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(Research Article.)

Study of pathological and biochemical connection of IL-10 gene polymorphism and Rheumatoid Arthritis and Atopic Dermatitis in Egyptian patients admitted in a local clinical setting

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Abstract: Rheumatoid arthritis (RA) is a chronic inflammation of the immune system that similarly affects the synovial joints. Atopic dermatitis (AD) is a chronic skin condition that is characterized by a complicated interaction of repeated exposures, host factors, and hereditary factors. Interleukin 10 (IL-10) is an immune-regulatory cytokine that is commonly utilized in RA to reduce the inflammatory response. An allelic variation in the IL-10 gene's promoter region may play a role in autoantibody production control. This study aims to identify Polymorphisms in the IL-10 gene in RA and AD patients and sequence the IL-10 gene fragment to elucidate the polymorphism (IL10-1082A/G) and determine the possible relationship between IL10 gene polymorphism and pathogenesis of RA and AD. Twenty-five blood samples were collected from RA Egyptian patients / 17 healthy control samples and 19 blood samples were collected from AD Egyptian patients / 12 healthy control. DNA has been extracted from blood samples and subjected to a Restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) test for the determination of the IL-10 genotype. PCR products were sent for sequencing. Genotyping revealed that both the patient and control groups had the same IL-10 allele sequencing results and showed silent mutation with no effect in the amino acid sequence. Our results showed that there is no association between IL10-1082 A/G single nucleotide polymorphism (SNP) and RA or AD pathogenesis. Environmental factors may have more effect on the pathogenesis of RA and AD.

Keywords: Genotyping, Inflammatory disorders, RFLP PCR, Sequencing, Pathogenesis

1. INTRODUCTION

RA is an autoimmune immunosuppressive illness with an unknown origin that mostly affects joints but can also show outside of them. Good RA care necessitates a multidisciplinary approach due to its complexity, which is based on a completely unknown degenerative physiological mechanism [1]. Approximately 0.5 to 1% of the population is affected by RA [2]. A cascade of immunological responses emerges from the combination of hereditary and environmental variables [2]. Patients with RA have a variety of extra-articular symptoms and comorbidities, which leads to an elevated death rate [3]. Females are more likely to develop RA than men, with a female-to-male ratio of 2:3 [4]. The causes of RA have mostly remained unclear till now. However, research has indicated that genetic - variants within immunosuppressive genes may impact the likelihood of developing RA, implying that the risk of passing on RA may be as high as 60% [5]. These disparities showed that hereditary factors may contribute to the development of RA.

RA is thought to be caused, in part, by the repetitive stimulation of immunological systems (both innate and adaptive) as a consequence of which the immune system is harmed (aberrant generation of autoimmune apoptosis, and activation of particular T

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and B cells) according to studies [6]. Additionally, cytokines are involved in the regulation of the immune system response, and cytokines gene polymorphisms contribute to immune response flexibility [7,8]. Numerous cells of adaptive and innate immune, including Th2 lymphocyte, B - lymphocyte, and macrophage, secrete IL-10, a very strong anti-inflammatory cytokine with the capacity to down-regulate antigen performance and activate macrophages [9]. Inhibition of B cell activation and antibody production has also been demonstrated with IL-10 [10] IL-10 had been shown to suppress pro-inflammatory cytokine in rheumatoid joints and also to induce joint swelling and deformity reduction, while also cartilage necrotic decrease [11]. Given the importance of IL-10 in the development of RA, various research had looked at IL-10 genetic diversity that might influence human association with RA, but the data have been mixed and inconclusive. Autoantibody status, such as rheumatoid factor (RF) and citrulline (anti-CCP) antibodies, has been used to separate RA patients into various hereditary groupings [12]. According to Lagha et al, the IL-10 (1082 G\A) genetic variation has been linked to an increased risk of RA developing in the population of Tunisians [13]. In another investigation, the authors concluded that single nucleotide polymorphisms (SNPs) in the IL-10 gene at codons 592 (C/A) and 1082 (G/A) showed no impact on susceptibility to RA in the Asian Indian community [14].

