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ORIGINAL ARTICLE

Insertion/Deletion Polymorphism of *ACE* Gene and Anti-Nuclear Antibodies Association Study as Diagnostic Markers in the Rheumatoid Arthritis Patients of Pakistan

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Authors' Contributions
1 Conception & Study Design, Data Collection, Data Analysis, Drafting, Critical Review.
2 Conception & Study Design, Data Analysis, Critical Review.

Acknowledgements
The authors hereby acknowledge the volunteer patients who provided their samples for the accomplishment of the study and Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan.

Article info.
Received: July 2, 2019
Accepted: September 24, 2019

Funding Source: *This work was supported by the Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan. This present study and the included research work did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.*

Conflict of Interest: *Nil*

Cite this article: *Sajid A, Hussain N. Insertion/Deletion Polymorphism of ACE Gene and Anti-Nuclear Antibodies Association Study as Diagnostic Markers in the Rheumatoid Arthritis Patients of Pakistan. RADS J. Pharm. Pharm. Sci. 2019; 7(3): 119-127.*

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ABSTRACT

***Objective*:** The main emphasis of the present study was to precisely assess the association of *I/D* polymorphism of *ACE* gene with Rheumatoid Arthritis. According to previously reported studies, there may be different causes of Rheumatoid Arthritis and the angiotensin enzyme has shown the correlation with different arthritis diseases. TRF, *HLA* genes and *ACE* gene *I/D* polymorphisms have been studied in an aspect of being involved in the manifestation of RA.

***Methods*:** For the purpose of research, general Pakistani Punjabi Population was selected. Immunoassays used for the analysis of samples included; Rheumatoid factor of the patients and C – Reactive protein levels in their blood. The immunofluorescence assay of the RA positive samples was also done to determine the anti-nuclear antibodies in the serum samples of RA subjects. Blood glucose levels in the patients were noted. Contemporarily, the *I/D* polymorphism of ACE gene was examined in the native Punjabi population of Pakistan.

***Results*:** As a result, significant association was found between RA and ACE gene Insertion/Deletion polymorphism. The RA patients showed high frequency of *D* allele (50%) and low frequency of *I* allele (12.5%), contrary to the controls. The comparison between present study and the previously studied populations showed that *D* allele has proved to be associated with the RA disease.

***Conclusion*:** The present study suggests that further studies are required on the *ACE* gene polymorphism association with other populations in Pakistan and other countries. The anti-nuclear antibodies could also be a diagnostic marker for Pakistani Rheumatoid Arthritis Patients.

Keywords: Rheumatoid arthritis (RA), anti-nuclear antibodies (ANA), immunofluorescence (IF) assay, I/D polymorphism of angiotensin-converting enzyme (*ACE*) gene, insertion (*I*) allele, deletion (*D*) allele, homozygous (*II* and *DD*), heterozygous (*ID*).

INTRODUCTION

Rheumatoid Arthritis (RA) is proven to be a long-lasting provocative disease and can mark more than unbiased of the joints. As RA is counted to be an autoimmune sickness, it generally arises when the body immunity falsely spasms body's own tissues and joints. This state of illness, in many people, can cause serious harm to the various body systems including skin, eyes, lungs, blood vessels and heart [1].

