of ESR levels between patients and controls.**

	ESR Mean ± SD	p-value
Patients n(40)	64.37±55.05	<0.001
Controls n(40)	8.45±2.85	

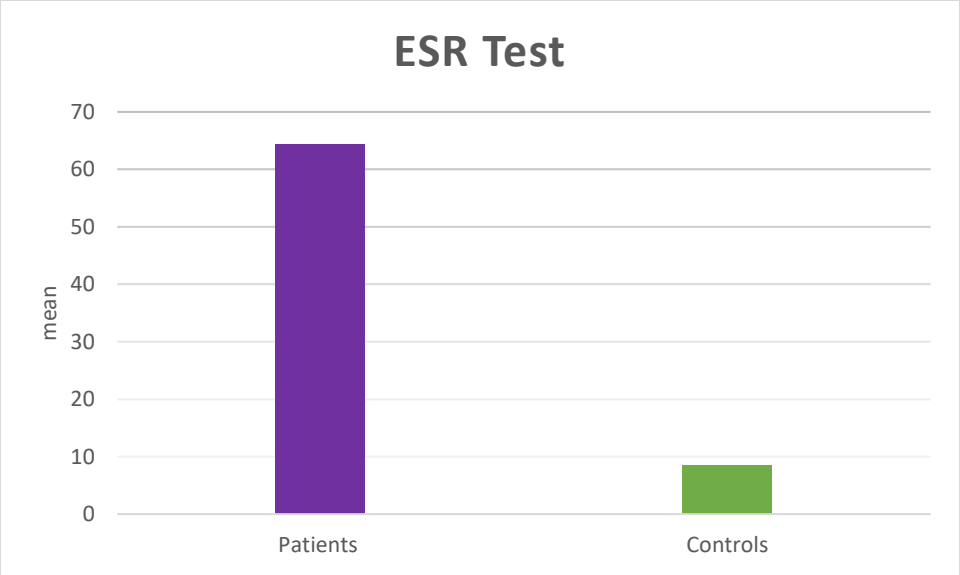

Figure 3: Mean ESR value in patients and controls.

Table 4 displays the mean value of the IL-38 concentration across the patients and controls. Patients' IL-38 levels indicated a mean value of 26.8621.70 with a minimum value of 12.47 and an average concentration of 133.60, while the mean value for the control group was 19.2712.92 with a minimum concentration of 6.50 and a maximum concentration of 64.78. IL-38 serum levels are higher in patients compared to healthy controls, although there is no statistically significant difference ($p > 0.005$).

Table-4: Serum levels of IL-38 in Cases and Controls.

	(IL-38) Concentration Mean ± SD	**P-value**	**Odds Ratio**
Patients n(40)	26.86±21.70	0.061	1.022
Controls n(40)	19.27±12.92		

When comparing the mean values of IL-38 between patients and controls who had the homozygous allele (AA), Table 5 revealed a significant difference (p-0.001). In contrast, there is

no difference in the means of IL-38 between patients and controls who had the homozygous allele (CC) and heterozygous allele (A/C), respectively, with a p-value of (p>0.005).

Table-5: Association of IL-38 SNPs with IL-38 levels in the studied population.

		group	**N**	**Mean ± SD**	**p-value**
AA allele	(IL-38 Concentration)	Patients	17	31.16 ± 17.70	0.001
		Controls	3	14.38 ± 1.04	
A/C allele	(IL-38 Concentration)	patients	9	32.51 ± 38.05	0.32
		Controls	16	18.89 ± 14.38	
CC allele	(IL-38 Concentration)	patients	14	18.02 ± 2.32	0.52
		Controls	21	20.27 ± 12.87	

Table 6 and figure 4 revealed that 3 (7.5%) of the controls and 17 (42.5%) of the patients have the homozygous allele (AA). Rheumatoid arthritis and the IL-38 SNP (Allele AA) are significantly associated (p 0.0001, OR = 9.11).

Nine (22.5%) of the case group's patients have the heterozygous allele (AC), compared to 16 (40%) of the control group's participants. Results revealed no difference in the non-significant AC allele between patients and controls. As a result, it was shown that there was no connection between the heterozygous allele of the IL-38 SNP (AC) and rheumatoid arthritis, but (p=0.09), (OR=0.43). In the case group, 14 (35%) patients carry the homozygous allele (CC), but in the control group, 21 (52.5%) controls do. Results revealed no variation in the frequency of the CC allele between patients and controls. It was established that there was no significant correlation between the homozygous allele of the IL-38 SNP (CC) and rheumatoid arthritis (p=0.012), (OR=0.48).