AD is a recurrent chronic skin condition that is more frequent during childhood. It's typically linked to skin barrier malfunction, allergic sensitivity, and recurring skin infections [15]. AD has a profound impact on the health and quality of life of patients both physically and physiologically. AD affects about 20% of children and 10% of adults 15. The pathogenesis of AD, like that of other complex chronic illnesses, is multi-factorial. According to the research, climate, UV exposure, air pollution, smoking, and stress may all have a role in AD [16]. Furthermore, AD may be a hereditary condition [17].

IL-10 is a key cytokine generated by a large number of immune cells that regulates immunological activity in the skin in different ways. T1/T2 differentiation, antigen-presenting cell activity, cell-mediated antigen presentation activation, T cell, B cell, and mast cell development and differentiation are all abnormal in the progression of many disorders [18]. The relevance of this cytokine in the development and maintenance of AD was due to the concept that polymorphisms in the IL-10 gene might be a genetic marker for AD in childhood [19]. There is evidence that a significant number of SNPs have a key role in AD susceptibility [20]. The IL-10 gene has been the subject of the most intensive research.

The IL-10 genes found on chromosome 1q31-32 express a class of widespread anti-inflammatory cytokines with regulatory function across the immune system [21]. One of these putative susceptibility polymorphisms is 1082 A / G (SNP; rs1800896) in the promoter region of IL10, which produces anti-inflammatory cytokines with multi-targeted effects [22]. The best distinct SNP for the IL10 gene is IL-10-1082 A / G. This polymorphism is characterized by the substitution of guanine (G) to adenine (A) nucleotides. The resulting IL-10−1082 AA genotype is marked by lower IL-10 production, which has been studied in various AD candidate gene association studies, although the findings have been found mainly negative [23].

The purpose of this research was to establish if the IL-10 (1082 A / G) genetic polymorphisms might affect the development of RA and AD diseases in the Egyptian population. See the end of the document for further details on references.

2. METHODS

2.1. Collection and processing of clinical samples

The Faculty of Pharmacy - Suez Canal University's Research Ethics Committee accepted this study. The current research included 73 individuals and separated them into three groups: the first group (I): contained 25 patients with RA, the second group (II): contained 19 patients with AD, and the third group (III): contained 29 healthy controls (17 samples matched with RA patients and 12 samples matched with AD patients).

Under aseptic conditions, samples were collected from the departments of Dermatology at Mansoura University Hospitals for group II and their matched healthy control, and samples for group I and their matched healthy control were collected from the department of Rheumatology at Benha Educational Hospital. Healthy Controls matched the sex and age of patients.

2.2. Criteria for patient inclusion:

Diagnosis of the RA patients was established according to the American College of Rheumatology's criteria, at which the patients suffered swelling of the joints and the serum Anti-cyclic citrullinated peptide (CCP) and anti-rheumatoid factor (RF) autoantibodies were found to be high. In addition, the elevation of the plasma level of erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP).

AD patients were diagnosed according to the criteria proposed by Hanivin and Rajka [24] which included.
Three or more fundamental features: Pruritus, Typical morphology and distribution (Flexural lichenification or linearity in adulthood, Facial and extensor involvement in newborns and children), Personal or familial history of allergy (asthma, allergic rhinitis, atopic dermatitis).
Plus 3 or more minor features:
Xerosis, Ichthyosis/palmar hyperlinearity/keratosis, pilaris Immediate (type I) skin test reaction, Elevated serum IgE Early age of onset, Tendency toward cutaneous infections (particularly, *Staph. aureus* and *Herpes simplex*), impaired cell-mediated immunity. The tendency toward non-specific hand or foot dermatitis, Nipple eczema, Cheilitis, Conjunctivitis, Keratoconus, Cataract (anterior subcapsular), Darkening of the orbit, Pallor/erythema of the face, alba, Pityriasis, Itching, Intolerance to wool and solvents derived from lipids, Intolerance to foods. Environmental/emotional variables affect the course of treatment.