Rheumatoid Arthritis moves the lining of the joints, contrasting the wear-and-tear mutilation of osteoarthritis, and triggers a painful puffiness that can eventually result in bone and joint defect. Rheumatoid Arthritis leads to permanent damage and problems, and the age-related mortality of affected people exceeds as compared to the general population [2]. New criteria demonstrated the 89% specificity and 91–94% sensitivity for Rheumatoid Arthritis, when compared with non-Rheumatoid Arthritis (control) subjects. The existence of these complete manifestations is foremost interpreter of death rate in patients with Rheumatoid Arthritis [3]. Rheumatoid Arthritis can occur due to both genetic and environmental factors [4]. If not treated properly Rheumatoid Arthritis can lead to long lasting damage to the body with severe pain and disability [5]. For 25 years almost, the major role of *HLA-DRB1* alleles are considered as the threat to Rheumatoid Arthritis patients [6]. Angiotensin-converting enzyme (*ACE*) was reported to be pathogenic factor of RA, and greater level *ACE* has been accepted to be present in RA synovial fluid and pleural effusions. *ACE* suppressors have the tendency of treating the hypertension in the body [7]. The suppression of renin angiotensin system is a best method to lower the pathogenesis of cardiovascular disorders [5, 8, 9]. A polymorphism of the mankind's angiotensin changing over compound (*ACE*) gene needs have been recognized previously [10, 11]. In the European RA patients, the proportion of the existence of the *ID*, *DD* and *II* genotypes was 2:1:1. Contrary to this, there was an inclination towards *D* allele had a higher rate in the Nigerian Population. On the other hand, *I* allele had much higher frequency in the Samoans and the Yanomami Indians [12]. Comprising of the absence or presence of a DNA piece of 250-bp; a polymorphism was identified inside the angiotensin I-changing over chemical quality (*ACE*) utilizing the endothelial *ACE* cDNA test. In all of the three genotype classes of ACE gene, a contrast in serum *ACE* levels in the serum were seen between subjects. The insertion/deletion polymorphism represented the 47% aggregate phenotypic fluctuation of serum *ACE*, demonstrating that the quality locus of *ACE* is the significant locus that decides serum *ACE* level [13]. Antibodies that attack healthy proteins in the nucleus of cells are called antinuclear antibodies (ANA) [5]. When the body receives signals to attack the body itself, autoimmune inflammatory diseases such as scleroderma, lupus, arthritis, mixed connective tissues and other diseases appear in the body. A huge overrepresentation of the *D* allele the *DD* genotype in patients with RA was observed. The men with RA displayed a higher recurrence of the *DD* genotype and *D* allele. By strategic relapse investigation the genotype *DD*, gives a relative pathogenic risk for improvement of RA [14]. In present study, a significant association was found between Insertion/Deletion polymorphism of *ACE* gene ('*D*' allele) and Rheumatoid Arthritis, among Pakistani Punjabi Population. Based on visualizations, anti-nuclear antibodies are also hypothesized [15] to be a diagnostic marker of Rheumatoid Arthritis.

MATERIALS AND METHODS

Ethical Approval and Patients' Consent

The study was ethically approved by the Research Ethics and Biosafety Committee at the Department of Microbiology and Molecular Genetics, University of the Punjab, Pakistan. Patients' consent, family history and the personal information of the subject were recorded simultaneously in the consent form and the proforma. All subjects signed the written and postulated consent form as it was in accordance of the study protocol approved by the University of the Punjab, Department of Microbiology and Molecular Genetics, Lahore, Pakistan. Patients were completely evaluated clinically by the laboratories. Consent form included age, disease duration, deformed joints, *etc*.

Study Subjects

The study was carried out on the 80 subjects (40 RA subjects and 40 controls). Subjects were randomly chosen from native Punjabi population of Pakistan. The mean age of male patients was 50.833 ± 0.17 and that of female patients was 55.90 ± 1.65. Consent from all the subjects, was taken, who met the Rheumatoid Arthritis criteria (Table **1**) prior to blood sample collection. 40 RA subjects and 40 controls were randomly sampled from the population.

After collection of the blood samples, the samples were stored at the temperature as low as 4ºC for further purposes. For screening of the blood samples, the serum samples were also collected from the RA subjects. Screening of the blood samples was done for HIV, HBV, HCV and Malaria Pf/Pv. The immunofluorescence assay was also done and pattern for Anti-Nuclear antibodies was mostly

speckled and homogenous. Glucose levels of the RA positive and diabetic patients was also recorded.

Table 1. Percentage of the parameters of the RA subjects.