Table-6: Association of IL-38 SNPs with rheumatoid arthritis.

	Patients n(40)	**Controls n(40)**	**Chi-Square**	**p-value**	**Odds Ratio (95% CI)**
AA allele n (%)	17(42.5%)	3(7.5%)	13.067	< 0.001	9.11 (2.40-34.58)
A/C allele	9(22.5%)	16(40%)	2.85	0.091	0.43

n (%)					(0.16-1.55)
CC allele n (%)	14(35%)	21(52.5%)	2.48	0.12	0.48 (0.19-1.17)

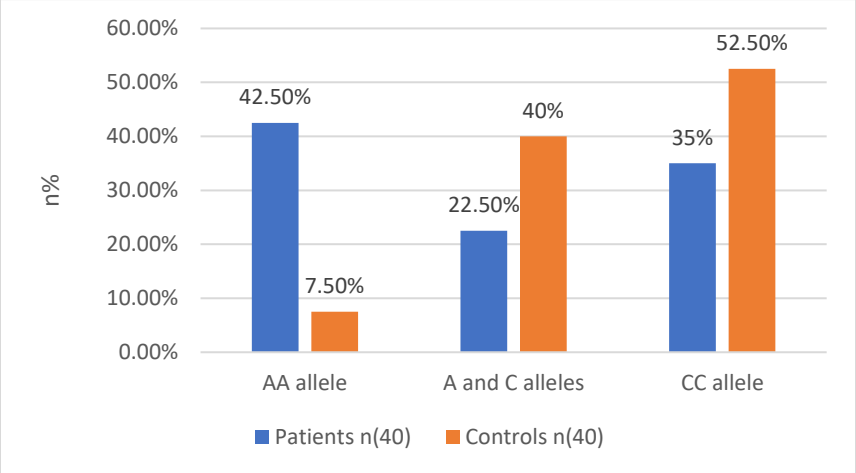

Figure 4: The percentage of each allele in patients and controls.

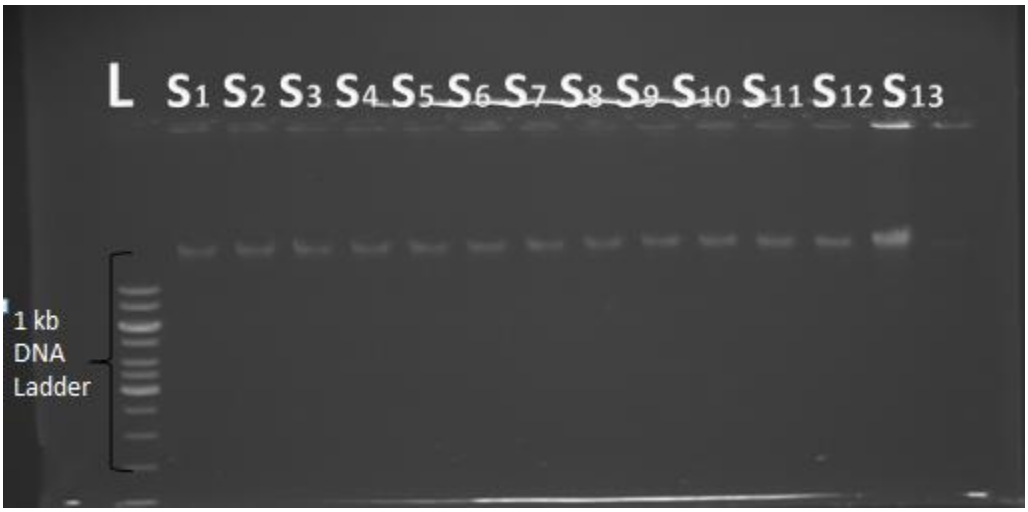

Figure 5: Isolated genomic DNA from blood sample: According to gel electrophoresis, all of the samples for the IL-38 gene have DNA that is the same length (1359 bp). (1000 bp) DNA Ladder.