2.2.1. Patients Exclusion Criteria:
There have been exceptions made for RA patients. Smokers, diabetics, acute infections, endometriosis, chronic obstructive pulmonary disease, and pregnant women were excluded.

2.3. Inflammatory markers for RA:
The Westergren technique was used to determine ESR, anti-CCP, RF autoantibodies, and CRP were measured using conventional ELISA kits (Thermo Fisher Scientific, USA).

2.4. IL-10 –1082 A/G genotyping
We used the PCR-RFLP approach to determine the polymorphism of the IL-10 gene at SNP (1082 A/G). Restriction nucleases are enzymes that splice lengthy stretches of DNA into shorter segments. Each restriction nuclease recognizes a unique nucleotide sequence inside the DNA strand and hence cuts at a unique location. The distance between endonuclease's cleavage sites differs between people. As a result, the length of DNA fragments generated by a bound nuclease varies across organisms and between individual species.

2.4.1. Extraction of DNA
A DNA Isolation Kit (QIAamp DNA Mini Kit) was used to extract genomic DNA from peripheral blood (Thermo Fisher Scientific, USA).

2.4.2. The Polymerase Chain Reaction
Amplification of the IL-10 gene was accomplished using a thermal cycler (Thermo Fisher Scientific, USA). PCR reagents were provided by (Thermo Fisher Scientific, USA). The primers were used as follows; forward primer-F (5'-CCA AGA CAA CAC TAC TAA GGC TCC TTT-3) and reverse primer-R (5'GCT TCT TAT ATG CTA GTC AGG TA-3') for RA patients and control and forward primer- F(5'-TCTTACCTATCCCTACTTCC-3), reverse primer-R(5'CTCGCTGCACCACACACTGGC-3') for AD patients and controls.
The conditions for the PCR reaction for RA were as follows
Five minutes of denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 35 seconds, followed by a 48-second annealing step at 58 °C, and a final 1-minute extension at 72 °C. To finish the PCR, a final extension step of 7 minutes at 72 °C was performed.
The conditions for the PCR reaction for AD were as follows
Five minutes of denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 35 seconds, followed by a 48-second annealing step at 60 °C, and a two-minute extension at 72 °C. Then, for 7 minutes at 72 °C, perform the last extension step to finish the PCR. For 30 minutes at 100 V, the PCR product was loaded and electrophoresed on a 1% agarose gel (Sigma, USA) prepared in 1 TBE solution containing ethidium bromide (1.0 g/ml). The Gene Ruler 100 bp DNA ladder was commonly used in agarose gels as a molecular weight benchmark. On a computerized UV gel documentation system, the gel had been photographed.

2.4.3. The technique of Restriction Fragment Length Polymorphism (RFLP)

The PCR products from RA and control samples (377 bp) were treated with XagI restriction enzyme for 6 hours at 37 °C using the PCR-RFLP method to discover three distinct kinds of IL-10 (1082 A/G) SNPs. XagI restriction enzyme was obtained from (Thermo Fisher Scientific, USA). The DNA fragments were confirmed through gel electrophoresis on 2.5 % agarose gel and visualized under UV light after ethidium bromide staining.
The PCR products from AD and control samples (139 bp) were treated with mnII restriction enzyme for 6 hours at 37 °C using the PCR-RFLP method to discover three distinct kinds of IL-10 (1082 A/G) SNPs. The restriction enzyme mnII was purchased from (Thermo Fisher Scientific, USA). Gel electrophoresis of the digested PCR product on a 2.5 percent agarose gel (1.25 g agarose (Sigma, USA) was used to analyze the restriction pattern and see it under UV light after Ethidium bromide staining.

2.4.4. Purification of PCR Products

Purification of the IL-10 PCR product was performed according to the manufacturer's instructions using a PCR Product Purification Mini Kit (Thermo Fisher Scientific, USA).