Individual Characters	Patients (n=40) %	Controls (n=40) %
Sex		
Male n	25	20
Female n	75	89
RF (positivity)	100	0
CRP (positivity)	95	5
Deformed Joints		
≥2	35	-
≤2	65	-

Immunofluorescence Technique Procedure for the Detection of Antinuclear Antibodies

Before using the 10X Phosphate Buffer Saline (PBS), the contents of one tube of 10X PBS concentrate were diluted by using distilled water to raise the final volume up to 1liter. The filling of the dilution into squeeze bottle was preferred. The RA patient's serum sample was diluted (1:80) before the procedure; according to the population characteristics. The reagents of the assay were brought to the normal room temperature *i.e*., 15-30°C. One drop of the diluted patient's sample, *i.e*., 25µl, or the control are placed onto the wells of the slides. The slides were incubated in the moist chamber for 30 minutes. The drained samples were dropped off from the surface of the slide. The cross contamination of the sear samples was avoided. The slide was gently washed by diluted PBS. The process was done two times repeated for 5 minutes by dipping the slide in dilute PBS. The slide was carefully dried and the substrate was kept moist during the process. Fluorescein isothiocyanate (FITC), an extensively utilized fluorophore for antibody detection, was used. FITC-conjugate was placed drop by drop on each well and slide was incubated for 30 minutes in the moist chamber for 30 minutes. The slide was washed again two times by diluted PBS. Slide was dried carefully and mounting media (several drops) was placed onto the surface of the slide. The slide was covered with a coverslip and the bubble formation on the slide was avoided. The slide was immediately examined under the 250-400X lens of immunofluorescence microscope [16]. The well of the slide that contained the positive control was exactly on the side of the wells containing patients' samples according to the standard [17].

Methodology for the Interpretations of Results of IF

A specific staining procedure for the fluorescent microscopy is recommended at the dilution to observe the anti-nuclear antibodies ANA. The fluorescent patterns may vary due to coexistence in one sample. This pattern may be avoided by the modification of the dilution technique of the sample [18]. The major types of the ANA (anti-nuclear antibody) patterns are as given below:

i. Peripheral pattern: The pattern staining of the peripheral side of the nuclei is more strong at the inner edge of the nucleus and the homogenous id the remaining nuclei.

ii. Homogenous pattern: The observation of uniform and the homogenous fluorescence pattern all over the nucleus of interphase cell is the homogenous pattern and strong and bright fluorescence is observed in mitotic cells.

iii. Nucleolar pattern: It is of two main types:

a) Speckled pattern is the staining of the interphase cell's nucleoli. Scattered staining of the organizing regions of the nucleoli of the mitotic cellular chromosomes represents the speckled pattern.

b) Nucleolar pattern is the staining along with the homogenous pattern and most commonly found with low brightness of the homogenous patterns in the nucleus of the cell.

iv. Speckles of the nucleus of the cell are stained but, nucleoli are not and the pattern is known as speckled pattern. Depending on the reacting antigen, there is a variation of shapes and sizes of speckles.

v. Centromere pattern shows the discrete dots lined up with the metaphase chromosomes on the interphase cells *i.e*., multiple number or only 46 of them.

Positive: When the above mentioned staining pattern is observed at the recommended dilutions then, the result of the fluorescence should be considered positive. This shows that these ANA in Rheumatoid Arthritis patients *i.e*., autoimmune antibodies can be a diagnostic marker.

Negative: If there is no specific staining pattern obtained in the results, then samples should be

considered as negative for the autoimmune antibodies [17].

DNA Extraction and Genotype Determination

DNA extraction was completed from the whole blood samples (1 ml) using the organic method. The quality and quantity of obtained DNA was checked by agarose gel electrophoresis. The extracted DNA was also quantified by using Real Time-Polymerase Chain Reaction (RT-PCR). The nested PCR was run for the amplification of the *ACE* gene intron 16 (specified sequence portion). PCR for ACE gene insertion deletion polymorphism was done. The reactions were performed by using 10 pmol of each primer *i.e*., sense oligo: 5'CCCATCCTTTCTCCCATTTCTC3' and antisense oligo 5'CCATGCCCATAACAGGTCTTCA3' in a final volume of 25 µl. The template DNA was amplified for approximately 35 cycles with the conditions including; denaturation of the template DNA at 98°C for 1 min, annealing of the product at 57°C for 1 min 30 s, and final extension at 72°C for 1 min 15 s using a thermal cycler for PCR [19]. Agarose gel (2.3%), stained with ethidium bromide, was used to analyze the PCR products. In the absence of 287bp the PCR resulted in 190bp product (*D* allele). In the presence of the insertion allele, the product produced was a 490bp. The both bands appeared together as a result of heterozygous condition. For the validation of the method and result product, each subject was run alongside the internal control for each genotype. The PCR was repeated randomly for the selected samples, which didn't gave the clear results. The results obtained after second PCR were almost concordant.