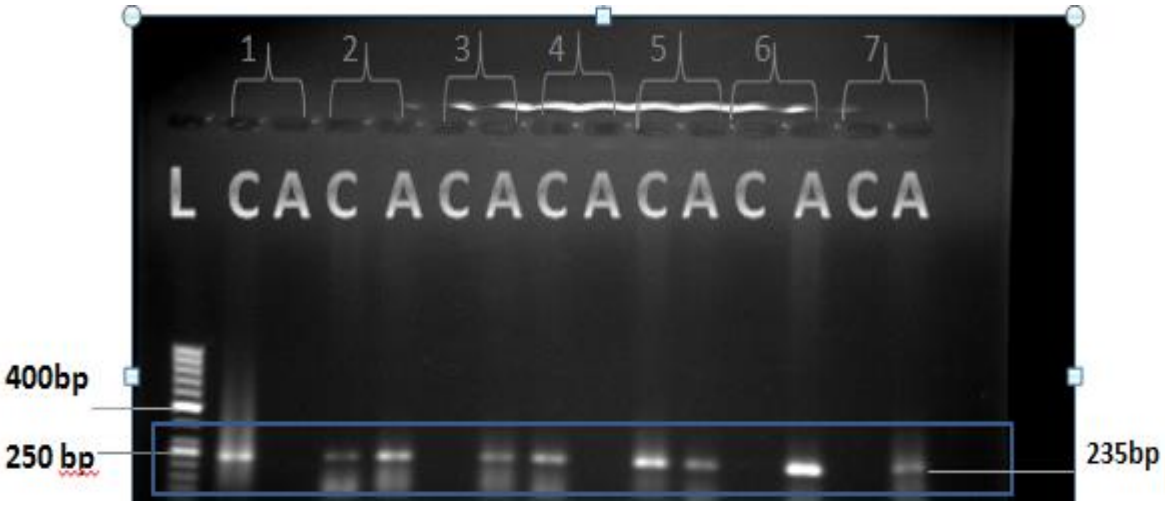

Figure 6: PCR amplified products (235 bp) for the SNP rs6743376 (C/A): 2% agarose gel following electrophoresis in Lane 1 with a 50 bp marker. Samples 1 and 4 have a homozygous CC allele, Samples 3 and 6 have a homozygous AA allele, and Samples 2 and 5 have heterozygous CA.

The results of the current study showed that all CRP, RF, and ACCP tests in the control group were negative, whereas in the case group, 30% of patients had positive CRP test results and 70% had negative results. The patient's RF and ACCP tests all came back positive. According to the current study, the mean ESR value among patients was (64.3755.05), whereas the mean ESR value among controls in the control group was (8.452.85). the IL-38 concentration mean values between patients and controls. The controls group had a mean IL-38 concentration of 19.2712.92, whereas the patient group had a mean IL-38 concentration of 26.8621 pg/mL. The RA group had higher levels of IL-38 than the control group, according to the results. The difference between the patients' and the control group's mean values for IL-38 was not statistically significant, as indicated by the p-value ($p>0.005$). The current study is the first to examine the relationship between IL-38 polymorphism and RA illness in the population of Pakistan. According to the results, 7.5% of controls and 42.5% of patients carry the homozygous allele (AA). The IL-38 SNP (AA) and rheumatoid arthritis are significantly associated. While 40% of the controls in the control group possessed heterozygous alleles (AC), just 22.5% of the case group's patients did. It proved that there was no connection between rheumatoid arthritis and the heterozygous allele (AC) of the IL-38 SNP. In the case group, 35% of patients had the homozygous allele (CC), whereas 52.5% of the

controls had the homozygous allele (AA), demonstrating that there was no conclusive evidence linking the homozygous allele (CC) of the IL-38 SNP to rheumatoid arthritis.

Conclusion

RA patients had greater levels of IL-38 in their serum than healthy controls, albeit there is no statistically significant difference. When compared to homozygous allele (CC) and heterozygous allele (AC), IL-38 rs6743376 (A/A) is related with a higher risk of developing RA illness. In contrast to heterozygous (A/A) allele, homozygous (C/C) allele and heterozygous (A/C) allele are protective in RA. IL-38 should be given more attention in future studies by lengthening the study's duration and participant pool.

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