2.4.5. DNA Concentration Measurement

The concentration of DNA in purified PCR products was determined using a (Thermo Scientific, USA) NanoDropTM 2000/2000c spectrophotometer. All items include serial DNA and have a concentration of more than 100 ng/mL.

2.4.6. Sequencing of DNA

The IL-10 gene PCR products were shipped to the Biovision facility in Egypt for DNA sequencing. The acquired sequences were aligned using https://blast.ncbi.nlm.nih.gov/Blast.cgi and https://blast.ncbi.nlm.nih.gov/Blast.cgi. Polymorphisms were evaluated using https://blast.ncbi.nlm.nih.gov/Blast.cgi.

2.5. Statistical analysis

The data were analyzed using the 2013 edition of IBM SPSS Corp. Version 22.0 of IBM SPSS Statistics for Windows. Armonk, New York: Armonk Publications. Quantitative data from IBM were expressed in terms of numbers and percentages. Quantitative data were characterized using the intermediate (min and max) values of unmarked and medium data, as well as the standard deviation of parametric data after the Kolmogorov-Smirnov/Shapiro-Welk test for normalcy. The significance level for the obtained findings was set at 0.05. To compare two or more sets of qualitative data, the Chi-Square test was utilized. The t-student test was used to compare two separate sets of parametric variables, while the Mann-Whitney U test was used to compare two independent sets of unmarked variables. The Spearman rank order correlation coefficient is used to measure the strength and direction of the linear link between two continuous variables that are not normally distributed and/or linear.

3. RESULTS

3.1. Patient Clinical Features

The RA group consisted of 24 women and one male with an average age of 50 years, whereas the normal control group consisted of 16 women and one man with an average age of 46 years.

Age and gender had no statistically significant difference between cases and controls as shown in Table. 1.

According to table 2, all of the patients in the RA group had been diagnosed with elevated ESR and positive RF and CRP. The average ESR for studied cases was 40 mm/h/l, the average CRP was 18.5 mg/l, the average RF was 34 u/Ml, and the average anti-CCP was 7.2 u/Ml, ranging from 1.5 to 41.5 u/ml, the average disease activity degree 28 was 4.07, the average disease duration was 4 ranging from 1 to 10 years. In all the study groups (RA patients and controls), the genotype frequencies of the IL10-1082 A/G SNP were AA allele.

For AD patients and the control group: Table 3 indicated that there was no significant difference in gender or age among patients and healthy controls. The average patient age of AD patients was 9 years and the average age of the controls group was 8 years. Among the patients under study, 26.3% were females versus 41.7% of females in the control group. The average disease activity score was 26.3, ranging from 14.5 to 44.5, and the average duration of the disease was 12 months, ranging from 1 to 48 months. All patients and control groups were AA genotypes.

3.2. IL 10 gene genotyping in RA patients and controls

The 377 bp PCR products were sized and matched on a one percent agarose gel and bands were seen under UV light for both RA samples and controls.

Figures 1 and 2 display the frequency of the IL-10 gene SNP at 280bp + 97 bp in RA patients and healthy controls.

3.3. IL 10 gene genotyping in AD patients and controls

All samples and controls had the wild-type homozygote AA genotype, according to the analysis of the IL-10 gene. Thus, all bands in the samples and controls had a length of 139 bp. As can be shown in Figure 3, there is still no difference in the IL-10 allele between AD cases and healthy controls.

The restriction pattern was observed using a 2.5% agarose gel, and bands were seen under a UV light following Ethidium bromide staining. The digestive byproducts of the control samples were displayed in the first and second wells, whereas those of the AD samples were displayed in the third and fourth wells.

3.4. Association of IL-10 gene polymorphism with RA

Multiple sequence alignment of the (IL-10 1082 G/A) gene fragment was carried out using the Blast function of the National Center of Biotechnology Information (NCBI). **Figure 4** showed the pairwise alignment of the RA sample with the query sequence. There are two gaps in which the nucleotide changes from C to T and T to A respectively, and since the nucleotide change didn't affect the amino acids, it is referred to as a silent mutation.