Statistical Analysis

Statistical analysis was carried out with the help of IBM SPSS Statistics V25.0 [20]. Chi-square test was applied *via* SPSS software and the results were calculated with $p < 0.05$ significance level.

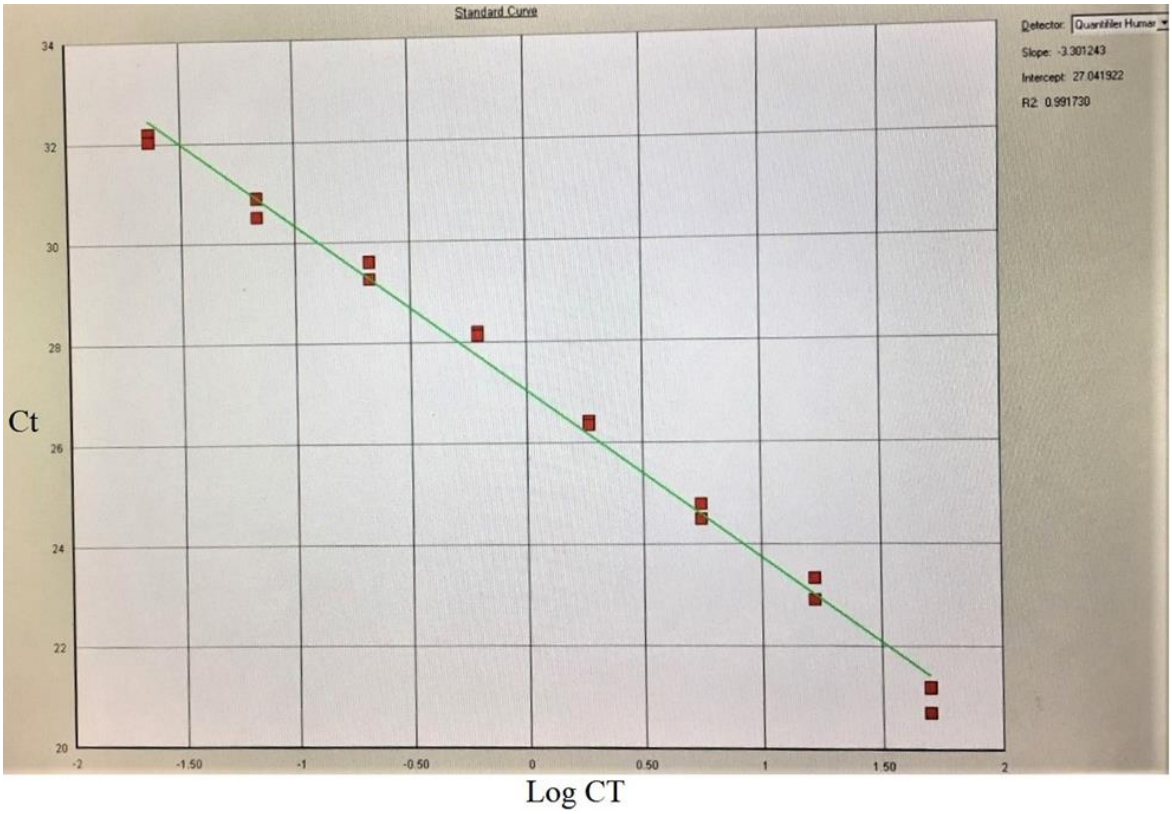

Figure 1. RT-PCR standard curve. (The standard curve shows the straight line obtained after the quantification of the extracted DNA samples on the Human identifier quantifier Real Time PCR. The diagram explains the standard curve for the DNA quantification against standard DNA).