3.5. Protein Multiple Sequence Alignments result for RA samples and control

There is one gap in the second sequence that doesn't seem to have a corresponding mate in the corresponding sequence, as shown in **Figure 5**'s alignment of amino acids between the query sequence and the RA sample. The dash indicates this location. Amino acids remain unchanged. This sequence alignment has a 99 percent identity rate. IL10 genotyping and genome sequencing data did not reveal variations between RA patients and controls.

Table 1. Sociodemographic characteristics of the RA studied groups.

	RA patients N=25	Control of RA N=17	Test of significance
Age/years	**50**	**46**	**t=1.29 p=0.202**
Sex\ Male	**1 (4.0)**	**1(5.9)**	**FET, P=1.0**
Female	**24 (96.0)**	**16(94.1)**	

Table 2. Genotyping distribution and clinical laboratory of RA patients and controls**.**

	RA patients N= 25 median (range)	RA Control N= 17
ESR (mm/1st hour)	**40 (8-125)**	**Negative**
CRP (mg/l)	**18.5 (3-50)**	**Negative**
RF(u/Ml)	**34 (3-158)**	**Negative**
Anti CCP(u/Ml)	**7.2 (1.5- 41.5)**	**Negative**
DAS 28	**4.07±0.519**	**Negative**
Disease Duration Years	**4.0 (1.0-10.0)**	**Negative**
Morning Stiffness /minutes	**15 (5-120)**	**Negative**
Allele AA	**25 (100%)**	**17 (100%)**

Table 3. Sociodemographic characteristics of the AD patients and control group.

	AD patients N=19	Control of AD N=12	Test of significance
Age/years median (min-max)	**9 (3-16)**	**8 (3-12)**	**z=0.347 p=0.729**
Sex/ Male Female	**14(73.7%) 5(26.3%)**	**7(58.3%) 5(41.7%)**	**χ^2=0.793 p=0.373**
Score median (min-max)	**26.3(14.5-44.5)**	**-**	**-**
Duration /months median (min-max)	**12(1-48)**	**-**	**-**
Allele AA	**19(100.0%)**	**12(100%)**	**-**

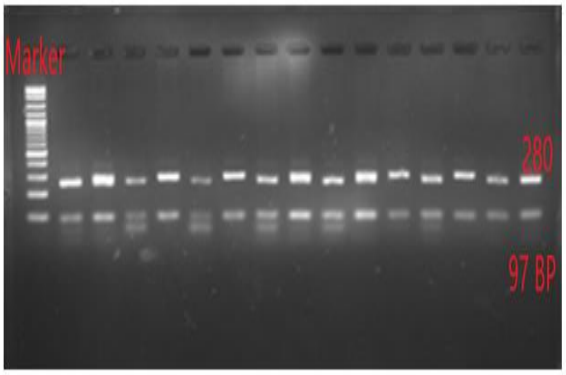

Figure 1. PCR- RFLP results for RA cases samples. Complete digestion of the wild-type homozygote gives the AA genotype (280 bp+97 bp).

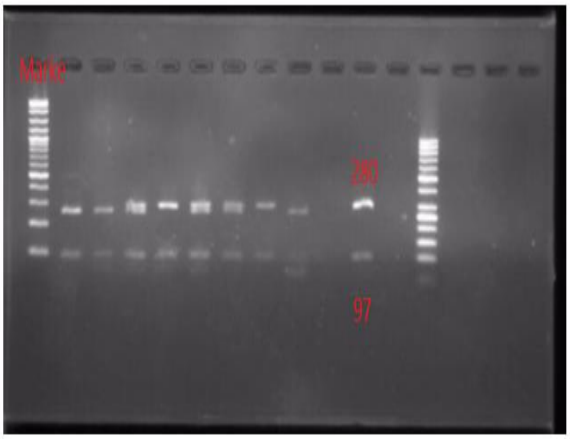

Figure 2. PCR- RFLP results for RA Control samples. Complete digestion of the wild-type homozygote gives the AA genotype (280 bp+97 bp).

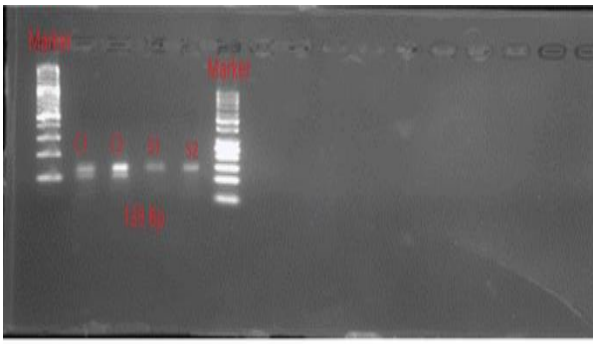

Figure 3. PCR- RFLP results for AD Samples and Control

Figure.4. Sequence alignments of (IL-10 1082 G/A) fragment of RA samples and control

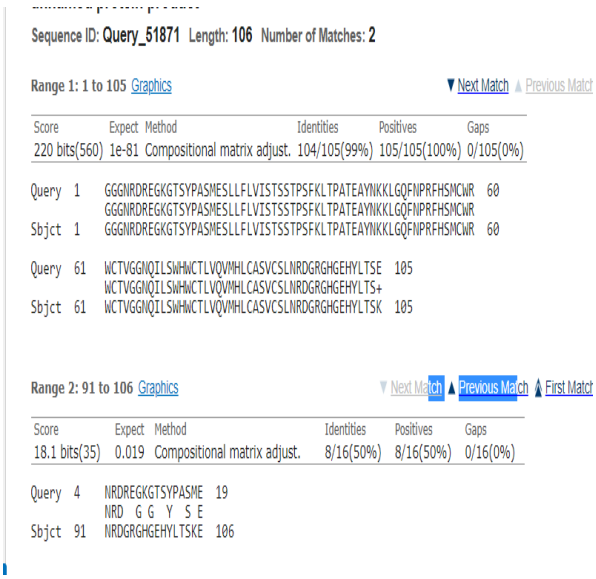

Figure 5. Protein alignments of (IL-10 1082 G/A) for RA samples and control**.**

4. DISCUSSION

RA and AD are chronic and autoimmune disorders. While RA is a systemic condition evidenced by synovial arthritis, which results in joint damage and deformity, AD is a complicated skin disorder with a significant hereditary component. Although the specific cause of AD is unknown, numerous studies had established that environmental and genetic variables have roles in the genesis and progression of chronic autoimmune illnesses such as RA.

Additionally, RA is characterized by a persistent imbalance between pro- and anti-inflammatory immunological processes, which results in chronic synovitis [25]. Thus, regulating the production and action of the proponent and anti-inflammatory substances, as well as examining the correlation of polymorphisms in these cytokines of genes with RA, represents a prospective therapeutic breakthrough. According to recent research, RA patients had significantly greater IL-10 gene expression than control [26], whereas another study revealed no statistically significant differences in blood levels of IL-10 between RA patients and controls [27].

IL-10 appears to have a conflicting function in RA, decreasing pro-inflammatory cytokines while simultaneously fostering a mixed immunological response, as indicated by contradicting findings regarding IL-10's therapeutic effectiveness in RA [28]. In recent years, genetic susceptibility to RA had been examined [29]. Numerous genes associated with RA have been discovered as risk factors for the disease, and the IL-10 gene is one of the most extensively examined. Numerous studies suggest that various IL-1082 A/G polymorphisms are related to a higher risk for RA disease. Our investigation showed that there is no correlation between (IL-10 1082 A / G) gene polymorphisms with the likelihood of developing RA or AD disorders in the Egyptian population since both the patient and control groups had the same AA genotype.