RESULTS

The basic physiognomies of the Rheumatoid Arthritis patients are given in Table **1**. All patients had positive rheumatoid factor. The standard curve to show the authentic quantification (Cq values) of the extracted DNA is shown in Figure **1**. The *ACE* gene *I/D* polymorphism PCR gel results are shown in Figure **2**. After the PCR, the genotype analysis depicted that, the *DD* genotype was comparatively more prevalent in controls. The *ACE* genotype was according to the expected results in the controls but not in accordance, in the RA subjects group. *I/D* genotype of *ACE* gene showed the results as represented in the Table **2**. The following calculations proved that there was no significant association either in the demographic sub-groups (Table **2**, **3**). The genotype distribution results were statistically significant between both control and RA group (Figure **3**). The RA patients showed high frequency of *D* allele (50%) and low frequency of *I* allele (12.5%), contrary to the controls.

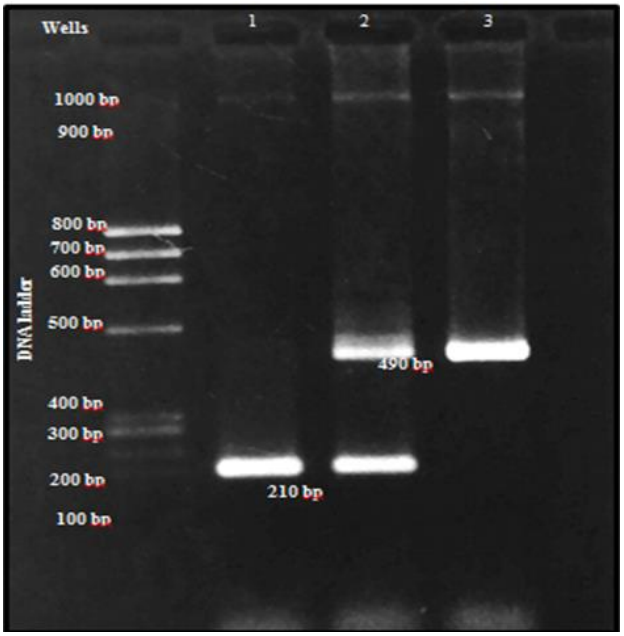

Figure 2. Gel picture obtained after PCR. (The picture shows the bands obtained after assaying the PCR of the sample DNA templates and the resultant products were observed by the gel electrophoresis. The gel was viewed on the UV illuminator and the results recorded showed the markers *i.e*., **490 bp**: marker *ID* genotype; **210 bp**: marker *DD* genotype).

Table 2. Statistical analysis of the distribution of II, ID, DD alleles in RA patients according to the demographic parameters.

Variables	Patients				P Value
	n=total	DD	ID	II	
Sex					
Females	30	14	13	3	0.4116
Males	10	6	2	2	
Age at Diagnosis					
≥40	35	19	12	4	0.3575
≤40	5	1	3	1	
Rheumatoid Factor Positive					
Positivity (n)	40	20	15	5	-
Deformed Joints					
≥2 (n)	14	11	2	1	0.0073
≤2 (n)	26	7	11	8	

Table 3. Distribution of ACE gene I/D polymorphism and frequencies of alleles between controls and RA subjects.

Genotype	Patients (n=40) (%)	Controls (n=40) (%)	X^2 Value	P Value
DD	20 (50)	16 (40)	2.146	0.3421
II	5 (12.5)	10 (25)		
ID	15 (37.5)	14 (35)		

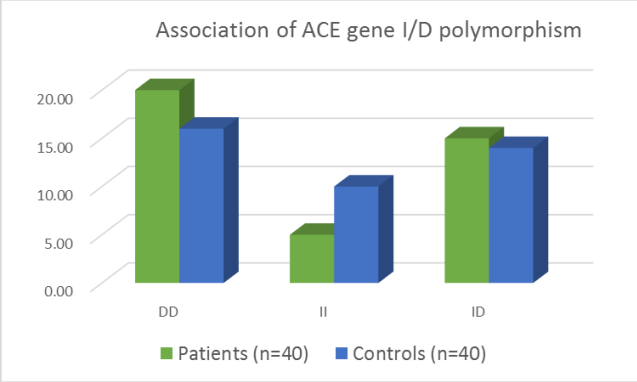

Figure 3. Association of *ACE* gene I/D polymorphism with Rheumatoid Arthritis. (The graph shows the association of the *ACE* gene I/*D* polymorphism with the rheumatoid arthritis disease in Pakistani subjects. The number of obtained markers for the respective genotypes and their association in controls and subjects is explained in the results).