Previously, inconsistent findings regarding the function of IL-10 polymorphisms in RA have been described [30]. A Turkish study showed a statistically significant connection between the (IL-10 1082) G allele with the occurrence of RA ($p = 0.008$; OR = 1.44, 95 percent CI1.11-1.86) [31]. Hajeer et al observed no significant variations in IL-10 polymorphism allele frequencies between patients of RA and healthy controls, however, they revealed that

genetic patterns that encode low IL-10 expression are connected with RA [32]. Additionally, three other investigations found no association between IL-10 and RA [33,34]. In a Chinese population, RA patients had a substantial drop in the frequency of an allele (A) in rs1800890 of IL-10, indicating that the A allele may be protective against RA. [35]. The association between IL-10 polymorphisms and RA risk was shown to be more significant in Caucasians than in Asians in meta-analysis research. This gap might be explained by more recent research that has concentrated on the Caucasian population [36]. The prevalent genotype at the IL-10 1082 BP gene was allele AA, in contrast to Caucasians, who had a higher prevalence of the GG genotype [37]. Antibodies against RF are the most well-known biochemist and immunologic markers for determining the extent of RA [38]. In a healthy population, the prevalence of RF is less than 5% but jumps to 70-90 percent in those with active RA [39].

The exact pathophysiology of AD disease is still unknown. Both intrinsic and extrinsic variables had been identified as possible causes of AD [40]. Our findings showed that there is no evidence of a genetic link between (IL-10 -1082 A / G) SNPs and the chances of getting AD. These results support Qi et al., who found no genetic association between (IL-10 -1082 AG) SNPs and the chances of developing AD [41]. Zhao et al. recently published a meta-analysis in which they observed a probable correlation between the (IL-10-1082 A/G) SNPs and AD risk within the association group using the recessive model. [42] Previously, the (IL-10-1082 A/G) polymorphism was linked with susceptibility to AD in Indian individuals [43]. On the other hand, our finding is in line with Sohn MH., et al and Lesiak A et al [44,45].

To gain a more objective understanding of the pathophysiology of AD, it is critical to investigate the combined effect of several interleukin gene variations on resistance or susceptibility to hereditary illnesses, including diverse skin conditions. However, this study examined the significance of a variant in the IL-10 gene, a part of the IL family of genes, in AD susceptibility. Linked to polymorphism's contextual effects and the heterogeneity of the heterogeneous response, additional research into the interleukin gene's association with additional modulating clinical or environmental factors will be required, at which point additional association investigation data will be accessed.

5. CONCLUSIONS

In conclusion, we found that there is no association between IL10-1082 A/G SNP and RA and AD in the Egyptian population. The results showed that the polymorphisms involved in the immune response are not related to some aspects of the development and course of AD and RA disease and, although not conclusive, support the immunological hypothesis of the origin of the inflammatory lesions of AD and RA. However, due to the conflicting nature of the published findings, further, large-scale studies are needed to determine the associations between genetic variations and AD, and RA pathogenesis and to identify candidate genes that may allow more accurate identification of all aspects of the two diseases and early identification of cases with a clearer prognosis.

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Conflicts of Interest: The authors declare that they have no competing interests.

Ethical Statement: This work was approved by the research ethics committee of the faculty of pharmacy at Suez Canal University. (201809MH1)

Author Contribution: Rahma runs all the experiments and writes the first draft. Dr Ali proposes the project idea, follows up the experiment and analyzes the results. Dr Ashraf collects samples of Atopic Dermatitis patients. Dr Samar proposes the project idea, follows up the experiment, analyzes the results and reviews the manuscript.

List of Abbreviations:
RA: Rheumatoid arthritis
AD: Atopic dermatitis
IL-10:Interleukin 10
RFLP-PCR: Restriction fragment length polymorphism-polymerase chain reaction
SNP: single nucleotide polymorphism
DNA: Deoxyribonucleic acid
RF:Rheumatoid factor
Anti-CCP Anti-cyclic citrullinated peptide antibody
ESR: Erythrocyte sedimentation rate
CRP: C-reactive protein
PCR: Polymerase chain reaction

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