The *D* allele has proved to be associated with the RA disease. There was a significant dissimilarity in the frequencies of *I* and *D* allele *i.e*., among the controls and the subjects. The present study depicts that, the *ACE* gene *I/D* alleles in RA subjects of Pakistani population are possibly playing an important character in the occurrence of Rheumatoid Arthritis. Moreover, there is a significant variation in the allele frequencies among the subjects and the controls (Table **3**, Figure **3**). In RA serum samples homogenous and speckled anti-nuclear patterns were commonly observed (Table **1**).

DISCUSSION

The present study demonstrates the correlation of Rheumatoid Arthritis with the *ACE* gene *I/D* polymorphism and specific patterns of antinuclear antibodies, principally for the first time in the Pakistani population. RA has been described as a prolonged inflammation and disability of the joints, generally leading to the early death of the patient [21]. The early death of RA patients is also considered to be a consequence of cardiac disorders that they come across during systematic inflammation of body tissues and synovia [22]. The genetic basis of the Rheumatoid Arthritis is a phenomenon which is yet not clear. Various studies have demonstrated that many genes are linked to the onset of Rheumatoid Arthritis. It is also calculated that the Rheumatoid Arthritis patients have lower cytokine production and higher T-cell deposition percentage in anti-CD28 + anti-CD3 mononuclear cells [23]. The important role of Angiotensin converting enzyme (ACE) has already been reported for the occurrence of the issues regarding inflammatory disorders, blood pressure and pulmonary vasculature illnesses [24]. The *ACE* gene *I/D* polymorphism has been reported as pathogenic in the Rheumatoid Arthritis. Consistent with the results of the present study, already reported results in Indian Gujrat population showed higher prevalence of *I* allele [25] whereas, the present study showed higher prevalence of *D* allele. The macrophage inhibitory factor has also found to be associated with the RA along with the *ACE* gene [26].

Table 4. Associated alleles of ACE I/D polymorphism with Rheumatoid Arthritis in altered populations of the world from previous databases.

Origin	Country	Linked Allele	Reference
Caucasian	Spain	'D' allele	[31]
Turkish	Turkey	'D' allele	[15]
Asian	Kuwait	'I' allele	[32]
Asian	Kuwait	'D' allele	[33]
Asian	UK	'D' allele	[34]
Asian	Pakistan	'I' allele	[35]
Asian	Indian (Gujrat)	'I' allele	[26]
Caucasian	UK	'D' allele	[34]
African	Egypt	'D' allele	[35]
Asian	Pakistan (Punjab)	'D' allele	[36]

Various studies have shown the association of *DD* genotype with RA. Table **4** shows that in most of the population *D* allele has proved to be associated with the RA disease. The *D* allele from ACE gene *I/D* polymorphism has found to be frequently associated with the RA in the populations all over the world. The present study suggests that further studies are required on the association of the polymorphism of the *ACE* gene with other populations in Pakistan and other countries. This would be helpful in the confirmation of these findings. Moreover, the *ACE*

gene pathogenesis regarding RA should also be studied in other study groups. Understanding the basics of technique and Rheumatoid Arthritis, IF technique was completed using the kit [17]. According to previous investigations, the antinuclear antibodies have been categorized as the auto-antigens. These are reported to be playing an important role in triggering the immunochemical response and mostly appear in the patients of autoimmune disorders [27]. A constant expression of ANA has already been proved in the subjects of Systemic Lupus Erythematosus (SLE) [28]. The significance and validation of the unusual patterns of ANA are yet to be clinically assessed for the development of the association with the autoimmunity related syndromes [29]. These ANA markers could be used as the identifying markers for the inflammatory diseases [30]. The IF technique revealed that ANA are actually the diagnostic markers for autoimmune diseases *i.e*., Rheumatoid Arthritis. There are various patterns for the visualization of ANA at specific wavelengths. The filter used in IF microscopy for the RA subjects' serum analysis was blue filter *i.e*., wavelength = 475 nm. In RA serum samples homogenous and speckled anti-nuclear antibody patterns were commonly observed (Figure **4**; Table **5**). The clinical results are shown in Table **6**.

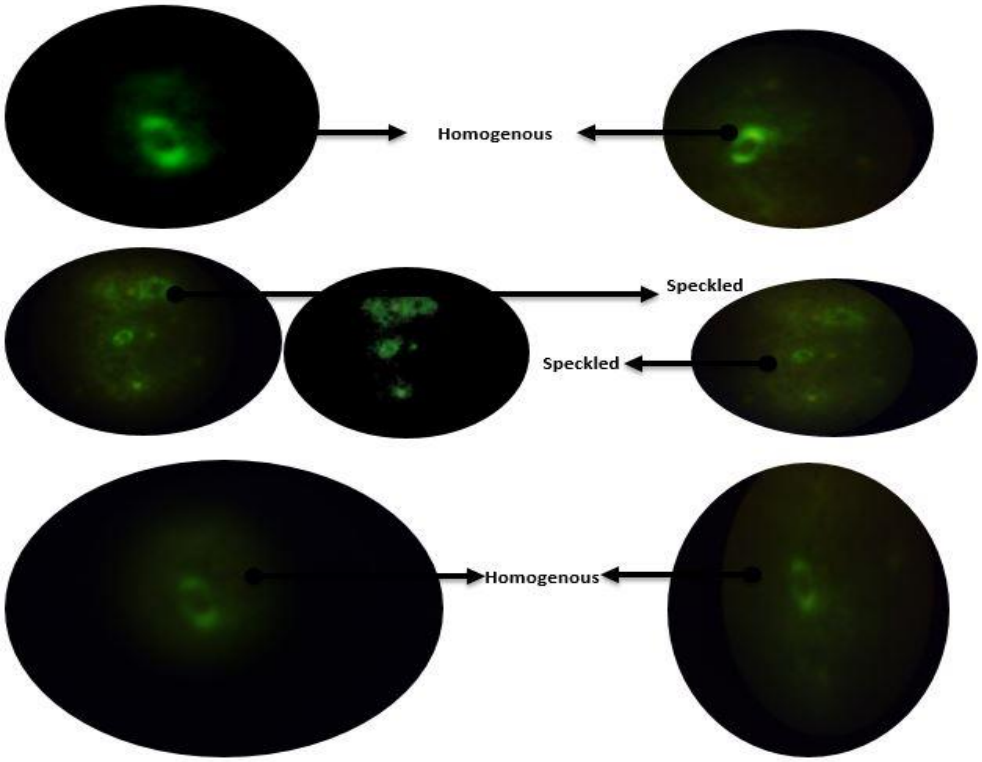

Figure 4. Commonly observed homogenous and speckled anti-nuclear patterns in RA serum samples via Immunofluorescence Assay. (The pictures show the original images obtained after the Immunofluorescence microscopy. The stained RA serum samples were diluted and were undergone the process of Immunofluorescence assay and the assay concluded to show that these ANA markers could be a diagnostic marker for Rheumatoid Arthritis).

Table 5. Results for the serum anti-nuclear antibodies in RA patients.

Study Population	Total Number of Patients (n)	Positive Results (n positive)	Positive Results (%)
RA patients	40	34	85%

Table 6. Comparison of ANA positive results among RA subjects and controls.

	Positive	negative
Positive samples	34	6
Negative/controls	0	40

CONCLUSION

The present study concludes that the associative analysis of *ACE* gene polymorphism and anti-nuclear antibodies with the samples of the Rheumatoid Arthritis in Pakistani patients has been proved to quite an extent. The idea of using these both markers in the diagnostics of RA should be studied and analyzed further. Moreover, this could help in the improvement and upgrading of the blurred genetic background data of the Rheumatoid Arthritis. The challenge of the ANA association with RA and other inflammatory syndromes is yet to be faced.

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